Neurobiology of Aging 34 (2013) 2585–2603

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

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

Clearance of the mutant androgen receptor in motoneuronal models of spinal and bulbar muscular atrophy☆

Paola Rusmini [a,b,1], Valeria Crippa [a,b,1], Elisa Giorgetti [a,b], Alessandra Boncoraglio [a,b], Riccardo Cristofani [a,b], Serena Carra [c], Angelo Poletti [a,b,*]

[a] Sezione di Biomedicina e Endocrinologia, Dipartimento di Scienze Farmacologiche e Biomolecolari (DiSFeB), Centro di Eccellenza sulle Malattie Neurodegenerative, Universita' degli Studi di Milano, Milan, Italy
[b] Centro InterUniversitario sulle Malattie Neurodegenerative, Universita' degli Studi di Firenze, Milano, Genova e Roma Tor Vergata, Sede di Milano, Italy
[c] Sezione di Fisiologia e Neuroscienze, Dipartimento di Scienze Biomediche, Metaboliche e Neuroscienze, Università degli Studi di Modena e Reggio Emilia, Modena, Italy

ARTICLE INFO

Article history:
Received 12 November 2012
Received in revised form 23 April 2013
Accepted 27 May 2013
Available online 28 June 2013

Keywords:
Spinal and bulbar muscular atrophy
Androgen receptor
Polyglutamine
CAG repeat
Motoneuron disease
Motoneuron
Neurodegeneration
Protein misfolding
Chaperones
HspB8

ABSTRACT

Spinal and bulbar muscular atrophy (SBMA) is an X-linked motoneuron disease caused by an abnormal expansion of a tandem CAG repeat in exon 1 of the androgen receptor (AR) gene that results in an abnormally long polyglutamine tract (polyQ) in the AR protein. As a result, the mutant AR (ARpolyQ) misfolds, forming cytoplasmic and nuclear aggregates in the affected neurons. Neurotoxicity only appears to be associated with the formation of nuclear aggregates. Thus, improved ARpolyQ cytoplasmic clearance, which indirectly decreases ARpolyQ nuclear accumulation, has beneficial effects on affected motoneurons. In addition, increased ARpolyQ clearance contributes to maintenance of motoneuron proteostasis and viability, preventing the blockage of the proteasome and autophagy pathways that might play a role in the neuropathy in SBMA. The expression of heat shock protein B8 (HspB8), a member of the small heat shock protein family, is highly induced in surviving motoneurons of patients affected by motoneuron diseases, where it seems to participate in the stress response aimed at cell protection. We report here that HspB8 facilitates the autophagic removal of misfolded aggregating species of ARpolyQ. In addition, though HspB8 does not influence p62 and LC3 (two key autophagic molecules) expression, it does prevent p62 bodies formation, and restores the normal autophagic flux in these cells. Interestingly, trehalose, a well-known autophagy stimulator, induces HspB8 expression, suggesting that HspB8 might act as one of the molecular mediators of the proautophagic activity of trehalose. Collectively, these data support the hypothesis that treatments aimed at restoring a normal autophagic flux that result in the more efficient clearance of mutant ARpolyQ might produce beneficial effects in SBMA patients.

1. Introduction

Spinal and bulbar muscular atrophy (SBMA) is an X-linked neuromuscular disease characterized by the loss of motoneurons in the spinal cord and in the bulbar regions of the brain stem. Degeneration of sensory neurons in the dorsal root ganglia has also been reported. SBMA is the result of a CAG triplet repeat expansion in the androgen receptor (AR) gene (La Spada et al., 1991) that results in an elongated polyglutamine (polyQ) tract in the N-terminal transactivation domain of the mutant AR protein (ARpolyQ). In unaffected individuals, the polyQ tract ranges in size from 9 to 37 Qs, and polyQ expansions that extend longer than 38 contiguous Qs lead to neuronal toxicity that is the cause of motoneuron death in SBMA. Eight other inherited neurodegenerative diseases have been found to be the result of similar elongated polyQ tracts and are classified as CAG/polyQ-diseases (Orr and Zoghbi, 2007; Pennuto et al., 2009). In each disease, the elongated polyQ tract is believed to act through a gain-of-function mechanism by inducing the formation of an aberrant protein conformation. This leads to protein misfolding and aggregation, and ultimately neuronal toxicity (motoneuronal in the case of SBMA). The fact that AR is an extremely well characterized member of the steroid receptor superfamily (Poletti, 2004; Vismara et al., 2009) makes SBMA a valuable model to study CAG/polyQ-related diseases because it allows for the discrimination between the physiological and

* Corresponding author at: Sezione di Biomedicina ed Endocrinologia, Dipartimento di Scienze Farmacologiche e Biomolecolari (DiSFeB), Universita' degli Studi di Milano - Via Balzaretti 9, 20133 Milano, Italy. Tel.: +39 02 5031 8215; fax: +39 02 5031 8204.
E-mail address: angelo.poletti@unimi.it (A. Poletti).
[1] Both authors equally contributed to this report.

0197-4580/$ – see front matter © 2013 The Authors. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2013.05.026

pathological activities of ARpolyQ. In addition, ARpolyQ toxicity strictly depends on the binding of the receptor to androgens (Chevalier-Larsen et al., 2004; Fischbeck, 2012; Katsuno et al., 2003a, 2012; Schmidt et al., 2002), which apparently converts ARpolyQ from a non-pathological to a pathological status (Katsuno et al., 2003a, 2003b), possibly via the induction of conformational changes in the mutant protein. These alterations could be linked to the release of accessory chaperones and/or to the AR nuclear translocation, because both processes have been linked to ARpolyQ neurotoxicity. Incorrect ARpolyQ folding during its activation results in the formation of nuclear aggregates in anterior horn spinal cord motoneurons and cytoplasmic aggregates in dorsal root ganglia (Adachi et al., 2005; Suzuki et al., 2008). Nuclear localization, but not aggregation, is apparently required for ARpolyQ neurotoxicity (Montie et al., 2009, 2011). In fact, cytoplasmic retention of ARpolyQ correlates with a decrease in its toxicity (Montie et al., 2009, 2011). However, non-nuclear ARpolyQ neurotoxic mechanisms have also been described (Morfini et al., 2006; Piccioni et al., 2002; Ranganathan et al., 2009; Schindler et al., 2012); for instance, misfolded cytoplasmic ARpolyQ can trigger a Bax-dependent apoptotic pathway (Young et al., 2009). In our motoneuronal SBMA models, testosterone induces the formation of cytoplasmic ARpolyQ aggregates (Simeoni et al., 2000) that we have already shown do not correlate with increased cell death (Rusmini et al., 2007; Simeoni et al., 2000). We postulated that, at least in the early stages of their formation, cytoplasmic ARpolyQ aggregation contributes to segregate the harmful proteins into physically defined intracellular compartments (Carra et al., 2012). At later stages, however, these aggregates might themselves become toxic because they might affect other cellular activities (e.g., protein clearance, axonal transport) that might also contribute to neurotoxicity (Piccioni et al., 2002; Poletti, 2004; Rusmini et al., 2010).

Apart from their role in neurotoxicity, aggregates represent a valuable "marker of misfolding" because their presence clearly correlates with defects in the misfolded protein clearance via proteolytic pathways (both the ubiquitin-proteasome system [UPS] or autophagy) (Carra et al., 2012; Rusmini et al., 2010, 2011; Sau et al., 2011). Indeed, cytoplasmic soluble ARpolyQ (testosterone-untreated) has been shown to impair UPS activity (Rusmini et al., 2007) because it might overwhelm its degradative capacity. In addition, it has been shown that the UPS poorly degrades long polyQs (Holmberg et al., 2004). Interestingly, ARpolyQ aggregation (induced by testosterone) correlates with UPS desaturation (Rusmini et al., 2007). In addition, ARpolyQ (with or without testosterone) activates autophagy (Rusmini et al., 2010) and autophagic inhibition increases ARpolyQ aggregation (Rusmini et al., 2010). Thus, cytoplasmic ARpolyQ aggregation might depend on autophagic flux failure (Rusmini et al., 2010) so that the stimulation of autophagy would have beneficial effects.

We recently showed that the small heat shock protein B8 (HspB8) facilitates autophagic removal of other proteins associated with motoneuron diseases (e.g., SOD1 and TDP-43) (Carra et al., 2012; Crippa et al., 2010a, 2010b). Moreover, it has been already shown that HspB8 exerts antiaggregation and/or prodegradative activity on ARpolyQ, but so far this has been evaluated only on the AR nontoxic conformational status (without testosterone) (Carra et al., 2005). Here, we evaluated and compared the HspB8 activities on ARpolyQ clearance in the presence and absence of testosterone and found that HspB8 restores the normal autophagic flux in motoneuronal cells expressing ARpolyQ (with or without testosterone). Interestingly, we also found that the trehalose proautophagic activity might be mediated by HspB8 action.

2. Methods

Chemicals, 3-methyladenine (3-MA), MG132, and trehalose were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.1. Plasmids

The plasmids AR.Q23 and AR.Q46, routinely used in our laboratory, have been previously described (Simeoni et al., 2000). The GFP-AR.Q22 and GFP-AR.Q48 were obtained by insertion of AR cDNA into the green fluorescent protein (GFP) vector, expressing chimeric fluorescent fusion proteins as previously described (Stenoien et al., 1999). The plasmid pCNEO-cMyc-HspB8 expressing the *myc* tagged-rat HspB8 was obtained from J. Landry, Université Laval, Québec, Canada (Chavez Zobel et al., 2003) and the plasmid pCI-HspB8 contains the sequence of human HspB8 cloned in pCI (Promega, Madison, WI, USA) (Carra et al., 2005). The plasmid GFPu, expressing a proteasome activity reporter based on a GFP fused to a constitutive degradation signal (CL-1), was obtained from Ron Kopito, Stanford University, Stanford, CA, USA (Bence et al., 2001). The plasmid pmRFP-LC3, expressing the autophagic reporter LC3 tagged with mRFP, was obtained from Aviva Tolkovsky, University of Cambridge, UK (Klionsky et al., 2012). The plasmid pDest-mCherry-p62, expressing a Cherry-tagged p62, was obtained from Terje Johansen, University of Tromsø, Norway (Pankiv et al., 2007). pDsRed-monomer-C1 encodes a monomeric mutant form of the *Discosoma sp*. Red fluorescent protein DsRed (Clontech Laboratories, Inc, Mountain View, CA, USA). The plasmid promB8 expresses the firefly luciferase gene under the control of the human HspB8 promoter (Crippa et al., 2010b). pRL-TK was from Promega. To silence LC3 expression we used an shRNA construct against *Mus Musculus* Map1LC3b in pGFP-V-RS-vector and a scrambled noneffective shRNA, as control (OriGene Technologies, Inc, Rockville, MD, USA), as previously described (Rusmini et al., 2011).

To silence endogenous HspB8 expression we used a custom siRNA duplex (CGG AAG AGC UGA UGG UAA AUU) (Dharmacon, Thermo Scientific Life Sciences Research, Waltham, MA, USA).

2.2. Cell cultures and transfections

The immortalized motoneuron cell line, NSC34 (Cashman et al., 1992), is routinely used in our laboratory (Crippa et al., 2010a, 2010b; Piccioni et al., 2001; Rusmini et al., 2011; Simeoni et al., 2000), and was transfected with lipofectamine (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and transferrin (Sigma-Aldrich), as previously described (Marron et al., 2005; Simeoni et al., 2000) using 1.0–1.5 μg of plasmid DNA, 3 μL of transferrin solution and 2 μL of lipofectamine.

HspB8 and scramble siRNA were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions.

In the experiments involving steroid hormone treatments, the fetal bovine serum (FBS) was replaced with charcoal stripped-FBS, to eliminate endogenous steroids (Poletti et al., 2001; Pozzi et al., 2003).

The following amounts of cDNAs were used: (1) 0.5 μg of AR.Q(n) or GFP-AR.Q(n); (2) 0.6 μg of pCNEO-cMyc-HspB8, pCIHspB8 or pCDNA3 as vector control; (3) 0.05 μg of GFPu; (4) 0.2 μg of DsRed Monomer; (5) 0.2 μg of mRFP-LC3; (6) 0.2 μg of mCherry-p62; (7) 0.5 μg of shRNA against LC3 or shRNA scrambled control; (8) 0.25 μg of promB8; and (9) 0.25 μg of pRL-TK.

2.3. Fluorescence, immunofluorescence, and microscopy on NSC34 cells

NSC34 cells were plated in 12-well multiwell plates containing coverslips at a density of 70,000 cells per well and transiently