Blocking P2X receptors can inhibit the injury-induced proliferation of olfactory epithelium progenitor cells in adult mouse

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ABSTRACT

Objective: The olfactory epithelium (OE) is unusual for its remarkable regenerative capacity and sustained neurogenesis of olfactory receptor neurons (ORNs) throughout adult life. Regeneration of ORNs is accomplished by basal cells in the OE, including stem cells and progenitor cells. Although there is considerable knowledge about the roles of basal cells in ORN turnover, the molecular mechanism that regulates the proliferation and differentiation of adult OE basal cells is not fully understood. As intercellular signaling molecules, purines have been reported to mediate proliferation, differentiation and migration of many kinds of neural stem cells. However, it is still unclear whether ATP, which could be released by injured ORNs, plays a role in regulating neurogenesis in ORN turnover.

Methods: RT-PCR and immunohistochemistry were used to detect the expression of ionotropic purinergic receptors-P2X receptors in adult mouse OE. By using the olfactory bulbectomy model and in vivo administration of P2X receptors antagonists, the function of P2X receptors in regulating the proliferation of OE progenitor cell was evaluated.

Results: We found that basal cells in the adult mouse OE express functional P2X receptors, and blocking the activities of P2X receptors can significantly inhibit the injury-induced proliferation of OE basal cells.

Conclusion: Our research provides evidence in support of the hypothesis that purinergic signaling can serve as a paracrine signal in regulating the neurogenesis of OE in adult mouse.

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1. Introduction

The olfactory epithelium (OE) is unusual for its remarkable regenerative capacity and sustained neurogenesis of olfactory receptor neurons (ORNs) throughout adult life [1]. Regenerated ORNs are differentiated from the basal cells in OE. OE basal cells can be morphologically divided to horizontal basal cells (HBCs) and global basal cells (GBCs) [2]. HBCs are very quiescent but with multipotency. HBCs are the stem cells of OE and can be activated when OE is damaged severely [3]. In contrast, in normal turnover process of ORNs, GBCs are active and are the main source of newly generated ORNs [4]. Based on these characters, GBCs are called as the olfactory epithelium progenitors or olfactory progenitor cells [2]. In the turnover of ORNs, neurogenesis is prompted by injury of ORNs [5]. Many factors participate in the regulation of this process.

It has been reported that leukemia inhibitory factor (LIF), bFGF, EGF, and TGF-α all serve as proliferative factors in stimulating the injury-induced proliferation of adult OE progenitors [6,7]. Purinergic signaling has been reported to play important roles in mediating proliferation, differentiation and migration of neural stem cells (reviewed in [8]). But it is still unclear whether purinergic signaling can function in ORN turnover.

Two recent publications suggest that purinergic signaling is involved in regulating the proliferation of OE progenitor cells. Hassenklo¨ver et al. reported that nucleotides could induce Ca2+ signaling and regulate cell turnover of OE progenitor cells in larval Xenopus laevis [9]. Another publication indicates that nasal instillation of ATP can increase the proliferation of OE progenitor cells in normal adult mouse [10]. But it still remains unclear whether purinergic signaling is involved in regulating injury-induced proliferation of OE basal cells in adult mouse.

Purinergic receptors were separated to P1 and P2 receptors (P1R and P2R) based on their different ligands adenosine and ATP/ADP, respectively. P2R were subdivided into P2X and P2Y subtypes on the basis of pharmacology. P2X receptors are ionotropic purinergic receptors and permeable to Na+, K+ and Ca2+ when
2.3. Immunohistochemistry

Receptor PCR primers are described in Table 1. Primers for GAPDH [12] were designed as reported [12]. In order to exclude contamination with genomic DNA, a primer pair for the immunoglobulin heavy chain binding protein (BiP) was used as reported previously, and the binding sites of these primers were separated by an intron in the BiP gene [12].

2.4. In vivo administration of P2X antagonists

Suramin and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) are functional selective antagonists for P2 receptors [15]. To investigate the functions of P2X receptors in regulating OE progenitor proliferation, we used a modified method from a previous study [16], such that bilateral OBX adult C57BL/6 male mice were injected twice daily with 50 μmol/kg suramin + PPADS or an equivalent volume of Ringer’s solution.

2.5. Bromodeoxyuridine incorporation and detection

Bromodeoxyuridine (BrdU) incorporation assay was carried out as reported previously [17]. Mice were injected with 30 mg/kg BrdU at 3 and 1 h before perfusion. BrdU detection was processed according to a standard immunohistochemistry protocol with the exception that a 30 min pretreatment in 3 M HCl before the incubation with monoclonal mouse anti-BrdU (1:200, NeoMarkers), OE within the third–fourth endoturbinates and nasal septum (Fig. 3A) were used to assess the OE thickness and the number of basal BrdU positive cells as reported previously [17]. Each group contained four mice, and four non-serial sections from each mouse were used for immunohistochemistry. Statistical analysis was performed using an unpaired Student’s t-test. All data are presented as mean ± s.d.

3. Results

3.1. P2X1–3 are expressed in the basal progenitors of adult mouse OE

Our RT-PCR results revealed that all of the ionotropic purinergic receptors P2X1–3 were expressed in the adult mouse olfactory mucosa (Fig. 1A). The PCR product of spliced BiP mRNA was 560 bp and product of non-spliced genome was 876 bp [12]. BiP was amplified from the OE cDNA and only the 560 bp PCR product was amplified to the OE genomic DNA (gDNA), a 876 bp sequence was amplified. With olfactory mucosa genomic DNA (gDNA), a 876 bp PCR product was amplified.

Fig. 1. RT-PCR analysis of P2X receptor expression in the adult mouse mucosa. (A) Adult mouse OE expresses mRNA for P2X1–3 receptors and (B) GAPDH as a positive control. Using the cDNA of olfactory mucosa, a 560 bp sequence was amplified. With olfactory mucosa genomic DNA (gDNA), a 876 bp PCR product was amplified.

Table 1
Details of primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primers (5′ → 3′)</th>
<th>Product length (bp)</th>
<th>Tm (°C)</th>
<th>GenBank accession number</th>
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<td>60</td>
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</tbody>
</table>

2. Materials and methods

2.1. RT-PCR analysis

All protocols of animal experiments followed were approved by the Committee of Animal Use for Research and Education of the Second Medical Military University. Adult C57BL/6 male mice (8 weeks old) were euthanized with a lethal dose of pentobarbital sodium. Total RNA was isolated from the olfactory mucosa using TRIzol (Gibco BRL) and the RNA concentration was measured photometrically. After RNA extraction, samples were digested with TRIzol (Gibco BRL) and the RNA concentration was measured photometrically. After RNA extraction, samples were digested with TRIzol (Gibco BRL) and the RNA concentration was measured photometrically. After RNA extraction, samples were digested with TRIzol (Gibco BRL) and the RNA concentration was measured photometrically.
obtained (Fig. 1B), indicating that the mRNA was not contaminated with genome DNA. We also found mRNA expression of P2X4-7 in the adult mouse olfactory mucosa (data not shown).

To identify the localization of P2X receptors in OE, tissue sections were co-stained with P2X1-3 and TuJ1 antibodies. TuJ1 is expressed in the mature and immature ORNs [17]. Our results showed that P2X1, P2X2 and P2X3 receptors were localized at the basal part of OE. P2X1 and P2X2 were mainly localized to the soma of sustentacular cells and basal cells (Fig. 2A and C). No significant colocalization with TuJ1 was detected (Fig. 2B and D). P2X3 receptors were widely localized in the OE, including the sustentacular cells, ORNs and basal cells (Fig. 2E). Strong colocalization of P2X3 with TuJ1 was detected in the olfactory nerve (Fig. 2F). We also found immunoreactivity to P2X4-7

Fig. 2. Expression pattern of P2X1, P2X2 and P2X3 receptors in adult mouse OE. (A, C) P2X1 and P2X2 receptors are expressed in the basal cells (arrow heads), soma of sustentacular cells, and mesenchymal cells of the lamina propria. (B, D) No detectable P2X1-2 expression was seen in the ORNs when merged with TuJ1. (E) P2X3 receptors are expressed in the basal cells (arrow heads) and sustentacular cells and (F) high expression of P2X3 receptors can be detected in the olfactory nerves (indicated by arrows). Scales bar in (A-F) = 50 μm, dashed lines indicate the basal lamina of olfactory mucosa.

Fig. 3. Expression pattern of cleaved caspase-3 in the OE after OBX. (A) Cleaved caspase-3 was detected in the lesioned lateral OE 48 h after OBX and when merged with TuJ1, is localized in the ORNs. (B) No cleaved caspase-3 was detected in the unlesioned lateral. (C) Cleaved caspase-3 is expressed in the soma of ORNs and olfactory nerves (indicated by arrows). OE: olfactory epithelium, LP: lamina propria, AX: axons and (D-I) cleaved caspase-3 started to express in the OE 24 h after OBX (E), and reached expression peak at 48 h (G), only expression in olfactory nerves was detected 3d after OBX (H), no cleaved caspase-3 was detected 4 days after OBX (I). Dashed lines indicate the basal lamina of olfactory mucosa. Scale bar in (A) = 500 μm, in (B-I) = 40 μm.
3.2. P2X receptors antagonism inhibits the proliferation of the OE basal progenitors after OBX

An olfactory bulbectomy (OBX) model was used to investigate neurogenesis in adult mouse OE. After removal of olfactory bulbs, the ORNs undergo a process of synchronizing apoptosis, and the olfactory neural progenitor cells start to proliferate and differentiate into new ORNs [2]. Firstly, immunohistochemistry of cleaved caspase-3 was performed to investigate the time course of ORN apoptosis after OBX. Immunoreactivity to cleaved caspase-3 was detected in the OE at 24 h following OBX (Fig. 3E), and reached its peak 48 h after OBX (Fig. 3G). At 4 days after OBX, no expression of cleaved caspase-3 was detected in the OE and the OE thickness reached a minimal level (Fig. 3I). Our data indicated that after 4 days OBX, most of the ORNs in the OE had apoptosed. This result was identical to the western blotting analysis of caspase-3 in the OE after OBX as seen in previous studies [18].

Suramin and PPADS are general P2R antagonists and can block the activities of P2X2 and P2X3 receptors [15]. Injured ORNs can release ATP as reported previously [16], and all the injured ORNs had apopptosed after 4 days OBX as mentioned before. So we administered P2R antagonists to the mice for 4 days after OBX, and our data revealed that in vivo administration of P2R antagonists for 4 days could inhibit the proliferation of progenitor cells significantly (Fig. 4A and B). After 4 days OBX, the basal BrdU positive cells were 20 cells/mm OE in the control group, which had only been injected with saline buffer (Fig. 4C), and this value is equal to the published one [17]. However, in the group where antagonists were administered, the basal BrdU positive cells were significantly reduced to 12 cells/mm OE (Fig. 4C, \(P < 0.05, n = 4\)). The OE thickness of the test group was thinner than the control group (Fig. 4D), and it suggested that less cells were generated after OBX in the antagonists administration group.

4. Discussion

This study has demonstrated that adult mouse OE progenitor cells express P2X1–3 receptors. No significant immunoreactivity for P2X1 receptors could be detected in the ORNs when co-stained with the TuJ1 antibody. These differences to previous reports may be due to the distinctions between the age and genus of animals used for experiments [19,20]. Immunoreactivity of cleaved caspase-3 was detected in the OE at 24 h after OBX and reached its peak at 48 h after OBX. At 3 days after OBX, cleaved caspase-3 was only expressed in the olfactory nerves. No expression of cleaved caspase-3 was detected in the OE and the OE thickness reached the minimal level at 4 days after OBX. Blocking the activities of P2X2,3 receptors by in vivo suramin and PPADS administration significantly inhibited the injury-induced proliferation of OE progenitors. As suramin and PPADS cannot block the activity of P2X1, the function of P2X2, in regulating OE progenitor proliferation needs further investigation.

Purines as intercellular factors have been reported to play important roles in mediating proliferation, differentiation and migration of neural stem cells [8]. In the retinal pigment epithelium, ATP is released through efflux by gap junction connexin 43 hemichannels. The released ATP increased the proliferation and stimulated DNA synthesis in neural retinal progenitor cells [21]. ATP application to human neural stem cells induced Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, which in turn promoted NSC proliferation [22]. The adult subventricular zone (SVZ) expresses defined P2 receptors, whose activation can elevate cytosolic Ca\(^{2+}\) concentrations and augment cell proliferation in a synergistic manner when combined with mitogenic growth factors [12]. HassenklÜver et al. recently reported that in larval X. laevis, nucleotide-induced Ca\(^{2+}\) signaling in OE progenitor cells is involved in the regulation of cell turnover in OE [9]. Whether purines can induce Ca\(^{2+}\) signaling in OE progenitor cells of adult mice and what is the downstream effector of purinergic signaling in regulating OE progenitor cells proliferation has yet to be determined.

In physiological condition, ORNs undergo unceasing regeneration in adult vertebrates, and injury of ORNs is a sufficient condition for neurogenesis of adult OE progenitor cells to occur [5]. Previous work has shown that systemic injection of EGF, transforming growth factor-\(\alpha\) (TGF-\(\alpha\)) or olfactory marker protein (OMP) peptides enhance cell proliferation in OE [23,24]. Jia et al. reported that nasal instillation of ATP can increase the proliferation of OE progenitor cells in normal adult mice [10]. But until now, only LIF has been found as a proliferative factor which is released from injured ORNs and can induce neurogenesis of OE progenitors in the turnover of ORNs [6]. Here we show that ATP, which is released from the injured ORNs [16], can serve as such a factor too.

Taken together, our study supports the notion that purinergic signaling contributes to the injury-induced proliferation of OE progenitor cells in adult mice. In the turnover of ORNs, purines released from damaged ORNs can activate nearby OE progenitors through the activation of P2X receptors. Thus, in physiological
conditions, purinergic signaling may serve as a paracrine signal in regulating injury-induced neurogenesis of OE in adult mammals.

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