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Stimulation of adenosine A_{2A} receptors reduces intracellular cholesterol accumulation and rescues mitochondrial abnormalities in human neural cell models of Niemann-Pick C1

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ABSTRACT

Niemann Pick C 1 (NPC1) disease is an incurable, devastating lysosomal-lipid storage disorder characterized by hepatosplenomegaly, progressive neurological impairment and early death. Current treatments are very limited and the research of new therapeutic targets is thus mandatory. We recently showed that the stimulation of adenosine A_{2A} receptors (A_{2A}Rs) rescues the abnormal phenotype of fibroblasts from NPC1 patients suggesting that A_{2A}R agonists could represent a therapeutic option for this disease. However, since all NPC1 patients develop severe neurological symptoms which can be ascribed to the complex pathology occurring in both neurons and oligodendrocytes, in the present paper we tested the effects of the A_{2A}R agonist CGS21680 in human neuronal and oligodendroglial NPC1 cell lines (i.e. neuroblastoma SH-SY5Y and oligodendroglial MO3.13 transiently transfected with NPC1 small interfering RNA). The down-regulation of the NPC1 protein effectively resulted in intracellular cholesterol accumulation and altered mitochondrial membrane potential. Both effects were significantly attenuated by CGS21680 (500 nM). The protective effects of CGS were prevented by the selective A_{2A}R antagonist ZM241385 (500 nM). The involvement of calcium modulation was demonstrated by the ability of Bapta-AM (5–7 μM) in reverting the effect of CGS. The A_{2A}-dependent activity was prevented by the PKA-inhibitor KT5720, thus showing the involvement of the cAMP/PKA signaling. These findings provide a clear *in vitro* proof of concept that A_{2A}R agonists are promising potential drugs for NPC disease.

1. Introduction

Niemann Pick C (NPC) disease is a fatal neurovisceral syndrome caused, in 95% of cases, by the loss of the NPC1 protein (Vanier and Millat, 2003). Although the function of this protein is not yet fully understood, recent evidence indicates a possible role in regulating the efflux of sphingosine from the lysosomal compartment (Lloyd-Evans et al., 2008). The leading hypothesis is that the mutation of the protein causes an abnormal sphingosine storage and, as a consequence, a reduction of calcium levels in lysosomes; in turn, these events disrupt the vesicular transport inside the cells and cause the accumulation of unesterified cholesterol and other lipids in the lysosomal and/or late endosomal compartment (Chang et al., 2005).

Even though NPC1 is a visceral condition that involves peripheral organs such as liver, spleen and lung (Vanier and Millat, 2003), the brain is the only organ in which progressive cell death ensues (Higashi et al., 1993; Sarna et al., 2003; Li et al., 2005).

The complex pathology resulting from the deficiency in both neurons and glial cells is the cause of the devastating neurological symptoms of NPC1 disease, which include progressive ataxia, cataplexy, epilepsy, supranuclear gaze palsy, and impairment of the swallowing reflex (Fink et al., 1989; Vanier and Millat, 2003). While the cerebellum is particularly affected by an early loss of Purkinje neurons, pathological changes are also present in the neocortex, hippocampus and neostriatum (Yamada et al., 2001). Moreover, considering that cholesterol is a major lipid of myelin and that its incorporation is rate-limiting for myelin membrane growth (Saher et al., 2005), the impairment of intracellular vesicle transport and

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the redistribution of cellular cholesterol profoundly affect oligodendrocyte functions. Indeed, myelination is compromised both in NPC1 patients (Palmeri et al., 1994; Walterfang et al., 2010) and in NPC1 mutant mice (Takikita et al., 2004; Yu et al., 2012; Yu and Lieberman, 2013).

Since the therapeutic options for NPC1 are extremely limited (miglustat is the only drug approved for the treatment of neurological symptoms of NPC1 patients, and it has only limited effects) new therapeutic targets are highly warranted. In a previous paper, we demonstrated that adenosine A_{2A} receptor ($A_{2A}R$) stimulation was able to restore calcium homeostasis, mitochondrial membrane potential (mMP) and cholesterol content in fibroblasts from NPC1 patients (Visentin et al., 2013). Since the normalization of cholesterol accumulation in fibroblasts from NPC1 patients is the main criterion currently used to identify a compound or pathway that could be beneficial for NPC1 disease (Karten et al., 2009), our data strongly supported $A_{2A}R$ agonists as a potential therapeutic option in this disease.

Although fibroblasts from patients are considered effective tools to identify new therapeutic strategies, considering the major involvement of the Central Nervous System (CNS) in the disease, it is mandatory that any potential new drug is tested also in cell types relevant to the neurological abnormalities. In this study, NPC1 phenotype was induced in both human neuronal and oligodendroglial cell lines by reducing the expression of NPC1 protein by small interfering RNA. In both lines, the down-regulation of NPC1 protein induced a clear NPC1 phenotype (namely cholesterol accumulation and altered mMP), which was normalized by the $A_{2A}R$ agonist CGS21680. These results suggest that $A_{2A}R$ agonists could represent promising drugs to ameliorate CNS abnormalities in NPC1.

2. Materials and methods

2.1. Cell cultures

Human neuroblastoma cell line SH-SY5Y was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum, 5% FetalClone II serum (Sigma, Italy), 2 mM L-glutamine and 1 U/ml penicillin/streptomycin (Euroclone, Italy). Human oligodendrocytic cell line MO3.13 was maintained in DMEM supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine and 1 U/ml antibiotics, in humidified 37 °C chambers with 5% CO_2. In these conditions MO3.13 cells exhibit the immature oligodendrocyte markers GalC (galactosylceramidase) and CNPase.

2.2. Inhibition of NPC1 expression by siRNA

To obtain human genetic models of NPC neurons and oligodendrocytes, neuroblastoma SH-SY5Y and oligodendroglial MO3.13 cell lines were transiently transfected with 100 nM and 70 nM NPC1 small interfering RNA (siRNA), respectively.

Cells were plated at a density of 25,000 cells/500 μl in 24wells-plate. siRNA duplex derived from the human NPC1 sequence and a non-targeting siRNA were purchased from Ambion (Pre-designed siRNA, ID8092; targets exon 2 of NPC1 and Negative Control #2). Cells were transfected using Lipofectamine 2000 (Invitrogen, Italy) according to the manufacturer's instructions. NPC1 protein down-regulation was assessed by western blotting at 24, 48, 72, 96 h after siRNA transfection; cholesterol accumulation was evaluated by Filipin III staining at the same time points. Cells transfected with siRNA for NPC1 will be hereafter indicated as siSH-SY5Y and siMO3.13. The non-targeting siRNA-transfected cells were considered as the controls in each experiments (hereafter indicated as CTR cells).

2.3. Cell treatments

Treatments with the $A_{2A}R$ agonist CGS21680 (100 and 500 nM) were performed for 24 h starting from 24 h or 48 h after siRNA transfection for MO3.13 and for SH-SY5Y, respectively. ZM241384 (500 nM) and BAPTA-AM (5 μM for SH-SY5Y and 7 μM for MO3.13) were applied 30 min before and then along with CGS21680.

The intracellular signaling elicited by $A_{2A}R$ stimulation was analyzed by treating cells with specific inhibitors of PKA and Erk1/2 cascade. In particular, the PKA inhibitor KT5720 (4 μM) and the MEK 1 inhibitor PD98059 (10 μM), were applied 30 min before and along with CGS21680.

2.4. Protein extraction and western blotting

For NPC1 and Erk1/2 detection, cells were lysed in RIPA buffer on ice for 30 min and centrifuged at 12000 × g for 20 min at +4 °C. For $A_{2A}R$ detection, cell membranes were extracted according to the protocol of Vachon and Costa (1987). In brief, cells were harvested with a scraper and washed twice in phosphate-buffer saline (PBS). Pellets were homogenized in 5 mM Tris–HCl containing 1 mM EGTA, 1 mM dithiothreitol and 0.32 mM sucrose with a Dounce homogenizer; homogenate was centrifuged at 1000 × g for 10 min at +4 °C; the pellet was discarded and the supernatant was centrifuged at 24000 × g for 20 min at +4 °C. The pellet was resuspended in the same buffer without sucrose, re-homogenized and stored at −80 °C. Protein content was determined by using the BCA method (Thermo Scientific).

Protein extracts (40 or 5 μg) were resolved on 6% (for NPC1 protein) and 8% (for Erk1/2 and $A_{2A}R$) SDS PAGE gels and transferred to PVDF membranes (BioRad, Italy). After blocking with 5% non-fat milk in T-TBS, membranes were probed with anti-NPC1 polyclonal antibody (1:1000, Novus Biologicals, DBA, Italy), anti-Phospho-Erk1/2, anti-Total-Erk1/2 (1:2000, Cell Signaling Technologies, Euroclone, Italy) and with anti-A_{2A} (1:1000, Merck Millipore, Italy) overnight at +4 °C. Anti-rabbit and anti-mouse HPR conjugated antibodies were used (Jackson laboratories, DBA, Italy). Immunoreactive bands were detected by enhanced chemiluminescence ECL (Amersham, Italy). Phospho-Erk1/2 bands were normalized with respect to Total-Erk1/2 by using ImageJ software.

2.5. Filipin III fluorescence and $A_{2A}R$ immunofluorescence

Cells were grown onto coverslips and were fixed in 3% formaldehyde in PBS for 15 min at room temperature (RT). For Filipin III staining, after washing in PBS, cells were incubated with 250 μg/ml Filipin III for 1 h at RT in the dark. Nuclei were stained with propidium iodide (Sigma, Italy). For $A_{2A}R$ immunodetection, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at RT and nonspecific sites were blocked by incubation with 2.5% horse serum in PBS for 25 min at RT. Cells were incubated with an antibody against $A_{2A}R$ (1:50, Merck Millipore) for 1 h at 37 °C; after extensive washing in PBS they were incubated with a secondary antibody conjugated to the Alexa Fluor dye 555 or 546 (1:200, Invitrogen) 45 min at 37 °C. Nuclei were stained with Hoechst 23558 (Sigma, Italy). Coverslips were mounted with PBS-Glycerol (1:1) and observed using an Eclipse 80i Nikon (Amsterdam, Netherlands) Fluorescence microscope equipped with a VideoConfocal (ViCo) system.

2.6. Mitochondrial inner membrane potential measurement

Mitochondrial inner membrane potential (mMP) was measured, using the potentiometric dye tetramethylrhodamine ethyl ester perchlorate (TMRE) as previously described (Visentin et al., 2013).