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Neuroprotection elicited by $P2Y_{13}$ receptors against genotoxic stress by inducing DUSP2 expression and MAPK signaling recovery

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ABSTRACT

Nucleotides activating $P2Y_{13}$ receptors display neuroprotective actions against different apoptotic stimuli in cerebellar granule neurons. In the present study, $P2Y_{13}$ neuroprotection was analyzed in conditions of genotoxic stress. Exposure to cisplatin and UV radiation induced caspase-3-dependent apoptotic cell death, and p38 MAPK signaling de-regulation. Pre-treatment with $P2Y_{13}$ nucleotide agonist, 2methyl-thio-ADP (2MeSADP), restored granule neuron survival and prevented p38 long-lasting activation induced by cytotoxic treatments. Microarray gene expression analysis in 2MeSADP-stimulated cells revealed over-representation of genes related to protein phosphatase activity. Among them, dual-specificity phosphatase-2, DUSP2, was validated as a transcriptional target for $P2Y_{13}$ receptors by QPCR. This effect could explain 2MeSADP ability to dephosphorylate a DUSP2 substrate, p38, reestablishing the inactive form. In addition, cisplatin-induced p38 sustained activation correlated perfectly with progressive reduction in DUSP2 expression. In conclusion, $P2Y_{13}$ receptors regulate DUSP2 expression and contribute to p38 signaling homeostasis and survival in granule neurons.

1. Introduction

Mitogen-activated protein kinases (MAPKs) regulate a great variety of physiological and pathological processes at the central nervous system including survival/apoptosis, proliferation or differentiation. Among them, the extracellular signal-regulated kinases (ERK) are main targets of growth factors and neurotrophins [1–3]. In general, ERK1,2 transient activation mainly trigger neuroprotective actions against apoptotic stimuli [4,5]. Concerning the stress related kinases, p38 and JNK, they mainly function as cell death mediators in response to environmental, inflammatory and danger stimuli [6,7]. Despite this general rule for MAPKs, their real contribution to neural function is not yet totally clarified. In this respect, long-lasting activation of MAPKs, including ERK1/2, is associated to cell death under damaging conditions, such as cytotoxicity, hypoxia/ischemia, oxidative and genotoxic stress [8,9]. Therefore, it is becoming clear that cell fate as a result of MAPK activation seems to be strongly dependent on different factors, such as the cellular environment, type of stimuli and signal duration.

MAPK pathways entail several phosphorylating events that work in cascade. They are tightly regulated at multiple levels, and among them, modulation of protein phosphatase activity plays a key role in the regulation of both duration and magnitude of MAP kinase signaling [10,11]. Different families of protein phosphatases, serine-threonine (PSPs) and tyrosine phosphatase proteins (PTPs and DUSPs), have been identified to operate coordinately to inactivate MAPK signaling, acting through both positive and negative feedback regulatory mechanisms. Among them, dual-specificity protein phosphatases, DUSPs, exhibit activity towards both Thr and Tyr residues. Some members of this family belong to the group of MAPK phosphatases or MKPs, and are emerging as major mediators of the inactivation of sustained MAPK signaling induced by growth factors. Both constitutive and inducible DUSPs can coexist in a cellular model and are transcriptionally regulated by growth

Abbreviations: CaMKII, calcium/calmodulin kinase II; CREB, (cAMP-response-element-binding protein)-binding protein; DAPI, 2-(4-amidinophenyl)-1H-indole-6-carboxamidine; DUSP, dual-specificity phosphatase; ERK, extracellular-signal-regulated kinase; JNK, c-Jun Kinase; 2MeSADP, 2methyl-thio-ADP; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MRS-2179, 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate tetrasodium salt; MRS-2211, 2-[(2-Chloro-5-nitrophenyl) azo]-5-hydroxy-6-methyl-3-[(phosphonooxy) methyl]-4 pyridine carboxaldehyde disodium salt; RT, reverse transcription; NB, Neurobasal-A medium; PI3-K, phosphatidyl inositol-3 kinase; U-0126, 1,4-Diamino-2,3-dicyano-1,4-*bis*[2-aminophenylthio]butadiene

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factors in a rapid manner as immediately early inducible genes (IEGs) [10,12,13]. They exhibit different substrate selectivity towards a particular MAPK protein, and their expression or activation is normally dependent on the same MAP kinase under regulation [14,15]. Among the most representative ones, DUSP1 and DUSP2 belong to the group of nuclear inducible phosphatases with broad specificity for ERK1/2 and stress kinases, JNK and p38. In addition, DUSP4 and DUSP6 belong to the group of cytoplasmic and constitutive phosphatases more selective for ERK1/2. However, not many pharmacological tools are available to properly characterize protein phosphatases.

Extracellular nucleotides play relevant roles at the nervous system through their coupling to MAP kinase signaling [16,17]. They activate two major families of nucleotide receptors, both ionotropic (P2X) and metabotropic (P2Y) (see recent reviews) [18,19]. In recent works, we described neuroprotective actions displayed by $P2Y_{13}$ and P2X7 receptors in cerebellar granule neurons (CGNs). The survival promoting effect against glutamate excitotoxicity proved to be dependent on the activation of the ERK1/2 target that is the CREB transcription factor. $P2Y_{13}$ and P2X7 receptors were differently coupled to ERK1/2 activation through PI3K and CaMKII-dependent pathways, respectively [20]. In addition, $P2Y_{13}$ receptors prevented oxidative stress-mediated cell death through the up-regulation of the cyto-protective protein heme oxigenase-1 (HO-1), which is a transcriptional product of the master antioxidant regulator Nrf-2 transcription factor [21].

The aim of the present study was to deepen into the meaning of $P2Y_{13}$ receptors neuroprotective actions in granule neurons. Their role was investigated in conditions of genotoxic stress induced by exposure to UV radiation and cisplatin, a drug commonly used in chemotherapy with important neurotoxic side effects [22]. We described the impairment of MAP kinase signaling, especially at the level of p38 phosphorylated form accumulation, in response to cytotoxic treatments. This effect correlated with loss of function of a specific type of dual-specificity protein phosphatase, DUSP2, selective for p38. In addition, activation of $P2Y_{13}$ receptor with 2MeSADP nucleotide agonist was sufficient to prevent p38 over-activation as well as to restore basal DUSP2 expression. Finally, $P2Y_{13}$ activation induced by 2MeSADP contributed to promote neuronal survival against those cytotoxic treatments. Both, the induction of protein phosphatase and the neuroprotective effect were dependent on PI3K/ERK-dependent signaling triggered by $P2Y_{13}$ receptors in granule neurons [20].

2. Materials and methods

2.1. Antibodies and materials

Nucleotide receptor agonists and antagonists, and transducing inhibitors, 2MeSADP and ortho-vanadate, were purchased from Sigma Aldrich (St Louis, USA), MRS-2211 from Tocris Bioscience (Essex, UK), and Wortmannin and U-0126, from Calbiochem Co. (San Diego, USA). Specific antibodies for phosphor-ERK1/2 (Tyr204), ERK2 were from mouse, and for DUSP2 were from goat, and purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies specific for GAPDH, 17 kDa-Caspase-3 fragment, phosphor-p38, phosphor-JNK and p38 were from rabbit and purchased from Cell Signalling Technology (Beverly, MA, USA). Secondary horseradish peroxidase-conjugated antibodies were from Santa Cruz Biotechnology (anti-mouse), and Dako (Denmark) (anti-rabbit, anti-goat) and Synaptic Systems (donkey anti-rabbit IgG Cy3-conjugated). All other reagents not specified were routinely supplied by Sigma, Merck (Darmstadt, Germany) or Roche Diagnostics SL (Barcelona, Spain).

2.2. Cell culture and treatment conditions

All experiments carried out at the Universidad Complutense de Madrid followed the guidelines of the International Council for Laboratory Animal Science (ICLAS). Cerebellar cultures were performed according to the procedure described by Pons et al. with some modifications [23]. Cerebella from Wistar rat pups (P7) were aseptically removed, and submitted to digestion with papain 100 U/ml (Worthington, Lake Wood, NJ) (previously activated in EBSS buffer containing 5 mM L-Cys and 2 mM EDTA), in the presence of 100 U/ml of DNAse (Worthington, Lake Wood, NJ), 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The obtained cells were resuspended in neurobasal medium supplemented with B-27 (GIBCO, BRL, Paisley, Renfrewshire, UK) containing 21 mM KCl, 2 mM glutamine, and antibiotics, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma Aldrich, St Louis, USA), and plated onto glass coverslips or plastic Petri dishes (60 and 35 mm) (Falcon Becton Dickinson Labware, Franklin Lakes, USA) precoated with 0.1 mg/ml poly-L-lysine (Biochrom, AG, Berlin) at a density of 200,000 cells/cm^2. They were maintained in a humidified incubator at 37 °C in 5% CO_2. AraC (10 μM) was added to avoid the proliferation of glial cells.

Cultured cerebellar granule neurons were used at 8–10 DIV (days in vitro), the stimulation of cells with the nucleotide agonists or other agents was carried out adding the corresponding compounds to cells maintained in complete culture media. These stimulations were done at specified times before the addition of the cytotoxic drug, cisplatin at concentrations of 20 or 40 μg/ml. The exposition to UV radiation was performed for 2 min under transilluminator lamp (32 J/cm^2), after that, cells were maintained in the incubator until the times required.

2.3. Western blot experiments

In phosphorylation assays of MAPK proteins, cell extracts or lysates were obtained at different incubation periods. Stimulation was stopped by the addition of lysis buffer (20 mM MOPS, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, pH = 7.2, 1 mM PMSF and protease inhibitor cocktail (Complete, Roche)). Protein determination of the cell extracts was performed and then mixed with sample buffer 4× (50% glycerol, 125 mM Tris pH 6.8, 4% SDS, 1% bromophenol blue, 5% β-mercaptoethanol, 4.5% H_2O). The samples were heated to 99 °C and aliquots (25 μg) were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis (25 mM Tris, 200 mM glycine, 0.1% SDS, pH = 8.3) using 12% acrylamide gels. Immunotransference was performed in PVDF membranes (Amersham Biosciences Europe GmbH, Barcelona, Spain) (25 mM Tris, 192 mM glycine, 20% methanol). The TBS buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.5) containing 1% (v/v) Tween-20 and 5% BSA was employed as blocking medium and in subsequent incubations with the antibodies. Incubation with the antibodies was performed at the following dilutions: 1:1000 for phosphor-ERK1/2, total ERK (Santa Cruz), total p38, GAPDH, and caspase-3 (Cell Signalling), and 1:500 for phosphor-p38, phosphor-JNK (Cell Signalling) and DUSP2 (Santa Cruz). Primary antibodies were detected with horseradish peroxidase-conjugated antibodies, 1:4000 for anti-mouse (Santa Cruz) and anti-rabbit (Dako), and 1:2000 for anti-goat (Santa Cruz) visualized by the ECL method (kit Super Signal substrate Western Blotting, from Amersham Biosciences Europe GmbH, Barcelona, Spain). The chemiluminescence images were quantified by densitometry employing the Fluo-S Imager of Bio-Rad (Munich, Germany).

2.4. Microarray analysis

RNA was collected from cells treated with 1 μM 2MeSADP for 2 h. Samples were hybridized with a chip of genomic expression Agilent, capable of quantifying the expression of 28,000 rat genes. 4 replicates of each condition (Control or 2MeSADP) were performed and analyzed. The resultant expression data was normalized following the method described by Bolstad et al. [24] and implemented into the analysis package affy [25] for the statistic environment R (GNU project http://www.r-project.org/).

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