P2X7 receptors and Fyn kinase mediate ATP-induced oligodendrocyte progenitor cell migration

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Abstract Recruitment of oligodendrocyte precursor cells (OPCs) to the lesions is the most important event for remyelination after central nervous system (CNS) injury or in demyelinating diseases. However, the underlying molecular mechanism is not fully understood. In the present study, we found high concentrations of ATP could increase the number of migrating OPCs in vitro, while after pretreatment with oxidized ATP (a P2X7 receptor antagonist), the promotive effect was attenuated. The promotive effect of 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP) (a P2X7 receptor agonist) was more potent than ATP. After incubation with BzATP, the activity of Fyn, one member of the Src family of kinases, was enhanced. Moreover, the interaction between P2X7 and Fyn was identified by co-immunoprecipitation. After blocking the activity of Fyn or down-

regulating the expression of Fyn, the migration of OPCs induced by BzATP was inhibited. These data indicate that P2X7 receptors/Fyn may mediate ATP-induced OPC migration under pathological conditions.

Keywords P2X7 receptors · Src family kinase · Fyn kinase · Migration · OPCs · ATP

Introduction

In the adult central nervous system (CNS), oligodendrocyte progenitor cells (OPCs), which are NG2 positive, are widely distributed in both white and gray matter [1, 2]. After CNS injury, OPCs can quickly migrate to the lesion areas [3, 4], differentiate into mature oligodendrocytes, and form new myelin sheaths around the axons [5, 6] eventually improving functional recovery. These migrated NG2 positive cells have also been demonstrated to play a role in sealing the lesions and in scar formation [7–9]. However, the mechanism underlying OPC migration after injury remains largely unknown.

Adenosine 5'-triphosphate (ATP) is not only a neurotransmitter [10], but is also involved in cell proliferation [11], migration [12], survival [13], and apoptosis [14]. Extracellular ATP can activate P2 receptors, which include ligand-gated ion channel receptors (P2X receptors) and metabotropic receptors (P2Y receptors) [15]. P2X receptors are ATP-gated ion channels, mediating intracellular potassium efflux, and extracellular sodium and calcium ions influx [16]. P2X7 receptors, with a longer intracellular C-terminal sequence than other members of the P2X receptor family [17], take part in a variety of intracellular signaling to regulate cell proliferation, migration, differentiation, and apoptosis [18, 19]. P2X7 receptors have low affinity for ATP [20], and only when extracellular ATP concentrations are greater than 100 μ M, P2X7 receptors could

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be activated [21–23]. Following CNS injury, a large amount of cell death causes higher concentrations of ATP in the extracellular environment and trigger the activation of P2X7 receptors on neurons and glial cells [24], thereby playing important pathophysiological roles [25].

Many of studies have indicated that activation of P2X7 receptors can promote migration in different types of cells, such as monocytes [26], tumor cells [27], and epithelial cells [28]. P2X7 receptors have also been shown to promote the migration of cultured glial cells [29]. Moreover, P2X7 receptor activation was demonstrated to trigger extracellular calcium ion influx, then activate intracellular kinases, such as MAPK [26, 28] and Akt [30], or increase the expression of matrix metalloproteinase 9 [29], thereby facilitating cell migration. Moreover, Fyn kinase, one member of the Src family, is involved in OPC migration [31, 32]. In the present study, we examined the effect of a high concentration of ATP on OPCs in vitro, and showed that P2X7 receptors couple to Fyn kinase to mediate ATP-induced migration of OPCs.

Material and methods

Antibodies and reagents

Cytarabine (Ara-C), adenosine 5'-triphosphate disodium salt (ATP), oxidized ATP (oxATP), 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate triethylammonium salt (BzATP), and poly-L-lysine (PLL) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies against p-Src (Tyr416) or p-Src (Tyr527) were purchased from Cell Signaling Technology (Beverly, MA, USA), anti-NG2 from Millipore (Billerica, MA, USA), anti-Fyn from Santa Cruz (Santa Cruz, TX, USA), anti-P2X7 from Alomone (Jerusalem, Israel), anti-Myc from Roche (Palo Alto, CA, USA), and anti-HA and Hoechst 33258 from Sigma-Aldrich. The HRP-conjugated secondary antibodies and β -Actin were purchased from KangChen (Shanghai, China), and immunofluorescence secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), neurobasal medium, B27, and trypsin were purchased from Life Technologies (Carlsbad, CA, USA). The other reagents and the Transwell assay were purchased from Corning (NY, USA), protein G-agarose beads were from Roche, and the 0.22- μ m membrane filters were from Millipore.

Purification and culture of OPCs

All animal procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University. Purification and culture of OPCs were performed as previously described [33, 34]. Briefly, mixed glial cell

cultures were generated from postnatal day 1 (P1) Sprague Dawley rat cerebrum and were plated on PLL-coated flasks. Cells were cultured at 37 °C with 5 % CO₂ for 10 days in DMEM with 10 % FBS. The purified OPCs were collected by a two-step shaking method. First, microglia was separated by shaking at 160 rpm for 1 h. Second, OPCs were separated by shaking at 220 rpm overnight. For the purpose of keeping purified OPCs in an undifferentiated state, they were maintained in NB/B27 supplemented with 30 % B104 CM [35].

Boyden chamber migration assay

Migration of OPCs was performed using a Transwell assay based on Boyden chambers system with 8- μ m pore-size, as previously described [36]. In brief, both sides of the transwell membrane were coated with PLL. Purified OPCs with a density of 5×10^4 cells per well were planted to the upper chamber cultured with B104/NB/B27 contained Ara-C (10 μ M). Agonists were added to the lower chamber. In some experiments, cells were pre-treated with antagonists or inhibitors before agonists. To establish if the migratory effect induced by the agonist was gradient-independent, an equal concentration of BzATP was added to the upper and lower chambers of the transwell. During the next 16 h, OPCs were allowed to migrate at 37 °C with 5 % CO₂. Then the non-migrated cells on the upper side of the membrane were wiped off with a cotton swab, then the migrating cells on the lower side of the membrane were fixed and stained with 4 % paraformaldehyde (PFA) and 0.25 % Coomassie Brilliant Blue, respectively. For each filter, the number of migrating cells was counted within eight random fields. Experiments were performed at least three times.

Immunocytochemistry

OPCs were fixed with 4 % PFA for 12 min at room temperature (RT) and subsequently rinsed with PBS, then incubated with primary antibodies for 16 h at 4 °C. The corresponding immunofluorescence secondary antibodies were used to stain the OPCs for an additional 1 h at RT. Cell nuclei were labeled with Hoechst 33258 (10 μ g/ml). Photographs were captured using a Leica SP2 confocal microscope. The dilution ratio of antibodies is as follows: anti-NG2 (1:100), anti-P2X7 (1:1,000), and anti-Fyn (1:100); all the immunofluorescence secondary antibodies were 1:100.

Immunoprecipitation

The cell lysates were harvested in RIPA lysis buffer (100 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 % TritonX-100, 1 % sodium deoxycholate, 0.1 % SDS, PH 7.4) and clarified by centrifugation at 18,000g for 20 min at 4 °C. The supernatant was incubated with 2–4 μ g of the corresponding primary

antibodies or control IgG overnight at 4 °C, kept continuously rotating. Protein G-agarose beads, pretreated with RIPA, were then added for another 3–4 h at 4 °C while rotating. The precipitates were washed with RIPA three times, boiled in SDS sample buffer at 100 °C for 5 min, and subjected to immunoblotting. The following antibodies were used: anti-Fyn, anti-Myc, and anti-HA.

To detect Fyn kinase activity, the lysates of OPCs stimulated with different concentrations of BzATP were immunoprecipitated with Fyn antibody, and the expression levels of p-Fyn(420) or p-Fyn(531) were detected with the antibodies against p-Src (Tyr416) or p-Src (Tyr527), respectively.

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to the nitrocellulose (NC) membranes. After blocking with 10 % non-fat milk, the NC membranes were incubated with primary antibodies overnight at 4 °C. The corresponding HRP-conjugated secondary antibodies were used for 1 h at RT. Finally, ECL was applied to visualize the immunoreactive bands. The following antibodies were used: anti-Myc (1:1000), anti-HA (1:4000), anti-p-Src(Tyr416) (1:1000), anti-p-Src(Tyr527) (1:1000), anti-Fyn (1:1000), anti-P2X7(1:1000), and the corresponding HRP-conjugated secondary antibody (1:10,000). Anti- β -Actin was used as a control.

Lentivirus generation and infection

The lentivirus, expressing both EGFP and a short-hairpin RNA (shRNA) sequence targeting rat Fyn, was generated by Shanghai Genechem. 5'-AACTACAACAACCTCCACGCA-3' was the nucleotide target sequence for rat Fyn [37]. To confirm the interference effect of Fyn shRNA, OPCs were infected with Fyn shRNA or control shRNA separately. After 72 h, OPC lysates were harvested in RIPA lysis buffer for immunoblotting. In the Boyden Chamber Migration Assay, infected after 48 h, OPCs were digested by 0.25 % trypsin, and counted with the density of 5×10^4 cells per well to perform the transwell assay.

Statistical analysis

All data are expressed as mean $\pm$ SEM from four independent experiments. Values were analyzed using one-way ANOVA followed by a Dunnett post hoc test, unless otherwise stated. $p < 0.05$ was considered statistically significant.

Results

P2X7 receptors mediate ATP-induced migration of OPCs in vitro

To test the effect of ATP on migration of OPCs, we used a Transwell assay based on Boyden chambers system. We found that after treatment with 500 μ M ATP for 16 h, the number of migrating OPCs was nearly 1.75 times greater than the control group (Fig. 1a, b). While OPCs pretreated with 100 μ M oxATP (a P2X7 receptor inhibitor) for 2 h before ATP (500 μ M) treatment, resulted in a significant decrease in the number of migrating OPCs; 71 % of migrating cells induced by ATP were prevented by oxATP (Fig. 1a, b).

Since ATP is rapidly degraded in the extracellular environment, we further examined the effect of BzATP, the P2X7 receptor specific agonist, which is resistant to degradation.

Fig. 1 ATP and BzATP-induced migration of cultured OPCs. **a** The migration of OPCs was performed with a Boyden chamber. **b** OPCs were incubated with or without 500 μ M ATP. The BzATP concentrations are as indicated. In the condition BzATP/BzATP, 100 μ M BzATP was added to both the upper and lower chambers. To verify the role of P2X7 receptors, before incubation with 500 μ M ATP or 100 μ M BzATP, OPCs were pretreated with 100 μ M oxATP for 2 h. After 16 h, the number of migrated OPCs was counted. Data are displayed as mean $\pm$ SEM and analyzed using one-way ANOVA followed by a Dunnett post hoc test. * $p < 0.05$, *** $p < 0.001$ vs. control. ### $p < 0.001$ vs. the corresponding treatments without oxATP. Bar 40 μ m

The results showed that BzATP treatment increased the migration of OPCs in a concentration-dependent manner, with the maximal effect at 50 μ M. After stimulation with 50 μ M BzATP for 16 h, the number of migrating cells increased 2.45 times compared to the control group (Fig. 1a, b). In order to establish if the migratory effect induced by BzATP was also gradient-independent (chemokinetic), we added an equal concentration of BzATP (100 μ M) to the upper and lower chambers of the transwell assay, to neutralize the chemical gradient. In this condition, we still found that the number of migrating OPCs increased about 2 times compared to the control group (Fig. 1a, b). We also evaluated the effect of BzATP on OPC migration in the presence of oxATP. The results indicated that pre-treatment with oxATP fully inhibited OPC migration induced by BzATP (Fig. 1a, b).

ATP can promote the proliferation of neural progenitor cells [38] and glial cells [39, 40]. To avoid the proliferative effect of ATP on OPCs, 10 μ M Ara-C was used in our transwell culture system. We further examined the proliferation and apoptosis of OPCs by Brdu and TUNEL staining. We found that BzATP did not show a significant effect on the proliferation and apoptosis of OPCs under our experimental system (data not show).

P2X7 receptors interact with Fyn in cultured OPCs

Fyn, a member of the Src family of kinases, has been reported to play a key role in regulating OPC proliferation and differentiation [41–43]. In primary cultured OPCs, we found that both P2X7 receptors and Fyn were expressed in OPCs (Fig. 2a–d). Double staining studies further confirmed the co-localization of P2X7 receptors and Fyn in OPCs (Fig. 2e, f).

To examine the association of P2X7 receptors with Fyn, we performed a co-immunoprecipitation (co-IP) assay. Myc-tagged P2X7 receptors (P2X7R-Myc, with a molecular mass of ~72 kDa) and/or HA-tagged Fyn (HA-Fyn, with a molecular mass of ~60 kDa) were transfected into HEK293T cells. When using the anti-Myc antibody to immunoprecipitate the cell lysates from HEK293T cells, only the group co-transfected with P2X7R-Myc and HA-Fyn could be detected by the anti-HA antibody (Fig. 3a). In contrast, using the anti-HA antibody to immunoprecipitate the transfected cell lysates, only the group co-transfected with P2X7R-Myc and HA-Fyn could be detected with the anti-Myc antibody (Fig. 3a). To further demonstrate that P2X7 receptors (~68 kDa) and Fyn (~59 kDa) are binding to each other in OPCs, we used cell lysates from cultured OPCs for co-IP. As showed in Fig. 3b,

Fig. 2 The expression of P2X7 receptors and Fyn in cultured OPCs. Cultured OPCs, placed onto PLL-coated dishes, were immunostained with NG2, P2X7R, or Fyn antibodies. NG2 is a marker for OPCs; Hoechst staining (blue) indicates the nucleus. **b, d, f** High magnification views of OPCs using a confocal laser scanning microscope

Fig. 3 Identification of the interaction between P2X7 receptors and Fyn. **a** P2X7-Myc and/or HA-Fyn were expressed in HEK cells. The lysates immunoprecipitated by anti-Myc or HA antibodies were subjected to Western blot analysis with antibodies against Myc or HA as indicated. **b** OPCs lysates were immunoprecipitated with Fyn antibody, with IgG as a control, and then blotted with antibodies against P2X7 or Fyn, as indicated. *WCL* whole cell lysate

P2X7 receptors were detected in the precipitates only when immunoprecipitated by the anti-Fyn antibody and not the IgG control. Taken together, these data indicated that P2X7 receptors interacted with Fyn in OPCs.

BzATP increases the activity of Fyn kinase

Fyn kinase activity is decided by the phosphorylation of tyrosines (Tyr) at two different sites: Tyr(420) phosphorylation (p-Tyr(420)) is an activated form, while Tyr(531) phosphorylation (p-Tyr(531)) is an inactivated form [44]. To examine the effect of BzATP on Fyn kinase activation, we used an anti-Fyn

antibody to immunoprecipitate the cell lysates from cultured OPCs, which had been incubated with BzATP for 16 h, and then immunoblotted with antibodies against different phosphorylated tyrosines. The results indicated that, following stimulation with concentrations of 10, 50, and 100 μ M BzATP, the levels of Fyn p-Tyr(420) were increased by 1.7, 2.3, and 2.4 times that of the control group, respectively (Fig. 4a, b). In contrast, stimulation with the same concentrations of BzATP, p-Tyr(531) levels of Fyn were decreased by 15, 43, and 41 %, respectively, versus the control group (Fig. 4a, c). Thus, these results indicate that BzATP is able to increase the activity of Fyn kinase.

Fig. 4 Fyn kinase activity was enhanced after stimulation with BzATP. **a** The lysates of OPCs stimulated with different concentrations of BzATP were immunoprecipitated with Fyn antibody, and expression levels of p-Fyn(420) or p-Fyn(531) were detected. With increasing BzATP concentrations, the levels of p-Fyn(420) were increased (**b**); conversely, the levels of p-Fyn(531) were decreased (**c**). Data are displayed as mean $\pm$ SEM and analyzed using one-way ANOVA followed by a Dunnett post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control, the group without BzATP

Inhibiting Src family kinase activity can block BzATP-induced migration of OPCs

As there is no specific inhibitor for Fyn, we used PP2, a broad-spectrum inhibitor of Src family kinases, in OPC migration experiments. Before stimulation with 100 μ M BzATP, OPCs were pretreated with different concentrations of PP2 for 20 min. Under these conditions, we found that PP2 treatment suppressed the facilitative effect of BzATP on OPC migration in a concentration-dependent manner, with the maximal effect at 10 μ M (Fig. 5a, b).

Fig. 5 PP2 inhibited BzATP-induced migration of OPCs in vitro. **a**, **b** OPCs were pretreated with different concentrations of PP2 for 20 min before incubation with 100 μ M BzATP. The group without PP2 was used as a control. Data are displayed as mean $\pm$ SEM and analyzed using one-way ANOVA followed by a Dunnett post hoc test. *** p <0.001 vs. control, the group without PP2. Bar 40 μ m

Fyn knock-down inhibits BzATP-induced migration of OPCs

To further clarify the role of Fyn in BzATP-induced migration of OPCs, we performed lentivirus-based Fyn knock-down experiments. After infection with the lentivirus expressing both EGFP and shRNA sequence targeting Fyn for 72 h, the expression of Fyn was down-regulated by 75 % (Fig. 6a). OPCs, infected with Fyn shRNA or control shRNA, were used to perform Transwell assay experiments at different concentrations (0, 10, 50, or 100 μ M) of BzATP. In the control shRNA group, similar to the uninfected group (Fig. 1a, b), stimulation with BzATP increased the migration of cells in a concentration-dependent manner, with the maximal effect at 50 μ M (Fig. 6b, c). Moreover, Fyn knock-down significantly reduced the number of migrating OPCs induced by BzATP to 56, 60.6, and 60.5 % of the control shRNA group at the same concentrations of BzATP, respectively (Fig. 6b, c), but did not affect the migration without BzATP (Fig. 6b, c).

Discussion

OPCs are distributed widely in normal adult brain. They not only form functional synapses with hippocampal interneurons [45] but also have direct close contact with Ranvier's nodes [46] and synapses [47], thus ensuring the OPCs can quickly detect physiological changes in axons and immediately respond to such changes. Following injury or demyelination, perifocal OPCs are able to react quickly, including proliferation [9] and migrating to lesion areas [48]. However, the distance for recruiting OPCs in the acute phase is strictly restricted [3]. Meanwhile, after the injury or demyelinating disease has occurred, local extracellular ATP concentrations were increased by the intracellular high concentrations of ATP being released from dead neurons and glial cells [25]. Thus, there is a high degree of overlap between the limited scope of recruiting OPCs in the acute phase and the range of local extracellular high concentrations of ATP. Studies have indicated that ATP gathered at lesions may be involved in recruiting OPCs during the acute stage.

Many previous studies have showed that P2Y-like receptor GPR17 was participated in OPC migration [49] and maturation [50]. What's more, ATP at 100 μ M could induce OPC migration through P2Y1 receptors [12]. The ATP concentration the authors used was much higher than that in the extracellular space under physiological conditions, which is in the nanomolar level [51]. But, as we mentioned earlier, only when the ATP concentration is greater than 100 μ M, can P2X7 receptors be activated [52]. After CNS injury or under other pathological conditions, intracellular ATP is instantly released into the extracellular surround due to cell death, and the extracellular local ATP concentration could reach the millimolar

Fig. 6 Knockdown of Fyn inhibited BzATP-induced migration of OPCs in vitro. **a**, To verify the interferential efficiency of Fyn shRNA, the lysates from infected OPCs were immunoblotted with Fyn and β -Actin antibodies. **b**, **c** OPCs infected with Fyn or control shRNA lentivirus were treated with different concentrations of BzATP (0, 10, 50, or 100 μ M) in Boyden chambers. Data are displayed as mean $\pm$ SEM and analyzed using one-way ANOVA followed by a Tukey post hoc test. *** p <0.01, **** p <0.001 vs. the control shRNA group without BzATP. # p <0.05, ## p <0.01 vs. the control shRNA group treated with the same concentration of BzATP. Bar 40 μ m

level [53]. In the present study, we found that 500 μ M ATP could significantly promote OPC migration. In the presence of oxATP, a P2X7 receptor antagonist, the migration of OPCs was inhibited, suggesting a crucial role of P2X7 receptors in the ATP-induced OPC migration. Interestingly, compared with the control group, oxATP could not fully eliminate the ATP-induced effect. These results indicate that in addition to P2X7 receptors, other P2 receptors might be involved in the high concentration of ATP-induced OPC migration. Moreover, after neutralizing the chemical gradient of BzATP, we still found that the number of migrating OPCs increased about 2 times compared to the control group (Fig. 1a, b), suggesting that BzATP stimulates random motility in OPCs.

OPCs express both P2X(1, 2, 3, 4, 7) and P2Y(1, 2, 4, 13) receptors [12, 54]. BzATP is also active at other P2 receptors, such as hP2X1, rP2X3, hP2X3, and rP2Y13 receptors [55, 52, 56]. In order to rule out the involvement of other P2 receptors, before incubation with 100 μ M BzATP, OPCs were pre-treated with 100 μ M oxATP for 2 h. Under these conditions, we found that the migration induced by BzATP was completely inhibited by oxATP (Fig. 1a, b). We also repeated the Transwell assay experiments with MRS2211, a selective antagonist for P2Y13 receptors. The results showed that blocking P2Y13 receptors did not affect OPC migration induced by BzATP (Suppl. Fig. 1).

Three Src family kinase members Fyn, Lyn, and Src are expressed in oligodendrocytes, and among them, Fyn is expressed at the highest level [42]. Moreover, Miyamoto et al. showed Fyn expression in primary cultured OPCs by using the technique of RT-PCR [32]. In addition, by using

western blot and electrophysiological methods, Agresti et al. reported the expression of P2X7 receptors in primary cultured OPCs [12]. Consistent with previous studies, our results also showed that P2X7 receptors and Fyn were co-expressed in cultured OPCs. P2X7 receptors have been suggested to interact with Src family kinase members in the lysates of J774 cell line macrophages by co-IP assay [57]. But so far, there is no direct evidence to identify which member of the Src family could interact with P2X7 receptors. In the present study, we demonstrated for the first time that Fyn kinase can interact with P2X7 receptors, suggesting Fyn might participate in downstream signaling mediated by P2X7 receptors.

Like other members of the Src family, Fyn kinase activity is associated with its phosphorylation level of tyrosine (Tyr) in two distinct positions. Under physiological conditions, the phosphorylation of Tyr(531) could result in Fyn kinase being in a “closed conformation,” resulting in no kinase activity, while dephosphorylation of Tyr(531) will result in autophosphorylation of Tyr(420), leading to the activation of Fyn [44]. Our results identified that after stimulation with BzATP, the phosphorylation of Fyn Tyr(420) was increased significantly, whereas the level of phosphorylated Tyr(531) was decreased markedly in OPCs, suggesting that BzATP could up-regulate Fyn kinase activity.

There is direct evidence demonstrating that in cultured OPCs stimulated with PDGF, Fyn kinase could promote the migration of OPCs through WAVE2 phosphorylation [32]. Our previous study also demonstrated that Slit2 could regulate the dispersal of OPCs through Fyn and RhoA signaling [31]. In the present study, we found PP2, an inhibitor of the Src

family kinases, could block the migration of OPCs induced by BzATP. We further used RNA interference to knock down the expression of Fyn. We found that at the same concentrations of BzATP, down-regulated, the expression of Fyn could significantly inhibit BzATP-induced OPCs migration. Taken together, our results suggest that P2X7 receptor-triggered Fyn kinase activation plays a crucial role in mediating ATP-induced OPC migration.

In summary, our experimental research has demonstrated that a high concentration of ATP activates P2X7 receptors and couple to Fyn signaling to promote OPC migration, suggesting a novel role of P2X7 receptors under pathological conditions.

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