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Oligodendrocyte differentiation and signaling after transferrin internalization: A mechanism of action

María Julia Pérez [a], Natalia Fernandez [b], Juana María Pasquini [a,*]

[a] Cátedra de Química Biológica Patológica, Departamento de Química Biológica, Facultad de Farmacia y Bioquímica (FFyB), Universidad de Buenos Aires (UBA), Instituto de Química y Fisicoquímica Biológica "Prof. Alejandro C. Paladini" (IQUIFIB), UBA-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Junin 965, CABA, Buenos Aires, Argentina

[b] Laboratorio de Farmacología de Receptores, Cátedra de Química Medicinal, Departamento de Farmacología, Facultad de Farmacia y Bioquímica (FFyB), Universidad de Buenos Aires (UBA), Junin 965, CABA, Buenos Aires, Argentina

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ABSTRACT

Oligodendrocytes are the cells producing the myelin membrane around the axons in the central nervous system and, although apotransferrin (aTf) is required for oligodendrocyte differentiation, the underlying mechanisms are not fully understood.

Fyn tyrosine kinase, a member of the Src family of proteins, has been shown to play an important role in myelination by up-regulating the expression of myelin basic protein; however, a molecular link between aTf and Fyn kinase signaling pathway during oligodendrocytes differentiation has not been established yet. Our aim was to investigate whether Fyn kinase, MEK/ERK and PI3K/Akt signaling pathways are required for aTf-stimulation of oligodendrocyte differentiation and also to determine if the transferrin receptor is involved in these mechanisms.

Treatment of primary cultures of oligodendroglial precursor cells with aTf leads to Fyn kinase activation by a mechanism that involves transferrin receptor. In turn, Fyn kinase activation promotes MEK-mediated transient phosphorylation of ERK1/2. On the other hand, transferrin receptor internalization also produces rapid and sustained activation of Akt, which involves phosphatidylinositol 3-kinase (PI3K) activation. Finally, aTf incorporated through clathrin-mediated endocytosis increases myelin basic protein, F3-contactin and β-tubulin through Fyn/MEK/ERK pathways, as well as an activation of the PI3K/Akt pathway. Our results also demonstrate that the activation of the pathways necessary for oligodendroglial precursor cell maturation is dependent on AP2 recruitment onto the plasma membrane for clathrin-mediated endocytosis of transferrin receptor.

Introduction

Oligodendrocytes (OL) are responsible for myelination around axons in the central nervous system. However, the molecular mechanisms underlying oligodendrocyte progenitor cell (OPC) differentiation and myelinogenesis are complex and still remain under study.

Transferrin (Tf) is a glycoprotein synthesized in the liver and whose principal function is to act as an iron transport protein. Many investigators have demonstrated that Tf is also necessary for cellular growth (Kawabata et al., 2000; Suzuki et al., 2006) and has bacteriostatic properties (Artini et al., 2012; Wally and Buchanan, 2007).

Tf accumulation by OL is associated with myelin production (Espinosa de los Monteros et al., 1999). Moreover, Tf is synthesized by OL, which also express abundant Tf mRNA (Bartlett et al., 1991; Bloch et al., 1985; Rouault and Cooperman, 2006) and can secrete Tf when cultured (Espinosa de los Monteros et al., 1990). It has also been reported that apotransferrin (aTf) accelerates the myelination process *in vivo* (Escobar Cabrera et al, 1994, 1997; Marta et al., 2000; Saleh et al., 2003). aTf also prevents hypomyelination produced by iron deficiency in rats (Badaracco et al., 2008), reduces hypoxic/ischaemic white matter injury in rats (Guardia Clausi et al., 2012) and stimulates remyelination in cuprizone-induced demyelination, an animal model of multiple sclerosis (Adamo et al., 2006). Similarly,

Abbreviations: Ab-TfR, mouse antibody against Tf receptor (Ab-2) clone 42/6; aTf, apotransferrin; Cyt B, cytochalasin B; DIII, pEGFP-C2-Eps15 DIII plasmid, Eps15 dominant negative mutant; DIIIΔ2, pEGFP-C2-Eps15 DIIIΔ2 plasmid, Eps15 irrelevant mutant; EH29, pEGFP-C2-Eps15 EH29 plasmid, Eps15 dominant negative mutant; F3, F3-contactin; Fyn, Fyn tyrosine kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LY, LY294002, PI3 kinase inhibitor; MBP, myelin basic protein; MDC, primary amine monodansylcadaverin; NG2, nerve/glia antigen 2; OL, oligodendrocytes; OPC, oligodendrocyte progenitor cell; PD, PD98059, MEK1/2 inhibitor; PDGFR-α, platelet-derived growth factor α; PI3K, phosphatidylinositol 3-kinase; PP2, Src family of tyrosine kinases inhibitor; Tf, transferrin; TfR, transferrin receptor; Tf-TR, Texas Red-labeled transferrin; U0126, MEK1/2 inhibitor; WM, wortmannin, PI3 kinase inhibitor; β-tub, β-tubulin.

* Corresponding author at: Cátedra de Química Biológica Patológica, Dpto. Química Biológica, FFyB, UBA, Junin 965, CABA, Buenos Aires, Argentina. Fax: +54 11 4964 8287/88.

E-mail addresses: mjuliaperez@gmail.com (M.J. Pérez), natyfernandez@hotmail.com (N. Fernandez), jpasquin@qb.ffyb.uba.ar (J.M. Pasquini).

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aTf induces OPC differentiation *in vitro* (Garcia et al., 2004; Paez et al., 2002).

Fyn tyrosine kinase (Fyn) is a protein belonging to the Src-family of non-receptor tyrosine kinases, among which Fyn, Lyn and Src are expressed by OL (Colognato et al., 2004; Umemori et al., 1992). Fyn expression and kinase activity have been identified as mediators of different OL processes such as migration, differentiation, axonal contact and myelination start-up (Baer et al., 2009; Krämer et al., 1999; Osterhout et al., 1999; Umemori et al., 1994). In addition, previous reports have shown the relevance of MEK/ERK (Fyffe-Maricich et al., 2011) and PI3K/Akt signaling cascades in OPC differentiation (Bibollet-Bahena and Almazan, 2009; Cui et al., 2005; Flores et al., 2008).

The aim of our study was to investigate the molecular mechanisms and signaling pathways by which aTf promote OPC differentiation. We observed that (i) aTf treatment of OPC activates Fyn; (ii) aTf stimulates a transient phosphorylation of ERK downstream Fyn activation, as well as rapid and sustained Akt phosphorylation independent of Fyn activation; (iii) the activation of these signaling pathways depends on Tf receptor (TfR) internalization by clathrin-coated pits; (iv) aTf induction of the expression of proteins involved in the myelination process, such as myelin basic protein (MBP), β-tubulin and F3-contactin, is mediated by Fyn/MEK/ERK and PI3K/Akt pathways.

Materials and methods

Materials

Human apotransferrin (aTf), cytochalasin B (Cyt B), paraformaldehyde (PFA), serum albumin, poly-L-lysine, triiodothyronine (T3), Triton X-100, Hoechst (bis-Benzamide H 33258), DMSO and wortmannin were obtained from Sigma-Aldrich (St Louis, MO). DMEM/F12, Lipofectamine 2000, human transferrin conjugated to Texas Red (Tf-TR) and TMB Single Solution (3,3′,5,5′-TetraMethyl-Benzidine) were from Life Technologies (Argentina). Fetal calf serum was from Natocor (Argentina). Mowiol and PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d]pyrimidine) were from Calbiochem (Nottingham, UK). PD 98059 (2′-amino-3′-methoxiflavone) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenyl-thio)butadiene) were from Promega (Madison, USA). LY 294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] was purchased from Cell Signaling Technology (Danvers, MA). Immobilon-P^{sq} (PVDF transfer membrane) was from Millipore (Temecula, CA), while Hyperfim ECL and ECL Plus Western Blotting Detection Reagents were purchased from GE Healthcare (Buckinghamshire, UK). Human platelet-derived growth factor-AA (PDGF) and basic fibroblast growth factor (bFGF) were purchased from Peprotech (Mexico City, Mexico). Antibodies used were as follows, mouse anti-Active Src (dephosphorylated-Tyr 529) (Invitrogen); rabbit anti-total Fyn, rabbit anti-ERK1/2 (Santa Cruz Biotechnology); rabbit anti-phospho-ERK1/2 (p-Thr202/p-Tyr204), rabbit anti-phospho-Akt (p-Ser 473), rabbit anti-Akt (Cell Signaling Technology); mouse anti-β-tubulin (Chemicon International); goat anti-Tf (ICN Biomedicals); mouse anti-GAPDH (Abcam); rabbit anti-myelin basic protein (MBP) and mouse anti-O4 (generous gift from A. Campagnoni-UCLA); mouse anti-GFAP and anti-neurofilament NF200 (Sigma-Aldrich); rabbit anti-NG2 Chondroitinsulfate proteoglycan (Millipore, Temecula, CA), goat anti-PDGFR α (Neuromics); mouse anti-CD71 OX-26 transferrin receptor (BD Biosciences Pharmingen); rabbit anti-F3-contactin (generous gift from Dr.Watanabe) and mouse anti-Tf receptor (Ab-2) clone 42/6 (Calbiochem, Nottingham, UK). Horseradish peroxidase, Cy2, DyLyght 488, Cy3 and DyLight 549-conjugated secondary antibodies used for immunoblotting and immunocytochemistry were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). All other chemicals were analytical grade reagents.

Oligodendrocyte progenitor cell primary culture

Primary cultures of OPC from newborn Wistar rats of either sex were performed according to McCarthy and de Vellis (1980). After removing the meningeal membranes, newborn rat cerebral hemispheres were mechanically dissociated by gentle repetitive pipetting in a mixture of DMEM/F12 (1, 1 v/v) containing 5 g/ml streptomycin and 5 U/ml penicillin, supplemented with 10% fetal calf serum. The cell suspensions were seeded in poly-L-lysine-coated 75 cm^2 tissue culture flasks and incubated at 37 °C in 5% CO_2, with changes of medium every 4 days. After 14 days in culture, when cells reached confluence, the subpopulation of OPC was obtained by using a differential cell adhesion protocol. After first shake at 140 rpm/min during 1 h, the medium containing microglia was discarded. Then cultures were shaken overnight at 240 rpm/min. The cell suspension obtained was filtered through a 15 μm mesh filter and plated on bacterial grade Petri dishes for 1 h. Astrocytes and microglia were attached to the plastic surface while OPC remained in suspension. Then OPC were centrifuged at 1500 rpm during 10 min and the pellet was resuspended in glial defined medium (GDM) (Casaccia-Bonnefil et al., 1996), without the addition of aTf. OPC in suspension were seeded either on 12-mm poly-L-lysine-coated coverslips or 30-mm poly-L-lysine-coated Petri dishes with GDM in the presence of PDGF (10 ng/mL) and bFGF (10 ng/mL) during 24 h. Oligodendroglial cell cultures were evaluated quantitatively with anti-O4, anti-neurofilaments NF200 and anti-GFAP antibodies were 95% pure (Pasquini et al., 2003). For experiments involving intracellular signaling, OPC medium was changed to GDM without PDGF and bFGF during 4 h before aTf (100 μg/mL) treatment in order to initiate a mitogen starvation condition. Then, for experiments with kinase inhibitors, cells were pretreated with DMSO, 5 μM PP2 (Src family of tyrosine kinases inhibitor, Lck and Fyn) (Hanke et al., 1996), 10 μM U0126 and 2.5 μM PD 98059 (MEK1/2 inhibitors), 1 μM wortmannin and 50 μM LY 294002 (PI3 kinase inhibitors) for 30 min before the addition of aTf (100 μg/mL) during different times.

SDS-PAGE and Western blot analysis

After treatment, cells were harvested in 150 μL of ice-cold lysis buffer which contained 20 mmol/L Tris-HCl (pH 8), 1% Nonidet P-40, 10% glycerol, 137 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L aprotinin, 0.1 mmol/L sodium vanadate, and 20 mmol/L NaF. Protein content of cell lysates was determined with the BIO-RAD Protein Assay Kit and the samples were adjusted with loading buffer containing 2% sodium dodecyl sulfate (SDS), 5% glycerol, 5% b-mercaptoethanol and 0.01% bromophenol blue and boiled for 5 min. Aliquots containing 20 μg of protein were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Membranes were blocked in 5% non-fat dried milk in 0.1% Tween 20 in TBS for 1 h at room temperature and incubated with an appropriate primary antibody overnight at 4 °C [mouse anti-Active Src (Tyr 529), rabbit anti-Fyn, rabbit anti-phospho-ERK1/2 (Thr202/Tyr204), rabbit anti-ERK1/2, rabbit anti-phospho-Akt (Ser 473), rabbit anti-Akt, goat anti-Tf, rabbit anti-MBP, rabbit anti-F3-contactin, mouse anti-β-tubulin, mouse anti-GAPDH]. After being washed, membranes were incubated in the corresponding horseradish peroxidase-conjugated secondary antibody. Bands were visualized by chemiluminescence with ECL Western Blotting Detection Kit on autoradiographic film. Films were scanned and quantified using Scion Image® software from National Institutes of Health (NIH). To normalize for sample loading and protein transfer, membrane-bound proteins were first exposed to anti-active or phospho-protein antibody, then stripped at 60 °C during 1 h, and finally incubated with anti-total antibody. The active or phospho-protein/total protein ratio was used to evaluate signaling activation.