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Original Articles

Dopamine receptor type 2 (DRD2) inhibits migration and invasion of human tumorous pituitary cells through ROCK-mediated cofilin inactivation

E. Peverelli [a,b,*], E. Giardino [a,b], D. Treppiedi [a,b], M. Locatelli [c], V. Vaira [d,e], S. Ferrero [d,f], S. Bosari [d,g], A.G. Lania [h], A. Spada [a,b], G. Mantovani [a,b]

[a] Endocrine Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[b] Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy
[c] Neurosurgery Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[d] Division of Pathology, Fondazione IRCCS Ca' Granda-Ospedale Maggiore Policlinico, Milan, Italy
[e] Istituto Nazionale Genetica Molecolare "Romeo ed Enrica Invernizzi" (INGM), Milan, Italy
[f] Department of Biomedical, Surgical and Dental Sciences, University of Milan Medical School, Milan, Italy
[g] Department of Pathophysiology and Transplantation, University of Milan, Milan, Italy
[h] Endocrine Unit, IRCCS Humanitas Clinical Institute, Rozzano, University of Milan, Milan, Italy

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ABSTRACT

Non-functioning pituitary tumors (NFPTs) frequently present local invasiveness. Dopamine receptor 2 (DRD2) agonists are the only medical therapy that induces tumor shrinkage in some patients. Invasion requires cytoskeleton rearrangements that are tightly regulated by cofilin pathway, whose alterations correlate with invasion in different tumors.

We investigated the effect of DR2D agonist on NFPT cells migration/invasion and the molecular mechanisms involved.

We demonstrated that DRD2 agonist reduced migration (−44 ± 25%, $p < 0.01$) and invasion (−34 ± 6%, $p < 0.001$) and increased about 4-fold Ser3-phosphorylated inactive cofilin (P-cofilin) in NFPT cells. These effects were abolished by inhibiting ROCK, a kinase that phosphorylates cofilin. The overexpression of wild-type or phosphodeficient S3A-cofilin increased HP75 cells migration (+49 ± 6% and +57 ± 9% vs empty vector, respectively, $p < 0.05$), while phosphomimetic mutant had no effect.

Interestingly, P-cofilin levels were lower in invasive vs non-invasive tumors by both western blot (mean P-cofilin/total cofilin ratio 0.77 and 1.93, respectively, $p < 0.05$) and immunohistochemistry (mean percentage of P-cofilin positive cells 17.6 and 45.7, respectively, $p < 0.05$).

In conclusion, we showed that the invasiveness of pituitary tumors is promoted by the activation of cofilin, which can be regulated by DRD2 and might represent a novel biomarker for pituitary tumors' invasive behavior.

Introduction

Non-functioning pituitary tumors (NFPTs), so called because they do not secrete active hormones, are epithelial tumors that, although benign in nature, frequently show local invasiveness. Erosion of the sella turcica, invasion of the sphenoid bone inferiorly and the cavernous sinus laterally, that are found in about 40% of NFPTs [1–3], strongly reduce the success of transsphenoidal neurosurgery, which is the treatment of choice for this type of tumor [4–6]. Although most tumors express dopamine (DA) receptor type 2 (DRD2) [7] and *in vitro* experiments have shown that DA analogs, including the DRD2-specific agonist BIM53097 [8,9], exert antiproliferative activity in cultured cells from NFPTs [9,10], medical therapy of NFPTs is still under debate. In fact, contrasting data on the effects of the DRD2 agonist cabergoline on tumor shrinkage or stabilization of post-surgery tumor remnants have been reported [7,11–17].

To date, no diagnostic biomarkers predicting the invasive behavior of NFPTs are available and the molecular mechanisms of invasion are poorly understood. The process of invasion of tumor cells, as well as of normal cells during physiological processes, requires adhesion, proteolysis of extracellular-matrix components and migration. To migrate, cells rearrange actin cytoskeleton for the formation of protrusive structures, such as lamellipodia, pseudopodia and filopodia. Actin dynamics are tightly regulated by cofilin, an actin binding protein that severs actin filaments and initiates actin-polymerization by increasing the number of actin-free barbed ends,

* Corresponding author. Fax: +39 02 55033361.
E-mail address: erika.peverelli@guest.unimi.it (E. Peverelli).

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from which actin filaments (F-actin) polymerize, and by providing actin monomers (G-actin) for polymerization. Cofilin activity is mainly regulated by phoshorylation at Ser3, a residue located in its actin binding domain, with a consequent loss of its ability to bind actin and to promote cell migration [18]. Small GTPases of the Rho family activate a cascade of kinases (ROCK, LIMK) culminating with cofilin phosphorylation, whereas different phosphatases, including slingshot, chronophin and phosphatases type 1, 2A and 2B participate in cofilin dephosphorylation. Alterations in these pathways have been correlated with invasion and metastasis in different tumors [19–24].

Recently published data indicate that DRD2 exerts antimigratory effects in mesenchymal stem cells [25] and couples to Rho/ROCK/LIMK signaling pathway leading to cofilin phosphorylation in primary striatal neurons [26]. These data prompted us to investigate a possible effect of DRD2 agonist BIM53097 on migration and invasion of primary cultured NFPT cells and human NFPT-derived cell line HP75, and to evaluate a possible role of cofilin pathway in the invasive behavior of NFPTs.

Materials and methods

Patients

The study was approved by the local ethics committee and each patient gave informed consent to the use of his/her tumor sample and clinical information. The study was carried on NFPTs removed from 15 patients by transphenoidal surgery (Table 1).

Diagnosis of NFPT was based on the presence of a sellar lesion in the absence of hormonal hypersecretion and confirmed by histology and immunostaining analysis. Magnetic resonance imaging (MRI) showed a macrotumor (diameter ≥ 10 mm) in all patients, with suprasellar extension in 12 patients. Invasion of the cavernous sinus was observed by radiological findings and confirmed during the surgical procedure in 8 cases and this was the only criteria considered to define a tumor as invasive (Table 1).

Pituitary cell culture

We have obtained a sufficient number of viable cells for further experiments from 10 NFPTs of 15 dispersed tumors. NFPT tissues were partially frozen for subsequent analysis and partially enzymatically dissociated in DMEM containing 2 mg/mL collagenase (Sigma Aldrich, St. Louis, MO) at 37 °C for 2 h, as previously described [27]. Dispersed cells were cultured in DMEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% FBS, 2 mM glutamine and antibiotics (Gibco, Invitrogen, Life Technologies Inc., Carlsbad, CA, USA).

Human non-functioning pituitary tumor cell line HP75 was kindly gifted by Prof. Anne Klibanski, from Massachusetts General Hospital, Boston. Cells have been tested and authenticated by genetic profiling using polymorphic short tandem repeat (STR) loci with the PowerPlex Fusion system (Promega, BMR Genomics Cell Profile service, Italy, analysis dated 04/02/2016), that allows to amplify 23 loci STR (D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, DYS391, D8S1179, D12S391, D19S433, FGA, D22S1045) and Amelogenin for gender determination. Results were compared to reference cell line databases (ATCC, DSMZ, JCRB and RIKEN). HP75 was grown in DMEM medium supplemented with 15% HS, 2.5% FBS, 2 mM glutamine and antibiotics.

Transwell migration and invasion assays

Pituitary cells suspension (300,000 NFPT cells/insert or 50,000 HP75 cells/insert) was plated in serum-free medium, with or without increasing concentrations of DRD2 agonist, into the upper chamber of a transwell insert (Merck Millipore, Darmstadt, Germany) with porous polycarbonate membrane (pore diameter 8 μm), and allowed to migrate (6 h for NFPTs and 18 h for HP75 at 37 °C) towards the lower compartment, filled with serum-containing medium.

BIM53097, with high in vitro selectivity for DRD2 (IC50 22 nM) [8] and was previously used to test DRD2-mediated signaling in pituitary tumor cells [9], was selected as DRD2 agonist.

A negative control with serum-free medium in lower chamber was used in each experiment and subtracted from each value. For primary NFPT cells growing in suspension, the cells that migrated into the lower chamber were collected by centrifugation, resuspended in 120 μl of Calcein AM (AnaSpec, Fremont, CA), incubated at 37 °C for 30 min and fluorescence was measured with plate reader (ex485/em535 nm). For HP75 cells, non-migratory cells were mechanically removed from the upper side of the porous membrane with cotton swabs whereas migrated cells on the lower side of the insert were stained with Crystal Violet solution, extracted with 10% acetic acid, and then measured using a plate reader at a wavelength of 560 nm. To measure cell invasion, the upper chamber of a transwell insert was coated with 0.25 mg/ml of Collagen IV (Sigma, St. Louis, MO), and cells that invaded on the lower surface of the membrane were quantified as described above.

Wound healing assay

HP75 cells were cultured over night (50,000 cells/well in a multiwell of 24-well) before two cross-shaped scratches were introduced to the confluent monolayer with a sterile pipette tip [28]. After PBS washing, cells were incubated in a complete medium with or without increasing concentrations of BIM53097. 2.5 μM Y27632 dihydrochloride ROCK inhibitor (Sigma, St. Louis, MO) was added 30 min before and during BIM53097 stimulation, when indicated. Pictures of the four cross points per well were taken immediately after scratch and after 18 h at 5× magnification (A-plan objective 5 × /0.12, Zeiss Axiovert 200M microscope, Zeiss AxioCam MRm camera, Carl Zeiss Microscopy, Jena, Germany), and the percentage of the open area was analyzed with the T-Scratch software [28].

Western blot analysis

Total proteins were extracted from cultured cells and NFPT tissues. Cells were incubated for 10 min with or without increasing concentration of BIM53097. A pre-treatment with 2.5 μM Y27632 dihydrochloride ROCK inhibitor for 30 min was performed when indicated prior to and during BIM53097 stimulation. Proteins were quantified by BCA, separated on SDS/polyacrylamide gels and transferred to a nitrocellulose filter. P-cofilin (Ser3) or total cofilin antibodies (Cell Signalling, Danvers, MA) were used at 1:1000, DRD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:200, phospho-LIMK1(Thr508)/LIMK2(Thr505) and total LIMK2 antibody (Cell Signalling, Danvers, MA) were used 1:1000. The membranes were stripped and reprobed with GAPDH antibody. Chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Upland, CA) and analyzed with NIH ImageJ.

Table 1
Clinical, neuroradiological and anatomopathological features of NFPTs.

	Gender (M/F)	Age at diagnosis (Years)	Tumor size (mm)	Cavernous sinus invasion	Sovrasellar extension	Ki67 (%)	% P-cofilin + cells (IHC)	% cofilin + cells (IHC)
1	M	72	32 × 27	+	+	2	0	40
2	M	78	28 × 26	+	+	<1	5	40
3	M	57	29 × 27	+	+	3	5	10
4	M	67	25 × 25	+	+	<1	20	70
5	F	62	32 × 25	+	+	<1	70	80
6	F	79	24 × 25	+	+	1/2	30	70
7	M	67	27 × 23	+	+	<1	10	30
8	M	54	46 × 65	+	+	5	1	20
9	M	39	13 × 13	–	–	1	80	80
10	M	63	16 × 20	–	+	<1	60	80
11	F	58	18 × 11	–	+	<1	60	50
12	M	34	30 × 25	–	+	<1	30	60
13	M	71	21 × 17	–	+	<1	60	80
14	F	30	28 × 20	–	–	1	10	80
15	F	72	18 × 10	–	–	<1	20	40