BIOCHEMICAL PHARMACOLOGY 74 (2007) 448–456

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Effect of diadenosine polyphosphates in achondroplasic chondrocytes: Inhibitory effect of Ap_4A on FGF9 induced MAPK cascade

Ana Guzmán-Aránguez [a], *Marta Irazu* [a], *Avner Yayon* [b,c], *Jesús Pintor* [a,*]

[a] *Departamento de Bioquímica y Biología Molecular IV, E.U. Óptica, Universidad Complutense de Madrid, c/Arcos de Jalón s/n, 28037 Madrid, Spain*

[b] *Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel*

[c] *ProChon Biotech Ltd., Kiryat Weizmann, Bldg. 12, P.O. Box 1482, Rehovot 76114, Israel*

ARTICLE INFO

Article history:
Received 26 February 2007
Accepted 25 April 2007

Keywords:
Achondroplasia
Chondrocytes
FGFR3
ERK1/2
Diadenosine polyphosphates
Dinucleotide receptor

ABSTRACT

Achondroplasia is characterised by a mutation in the gene that encodes for the FGF receptor type 3 (FGFR3), producing a hyperactivation of this receptor and a subsequent increase in MAPK activity. We have tested the ability of nucleotides to decrease the activation of MAPK in chondrocytes with achondroplasic FGFR3 receptor. Diadenosine tetraphosphate, Ap_4A, reduced the phosphorylation of pERK1/2 triggered by FGF9 (38% reduction). Ap_4A diminished the expression of achondroplasic FGFR3 receptor (65% reduction), stimulating FGFR3 receptor degradation. The action of Ap_4A seems to be mediated by a dinucleotide receptor rather than by any other ATP receptor.

1. Introduction

Achondroplasia, also known as dwarfism, is one of the most representative types of congenital skeletal dysplasias. This pathology is produced by a mutation in the gene that encodes for the FGFR3 receptor, being the change of Gly^{380} to Arg the most common mutation in achondroplasia.

FGFR3 is activated after the binding of FGF. This activation induces receptor dimerization and a subsequent receptor autophosphorylation at the intracellular kinase domain, thus, triggering a downstream activation of intracellular signalling [1]. In achondroplasia the ''pathological receptor'' shows a gain of function, leading to an alteration the normal equilibrium between proliferation and maturation and thereby inhibiting the normal growth of the bone [2].

Several mechanisms have been reported to explain how mutant FGFR3 enhances these signals. One of the mechanisms described suggests that the mutation stabilizes the dimeric

* *Corresponding author*. Tel.: +34 91 3946859; fax: +34 91 3946885.
E-mail address: jpintor@vet.ucm.es (J. Pintor).

Abbreviations: MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinases 1 and 2; FGFR3, fibroblast growth factor receptor 3; FGF9, basic fibroblast growth factor 9; Ap_3A, diadenosine triphosphate; Ap_4A, diadenosine tetraphosphate; Ap_5A, diadenosine pentaphosphate; ATPγS, adenosine 5′-3-O-thiotriphosphate; 2MeSADP, 2-(methylthio)-ADP; 2MeSATP, 2-(methylthio)-ATP; AMP-PCP, adenyl 5′-(beta, gamma-methylene)-diphosphonate; IP3, inositol triphosphate; cGMP, cyclic guanosine monophosphate; PLC, phospholipase C; PKC, protein kinase C

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.04.023

state of the receptor, thus, permitting its prolonged signalling [3]. On the other hand, a slow down-regulation of the mutant receptor [4], and a defective lysosomal targeting of mutant FGFR3, has also been observed [5].

Concerning the signal transduction pathways downstream of FGFR3, two main pathways seem to play an important role in the inhibition of the proliferation of chondrocytes: STAT-1 and ERK1/2 (MAPKs) cascades [6,7]. In the case of ERK1/2, this pathway is mainly involved in chondrocyte differentiation process [8]. In addition, MAPK pathway reduces the synthesis of the components of the extracellular matrix [9].

Nucleotides and dinucleotides are biologically active substances that can modify the physiology of many tissues. For this reason, mononucleotides such as ATP and specially dinucleotides like dinucleoside polyphosphates can be used for the treatment of some pathologies such as chronic bronchitis, cystic fibrosis, dry eye disease and atherosclerotic events [10].

Apart from mononucleotides, dinucleoside polyphosphates exert their actions through P2X, P2Y and dinucleotide receptors [11]. P2Y receptors are classically coupled to phospholipase C activation, IP3, diacylglicerol formation and intracellular calcium mobilization. Some subtypes, such as the $P2Y_{12}$ are negatively coupled to adenylate cyclase, and most of them are also able to stimulate MAPK cascade. It has been also described that in the endothelium $P2Y_1$ and $P2Y_2$ receptors can stimulate guanylate cyclase inducing the formation of cGMP [12]. cGMP, on the other hand, has been described as an inhibitor of the MAPK pathway in achondroplasic chondrocytes [9]. Recently, we have demonstrated the presence of $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{11}$ in achondroplasic chondrocytes [13], and how they modify intracellular calcium concentration. Apart from these metabotropic ATP receptors it is necessary to bear in mind the possible existence of specific dinucleotide receptors, which can be coupled to both ionotropic and metabotropic mechanisms [14,15].

The aim of the present experimental work is to see whether or not a group of dinucleotides, the diadenosine polyphosphates can modify the activity of the MAPK cascade which is stimulated in prolonged a way by mutated FGFR3 in achondroplasia, therefore, becoming a possible alternative for the treatment of this pathology.

2. Materials and methods

2.1. Reagents and antibodies

Tetracycline, α-MEM, heat-inactivated foetal bovine serum and antibiotics (penicillin, streptomycin and hygromicin) were purchased from Invitrogen (Carlsbad, CA, USA).

Nucleotides, dinucleotides and FGF9 were purchased from Sigma (St. Louis, MO, USA). Tris, NaCl, Triton X-100, phenylmethylsulphonylfluoride (PMSF), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), SDS, Tween 20, BSA, *p*-formaldehyde, aprotinin, pepstatin leupeptin, MG132 (N-CBZ-Leu-Leu-Leu-AL) and chloroquine were obtained from Sigma.

Antibodies against phospho-ERK1/2, ERK2, FGFR3, β-tubulin and horseradish peroxidase-conjugated goat anti-mouse and goat anti-mouse FITC-conjugated were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell lines and cell culture

Non-transformed rat chondrocytes (RCJ3.1C5.18) were transfected with full-length human wild type (WT) FGFR3 or mutant (ACH) FGFR3, $FGFR3^{G380R}$, as described elsewhere [4]. Expression of FGFR3 was regulated by a tetracycline suppression system, the receptor is expressed in the absence of tetracycline in the culture medium.

Standard culture medium was α-MEM supplemented with 15% heat-inactivated foetal bovine serum and antibiotics. Cells were incubated at 37 °C with 5% CO_2.

In order to analyze phosphorylation status of ERK1/2 or FGFR3 protein expression 2×10^4 cell/cm^2 cells were plated onto tissue culture dishes. Two days later, culture medium was replaced by fresh one without tetracycline and cells were incubated during 16 h. After starvation for 4 h with serum-free α-MEM, the cells were exposed for 30 min and at the indicated concentrations with the different nucleotides and dinucleotides either without or with FGF9 (25 ng/ml). After stimulation cells were lysed in buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, pepstatin and 2 μg/ml leupeptin). Lysates were clarified at $13{,}000 \times g$ for 20 min at 4 °C. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA, USA).

Cell proliferation was assessed by determining the number of cells after detachment with tripsin. For this purpose, chondrocytes were seeded at a density of 0.4×10^5cells/well (24-well plates) and maintained for 1 day. Culture media was then replaced by fresh one containing FGF9 (25 ng/ml) or FGF9 with Ap_4A (100 μM) for 72 h and counted in a Neuebauer chamber.

2.3. Western blot analysis

Protein extracts from each sample (45 μg) were subjected to 10% SDS-polyacrilamide gels and were transferred to nitrocellulose membranes (Amersham-Pharmacia-Biotech, Buckinghamshire, UK). Thereafter, membranes were blocked and incubated overnight in the primary antibody appropriately diluted in PBS containing 2% skimmed milk and 0.05% Tween 20. After washing, blots were incubated with peroxidase-conjugated secondary antibody. Development was performed using ECL system (Amersham-Pharmacia-Biotech, Buckinghamshire). Films were scanned and a densitometric analysis was performed using Kodak GL 200 Imaging System and Kodak Molecular Imaging Software (Kodak, Rochester, NY, USA). All the data shown are representative of three independent experiments.

2.4. Inmunofluorescence studies

Covers slips with cells were treated with 4% *p*-formaldehyde for 15 min. The fixed cells were washed and incubated overnight in PBS containing 1% BSA and diluted FGFR3 antibody. The covers slips were washed and incubated for 1 h with the secondary antibody. Samples were analyzed by confocal microscopy using a Zeiss Axiovert 200M microscope equipped with a LSM 5 Pascal confocal module (Zeiss, Oberkochen, Germany). All images were taken under the