Trehalose, a Novel mTOR-independent Autophagy Enhancer, Accelerates the Clearance of Mutant Huntingtin and α-Synuclein

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Trehalose, a disaccharide present in many non-mammalian species, protects cells against various environmental stresses. Whereas some of the protective effects may be explained by its chemical chaperone properties, its actions are largely unknown. Here we report a novel function of trehalose as an mTOR-independent autophagy activator. Trehalose-induced autophagy enhanced the clearance of autophagy substrates like mutant huntingtin and the A30P and A53T mutants of α-synuclein, associated with Huntington disease (HD) and Parkinson disease (PD), respectively. Furthermore, trehalose and mTOR inhibition by rapamycin together exerted an additive effect on the clearance of these aggregate-prone proteins because of increased autophagic activity. By inducing autophagy, we showed that trehalose also protects cells against subsequent pro-apoptotic insults via the mitochondrial pathway. The dual protective properties of trehalose (as an inducer of autophagy and chemical chaperone) and the combinatorial strategy with rapamycin may be relevant to the treatment of HD and related diseases, where the mutant proteins are autophagy substrates.

Trehalose is a non-reducing disaccharide found in many organisms, including bacteria, yeast, fungi, insects, invertebrates, and plants. It is the natural hemolymph sugar of invertebrates. It functions to protect the integrity of cells against various environmental stresses like heat, cold, desiccation, dehydration, and oxidation by preventing protein denaturation (1). Many of the stress-protecting properties of trehalose were discovered in yeast (2); however, it also has beneficial effects in mammals and environmental stresses like heat, cold, desiccation, dehydration, and oxidation by preventing protein denaturation (1). Many of the stress-protecting properties of trehalose were discovered in yeast (2); however, it also has beneficial effects in mammals.

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The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

3 The abbreviations used are: polypeptide, polyglutamine; Baf, Bafilomycin A1; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; EGFP-HDQ74, EGFP-tagged huntingtin exon 1 with 74 polyglutamine repeats; Glu, glutamate; HD, Huntington disease; LC3, microtubule-associated protein 1 light chain 3; Lact, lactacystin; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; MEF, mouse embryonic fibroblasts; PD, Parkinson disease; p70S6K, ribosomal S6 protein kinase; Rap, raffinose; Rapamycin, ribosomal S6 protein; Sor, sorbitol; suc, sucrose; α-syn, α-synuclein; Tre, trehalose; HA, hemagglutinin; DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein.
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where their contents are degraded (14). Induction of autophagy reduces the levels of mutant huntingtin and protects against its toxicity in cells and in transgenic Drosophila and mouse models of HD (10, 11, 13). The apparent clearance of huntingtin aggregates by autophagy is likely to be a consequence of removal of aggregate precursors (soluble and oligomeric species), rather than big inclusions, which do not appear to be membrane-bound and are also much larger than typical autophagosomes. Currently, the only suitable pharmacological strategy for upregulating autophagy in mammalian brains is to use rapamycin, or its analogues, that inhibit the mammalian target of rapamycin (mTOR), a negative regulator of autophagy (14).

Parkinson disease (PD) is another condition associated with aggregate formation. The intraneuronal Lewy body aggregates seen in PD have the protein α-synuclein as a major component. The A53T and A30P point mutations in α-synuclein cause autosomal dominant forms of PD (15, 16). The A53T and A30P α-synuclein mutants are substrates of autophagy, and the clearance of these mutant forms is retarded when autophagy is inhibited (12). While these forms of α-synuclein aggregate in vivo, we do not observe overt aggregation in our cell lines (12). Furthermore, unlike wild-type α-synuclein, these mutant forms are not cleared by the chaperone-mediated autophagy pathway (17), which is distinct from macroautophagy (which we call autophagy in this report). Hence, we have used these mutations as model autophagy substrates.

Here we identify a novel role for trehalose as an autophagy inducer. Trehalose, in aqueous solutions, leads to enhanced clearance of aggregate-prone proteins like mutant huntingtin and α-synuclein, and protects cells from subsequent apoptotic insults.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—HD gene exon 1 fragment with 74 polyQ repeats in pEGFP-C1 (Clontech) (EGFP-HDQ74) and HA-HDQ74 constructs were characterized previously (18). EGFP-LC3, Myc-LC3-HA (from T. Yoshimori), EGFP-Atg5 (from N. Mizushima), and EGFP-Bax (from R. Youle) constructs were obtained as kind gifts. PABPN1 A17 (from E. Wahlle) was subcloned into pHM6 (Roche Applied Science) to generate the HA-PABPN1 A17 construct.

Reagents—Compounds used were 100 mM D-(-)-trehalose dihydrate, 100 mM sucrose, 100 mM D-(-)-raffinose pentahydrate, 100 mM D-sorbitol, 0.2 mM rapamycin, 200 mM baflo- mycin A1, 10 mM 3-methyladenine, 10 μM lactacystin, 3 μM stau- rosporine, 200 μg/ml Congo Red (all from Sigma-Aldrich), and 100 mM D-(-)-glucose (BDH).

Mammalian Cell Culture and Transfection—African green monkey kidney cells (COS-7), human neuroblastoma cells (SK-N-SH), human cervical carcinoma cells (HeLa), stable HeLa cells expressing EGFP-LC3 (19) (kind gift from A. M. Tolkovsky), and wild-type Atg5 (Atg5+/+) and Atg5-deficient (Atg5−/−) mouse embryonic fibroblasts (20) (MEFs) (kind gift from N. Mizushima) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin, and 2 mM l-glutamine (Sigma) at 37 °C, 5% CO2.

Inducible PC12 stable cell lines expressing EGFP-HDQ74 or EGFP-HDQ23 (10, 21), and HA-tagged A30P or A53T α-synuclein mutants (12), previously characterized, were maintained at 75 μg/ml hygromycin B (Calbiochem) in Dulbecco’s modified Eagle’s medium with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM l-glutamine, and 100 μg/ml G418 (Invitrogen) at 37 °C, 10% CO2.

T-REX 293 (Invitrogen), derived from human embryonal kidney cell line HEK 293, was maintained in Dulbecco’s modified Eagle’s medium with 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin/streptomycin, 2 mM l-glutamine, and 5 μg/ml Blasticidin S (Invitrogen) at 37 °C, 5% CO2. QA1/12/9A and A21/12/2B (Tre) are stable transfectant cell lines produced from T-REX 293, which were maintained in similar medium and conditions as T-REX 293 along with the addition of 500 μg/ml G418 (Geneticin) and 250 μg/ml Zeocin (Invitrogen).

Cells were transfected with the constructs for 4 h using Lipofectamine or Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol, fixed with 4% paraformaldehyde (Sigma) after 48 h (EGFP-HDQ74) or 2 h (EGFP-LC3) post-transfection, and mounted in Citifluor (Citifluor Ltd.) containing 4’6-diamidino-2-phenylindole (DAPI; 3 μg/ml; Sigma-Aldrich).

Quantification of Aggregate Formation and Cell Death—Approximately 200 EGFP-positive cells were counted for the proportion of cells with EGFP-HDQ74 aggregates, as described previously. Only EGFP-positive cells were counted so that we count only the transfected cells. If an EGFP-positive cell has one or many aggregates, the aggregate score is one. If an EGFP-positive cell does not have any aggregate, the aggregate score is zero. Nuclei were stained with DAPI and those showing apoptotic morphology were considered abnormal. These criteria are specific for cell death, which highly correlate with propidium iodide staining in live cells (22). Similar assessment for cell death was done after apoptotic insults with staurosporine or Bax. Experiments were done in triplicate at least twice.

Quantification of Cells with EGFP-LC3 Vesicles—Similar analyses in triplicate were done for counting the proportion of EGFP-positive cells with EGFP-LC3 vesicles. Approximately 100 EGFP-positive cells were counted for the proportions of EGFP-positive cells with >5 LC3-positive vesicles. We considered an EGFP-positive cell as having a score of zero if there were 5 or fewer vesicles (as cells have basal levels of autophagy) and cells scored one if they had >5 LC3-positive vesicles (13).

Clearance of Mutant Huntingtin and α-Synucleins—Stable inducible PC12 cell lines expressing EGFP-HDQ74, EGFP-HDQ23, or α-synuclein mutants (A30P or A53T) were induced with 1 μg/ml doxycycline (Sigma) for 8 and 48 h, respectively (10, 12). Transgene expression was switched off by removing doxycycline from medium. Cells were treated with or without compounds for time points as indicated in experiments. Compounds were replenished every 24 h for EGFP-HDQ74 clearance. Clearance of soluble huntingtin or α-synuclein mutants was measured by immunoblotting with antibody against EGFP or HA, respectively.

Western Blot Analysis—Cell pellets were lysed on ice in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 5% β-mercapto-
ethanol, 10% glycerol, and 0.01% bromphenol blue) for 30 min in presence of protease inhibitors (Roche Applied Science). Primary antibodies include anti-EGFP (8362-1, Clontech), anti-HA (12CA5, Covance), anti-complex IV subunit IV (A-21348, Molecular Probes), anti-cytoschrome c (4272), anti-caspase 3 (9665), anti-mTOR (2972), anti-phospho-mTOR (Ser2448) (2971), anti-p70 S6 kinase (9202), anti-phospho-p70 S6 kinase (Thr389) (9206), anti-4E-BP1 (9452), anti-phospho-4E-BP1 (Thr37/46) (9459) (all from Cell Signaling Technology), anti-LC3 (kind gift from T. Yoshimori), and anti-tubulin (Clone DM 1A, Sigma). Blots were probed with anti-mouse or anti-rabbit IgG-horseradish peroxidase and visualized using ECL or ECL Plus detection kit (Amersham Biosciences).

Generation of Stable Human Cell Line Synthesizing Intracellular Trehalose—T-Rex 293 (Invitrogen), derived from the human embryonal kidney cell line HEK 293, expresses TetR, which represses transcription from promoters containing the tet operator unless tetracycline or derivatives are present in the medium. QA1/12/9A and QA1/12/2B (Tre) are stable transfectant cell lines produced from T-Rex 293, which contains a tetracycline-inducible form of the trehalose-6-phosphate synthase gene (otsA) of Escherichia coli, together with constitutively expressed otsB (trehalose-6-phosphate phosphatase gene). Intracellular trehalose concentration in QA1/12/9A (Tre) cell line, 24 h after tetracycline induction, is ~20 mM as determined by gas chromatography (23).

Immunocytochemistry—Cells were fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.6, immunolabeled with antibody against active caspase 3 (G7851, Promega) or HA (12CA5, Covance) and fluorophore-conjugated secondary antibody (Alexa Fluor 488 (green) goat anti-rabbit or Alexa Fluor 594 (red) goat anti-mouse (Molecular Probes, Cambridge Bioscience), respectively), and mounted in Citifluor (3 μg/ml; Sigma-Aldrich) to visualize nuclei. Approximately 200 cells were scored for abnormal apoptotic nuclei (cell death) and active caspase 3 (bright, fluorescent immunolabeling using the active caspase 3 antibody).

Microscopy—Transfected cells were analyzed on a Nikon Eclipse E600 fluorescence microscope (plan-apo 60×/1.4 oil immersion lens at room temperature) (Nikon, Inc.). Images of EGFP-LC3 HeLa stable or COS-7 cells were acquired on a Zeiss LSM510 META confocal microscope (63× 1.4 NA plan-apochromat oil immersion or fluor 40×/1.3 oil lens, respectively) at room temperature using Zeiss LSM510 v3.2 software (Carl Zeiss, Inc.), and Adobe Photoshop 6.0 (Adobe Systems, Inc.) was used for subsequent image processing.

Statistical Analysis—Aggregate formation, cell death, or EGFP-LC3 vesicles were expressed as percentages from triplicate samples, and the error bars denote S.E. p values were determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS 9 software (SPSS, Chicago). Densitometry analysis on the immunoblots was done by Scion Image Beta 4.02 software (Scion Corporation) from three independent experiments (n = 3). Significance for the clearance of mutant proteins was determined by factorial ANOVA test using STATVIEW software, version 4.53 (Abacus Concepts), where the control condition was set to 100%. The y-axis values are shown in percentage (%), and the error bars denote S.E. ***, p < 0.001; **, p < 0.01; *, p < 0.05; NS, nonsignificant.

RESULTS

Trehalose Reduces polyQ-mediated Aggregation and Cell Death and Enhances the Clearance of Soluble Mutant Huntingtin—We confirmed that trehalose reduced aggregation and cell death caused by EGFP-tagged huntingtin exon 1 with 74 polyQ repeats (EGFP-HDQ74) in COS-7 (non-neuronal) and SK-N-SH (neuronal precursor) cells (Fig. 1, A and B and supplemental Fig. S1A). This effect of 100 mM trehalose was not caused by osmotic stress and was not a general property of the disaccharides, as no such effect was seen with 100 mM sucrose (a disaccharide), raffinose (a trisaccharide), or sorbitol (a sugar alcohol) (Fig. 1, A and B).

We tested if the reduced aggregation of the huntingtin construct was partly because of enhanced clearance leading to lower levels (10, 18), using a stable doxycycline-inducible PC12 cell line expressing EGFP-HDQ74, where transgene expression is first induced by adding doxycycline, and then switched off by removing doxycycline from the medium. If the transgene expression level is followed at various times after switching off expression after an initial induction period, one can assess if specific agents alter the clearance of the transgene product, as the amount of transgene product decays when synthesis is stopped (10). Trehalose significantly reduced EGFP-HDQ74 aggregates at 48 and 72 h and enhanced the clearance of soluble EGFP-HDQ74 and insoluble mutant huntingtin (that gets retarded in the stacking gel) at 120 h (Fig. 1, C and D and supplemental Fig. S1B). Sucrose, raffinose, and sorbitol did not have any effects on the clearance of EGFP-HDQ74 when used at similar concentrations (Fig. 1E). The enhanced clearance of EGFP-HDQ74 is not simply a chaperone effect, as no clearance was observed with Congo Red at doses that do reduce EGFP-HDQ74 aggregation and toxicity (supplemental Fig. S1, C and D). However, trehalose did not influence the clearance of wild-type huntingtin exon 1 (EGFP-HDQ23) (Fig. 1F and supplemental Fig. S1E).

Trehalose Enhances the Clearance of α-Synuclein Mutants—We also assessed the clearance of A53T and A30P α-synuclein mutants using stable doxycycline-inducible PC12 cell lines, using similar switch-on/off paradigms to EGFP-HDQ74, as these are autophagy substrates (12). Trehalose significantly enhanced the clearance of A30P and A53T mutants of α-synuclein at 24 h (Fig. 2, A and B). However, it had no significant effect on the clearance of wild-type α-synuclein at 24 h (Fig. 2C). This is entirely consistent with previous observations that wild-type α-synuclein clearance is not obviously retarded when autophagy is blocked, in contrast to the mutants (12, 17).

Trehalose Reduces Mutant huntingtin Aggregates by Autophagic Route—We tested if the enhanced clearance of mutant huntingtin mediated by trehalose was by autophagy or the proteasomal route, using inhibitors of autophagy (3-methyl adenosine, 3-MA) and the proteasome (lactacystin). Both these
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FIGURE 1. Trehalose reduces mutant huntingtin aggregates and toxicity and enhances the clearance of its soluble forms. A and B, COS-7 cells transfected with EGFP-HDQ74 for 4 h were treated with or without 100 mM Suc, 100 mM Raf, 100 mM Sorb or 100 mM Tre for 48 h. The percentage of EGFP-positive cells with EGFP-HDQ74 aggregates (A) or apoptotic morphology (cell death) (B) was assessed. Control represents untreated cells. p = 0.918 (Suc), p = 0.399 (Raf), p = 0.225 (Sor), p < 0.0001 (Tre) for aggregation (A); p = 0.389 (Suc), p = 0.127 (Raf), p = 0.274 (Sor), p < 0.0001 (Tre) for cell death (B). C, stable inducible PC12 cells expressing EGFP-HDQ74 were induced with doxycycline for 8 h, and transgene expression was then switched off (by removing doxycycline) for 24, 48, or 72 h, with or without 100 mM trehalose. Cells were assessed at each time point for the percentage of cells with EGFP-HDQ74 aggregates, p = 0.253 (24 h), p = 0.024 (48 h), p < 0.0001 (72 h). D, stable inducible PC12 cells expressing EGFP-HDQ74 were induced with doxycycline for 8 h, and transgene expression was then switched off (by removing doxycycline) for 24, 72, or 120 h, with (+) or without (−) 100 mM trehalose. Clearance of aggregated (in stacking gel) and soluble EGFP-HDQ74 were analyzed by immunoblotting with antibody against EGFP (panel i) and densitometry analysis relative to actin (panel ii). p < 0.0001 (at 120 h). E, clearance of soluble EGFP-HDQ74 in stable PC12 cells as in D, treated with or without 100 mM sucrose, 100 mM raffinose, or 100 mM sorbitol, was analyzed by immunoblotting with antibody against EGFP (panel i) and densitometry analysis relative to actin (panel ii). p = 0.4476 (Suc), p = 0.5697 (Raf), p = 0.1381 (Sor). F, stable inducible PC12 cells expressing EGFP-HDQ23 were induced as in D and treated with or without 100 mM trehalose for 120 h. Clearance of soluble EGFP-HDQ23 was analyzed by immunoblotting with antibody against EGFP (panel i) and densitometry analysis relative to actin (panel ii). p = 0.1669. *, p < 0.05; ***, p < 0.001; NS, nonsignificant.

FIGURE 2. Trehalose enhances the clearance of the α-synuclein mutants. A and B, stable inducible PC12 cell line expressing A30P (A) or A53T (B) α-synuclein mutant was induced with doxycycline for 48 h, and expression of transgene was switched off for 24 h, with (+) or without (−) 100 mM Tre. Clearence of A30P (A) or A53T (B) α-synuclein (α-syn) was analyzed by immunoblotting with antibody against HA (panel i) and densitometry analysis relative to actin (panel ii). p = 0.0005 (A); p = 0.0072 (B). C, stable inducible PC12 cell line expressing wild-type α-synuclein was induced with doxycycline for 48 h, and the expression of the transgene was switched off for 24 h, with or without 100 mM trehalose. Clearance of wild-type α-synuclein was analyzed by immunoblotting with antibody against HA (panel i) and densitometry analysis relative to actin (panel ii). p = 0.8664. ***, p < 0.001; NS, nonsignificant.

inhibitors increased EGFP-HDQ74 aggregates and toxicity in COS-7 cells (Fig. 3A and supplemental Fig. S2, A and B), consistent with our previous observations that this protein is cleared both by autophagy and proteasome (10, 12). We further confirmed these data by comparing mutant huntingtin aggregation in autophagy-competent MEFs (Atg5+/−) or matched MEFs lacking the essential autophagy gene Atg5 (Atg5−/−) (20). In untreated Atg5−/− cells, the Atg5/autophagy

autophagy was inhibited by 3-MA, trehalose could not further reduce EGFP-HDQ74 aggregates (Fig. 3A and supplemental Fig. S2A). However, cells treated with the proteasome inhibitor lactacystin and trehalose had significantly reduced EGFP-HDQ74 aggregates, compared with cells treated with lactacystin alone (Fig. 3A and supplemental Fig. S2A). When 3-MA was used together with lactacystin, the beneficial effect of trehalose on aggregation was lost and inclusions were significantly increased (Fig. 3A and supplemental Fig. S2A). These data suggest that trehalose enhanced clearance of EGFP-HDQ74 through the autophagic route.
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When these cells were treated with trehalose, the mutant huntingtin aggregation in cells (Fig. 3, A) was significantly reduced in autophagy-deficient Atg5 \(^{-/-}\) cells, as this mutant protein is an autophagy substrate (Fig. 3, B and C). When these cells were treated with trehalose, the mutant huntingtin aggregation and toxicity were significantly reduced in Atg5 \(^{+/+}\) cells, but not in Atg5/autophagy-deficient \((\text{Atg5}^{-/-})\) cells, thus confirming that the ability of trehalose to induce autophagy is a major factor behind its ability to reduce mutant huntingtin aggregation in cells (Fig. 3, B and C).

**Trehalose Induces Autophagy**—We first assessed the effect of trehalose on autophagy in the Atg5 \(^{+/+}\) and Atg5 \(^{-/-}\) MEFs by measuring the levels of microtubule-associated protein 1 light chain 3 (LC3). Endogenous LC3 is processed post-translationally into LC3-I, which is cytosolic. LC3-I is converted to LC3-II, which associates with autophagosome membranes (24). LC3-II levels relative to actin/tubulin correlate with autophagosome number per cell (24, 25). As we are interested in autophagosome number per cell, we have not quantified LC3-II versus LC3-I, as some LC3-II can be converted back to LC3-I (26). Trehalose significantly increased LC3-II levels in the autophagy-competent Atg5 \(^{+/+}\) cells, but not in the autophagy-deficient Atg5 \(^{-/-}\) cells (Fig. 3D).

LC3-II levels were significantly increased also in COS-7 cells treated with trehalose for 24 h (Fig. 4A). HeLa cells stably expressing LC3 fused to EGFP (EGFP-LC3) (19) treated for 24 h with trehalose had significantly higher EGFP-LC3-II levels (Fig. 4B) compared with untreated cells. The increase in LC3-II by trehalose is similar to what has been observed previously when autophagy is induced (24). In this cell line, 100 mM trehalose (the concentration used in our experiments) induced autophagy (supplemental Fig. S3A).

Accumulation of LC3-II can occur because of increased autophagosome formation, but also if there is impaired autophagosome-lysosome fusion. We assayed LC3-II in the presence of bafilomycin A1, which blocks autophagosome-lysosome fusion (27). Bafilomycin A1 resulted in the expected increase in EGFP-LC3-II in stable HeLa cells (Fig. 4C). The dose of bafilomycin A1 used is saturating for LC3-II levels in this assay (data not shown). Further blockade of autophagosome-lysosome fusion via a bafilomycin A1-independent mechanism, using the dynein inhibitor erythrod-9-[3-(2-hydroxyxonyl)]adenine (EHNA) (28), along with this dose of bafilomycin A1, results in no increase in LC3-II compared with bafilomycin A1 alone (data not shown). However, trehalose significantly increased EGFP-LC3-II levels in the presence of bafilomycin A1, compared with bafilomycin A1 alone, strongly arguing that the increased autophagosomes induced by trehalose are upstream of autophagosome-lysosome fusion.

**FIGURE 3. Trehalose reduces mutant huntingtin aggregates by autophagic pathway.** A, COS-7 cells transfected with EGFP-HDQ74 construct for 4 h were treated for 48 h with or without 100 mM trehalose, 10 \(\mu\)M 3-MA, and 10 \(\mu\)M lactacystin and 100 mM trehalose, or 10 \(\mu\)M lactacystin with 10 mM 3-MA and 100 mM trehalose. The percentage of EGFP-positive cells with EGFP-HDQ74 aggregates was counted. \(p = 0.003\) (Tre), \(p < 0.0001\) (3-MA, Lact, 3-MA + Tre, Tre + 3-MA + Lact, Lact versus Lact + Tre, and Lact + Tre versus Tre + 3-MA + Lact), \(p = 0.091\) (Lact + Tre), \(p = 0.3527\) (3-MA versus 3-MA + Tre). Representative microscopic images are shown in supplemental Fig. S2A. B and C, wild-type (Atg5 \(^{+/+}\)) and knock-out (Atg5 \(^{-/-}\)) Atg5 MEFs were transfected with EGFP-HDQ74 construct for 4 h and treated with either 100 mM trehalose or left untreated (Control) for 24 h. The percentage of EGFP-positive cells with EGFP-HDQ74 aggregates (B) and cell death (C) was assessed. \(p = 0.012\) (Tre in Atg5 \(^{+/+}\)), \(p = 0.153\) (Tre in Atg5 \(^{-/-}\)), \(p < 0.0001\) (Control in Atg5 \(^{+/+}\) versus control in Atg5 \(^{-/-}\)) for aggregation (B); \(p < 0.0001\) (Tre in Atg5 \(^{-/-}\)), \(p = 0.514\) (Tre in Atg5 \(^{+/+}\)), \(p < 0.0001\) (Control in Atg5 \(^{-/-}\) versus control in Atg5 \(^{+/+}\)) for cell death (C). D, Atg5 \(^{+/+}\) and Atg5 \(^{-/-}\) MEFs were treated with (+) or without (−) 100 mM trehalose for 24 h and analyzed for the levels of endogenous LC3-II by immunoblotting with antibody against LC3 (panel i) and densitometry analysis relative to actin (panel ii). \(p = 0.0047\) (Tre in Atg5 \(^{+/+}\) cells). ***, \(p < 0.001\); **, \(p < 0.01\); *, \(p < 0.05\); NS, nonsignificant.
some fusion, at the level of autophagosome formation (Fig. 4C). This result was confirmed in neurally differentiated non-mitotic PC12 stable cells by analyzing endogenous LC3-II (Fig. 4D).

We next assessed the effect of trehalose on autophagy by transfecting COS-7 cells with EGFP-LC3, which localizes only to autophagic membranes but not on other membrane structures (24). As EGFP-LC3 overexpression does not affect autophagic activity, numbers of EGFP-LC3 vesicles have frequently been used to assess autophagic activity. Assessment of autophagosome number with LC3 is both more sensitive and specific than electron microscopy (25). A significantly greater proportion of trehalose-treated cells expressing EGFP-LC3 had overt vesicle formation compared with untreated cells (Fig. 4E and supplemental Fig. S3B). Rapamycin, an inducer of autophagy, also increased the proportion of cells containing EGFP-LC3 vesicles (Fig. 4E). Trehalose also increased LC3 vesicle numbers in stable EGFP-LC3 HeLa cells (Fig. 4F).

**Stable Human Cell Lines Synthesizing Intracellular Trehalose Have Increased Autophagy**—To test whether intracellular trehalose has the same effect as trehalose in the medium, we used a human cell line engineered to produce trehalose endogenously. The cell line, QA1/12/9A (Tre), was derived from the human kidney cell line, T-REx 293, and carries endogenously the QA1/12/9A (Tre) cells have significantly more EGFP-LC3 vesicles compared with the control cell line (T-REx 293), suggesting that trehalose enhances autophagy by acting on intracellular targets (Fig. 5, A and B). Similarly, increased autophagy was seen in another clonal cell line QA1/12/2B (Tre) synthesizing intracellular trehalose, compared with T-REx 293 cells (Fig. 5C).

In uninduced QA1/12/9A (Tre) cells, trehalose is present at ~1 mM (probably due to leaky expression of *otsA*). Even this low intracellular trehalose concentration increased LC3-II levels, compared with uninduced T-REx 293 cells (Fig. 5D). The QA1/12/9A (Tre) cells have significantly more...
LC3-II after induction, compared with uninduced cells (supplemental Fig. S3C).

Trehalose Protects against Subsequent Pro-apoptotic Insults—We recently described that autophagy induction by rapamycin leads to protection against subsequent pro-apoptotic insults acting via the mitochondrial cell death pathway. We provided a plausible mechanism for the protective effect exerted by rapamycin. We observed decreased levels of several mitochondrial proteins after rapamycin pretreatment, suggesting enhanced clearance of mitochondria, which are degraded via autophagy (14). After pro-apoptotic insults, we observed decreased levels of cytosolic cytochrome c and activated caspases in cells pretreated with rapamycin, consistent with reduced mitochondrial load. We proposed that the protective effect of rapamycin can be accounted for by enhanced clearance of mitochondria by autophagy, thereby reducing cytosolic cytochrome c release and downstream caspase activation after pro-apoptotic insults (29).

Accordingly, we tested whether trehalose had similar protective effects. Trehalose reduced the levels of mitochondrial complex IV and cytochrome c in these cells, like rapamycin (Fig. 6, A and B). These proteins accumulate when autophagy is inhibited (29). Neither intracellular (30) nor extracellular (Fig. 6C) trehalose cause enhanced cell death in the cell types and paradigms we studied. Trehalose protected against subsequent toxicity of Bax, a pro-apoptotic protein that acts on mitochondria, against staurosporine toxicity and lowered levels of cleaved (active) caspase 3 (Fig. 6, D–G). No benefit was observed with short term incubation (Fig. 6, E and G), consistent with a model requiring prolonged autophagy to reduce mitochondrial load. These effects correlated with induction of LC3-II levels by long term trehalose treatment (Fig. 6H).

To test if these effects were dependent on autophagy, we tested if trehalose incubation protected against subsequent pro-apoptotic insults in autophagy-incompetent Atg5-deficient MEFs (20). No protective effects of trehalose against staurosporine-induced cell death were seen in the Atg5-deficient cells, while protective effects were seen in wild-type MEFs, or if Atg5 was transfected into the Atg5-deficient cells (Fig. 6, I and J). Thus, enhancing autophagy with trehalose protects against subsequent pro-apoptotic insults.

Trehalose-induced Autophagy Is mTOR-independent—We next studied whether induction of autophagy by trehalose was dependent on the known pathway that is negatively regulated by mTOR in mammalian cells. mTOR kinase activity can be inferred by the levels of phosphorylation of its substrates, ribosomal S6 protein kinase (S6K1, also known as p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) at Thr389 and Thr37/46, respectively, and the phosphorylation of ribosomal S6 protein (S6P) at Ser235/236, a substrate of p70S6K (31). While rapamycin (a specific mTOR inhibitor) reduced phosphorylation of p70S6K, S6P and 4E-BP1, trehalose had no
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Trehalose and Rapamycin Have an Additive Effect on the Clearance of Mutant Aggregate-prone Proteins—Because trehalose induces autophagy independently of mTOR inhibition, we confirmed the prediction that trehalose and rapamycin would have additive effects in reducing EGFP-HDQ74 aggregates and cell death in COS-7 cells, compared with the single treatments of trehalose or rapamycin (Fig. 8, A and B). Furthermore, trehalose and rapamycin together significantly facilitated greater clearance of aggregated and soluble EGFP-HDQ74 at 72 h and A30P α-synuclein at 16 h, compared with single either compound alone (Fig. 8, C and D). Here we chose early time points when we do not see dramatic reductions of the levels of these proteins when the cells are treated with either of the compounds alone. We have used saturating doses of rapamycin (0.2 μM) (13) and trehalose (supplemental Fig. S3E). COS-7 cells treated with both trehalose and rapamycin significantly had more EGFP-positive cells containing EGFP-LC3 vesicles than either of the compounds treated alone (Fig. 8E). Thus autophagic activity can be further enhanced through simultaneous induction of an mTOR-dependent and -independent route, compared with each of the route induced individually.

Trehalose Effects on Autophagy Are Not Caused by Glucose—Previously, we described that glucose-induced autophagy in an mTOR-dependent fashion, in contrast to trehalose (32). Whereas PC12 and other neuronal cells are not known to express trehalase (33), we further confirmed that our trehalose effects were not caused by glucose contamination by showing that combined glucose and trehalose treatment (at saturating doses) (32) (Fig. S3E) resulted in far more EGFP-HDQ74 clearance, compared with either compound alone (Fig. 8F). HEK-293 cells, from which the T-Rex 293 cells and stable inducible trehalose cells were generated, do not metabolize intracellular trehalose (30), consistent with suggestions that HEK-293 cells are derived from a rare neuronal cells present in the HEK primary culture.

**DISCUSSION**

We have demonstrated that trehalose induces autophagy. This has a number of beneficial consequences, including enhancing the clearance of disease-associated intracytosolic...
aggregate-prone proteins, and also protection against certain subsequent pro-apoptotic insults.

Trehalose reduced polyQ-mediated aggregation and toxicity caused by mutant huntingtin fragments (Fig. 1, A–C and supplemental Fig. S1A). Although this protective effect was previously thought to be caused by trehalose binding to expanded polyQ and stabilizing the partially unfolded mutant protein (7), here we show that a major consequence of trehalose is to induce autophagy thereby enhancing clearance of the mutant protein (Figs. 1D, 4, A–F, 5, B–D, and supplemental Fig. S3, A–C), because the ability of trehalose to reduce mutant huntingtin aggregates was abolished when autophagy was inhibited (Fig. 3, A–D and supplemental Fig. S2A).

Trehalose may have different effects on different aggregate-prone proteins. We recently described that trehalose protected against the toxicity of the polyalanine (poly(A))-expanded form of the polyadenine-binding protein nuclear 1 (PABPN1) that causes oculopharyngeal muscular dystrophy (OPMD) (35). Unlike mutant huntingtin, which forms both nuclear and cytosolic aggregates and is largely intracytosolic when soluble, mutant PABPN1 is essentially entirely intranuclear at steady state (8, 35). Trehalose enhanced proteasome-dependent clearance of mutant PABPN1 (35), in contrast with the current data which shows for the first time that trehalose induces autophagy, which is responsible for the enhanced clearance of autophagy substrates like mutant huntingtin and α-synuclein. Also mutant huntingtin aggregation was not reduced by trehalose in cells treated with the autophagy inhibitor 3-MA (Fig. 3A and supplemental Fig. S2A), while mutant PABPN1 aggregation was reduced by the sugar even when protein clearance pathways were blocked (35).

The differences that we observed with the longer expansions that cause polyQ disease (>37 repeats) versus the short (17 alanine) expansion causing OPMD are likely to be caused by the following factors. As expected for a nuclear protein, PABPN1 clearance is mediated by the proteasome, and autophagy has minimal influence (35). Autophagy can only act on extranuclear proteins and the significant cytosolic localization of huntingtin explains why it is efficiently cleared by autophagy. Indeed, Iwata et al. (36) reported that the clearance of polyQ expanded ataxin-1, which is largely nuclear, showed no obvious dependence on autophagy, while a major dependence on autophagy seen with the identical construct where the nuclear localization signal was mutated. Consistent with this paradigm, mutant huntingtin clearance is still enhanced by inducing autophagy with trehalose (or other autophagy-inducing agents like lithium or inositol monophosphatase inhibitor (13)) in cells treated with the proteasome inhibitor, lactacystin (Fig. 3A and supplemental Fig. S2A). However, mutant PABPN1 clearance induced by trehalose is blocked by this proteasome inhibitor (35).

We have further confirmed these data by comparing mutant huntingtin and mutant PABPN1 aggregation in autophagy-competent (Atg5+/+) and -incompetent (Atg5−/−) MEFs. In untreated Atg5−/− cells, mutant huntingtin aggregation was significantly increased as compared with Atg5+/+ cells; however, no significant effect on mutant PABPN1 aggregation was observed (Fig. 3B and supplemental Fig. S4A). When these cells were treated with trehalose, the PABPN1 aggregation was significantly reduced even in Atg5−/− cells (suggesting that trehalose acts independently of autophagy for PABPN1), while no significant reduction of huntingtin aggregation was seen in Atg5−/− cells (confirming that the autophagy-inducing ability of trehalose is a major factor behind its effect in reducing mutant huntingtin aggregation in cells) (Fig. 3B and supplemental Fig. S4A). Trehalose appears to induce autophagy efficiently in cells containing either mutant huntingtin or mutant PABPN1 (supplemental Fig. S4, B–D).

The ability of trehalose to attenuate poly(A) aggregation but not polyQ aggregation in cells treated with inhibitors of their clearance may be due to the different structures (37, 38) and detergent resistance of these aggregates. PolyQ, but not poly(A), aggregates formed in cells are resistant to 4% SDS and 4% Triton (39).

Enhanced autophagy occurred in cells that synthesize intracellular trehalose at ~20 mM (Fig. 5, A–C and supplemental Fig. S3C), and in even uninduced QA1/12/9A (Tre) cells where trehalose is present at ~1 mM (probably because of basal levels of otsA expression) compared with uninduced T-REx 293 cells (Fig. 5D). Although 100 mM extracellular trehalose had autophagy-inducing effects similar to endogenous trehalose, no increases in LC3-II were seen at extracellular concentrations of 10 mM or lower (supplemental Fig. S3A). Thus, it is likely that the trehalose is acting intracellularly, and not because of secretion of intracellular trehalose.

Trehalose does not readily cross cell membranes, but can be efficiently loaded into mammalian cells via fluid-phase endocytosis and pinocytosis (3, 40) like other small molecules that do
not readily cross membranes (41). Extracellular trehalose is likely acting in the cytosol in the same compartment(s) as the trehalose in the stable cells. There are many precedents for cells taking up extracellular material that does not readily cross cell membranes by pinocytosis or endocytosis and then releasing it into the cytosol, including MHC class II antigen presentation (42), and DNA transfection methods (43, 44). Thus, it is entirely plausible that high concentrations of extracellular trehalose can act via effects in the same cytosolic compartments as plasmid-synthesized trehalose, outside of membrane-bound pinosomes or endosomes.

The failure of trehalose to accelerate the clearance of wild-type α-synuclein and EGFP-HDQ23 (Figs. 1F and 2C and supplemental Fig. S1E) is entirely consistent with previous data suggesting that the dependence of proteins on the macroautophagy pathway for their clearance correlates with their propensity to aggregate (10, 12). The non-aggregate prone wild-type proteins are efficiently cleared by the proteasomes. For proteins that are accessible to both pathways, the greater efficiency of the ubiquitin–proteasome system compared with basal levels of macroautophagy makes the proteasomes the favored and dominating clearance route. When a cytosolic protein is aggregate-
prone and a poor proteasome substrate, then macroautophagy becomes a major clearance route by default. Under these circumstances, the macroautophagy route becomes more effective than the proteasome.

Our data suggesting that trehalose acts independently of mTOR (Fig. 7, A–G and supplemental Fig. S3D) may be explained by two possibilities. The two pathways may be truly independent and may act even on different components of the autophagy machinery. Alternatively, trehalose may act on a component in the pathway between mTOR and autophagy. Unfortunately, this is impossible to test without knowing what these components are, and they are currently not characterized in the context of mammalian autophagy. Trehalose is not simply increasing the levels of autophagy regulators like beclin-1/Atg6, Atg12, Atg7, or Atg5 (supplemental Fig. S5, A–D). The new role of trehalose as an autophagy inducer could have contributed to its protective effect in HD mice (7) because mutant huntingtin is an autophagy substrate and upregulating autophagy reduces the levels of mutant huntingtin and protects against its toxicity in cells, transgenic Drosophila, and mouse models of HD (10, 11).

Trehalose may also protect against secondary apoptotic effects (Fig. 6, A–J) occurring in HD in an autophagy-dependent manner. Furthermore, the ability of trehalose to protect against diverse pro-apoptotic insults in an autophagy-dependent manner may account for some of its previously poorly understood beneficial properties in a diversity of contexts (1). As far as we are aware, these anti-apoptotic effects of trehalose have not been reported previously. They also add to our previous data showing that rapamycin-induced autophagy is protective against subsequent pro-apoptotic insults and argue that autophagy induced by either mTOR-dependent or mTOR-independent pathways may be beneficial in this context.

It is difficult to be certain that prolonged up-regulation of autophagy would be without risks. One theoretical concern is that enhanced mitochondrial turnover might be deleterious. Increasing autophagy decreases the mitochondrial load to a lower steady-state level but will not result in eventual total depletion of mitochondria (29, 45). Very large decreases in mitochondrial load may be associated with deleterious effects due to a decrease in oxidative phosphorylation. However, it should be noted that although such relationships are complex and tissue-dependent, the activities of some respiratory complexes can be reduced by 25–80% before respiration or ATP synthesis in brain mitochondria are affected, and the activity of rat liver complex III can be decreased by 45% before respiration is affected (46). Thus, it is likely to be possible to induce autophagy and reduce mitochondrial load to levels that have substantial protective effects against proteinopathies but do not affect respiration. It is also worth noting that rapamycin is used chronically in people, and we are not aware of side effects that are caused by autophagy. Also, rapamycin attenuates mutant huntingtin toxicity in HD fly and mouse models (11).

Our data also raise the possibility of combination therapy using trehalose and rapamycin, which may be more effective in such diseases (Fig. 8, A–E). This new role of trehalose coupled with its anti-aggregation property and lack of toxicity increases its efficacy for treating a spectrum of protein conformational disorders like HD and PD. Recent data suggest that autophagy induction may be effective in certain infectious diseases, because mycobacteria and group A streptococci are cleared by this process (34, 47, 48). Therefore, it would be interesting to test the efficacy of trehalose as a possible safe addition to the armoury for treating such disorders.

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