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Research Article

GADD34 mediates cytoprotective autophagy in mutant huntingtin expressing cells via the mTOR pathway

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ABSTRACT

Increased protein aggregation and altered cell signaling accompany many neurodegenerative diseases including Huntington's disease (HD). Cell stress is counterbalanced by signals mediating cell repair but the identity of these are not fully understood. We show here that the mammalian target of rapamycin (mTOR) pathway is inhibited and cytoprotective autophagy is activated in neuronal PC6.3 cells at 24 h after expression of mutant huntingtin proteins. Tuberous sclerosis complex (TSC) 1/2 interacted with growth arrest and DNA damage protein 34 (GADD34), which caused TSC2 dephosphorylation and induction of autophagy in mutant huntingtin expressing cells. However, GADD34 and autophagy decreased at later time points, after 48 h of transfection with the concomitant increase in mTOR activity. Overexpression of GADD34 counteracted these effects and increased cytoprotective autophagy and cell survival. These results show that GADD34 plays an important role in cell protection in mutant huntingtin expressing cells. Modulation of GADD34 and the TSC pathway may prove useful in counteracting cell degeneration accompanying HD and other neurodegenerative diseases.

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Abbreviations: ACC, Acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ATF4, activating transcription factor 4; Bcl-2, B-cell CLL/lymphoma 2; CHOP, CCAAT/enhancer binding protein (C/EBP) homologous protein also called for growth arrest and DNA-damage-inducible protein GADD153; eIF2α, elongation initiator factor 2 alpha; ER, endoplasmic reticulum; ERAD, ER associated degradation; GADD34, Growth arrest and DNA damage-inducible protein 34; CAG, glutamine; FL, full-length; GFP, green fluorescent protein; HD, Huntington's disease; IRE1α, inositol requiring enzyme 1 alpha; JNK, jun-N-terminal kinase; kDa, kilodalton; LAMP1, Lysosomal-associated membrane protein 1; LC3, light chain 3 protein; PC6.3, pheochromocytoma cell line subline 6.3; PCR, polymerase chain reaction; PERK, PKR-like ER-localized eIF2α kinase; polyQ, polyglutamine; PP1, protein phosphatase 1; RFP, red fluorescence protein; S6K, RPS6-p70-protein kinase; TBS, Tris buffered saline; mTOR, mammalian target of rapamycin; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; UPR, unfolded protein response; Q, glutamine

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Introduction

Huntington's disease (HD) is an autosomally dominantly inherited disease characterized by severe motor and cognitive symptoms due to neurodegeneration in striatum and other parts of the brain [1]. HD is caused by a CAG-repeat expansion in exon I of the *IT15* gene that encodes huntingtin [2]. Increased CAG-repeats in huntingtin cause accumulation of intracellular protein aggregates in cells, including neurons [3,4]. Inhibition of the autophagy–lysosome pathway has been shown to impair the degradation of the huntingtin aggregates [5]. As shown in HD models in mice and *Drosophila*, blocking of the mammalian target of rapamycin (mTOR) pathway by rapamycin induces autophagy and reduces mutant huntingtin toxicity [6,7]. This suggests a role for the mTOR pathway and autophagy in cytoprotection in HD that warrants further studies.

mTOR is a serine/threonine kinase that is important in the control of cell growth, proliferation and survival [8]. The activity of mTOR is regulated by various growth factors and cell signals, as well as by the supply of amino acids and nutrients sensing the general energy state of the cell. mTOR inhibits autophagy by phosphorylating proteins encoded by the autophagy-related genes (Atgs), whereas phosphorylation of the ribosomal protein S6 kinases (S6K1 and S6K2) and the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) stimulates translation of specific proteins involved in cell repair after stress [9–11]. The precise links between altered protein synthesis and the process of autophagy under different cell conditions are not fully understood.

Growth arrest and DNA damage-inducible gene 34 (GADD34) is a protein induced by cell damage and it is a major regulator of translation during cell stress [12–16]. GADD34 forms a complex with the protein phosphatase 1 (PP1) to dephosphorylate the eukaryotic initiator factor-2α (eIF2α) [15,17]. Phosphorylation of eIF2α decreases global protein synthesis but is required for translation of a subset of specific mRNAs encoding proteins that are involved in cell repair [17,18]. Studies of GADD34 gene deficient mice have shown that GADD34 promotes cell survival and the recovery from protein synthesis inhibition induced by endoplasmic reticulum (ER) stress [19]. However, the possible roles played by GADD34 in human neurodegenerative diseases and in the regulation of autophagy in cells are so far less understood.

We have recently shown that mutant N-terminal huntingtin fragment proteins as well as disease-causing full-length (FL) huntingtin proteins trigger ER stress in neuronal PC6.3 cells with an increase in the phosphorylation of eIF2α [20,21]. eIF2α is a target for GADD34 in regulation of protein synthesis but GADD34 may have additional functions during cell stress [22]. In the present study, we showed that GADD34 induced cytoprotective autophagy in mutant huntingtin expressing cells via influencing the mTOR pathway. GADD34 interacted with Tuberous sclerosis complex 1 (TSC1), which caused dephosphorylation of TSC2. However, at later time points the mTOR inhibition was reversed and correlated with decreased levels of GADD34 and an inhibition of autophagy. Overexpression of GADD34 induced cytoprotective autophagy and increased cell viability in mutant huntingtin expressing cells. Interfering with the levels or the function of the GADD34 may represent novel targets to combat cell stress and the deleterious effects of mutant huntingtin proteins.

Materials and methods

Cell culture and transfections

Rat pheocromocytoma cells (PC6.3) were cultured in RPMI 1640 (Biochrom AG) medium supplemented with 5% fetal calf serum and 10% horse serum. Cells were transfected with expression vectors encoding for different CAG-repeat lengths of huntingtin exon-1 fused to EGFP, as well as full-length (FL) huntingtin with 17- and 75 polyglutamine-repeats as described [20,21]. LC3-RFP (light chain 3 protein-red fluorescent protein), LC3-EGFP (LC3-enhanced green fluorescent protein), LAMP1-RFP (Lysosomal-associated membrane protein 1-RFP) and GADD34 expression plasmids were obtained from Addgene. Cells were transfected using the Transfectin reagent (BioRad) with the above plasmids or with the control EGFP plasmid (Clontech). In some experiments, 1 mM 3-methyladenine (3-MA, Sigma), 200 nM Rapamycin (Calbiochem), 2 μM thapsigargin (Sigma) or 1 μg/ml tunicamycin (Sigma) was added. Rapamycin was added 4 h after transfection, 3-methyladenine for 6 h, thapsigargin and tunicamycin for 24 h. LC3 immunoblotting was done also in the presence of lysosomal proteasome inhibitors: 5 μM E64d (Sigma) and 10 μM pepstatin (Sigma).

Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Calbiochem) assay as described previously [21,23].

Silencing of GADD34 expression using siRNAs

GADD34 expression was specifically silenced in neuronal PC6.3 cells using siRNA (small interfering RNAs) duplexes (Silencer®, Applied biosystems). Ppp1R15a (GADD34) siRNAs (P/N:s 4390815 and 4390815) and siRNA universal negative control (Mission®, Sigma) were transfected by using the Transfectin reagent (BioRad) following the manufacturer's instructions.

Immunocytochemistry

PC6.3 cells plated on polylysine-laminin-coated coverslips were fixed for 20 min using 4% paraformaldehyde or methanol (staining of endogeneous LC3). Cells were incubated overnight with primary GADD34 (1:1000, Santa Cruz Biotechnology) or LC3 antibody (LC3 (1:100, Cell Signaling) followed by 1 h incubation with Alexa594-conjugated secondary antibodies (1:500, Molecular Probes). Stainings were analyzed with Zeiss LSM 5 duo confocal microscope at Molecular Imaging unit, Biomedicum Helsinki.

Immunoblots

Cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl and 1% SDS, pH 8.0) containing phosphatase inhibitor cocktail (Roche), or in Poly (ADP-ribose) polymerase (PARP) lysis buffer (62.5 mM Tris, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue and 5% 2-mercaptoethanol). Equal amounts of protein (40 μg) were separated by SDS-PAGE, and transferred to a nitrocellulose filters (Amersham Biosciences, Helsinki, Finland).

Filters were blocked for 1 h in 5% milk-TBS or 5% BSA-TBS, followed by an overnight incubation at +4 °C using primary