External Dentin Stimulation Induces ATP Release in Human Teeth

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Abstract

ATP is involved in neurosensory processing, including nociceptive transduction. Thus, ATP signaling may participate in dentin hypersensitivity and dental pain. In this study, we investigated whether pannexins, which can form mechanosensitive ATP-permeable channels, are present in human dental pulp. We also assessed the existence and functional activity of ecto-ATPase for extracellular ATP degradation. We further tested if ATP is released from dental pulp upon dentin mechanical or thermal stimulation that induces dentin hypersensitivity and dental pain and if pannexin or pannexin/gap junction channel blockers reduce stimulation-dependent ATP release. Using immunofluorescence staining, we demonstrated immunoreactivity of pannexin 1 and 2 in odontoblasts and their processes extending into the dentin tubules. Using enzymatic histochemistry staining, we also demonstrated functional ecto-ATPase activity within the odontoblast layer, subodontoblast layer, dental pulp nerve bundles, and blood vessels. Using an ATP bioluminescence assay, we found that mechanical or cold stimulation to the exposed dentin induced ATP release in an in vitro human tooth perfusion model. We further demonstrated that blocking pannexin/gap junction channels with probenecid or carbenoxolone significantly reduced external dentin stimulation-induced ATP release. Our results provide evidence for the existence of functional machinery required for ATP release and degradation in human dental pulp and that pannexin channels are involved in external dentin stimulation-induced ATP release. These findings support a plausible role for ATP signaling in dentin hypersensitivity and dental pain.

Keywords: dentin sensitivity, pain, pannexins, NTPDases, odontoblasts, dental pulp

Introduction

Dentin hypersensitivity results from exposed dentin in which environmental stimuli—including thermal, chemical, and mechanical factors—evoke transient, sharp dental pain (Cummins 2010). The hydrodynamic theory of dentin hypersensitivity proposes that external stimuli induce fluid movements in the dentin tubules that initiate nociceptive transduction in adjacent pulpal nerve fibers (Brannstrom 1986; Charoenlarp et al. 2007). However, direct evidence that dentin fluid movement induces nociceptive sensory impulses is lacking. Also, dentin hypersensitivity can persist even following adequate obturation of the exposed dentin tubules. Thus, in addition to direct activation of the nerve fibers by tubule fluid movements, other nociception transduction mechanisms likely contribute to dentin hypersensitivity and dental pain.

Odontoblasts may act as sensory cells that mediate or modulate nociceptive transduction in dental pulpal nerve fibers. For example, odontoblasts express thermosensitive transient receptor potential (Son et al. 2009; El Karim et al. 2011; Tsumura et al. 2012), acid-sensitive (Sole-Magdalena et al. 2011), and mechanosensitive (Magloire et al. 2009) ion channels. Opening of these cation-permeable ion channels depolarizes the membrane potential by producing inward currents. In addition, odontoblasts also express voltage-dependent Na⁺ channels (Allard et al. 2006). However, the role of odontoblasts in mediating/regulating dentin hypersensitivity and the

underlying cellular and molecular mechanisms for dental nociception initiation and transduction have yet to be fully elucidated.

Extracellular ATP is an important molecule involved in peripheral pain transduction (pain mediator). ATP also acts as both a neuronal transmitter and a modulator for pain signal transmission in the nervous system (Burnstock 2009). ATP signaling is dictated by ATP release, purinergic receptor activation, and subsequent ATP degradation by selective ectonucleotidases (ecto-ATPases; Zimmermann and Braun 1996). Millimolar concentration of ATP exists in vital cells, and

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A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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stimulation-dependent ATP release through gap junctions/hemichannels has been demonstrated (Zhao 2005; Gomes and Lambiase 2009). Our previous work and that of others have demonstrated that gap junction connexins are present in dental pulpal odontoblasts (Ikeda and Suda 2006; Liu, Yu, et al. 2012). These observations suggest that odontoblasts may mediate nociceptive signaling via ATP release through the undocked connexin hemichannels. Similar to connexins, pannexins function as large transmembrane channels that allow for the passage of ions and small molecules such as ATP (Ishikawa et al. 2011). The gating of pannexin channels is sensitive to mechanical stimulation and is controlled by membrane depolarization. As a result, pannexin channels can mediate ATP release in response to mechanical stimulation and membrane depolarization (Bao et al. 2004). This property enables pannexin channels to potentially bridge the gap between the dentin tubule fluid movements and ATP receptor activation of nociceptive nerve fibers that control dentin hypersensitivity and dental pain. Consistent with this notion, it was recently shown that the mechanosensitive pannexin channel mediates intercellular ATP signaling in cultured odontoblasts and trigeminal ganglia neurons (Shibukawa et al. 2014). However, expression of pannexins in human dental pulp has yet to be demonstrated.

Ecto-ATPases hydrolyze extracellular ATP to its respective metabolites, which is essential for termination of ATP signaling. Because of their dynamic catalytic activities under physiologic conditions, ectonucleoside triphosphate diphosphohydrolases (NTPDases) play a dominant role in the hydrolysis of extracellular ATP (Zimmermann and Braun 1996; Wink et al. 2006). All 3 members of NTPDase 1, 2, and 3 exhibit cytoplasmic N- and C-termini separated by 2 transmembrane domains and a large extracellular loop that contains the NTP catalytic site. We previously demonstrated that NTPDase 2 is expressed in the dental pulp odontoblast layer, Schwann cells, and satellite cells in the trigeminal ganglia. In addition, immunoreactivity for NTPDase 1 and 3 was also detected in human dental pulp (Liu, Yu, et al. 2012). However, direct evidence for the existence and distribution of functional ecto-ATPase activity within human dental pulp is lacking.

The hypothesis that ATP signaling is involved in pain signal transmission from odontoblasts to nerve fibers in dental pulp has recently emerged (Magloire et al. 2010; Liu, Yu, et al. 2012; Chung et al. 2013). However, direct evidence for ATP release from dental pulp upon external dentin stimulation is lacking and represents a critical missing piece of information needed to bridge the gap between odontoblasts and nerve fiber activation. In this study, we detected the presence and distribution of pannexins and functional ecto-ATPase activity in human dental pulp. Furthermore, we developed an *in vitro* human tooth perfusion model to determine if ATP is released via pannexin/gap junction channels in dental pulp upon external mechanical and cold stimulation to the exposed dentin. Our results provided evidence supporting the involvement of intercellular ATP signaling in mediating dentin hypersensitivity and dental pain.

Methods

Tooth Sample Preparation

Extracted third molars or premolars were collected from patients at the Eastman Institute for Oral Health at University of Rochester with the approval of the institutional research subject review board. The ages of the patients ranged from 16 to 28 y old. For immunostaining, the teeth were fixed for 12 h with 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) and then dehydrated in 30% sucrose for 12 h. The dental pulp was collected after splitting the tooth, embedded in optimal cutting temperature compound, and then stored at -80°C . Twenty-micrometer-thick longitudinal sections were made with a cryostat microtome (Leica 2000) and mounted on gelatine-coated microscope slides. For histochemical examination, the teeth were immediately chopped after extraction. Dental pulps were then isolated and sectioned into 30- μm slices under cryostat.

Immunofluorescence Staining

Prepared dental pulp sections were first incubated with blocking solution (5% normal goat serum / 0.3% Triton X-100 in $1\times$ PBS) for 2 h at 4°C and then incubated overnight with antibodies against Tuj-1 (1:1,000; Sigma-Aldrich) and/or pannexin 1, 2, 3 (1:500; Sigma-Aldrich). After washing with $1\times$ PBS, the sections were incubated for 2 h with secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 543 (1:200; Molecular Probes). Sections were then rinsed with $1\times$ PBS and cover slipped with DAPI (4',6-diamidino-2-phenylindole) mounting medium. Control experiments were performed using the same protocol in the absence of primary antisera. Sections were viewed and captured using a confocal Microscope (Zeiss 510, Germany). To demonstrate that Tuj-1 is expressed in human dental odontoblasts, we also made coimmunostaining with vimentin, a generally accepted marker for odontoblasts (Sigal et al. 1985). We found that Tuj-1 staining is colocalized with vimentin staining in odontoblasts (see Appendix and Appendix Fig. 1). A total of 12 dental pulps were used in these experiments, all of which displayed similar staining.

Nucleotidase Histochemistry

To demonstrate functional ATPase activity in dental pulp, we used a lead phosphate method with ATP (Sigma-Aldrich) as substrate as previously described (Braun et al. 2003). In brief, frozen sections were warmed to room temperature and fixed with 4% paraformaldehyde in PBS for 30 min. After washing with $1\times$ PBS, sections were preincubated for 30 min at room temperature with Tris-maleate-sucrose buffer (0.25M sucrose, 50mM Tris-maleate, pH 7.4) containing 2mM CaCl_2 . The enzyme reaction was performed for 60 min at room temperature in a Tris-maleate-sucrose-buffered substrate solution (1mM ATP, 2mM $\text{Pb}(\text{NO}_3)_2$, 5mM MnCl_2 , 2mM CaCl_2 , 50mM

Tris-maleate, pH 7.4, plus 0.25M sucrose) stabilized with 3% dextran T250 (Roth, Karlsruhe, Germany). After washing with demineralized water, the lead orthophosphate, which was precipitated from ATP as a result of nucleotidase activity, was visualized as a brown deposit by incubating sections in an aqueous solution of $(\text{NH}_4)_2\text{S}$ (1% v/v) for 30 s. Subsequently, the sections were dehydrated in graded ethanol and mounted with Permount mounting medium (Fisher Scientific). To account for nonspecific phosphatase activity, sections were incubated with para-nitrophenyl phosphate (1 mM; Sigma-Aldrich) as a substrate. In some experiments, levamisole (10 mM) and ouabain (5 mM) were included to inhibit alkaline phosphatase and sodium/potassium (Na^+/K^+) ATPases, respectively. To control for nonspecific lead precipitation, substrate was omitted from the incubation solution. Under our experimental condition (pH 7.4), it is expected that this assay will detect the activity of all nucleotidases (NTPDase 1, 2, 3, 8; Zimmermann and Braun 1996). A total of 10 sections from 4 pulps were used in these experiments, all of which displayed similar staining.

In Vitro Tooth Perfusion and ATP Bioluminescence Assay

We have developed an in vitro human tooth perfusion model. Briefly, immediately after tooth extraction, the root tips were cut off at the middle of roots, and the resident root pulps were removed with a broacher. The tooth pulp chamber was then connected by 2 polyethylene tubules and sealed with dental resin. The teeth were continuously perfused with modified Tyrode solution (140mM NaCl, 5mM KCl, 2.5mM CaCl_2 , 1mM MgCl_2 , 10mM glucose, 10mM HEPES, and pH 7.4.) at a rate of 0.3 mL/5 min via a microperfusion pump (Biopetech). The dentin was exposed by removal of the enamel in the central part of the occlusal surface. To reduce possible acute effects of the tooth manipulation on ATP release, the teeth were perfused for at least 1 h before experimental stimulation was applied.

Mechanical stimulation (scratch) with an explorer or cold stimulation with endo-ice (20 s each time and 3 times within

Figure 1. Pannexin 2 is expressed in odontoblasts (OBs). **(A)** Punctate immunoreactivity for pannexin 2 was exclusively detected in the OB layer in human dental pulp. Colocalization staining with Tuj-1 indicates that pannexin 2 is expressed in OBs (insets). **(B)** Pannexin 2 is detectable in the processes of OBs. Positive staining for both pannexin 2 and Tuj-1 was detected in the processes sprouting from OBs. **(C)** No obvious pannexin 2 immunoreactivity was detected in the nerve bundles (NBs) of the dental pulp. Tuj-1-positive staining was also detected in the nerve fibers of the dental pulp NB. Scales are 20 μm (A, C) and 10 μm (B).

5 min) was applied to the exposed dentin for external dentin stimulation. During the experiment, the perfusate was continuously collected from the outlet tubule every 5 min for ATP assay. The ATP concentration in the collected perfusate was determined with an ATP bioluminescence detection kit (ENLITEN ATP Assay System; Promega) as previously described (Liu et al. 2010). Exposure to 0mM Ca^{2+} Tyrode solution—which could induce pannexin/connexin channel opening and intracellular ATP release in live cells—was used to perfuse the tooth for 5 min to confirm the vitality of dental pulp cells by ATP release. To confirm the channels responsible for ATP release during 0mM Ca^{2+} perfusion or external dentin stimulation, the pannexin channel blocker probenecid (150 μM ; Silverman et al. 2008) or the pannexin/gap junction channel blocker carbenoxolone (100 μM) was added into the perfusion

Figure 2. Pannexin 1 is expressed in human dental pulp. **(A)** Immunoreactivity signal for pannexin 1 was detected in the odontoblast (OB) layer and also in cells in the dental pulp. Colocalization of pannexin 1 staining with Tuj-1 staining in the OB layer indicates that pannexin 1 is expressed in OBs (insets) and their processes. The arrows indicate the expression of pannexin 1 in the processes of OBs. **(B)** Immunoreactivity of pannexin 1 was also detected in the nerve bundles (NBs) within the dental pulp. Tuj-1-positive staining was also detected in the nerve fibers of the pulp NB. Scales are 30 μm (A) and 40 μm (B).

solution. As both probenecid (with 1% DMSO as solvent) and carbenoxolone were found to enhance ATP bioluminescence, different standard curves were applied to the corresponding samples. A total of 24 teeth were collected in this study. Seventeen teeth displayed ATP release when exposed to 0mM Ca^{2+} perfusion and were therefore selected for statistic analyses. Data were given as mean $\pm$ SE, and paired Student's *t* tests were used to determine statistical significance.

Results

Expression of Pannexins in Human Dental Pulp

Pannexins are integral membrane proteins that form channels optimized to facilitate ATP release. Here we tested if pannexins are expressed in the dental pulp of human teeth. As shown in Figure 1A, positive immunoreactivity for pannexin 2 was exclusively detected in the odontoblast layer of the dental pulp. Punctate pannexin 2 staining—characteristic for integral membrane protein immunostaining—was observed in odontoblasts

as demonstrated by the colocalization with the staining of Tuj-1 (β -tubulin), which has been identified as a marker for odontoblast and its processes (Sigal et al. 1985). Odontoblasts send processes into dentin tubules that may detect tubular fluid movements. We then tested if pannexin 2 was detectable in odontoblast processes. In isolated dental pulpal samples, 10- to 20- μm odontoblast processes are usually preserved. As shown in Figure 1B, pannexin 2 was detected in processes sprouting out from odontoblasts, which was also colocalized with Tuj-1 staining. Pannexin 2 immunoreactivity signal was not detected in other dental pulpal structures, including the subodontoblast layer (Fig. 1A) and nerve trunks and bundles (Fig. 1C).

Immunoreactivity for pannexin 1 was also detected in the odontoblast layer in human dental pulp (Fig. 2A), which is consistent with a recent observation in neonatal rat tooth (Shibukawa et al. 2014). Colocalization of staining for pannexin 1 and Tuj-1 indicates that pannexin 1 is expressed in odontoblasts and their processes (Fig. 2A). In contrast to the expression pattern of pannexin 2 in dental pulp, pannexin 1 immunoreactivity signal was also detected in other cells within the dental pulp

(Fig. 2A), as well as in nerve trunks and bundles (Fig. 2B). We also tested if pannexin 3 was expressed in human dental pulp. No positive immunoreactivity to pannexin 3 was detected in odontoblasts or other cells in human dental pulp (data not shown).

Detection of Functional Ecto-ATPase Activity in Human Dental Pulp

Our previous work demonstrated the presence of NTPDase 2 in the odontoblast layer and Schwann cells that encapsulate the nerve fibers in human dental pulp (Liu, Yu, et al. 2012). Immunoreactivity for NTPDase 1 and 3 was also detected in human dental pulp (Liu, Yu, et al. 2012). However, verification of functional NTPDase enzymatic activity to efficiently degrade extracellular ATP in dental pulp is lacking. Using enzyme histochemistry, we confirmed the presence of functional ecto-ATPase activity in human dental pulp. As showed in Figure 3A, robust ecto-ATPase activity was observed, as illustrated by positive brown deposits within different

structures (e.g., nerve bundles and blood vessels) of the dental pulp. In accordance with our previous finding that NTPDase 2 is expressed in the odontoblast layer, functional ecto-ATPase activity was also detected in odontoblasts and their processes (Fig. 3A, B). Strong ecto-ATPase activity was also detected in nerve bundles and nerve trunks within the dental pulp (Fig. 3A, C), consistent with our previous observation that NTPDase 2 is expressed in Schwann cells encapsulating dental pulp nerve fibers. Furthermore, strong ecto-ATPase activity was also detectable in the subodontoblast layer (Fig. 3A, B). In contrast, only slight ecto-ATPase activity was detected in the relatively large blood vessels in other regions of the dental pulp (Fig. 3A, C). Figure 3D shows the negative control for the absence of ecto-ATPase staining when ATP was not included in the incubation solution.

External Dentin Stimulation Induces ATP Release from Dental Pulp

To test if environmental stimulation that mimics dentin hypersensitivity induces ATP release from human dental pulp, we developed an *in vitro* tooth perfusion system (Fig. 4A). While teeth were continuously perfused, the ATP that was released from the dental pulp was assayed in the perfusate. To confirm the vitality of the dental pulp, we perfused the teeth with 0mM Ca^{2+} Tyrode solution, which induces ATP release by opening pannexin/connexin channels in vital cells (Liu et al. 2010). Figure 4B shows the ATP level in the perfusate before, during, and after external dentin stimulation from representative teeth that exhibited either positive or no obvious response to 0mM Ca^{2+} perfusion. As illustrated in Figure 4B, the tooth that responded to 0mM Ca^{2+} perfusion (purple lines) also displayed an increase in ATP release upon external cold and mechanical dentin stimulation. In contrast, the tooth with no obvious response to 0mM Ca^{2+} perfusion (red lines) also lacked responses to external dentin stimulation. After termination of stimulation, ATP release quickly returned to the baseline (Fig. 4B2, B3). The concentrations of ATP in the perfusate before and during the stimulation were used for statistical analysis. As illustrated in Figure 4C, both mechanical and cold external dentin stimulation induced significant ATP release in dental pulps that were positive in response to 0mM Ca^{2+} perfusion.

Pannexin/Gap Junction Channel Blockers Reduce External Dentin Stimulation–induced ATP Release

To determine the channels responsible for ATP release during external dentin stimulation, we tested the effects of the pannexin channel blocker probenecid and the pannexin/gap junction channel blocker carbenoxolone. We first tested the blocking effects of probenecid and carbenoxolone on 0mM Ca^{2+} perfusion–induced ATP release. As illustrated in Figure 5A, both probenecid and carbenoxolone significantly reduced ATP release during 0mM Ca^{2+} perfusion. The percentage

Figure 3. Histochemical determination of ecto-ATPase activity in human dental pulp. (A) Brown staining in different structures of dental pulp indicates the release of phosphate from ATP via functional ecto-ATPase activity in that region. Positive staining was observed in the following structures: odontoblast (OB) layer, subodontoblast (sub-OB) layer, nerve bundles (NBs), and blood vessels (BVs). (B) Positive staining for ecto-ATPase activity in the OB and sub-OB layers at higher magnification. Note that positive staining was also detectable in the processes of odontoblasts. (C) Positive staining for ecto-ATPase activity in dental pulp NBs at high magnification. Brown deposit was also detectable in dental pulp BVs. (D) Negative control staining in the absence of ATP. No obvious positive staining was noted in dental pulp when ATP was omitted from the incubation solution. Scale bars 40 μm (A), 15 μm (B), 10 μm (C), and 50 μm (D).

reduction in ATP release by probenecid and carbenoxolone during 0mM Ca^{2+} perfusion was 87% and 82%, respectively. The inhibitory effects of probenecid or carbenoxolone were not significantly different. We further tested the inhibitory effects of probenecid and carbenoxolone on external dentin stimulation–induced ATP release. As showed in Figure 5B and C, both probenecid and carbenoxolone significantly reduced ATP release evoked by cold or mechanical stimulation. The percentage reduction in cold stimulation–induced ATP release by probenecid or carbenoxolone was 81% and 78%, respectively. For mechanical stimulation–induced ATP release, the percentage reduction by probenecid or carbenoxolone was 73% and 79%, respectively. Again, there is no significant difference between the inhibition effects of probenecid and carbenoxolone on cold or mechanical stimulation–induced ATP release.

Discussion

ATP is a key mediator for nociception through activation of purinergic receptors expressed on peripheral sensory nerve fibers (Hamilton 2002; Wirkner et al. 2007). Inotropic purinergic P2X3 receptors are expressed in dental pulpal nerve fibers (Alavi et al. 2001), which supports a role for ATP signaling in

Figure 4. ATP is released from the dental pulp upon external cold or mechanical dentin stimulation. **(A)** In vitro tooth perfusion system: the dental pulp was continuously perfused with Tyrode solution (0.3 mL / 5 min) by a pump. **(B)** External dentin stimulation induced ATP release from dental pulp in a representative tooth that responded to 0mM Ca²⁺ perfusion or did not respond to 0mM Ca²⁺ perfusion. B1, 0mM Ca²⁺ perfusion; B2, cold stimulation; B3, mechanical stimulation. **(C)** Changes of ATP concentration in the perfusates before and during cold/mechanical stimulation in teeth with positive response to 0mM Ca²⁺ perfusion. Paired Student's *t* test was used for the statistical analysis. Mean $\pm$ SE, *n* = 9 for each group.

mediating dentin hypersensitivity and dental pain (Magloire et al. 2010). Previous work found that odontoblasts express the gap junction/hemichannel protein connexin 43, which mediates ATP release upon intracellular and extracellular stimulation with 0mM Ca²⁺, CO₂, IP₃, and NO signals (Retamal et al. 2009; Liu et al. 2010; Nakagawa et al. 2010; Liu, Sun, et al. 2012). Therefore, ATP released from odontoblasts could then activate nearby P2X3 receptors on dental pulp nerve fibers to depolarize the membrane potential and initiate the nociception signals for dentin hypersensitivity. However, the coupling mechanism between external dentin stimulation and the initiation of nociceptive nerve impulses remains an enigmatic.

Pannexin channels are permeable to small molecules up to 1 kDa in size (Dubyak 2009) and thus allow extracellular efflux of signaling nucleotides such as ATP and UTP (Elliott et al. 2009; Chekeni et al. 2010). Interestingly, pannexin channels are sensitive to both membrane depolarization (Clarke et al. 2009; Dubyak 2009; Li et al. 2011) and mechanical stimulation (Bao et al. 2004), which enables cells to release ATP via pannexin channels upon stimulation. In addition, cultured odontoblasts express thermosensitive transient receptor potential channels (Egbuniwe et al. 2014), and mechanical stimulation induces Ca²⁺ influx and cell depolarization via transient receptor potential channel opening (Egbuniwe et al. 2014; Shibukawa et al. 2014), which will further facilitate ATP release through pannexin channels. Our results also suggested that pannexins are expressed in odontoblasts and their processes that extend into the dentin tubules. Thus, external dentin stimulation that induces tubular fluid movements may activate pannexin channels to induce ATP release from odontoblasts. Using an in vitro tooth perfusion model, we demonstrated that external cold or mechanical stimulation of the dentin induces ATP release from the dental pulp. Surprisingly, even though connexin/gap junction channels are also present in dental pulp (Ikeda and Suda 2006; Liu, Yu, et al. 2012), we found that the pannexin channel blocker probenecid along could block dentin stimulation-induced ATP release, suggesting that pannexin channels—but not connexin hemichannels—mediate ATP release from

dental pulp during external dentin stimulation. Indeed, mechanical stimulation induced ATP release from cultured odontoblasts, and intercellular odontoblast-neuron communication has been linked to the presence of pannexin channels but not connexin hemichannels (Shibukawa et al. 2014). However, by forming a functional syncytium in odontoblasts, the potential role of connexin/gap junction channels in contributing to dentin hypersensitivity and dental pain remains unclear.

There is increasing evidence that extracellular metabolites of ATP, including nucleotides and adenosine, also play a role in pain signal initiation, transmission, modulation, sensitization, and inhibition (Hamilton 2002; Wirkner et al. 2007). ATP and its

metabolites may produce different actions via activation of distinct inotropic (P2X, permeable for Na^+ , K^+ , and Ca^{2+}), metabotropic (P2Y, G-protein coupled), and adenosine (P1, G-protein coupled) receptors. For example, ATP depolarizes the membrane potential and excites sensory neurons by activating inotropic purinergic (P2X) receptors (Hamilton 2002; Wirkner et al. 2007), while adenosine promotes analgesia through activation of peripheral nerve P1 receptors (A1; Goldman et al. 2010). These observations suggest that action of ATP and its metabolites will be significantly affected by the presence and activity of ectonucleotide degradation enzymes within the dental pulp.

Three cell surface-located ATP-hydrolyzing members of the E-NTPDase family (NTPDase 1, 2, and 3) have been identified in multiple human tissues. NTPDase 1 and 3 hydrolyze both ATP and ADP, while NTPDase 2 hydrolyzes primarily ATP with minimal ADP hydrolysis (Zimmermann and Braun 1996). Thus, NTPDase 1 and 3 produce AMP and adenosine following further degradation, while NTPDase 2 primarily produces extracellular ADP, the endogenous agonist of P2Y₁, 12, 13 receptors. Depending on the purinergic receptor subtype, ATP and its degradation metabolites can differentially function as agonists on P2X, P2Y, and P1 receptors. Our previous work showed pronounced positive staining for NTPDase 2 in dental pulp nerve bundles, Raschkow nerve plexus, and the odontoblast layer (Liu, Yu, et al. 2012). Immunoreactivity for NTPDases was also detected in structures outlining blood vessels (NTPDase 1) and the subodontoblast layer (NTPDase 3). These findings indicate that machinery required for extracellular ATP hydrolysis is present in the dental pulp and thus may play a significant role in modulating the magnitude and duration of pain signal. Consistent with the expression profile of NTPDases, our results confirmed robust ecto-ATPase activity within the odontoblast layer, subodontoblast layer, and nerve bundles in human dental pulp. While P2X receptors are detected in nerve fibers within dental pulp (Alavi et al. 2001), the cellular expression and functional profiles of the other purinergic receptors within the dental pulp remain largely unknown. Thus, a more definitive role for purinergic signaling in the process of nociceptive initiation, transduction, and modulation in mediating dentin sensitivity and dental pain awaits further exploration.

The results of this study support the hypothesis that extracellular ATP release and intercellular ATP signaling play a role in dentin hypersensitivity and dental pain. We propose that external stimuli promote ATP release from odontoblasts through activation of pannexin channels, which then initiates pain signaling via activation of nerve fiber P2X receptors. Functional NTPDases in odontoblasts and Schwann cells surrounding the nerve fibers terminate and modulate pain transmission by reducing the local concentration of extracellular ATP, correspondingly increasing the concentration of ADP, AMP, and adenosine. ATP and its metabolites may also activate the P2Y and P1 receptors via a paracrine mechanism to trigger intracellular Ca^{2+} signals that further promote ATP release and regulate the expression of pannexin/connexin channels, purinergic receptors, and ecto-ATPases. These intercellular and intracellular signaling mechanisms represent important

Figure 5. Pannexin/connexin channel blockers reduce ATP release in perfused human teeth. **(A)** Both the pannexin blocker probenecid (150 μM) and connexin/pannexin channel blocker carbenoxolone (100 μM) reduce ATP release induced by 0mM Ca^{2+} perfusion. *Compared with the basal level of ATP release before 0mM Ca^{2+} perfusion (before); #compared with 0mM Ca^{2+} perfusion. Mean $\pm$ SE, $n = 7$. **(B)** Probenecid (150 μM) and carbenoxolone (100 μM) reduce ATP release induced by external cold stimulation. The data are presented as percentage of basal level of ATP release before cold stimulation (before). *Compared with the before; #compared with cold stimulation. Mean $\pm$ SE, $n = 4$. **(C)** Probenecid (150 μM) and carbenoxolone (100 μM) reduce ATP release induced by external mechanical stimulation. The data are presented as percentage changes of basal level of ATP release before mechanical stimulation (before). *Compared with the before; #compared with mechanical stimulation. Mean $\pm$ SE, $n = 4$.

potential therapeutic targets for interventions designed to ameliorate ATP-mediated dentin hypersensitivity and dental pain.

Author Contributions

X. Liu, contributed to conception, design, and data acquisition, drafted and critically revised the manuscript; C. Wang, T. Fujita,

contributed to data acquisition, critically revised the manuscript; H.S. Malmstrom, M. Nedergaard, Y.F. Ren, contributed to design, critically revised the manuscript; R.T. Dirksen, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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