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In vitro drug treatments reduce the deleterious effects of aggregates containing polyAla expanded PHOX2B proteins

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ABSTRACT

Heterozygous in frame duplications of the *PHOX2B* gene, leading to polyalanine (polyAla) expansions ranging from +5 to +13 residues of a 20-alanine stretch, have been identified in the vast majority of patients affected with Congenital Central Hypoventilation Syndrome (CCHS), a rare neurocristopathy characterized by absence of adequate autonomic control of respiration with decreased sensitivity to hypoxia and hypercapnia. Ventilatory supports such as tracheostomy, nasal mask or diaphragm pacing represent the only options available for affected. We have already shown that the severity of the CCHS phenotype correlates with the length of polyAla expansions, ultimately leading to formation of toxic intracytoplasmic aggregates and impaired PHOX2B mediated transactivation of target gene promoters, such as *DBH*. At present, there is no specific treatment to reduce cell aggregates and to ameliorate patients' respiration. In this work, we have undertaken *in vitro* analyses aimed at assessing the effects of molecules on the cellular response to polyAla PHOX2B aggregates. In particular, we tested 17-AAG, ibuprofen, 4-PBA, curcumin, trehalose, congo red and chrysamine G for their ability to i) recover the nuclear localisation of polyAla expanded PHOX2B, ii) rescue of PHOX2B mediated transactivation of the *DBH* promoter, and iii) clearance of PHOX2B (+13 Ala) aggregates. Our data have suggested that 17-AAG and curcumin are effective *in vitro* in both rescuing the nuclear localization and transactivation activity of PHOX2B carrying the largest expansion of polyAla and promoting the clearance of aggregates of these mutant proteins inducing molecular mechanisms such as ubiquitin–proteasome (UPS), autophagy and heat shock protein (HSP) systems.

Introduction

Congenital central hypoventilation syndrome (CCHS) is a rare neurocristopathy characterized by decreased sensitivity to hypoxia and hypercapnia with absence of adequate autonomic control of breathing, especially during sleep (Coleman et al., 1980; Patwari et al., 2010). In particular, patients with CCHS show an adequate ventilation while awake but hypoventilate during sleep (Patwari et al., 2010). CCHS is a chronic disorder, with no effective treatment available, except ventilatory supports such as tracheostomy, nasal mask or diaphragm pacing. CCHS is inherited as an autosomal dominant trait with reduced penetrance, associated with mutations of the *PHOX2B* gene. This gene encodes for a transcription factor expressed in several different districts of the autonomic nervous system, in particular in the developing hindbrain and peripheral nervous system as well as in all noradrenergic centers and in specific neuronal groups, such as those involved in the medullary control reflexes of autonomic functions (Brunet and Pattyn, 2002; Pattyn et al., 1997, 1999).

The most frequent mutations found in CCHS patients are heterozygous in-frame duplications within a 20 alanine stretch, leading to expansions from +5 to +13 alanine residues, though frameshift, nonsense and missense mutations, frequently reported in association with Hirschsprung disease and tumors of neural crest origin, are also identified in small subsets of patients (Amiel et al., 2003; Matera et al., 2004; Sasaki et al., 2003; Weese-Mayer and Berry-Kravis, 2004). A correlation between length of alanine expanded stretches and phenotype severity has already been reported in CCHS patients (Matera et al., 2004; Patwari et al., 2010); moreover, functional studies have correlated polyalanine (polyAla) expansions with decreasing PHOX2B-mediated activation of the DBH and PHOX2A promoters, due to polyAla length-dependent formation of cytoplasmic aggregates which prevent the protein from entering the nucleus, binding DNA and activating transcription (Bachetti et al., 2005, 2007; Trochet et al., 2005).

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PolyAla, as well as polyglutamine (polyQ) expansions belong to a class of defective trinucleotide repeats known to associate with many neurodegenerative diseases (Albrecht and Mundlos, 2005) and to share typical features such as formation of amyloid-like fibrils characterized by a β-sheet conformation thus leading to insoluble protein aggregates (Blondelle et al., 1997; Forood et al., 1995; Scheuermann et al., 2003; Shinchuk et al., 2005). To reduce aggregate formation and cell death, therapeutic strategies, tested so far in *in vitro* models of neurodegenerative disorders, have targeted protein misfolding and chaperones expression. Indeed, several chaperones, members of the heat shock protein (HSP) family, have been shown to interact with huntingtin proteins containing different polyQ lengths, and to co-localize with intracellular aggregates. Furthermore, transient overexpression of these HSPs does reduce aggregate formation as well as cellular toxicity induced by expanded polyQ tracts (Abu-Baker et al., 2003; Bachetti et al., 2007; Bao et al., 2004; Cummings et al., 1998; Hay et al., 2004; Jana et al., 2000; Wang et al., 2005). Therefore, compounds that either induce HSPs expression, interact with the secondary configuration of mutated proteins, or reduce aggregates formation may be beneficial to rescue the cellular phenotype in association with polyAla expansions. To this end, we have investigated the effects of the following molecules on the cellular response to polyAla PHOX2B aggregates: 1) the antibiotic geldanamycin (GA), already shown to activate a heat shock response and to inhibit huntingtin aggregation in a cell culture model of Huntington's disease (Sittler et al., 2001), in addition to promoting nuclear localisation and clearance of PHOX2B misfolded proteins (Bachetti et al., 2007) and, for this reason, used as positive control in our cellular model; 2) the GA analog 17-AAG (17-(Allylamino)-17-demethoxygeldanamycin), which is currently in clinical trials as an anticancer drug, and specifically binds to and inhibits HSP90 (Neckers and Neckers, 2002); 3) ibuprofen, known to induce HSP70 expression and to reduce poly(A) binding protein, nuclear 1 (PABPN1) aggregation (Wang et al., 2005; Wang and Bag, 2008); 4) curcumin (diferuloylmethane), a major component of turmeric (*Curcuma Longa*) at present under phase II clinical investigation for the treatment of various tumors, and already investigated in different neurological disorders (Cheng et al., 2001; Sharma et al., 2004) for its potent anti-inflammatory, antioxidant, and anti-protein-aggregation activities (Cole et al., 2007); 5) sodium 4-phenylbutyrate (4-PBA), a histone deacetylase inhibitor, FDA approved drug for management of urea cycle disorders, has been shown to improve survival and attenuate striatal atrophy in the R6/2 transgenic mouse model of Huntington's disease (Gardian et al., 2005); 6) trehalose, already shown to reduce aggregation and toxicity of mutant PABPN1 *in vitro* and *in vivo*, and to delay the onset of muscle weakness in OPMD transgenic mice, that develop a progressive neuro-muscular phenotype accompanied by the formation of aggregates in skeletal muscle nuclei (Davies et al., 2006); 7) congo red, and its analog chrysamine G, acting by inhibiting aggregation of amyloid β-peptide and its neurotoxic effect in cultures of rat hippocampal neurons (Burgevin et al., 1994; Fraser et al., 1992; Lorenzo and Yankner, 1994) and also huntingtin aggregation *in vitro* (Heiser et al., 2000). Following treatments with these molecules, we have observed that 17-AAG and curcumin exerted the major effects on both rescue of the PHOX2B (+13Ala) nuclear localization and transactivation activity, thus disclosing some of the mechanisms underlying polyAla expansion pathogenesis and drug mediated effects.

Material and methods

Cell line and cell cultures

COS-7 cells were cultured in Dulbecco's modified essential medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco, New Zealand), 1% L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin in an atmosphere of 95% air and 5% CO_2 at 37 °C. HeLa cells were cultured in minimal essential medium (MEM, Euroclone) supplemented with 10% fetal bovine serum (FBS) (Gibco, New Zealand), 1% L-glutamine, 1% non-essential aminoacids, 100 U/ml penicillin and 100 g/ml streptomycin in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Fluorescence microscopy analysis

10^5 COS-7 cells were plated in 35 mm dishes 24 h prior to transfection. Cells were transiently transfected with 700 ng of the GFP reporter plasmids pcDNA3.1/CT-GFP-TOPO [PHOX2B wt] and pcDNA3.1/CT-GFP-TOPO [dup39], encoding the wild type and the mutant PHOX2B (+13 Ala), this latter carrying 13 extra alanine residues in the 20 alanine stretch, respectively. Transfection was performed with Fugene HD transfection reagent (Roche). At the time of transfection, cells were also added with different concentrations of compounds for further 48 h. 48 h after transfection, cells were washed once with PBS 1x (Phosphate Buffered Saline Tablets, Dulbecco's Formula, 1X; pH = 7.4, MP Biomedical) and then fixed with MeOH/Acetone 1:1 for 3 min at room temperature. Nuclei were stained with DAPI (Roche). For quantification of PHOX2B aggregates at least 100 cells were examined with a Zeiss Axiophot fluorescence microscope and the GFP fluorescence allowed to assess the subcellular PHOX2B localization thus classified as "nuclear" or "nuclear and cytoplasmic". The most effective concentration was assessed for each compound by at least three independent experiments.

Analysis of LC3B localisation

10^5 HeLa cells were plated in 35 mm dishes 24 h prior to transfection. 48 h after co-transfection with the pcDNA3.1/CT-GFP-TOPO [dup39] expression construct and the pEX-HcRed-hLC3WT (Addgene plasmid 24991; Tanida et al., 2008), cells were washed with PBS 1x and then fixed with MeOH/Acetone 1:1 for 3 min at room temperature. Nuclei were stained with DAPI (Roche) and analyzed with a Zeiss Axiophot fluorescence microscope.

Evaluation of proteasome activity

HeLa cells were transiently transfected with the ZsProSensor-1 plasmid (Clontech), expressing the fluorescent ZsGreen protein, and added with MG132 for 24 h. Expression of the ZsGreen protein, a C-terminal fusion of ZsGreen, a naturally occurring green fluorescent protein, with the mouse ornithine decarboxylase degradation domain, normally targeted for rapid degradation by the proteasome, was evaluated by direct microscope fluorescence.

Transcriptional activity assay

5×10^4 HeLa cells were seeded in 24-wells plate 24 h prior to transfection and transiently co-transfected, using Lipofectamine2000, with 370 ng of pcDNA3.1TOPO-PHOX2B wild type and pcDNA3.1-TOPO-dup39 mutant PHOX2B expression constructs (this latter carrying 13 extra alanine residues in the 20 alanine stretch) (Bachetti et al., 2005) together with 70 ng of a construct containing four copies of domain II of the *DBH* promoter, already known to be a PHOX2B target sequence, cloned upstream of the *Luciferase* reporter gene (Adachi et al., 2000; Benfante et al., 2007). The pRL-CMV plasmid, expressing the Renilla Luciferase gene, was co-trasfected and used as an internal control. Luciferase activity (Dual-Luciferase Reporter Assay System, Promega) was performed 48 h after transfection with a TD-20/20 Luminometer following manufacturer's instructions.

Compounds and treatment

17-(Allyloamino)-17-demethoxygeldanamycin (Sigma) (17-AAG) was prepared at 1 mM stock solution in DMSO and diluted in fresh medium at the final concentration of 25 nM, 50 nM, 100 nM and 300 nM at the time of transfection. Curcumin (Sigma) was prepared