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Rotenone down-regulates HSPA8/hsc70 chaperone protein *in vitro*: A new possible toxic mechanism contributing to Parkinson's disease

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ABSTRACT

HSPA8/hsc70 (70-kDa heat shock cognate) chaperone protein exerts multiple protective roles. Beside its ability to confer to the cells a generic resistance against several metabolic stresses, it is also involved in at least two critical processes whose activity is essential in preventing Parkinson's disease (PD) pathology. Actually, hsc70 protein acts as the main carrier of chaperone-mediated autophagy (CMA), a selective catabolic pathway for alpha-synuclein, the main pathogenic protein that accumulates in degenerating dopaminergic neurons in PD. Furthermore, hsc70 efficiently fragments alpha-synuclein fibrils *in vitro* and promotes depolymerization into non-toxic alpha-synuclein monomers.

Considering that the mitochondrial complex I inhibitor rotenone, used to generate PD animal models, induces alpha-synuclein aggregation, this study was designed in order to verify whether rotenone exposure leads to hsc70 alteration possibly contributing to alpha-synuclein aggregation. To this aim, human SH-SY5Y neuroblastoma cells were treated with rotenone and hsc70 mRNA and protein expression were assessed; the effect of rotenone on hsc70 was compared with that exerted by hydrogen peroxide, a generic oxidative stress donor with no inhibitory activity on mitochondrial complex I. Furthermore, the effect of rotenone on hsc70 was verified in primary mouse cortical neurons. The possible contribution of macroautophagy to rotenone-induced hsc70 modulation was explored and the influence of hsc70 gene silencing on neurotoxicity was assessed. We demonstrated that rotenone, but not hydrogen peroxide, induced a significant reduction of hsc70 mRNA and protein expression. We also observed that the toxic effect of rotenone on alpha-synuclein levels was amplified when macroautophagy was inhibited, although rotenone-induced hsc70 reduction was independent from macroautophagy. Finally, we demonstrated that hsc70 gene silencing up-regulated alpha-synuclein mRNA and protein levels without affecting cell viability and without altering rotenone- and hydrogen peroxide-induced cytotoxicity.

These findings demonstrate the existence of a novel mechanism of rotenone toxicity mediated by hsc70 and indicate that dysfunction of both CMA and macroautophagy can synergistically exacerbate alpha-synuclein toxicity, suggesting that hsc70 up-regulation may represent a valuable therapeutic strategy for PD.

1. Introduction

HSPA8/hsc70 (70-kDa heat shock cognate) protein represents a constitutively expressed protein belonging to the heat shock protein 70 (hsp70) chaperone family (Liu et al., 2012). Hsc70 is mainly localized in the intracellular space, possesses a highly

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conserved amino acid sequence and plays a critical role in a variety of cellular mechanisms including endocytosis, protein folding and degradation (Stricher et al., 2013).

Recent studies reported that hsc70 is able to confer to the cells resistance against metabolic stress, hyperthermia and oxidative challenges (Chong et al., 2013; Wang et al., 2013a). Furthermore, particularly relevant is the involvement of hsc70 protein in the autophagic pathway known as chaperone-mediated autophagy (CMA), a selective device for the degradation of aberrant proteins containing the consensus peptide sequence KFERQ, which are directly transported to the lysosomes by a translocation system constituted by specific carrier proteins including cytosolic hsc70. Hsc70 is also localized into the lysosomal lumen where it allows the translocation of the substrate protein across the lysosomal membrane (Cuervo and Wong, 2014). Dysfunction of the CMA pathway is known to be closely associated with Parkinson's disease (PD) (Alvarez-Erviti et al., 2010; Cuervo et al., 2004; Kabuta et al., 2008; Xilouri et al., 2009); in particular, a significant reduction of hsc70 levels was evidenced in the substantia nigra pars compacta and amygdala of PD brains (Alvarez-Erviti et al., 2010) and in lymphomonocytes obtained from sporadic PD patients (Sala et al., 2014).

A significant downregulation of HSPA8/hsc70 was also observed in Alzheimer's disease post-mortem brain tissues (Silva et al., 2014), suggesting that loss of expression of this molecular chaperone should play a critical role in the neuronal death associated not only with PD but also with other neurodegenerative diseases.

Since a crucial pathogenic role in PD is recognized to be played by intraneuronal accumulation and aggregation of alpha-synuclein, the demonstration that CMA represents the main catabolic system for alpha-synuclein (Cuervo et al., 2004; Mak et al., 2010) has strengthened the link between CMA dysfunction and PD pathology. Further reinforcing the connection between hsc70 and PD, hsc70 has been demonstrated *in vitro* to bind to both soluble alpha-synuclein, slowing down its assembly into fibrils, and fibrillar form even with higher affinity (Pemberton et al., 2011; Pemberton and Melki, 2012), thus limiting the prion-like alpha-synuclein spreading known to amplify PD-associated neurodegeneration. Lastly, a very recent study demonstrated that HSPA8/hsc70 represents the main constituent of a disaggregase system that efficiently fragments alpha-synuclein fibrils *in vitro* into shorter fibrils and promotes their depolymerization into non-toxic alpha-synuclein monomers (Gao et al., 2015). These findings identify other protective mechanisms exerted by hsc70 against the cytotoxicity associated with alpha-synuclein aggregation and inter-neuronal propagation occurring in PD.

It is well known that exposure to rotenone, an inhibitor of the mitochondrial complex I, is able to reproduce PD pathology both in animal and cellular models, as indicated by the degeneration of nigrostriatal dopaminergic neurons and the formation in nigral neurons of alpha-synuclein-positive cytoplasmic inclusions (Betarbet et al., 2000; Gao et al., 2002; Sherer et al., 2003), although with some important limitations (Höglinger et al., 2006). Considering that rotenone induces alpha-synuclein aggregation and that hsc70 has a disaggregant effect on alpha-synuclein, this study was designed in order to verify whether rotenone exposure leads to hsc70 alteration possibly contributing to alpha-synuclein aggregation. To this aim, human SH-SY5Y neuroblastoma cells were treated with rotenone and hsc70 mRNA and protein expression was assessed. The protein levels of other heat shock proteins, hsp70 and hsp90, were evaluated to examine the specificity of rotenone-induced hsc70 reduction; the effect of rotenone on hsc70 was compared with that exerted by hydrogen peroxide, a generic oxidative stress donor with no inhibitory activity on mitochondrial complex I. Furthermore, the effect of rotenone on hsc70 was confirmed in primary mouse cortical neurons. As we observed a rotenone-induced autophagosome accumulation, the possible contribution of macroautophagy to rotenone-induced modulation of hsc70 was explored. The influence of hsc70 reduction on neurotoxicity was verified through HSPA8/hsc70 gene silencing.

2. Material and methods

2.1. Cell cultures

Human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium-F12 (EuroClone) supplemented with 10% fetal bovine serum (EuroClone), 100 U/mL penicillin (EuroClone), 100 μg/ml streptomycin (EuroClone) and 2 mM L-glutamine (EuroClone), at 37 °C in an atmosphere of 5% CO_2 in air. SH-SY5Y cells were used at a number of passages of growth ranging from thirteen to seventeen.

Cortical neurons were prepared as previously described (Cirillo et al., 2014). Animal experiments were carried out using protocols approved by the University of Milano-Bicocca Animal Care and Use Committee and by the Italian Ministry of Health (protocol number 14-2011). This study complies with the ARRIVE guidelines. Briefly, cortices were dissected from neonatal (P1-P2) C57BL/6J mice (Charles River Laboratories), washed in dissociation medium and digested by trypsin (0.15%) with deoxyribonuclease (DNAse, 1 mg/ml, Sigma-Aldrich) at 37 °C for 20 min. After mechanical dissociation, cells (1×10^6/ml) were plated onto poly-D-lysine (1 mg/ml) coated dishes in Neurobasal medium (NB; Invitrogen) containing B27 (Invitrogen), bFGF 10 ng/ml (Invitrogen), glutamine 1 mM (Sigma-Aldrich) and antibiotics (Sigma-Aldrich). Cultures were maintained at 37 °C in 5% CO_2 and used after 8 days *in vitro* (DIV). To evaluate purity of cultures (99–99.5%), cells were plated onto 12 mm poly-D-lysine coated coverslip (5000/well) and assessed by immunocytochemistry using anti-βIII-tubulin (Cell Signaling), as previously described (Cirillo et al., 2014). Neurons were imaged under a reversed microscope Olympus CX40 (X20) equipped with an Olympus camera.

2.2. Cytotoxicity assays

The effect of rotenone or hydrogen peroxide on cell viability was assessed by the MTT assay based on reduction of the yellow tetrazolium salts (MTT) to the purple formazan by mitochondrial dehydrogenases. After exposure to rotenone (from 100 to 800 nM) or hydrogen peroxide (from 50 to 200 μM) for 6 or 24 h, SH-SY5Y cells were incubated with 0.5 mg/ml MTT (Sigma-Aldrich) in standard medium for 45 min at 37 °C in an atmosphere of 5% CO_2 in air. The effect on cell viability of 5 mM 3-methyladenine (3-MA) for 24 h, alone or in combination with 200–400 nM rotenone, was also assessed in SH-SY5Y cells. Similarly, cortical neurons (5000 cells/well) at DIV8 were treated with rotenone (from 100 to 800 nM) for 6 or 24 h and incubated with MTT (0.5 mg/ml) for 4 h. After cell solubilization with DMSO, absorbance was quantified (wavelength 570 nm) using a multimode microplate reader (FLUOstar Omega, BMG LABTECH) and cell viability expressed as% vs. vehicle-treated cells. Since rotenone affects mitochondrial function, rotenone-induced cell death was also evaluated with the Trypan blue exclusion test in order to independently confirm results obtained at MTT assay.

2.3. Whole-cell reactive oxygen species (ROS) levels

The dye 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) was used to quantify the levels of whole-cell ROS. After medium removal, cells were exposed to 10 μM DCF-DA