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Trehalose impairs aggregation of PrP^{Sc} molecules and protects prion-infected cells against oxidative damage

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ABSTRACT

Neurodegenerative disorders such as Alzheimer's, Huntington's, and prion diseases are characterized by abnormal protein deposits in the brain of affected patients. In prion diseases, a key event in the pathogenesis is the conversion of the normal prion protein (PrP^{c}) into abnormal protease resistant PrP^{Sc} deposits, a phenomenon associated with a higher sensitivity to oxidative stress *in vitro*. In cellular models of Alzheimer and Huntington diseases, the disaccharide trehalose has been shown to be effective in inhibiting huntingtin and Aβ peptide aggregates and reducing their associated toxicity. We show in this study that trehalose treatment of prion-infected cells decreases the size of *de novo* produced PrP^{Sc} aggregates and modify their subcellular localization. Despite the fact that trehalose does not modify the protease resistance properties of PrP^{Sc} molecules, it significantly protects prion-infected cells from induced oxidative damage, suggesting that this compound is of therapeutic interest.

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Neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington diseases, and prion diseases are brain proteinopathies characterized by the accumulation of abnormal protein deposits in the brain of affected patients. Prion diseases are fatal neurodegenerative disorders where abnormal deposits are rich in PrP^{Sc}, a protease-resistant isoform of the cellular prion protein PrP^{C} [1,2]. According to the Prion hypothesis, PrP^{Sc} convert the normal, protease-sensitive form PrP^{c} into its protease-resistant PrP^{Sc} counterparts [3,4]. Whether the accumulation of such aggregates in prion proteinopathies is the prime cause of the disease is a question still to be resolved. The attempts to discover aggregation inhibitors remained poorly efficient in animal models. However, recent *in vitro* screening studies of inhibitors of polyglutamine-mediated protein aggregation in models of Huntington disease demonstrated that various disaccharides reduced the formation of polyglutamine aggregates. Trehalose was the most effective of all the disaccharides tested, and its effect was correlated with a decrease of cell death and an extended lifespan of mouse models of Huntington disease [5]. Trehalose treatment could prevent the formation of huntingtin aggregates, and delayed the progress of symptoms such as motor dysfunction [5]. Trehalose has also been shown to be very effective in inhibiting aggregation of the Alzheimer's related β-amyloid peptide Aβ and in reducing its cytotoxicity [6]. Other studies revealed that it can accelerate the clearance of α-synuclein [7] and inhibit the formation of fibrillar aggregates of insulin [8].

Trehalose is composed of two molecules of glucose connected through an α,α-1,1 linkage. It has found numerous applications in the food industry and is widely used in bakery goods, beverages, confectionery, fruit jam, breakfast cereals...as a texturizer, stabilizer, or formulation aid with a low sweetening intensity. Trehalose was first isolated from ergot of rye. Found extensively but not abundantly in nature, it is produced naturally in a wide number of micro organisms such as fungi, plants, and invertebrate animals. It is implicated in anhydrobiosis, the ability of plants and animals to withstand prolonged periods of desiccation. As an extension of its natural capability to protect biological structures, trehalose has been used for the preservation and protection of biological materials during dehydration [9]. Trehalose also serves as a bioprotectant against various types of stress [10]. In yeast, trehalose has been shown to protect cells from oxidative damage and to prevent aggregation [10,11]. Following an extensive study that describe its ability to inhibit protein misfolding [12], trehalose biological properties suggest a number of potential uses in neurodegenerative disorders characterized by protein misfolding and protein aggregation.

Concerning prion disorders, whether trehalose is able to impair PrP^{Sc} aggregation is the question which has been addressed in this study. We have shown that trehalose treatment of prion-infected cells decreases the size of *de novo* produced PrP^{Sc} aggregates and modifies their subcellular localization. Prion infection results in an alteration of the molecular mechanisms promoting cellular

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resistance to reactive oxygen species in prion infected cells. Prion-infected cells displayed a higher sensitivity to induced oxidative stress when compared with non-infected cells [13]. We show in this study that trehalose protects prion-infected cells against induced oxidative stress.

Results and discussion

Trehalose treatment modifies the size and the subcellular localization of PrP^{Sc} aggregates

To study the effect of trehalose treatment on prion-infected cells, ScN2a were treated for 3 or 6 days with PBS, 50 μM trehalose or 50 μM glucose, and then analyzed by indirect immunofluorescence. After fixation and permeabilisation, a 3 M guanidine thiocyanate treatment was performed to expose epitopes which were previously hidden in PrP^{Sc} [14]. Immunofluorescence microscopy studies were carried out using an antibody specific for the C-terminus extremity of PrP^{Sc}.

In control cells, most PrP^{Sc} was found in large intracellular aggregates localized to perinuclear structures reminiscent of the Golgi apparatus (Fig. 1A) as described [14] [15]. In trehalose-treated cells, PrP^{Sc} aggregates were smaller, more diffuse and dispersed to the periphery of the cells (Fig. 1A). As a control, we also tested whether other saccharides could have the same effect on PrP^{Sc} aggregation. Treatment with glucose (Fig. 1A) and saccharose (not shown) did not modify PrP^{Sc} aggregation, suggesting that this effect is not universal with all saccharides. In addition, we investigated whether the effect on PrP^{Sc} aggregates could be observed after shorter incubation times of the cells with trehalose. Cells were treated with 50 μM trehalose for 48 or 72 h and PrP^{Sc} was detected by indirect immunofluorescence. The effect of trehalose on PrP^{Sc} aggregates could not be detected with incubation times shorter than 72 h (Fig. 1B). For studies on huntingtin aggregates, it has been suggested that trehalose may reduce aggregation by preventing further assembly of truncated huntingtin rather than by reversing aggregates formation after it has occurred [5]. The half-life of PrP^{Sc} is thought to exceed 24 h [16]. Because the trehalose effect on PrP^{Sc} aggregates could not be detected with treatments shorter than 72 h, our results suggest that trehalose can not dissociate pre-existing PrP^{Sc} aggregates but rather prevents the formation of *de novo* aggregates.

Several hypotheses have been proposed to explain protein folding stabilization by trehalose. Trehalose molecules can form a "coating layer" around the protein, thus reducing the number of protein-solvent hydrogen bonds and accompanying electrostatic solvation forces. This could lead to an increase in intra protein interactions, and a stabilization of the protein native structure [17]. In another hypothesis, water molecules could be replaced by trehalose molecules providing the required interactions to stabilize protein folding [18], or be trapped on the protein surface by a cage of sugar molecules forming a viscous layer around the protein [19]. Whatever the mechanism involved, trehalose could stabilize proteins in their native state after heat shock, and reduced aggregation of proteins which have been denatured [12]. In the case of PrP^{Sc} molecules, one could imagine that the trehalose molecules alter the environment of surrounding water molecules, making aggregation less energetically favourable, such as described in yeast [6,12].

Trehalose treatment does not interfere with PK resistance or detergent insolubility of PrP^{Sc}

Based on the assumption that PrP^{Sc} was the pathogenic entity of prion diseases, search for therapeutics agents has been aimed at destabilizing PrP^{Sc} or prevent conversion of PrP^{c} into PrP^{Sc} [31]. Several compounds such as Bis-ANS [20], beta-sheet breaker peptides [21], branched polyamines [22] and Congo red [23,24] partially disassembled PrP^{Sc} to a protease-sensitive form. Because trehalose treatment modified the pattern of PrP^{Sc} aggregates, we wondered whether trehalose could modify the biochemical properties of PrP^{Sc} i.e. proteinase K resistance and detergent insolubility. Cells were treated for 6 days in the presence of different concentrations of trehalose or with Congo red (5 μg/ml). When tested on prion-infected cultures, Congo red interfered with the generation of PrP^{Sc} and "cured" prion-infected cells [25]. After trehalose or Congo red treatment, cells were lysed and equal amounts of proteins were digested with proteinase K. As shown on Fig. 2A, trehalose treatment did not have any major effect on PrP^{Sc} production, even at higher concentrations of trehalose, whereas Congo

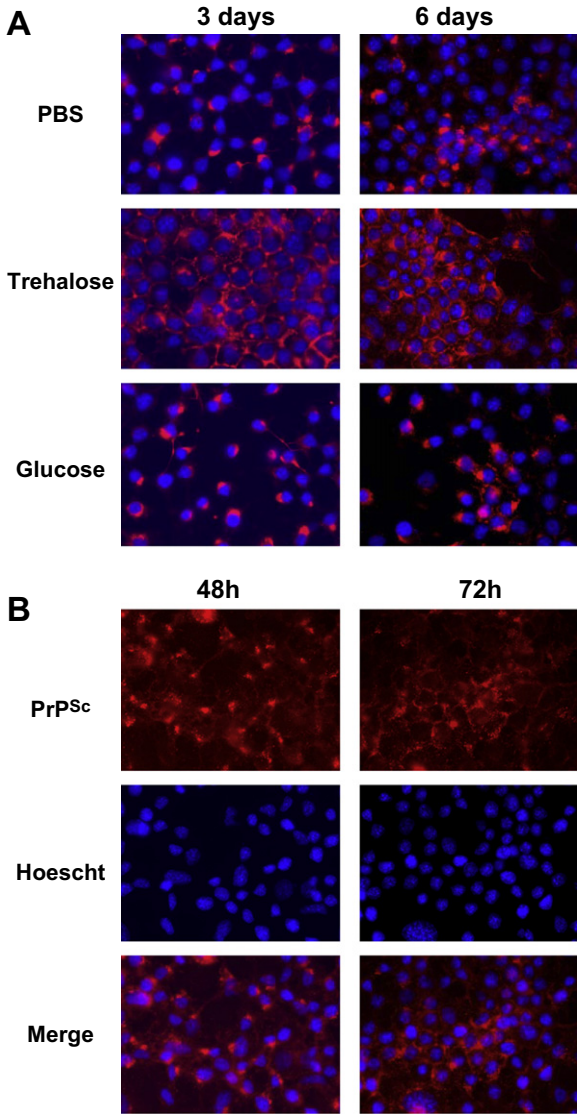

Fig. 1. Immunofluorescence detection of PrP^{Sc} ScN2a cells were incubated in 50 μM trehalose or glucose for the indicated times, fixed, permeabilised, treated with 3 M guanidine thiocyanate and processed for indirect immunofluorescence labelling with antibodies against PrP (SAF61 antibody), followed by Alexa555-conjugated anti-mouse secondary antibodies. Cells were viewed on a Leica microscope with blue excitation/emission settings to detect Hoechst staining of the nuclei (B) and with red excitation/emission settings to detect PrP (A,B). (A) 3 and 6 days treatment with trehalose, (B) 48–72 h treatment with trehalose

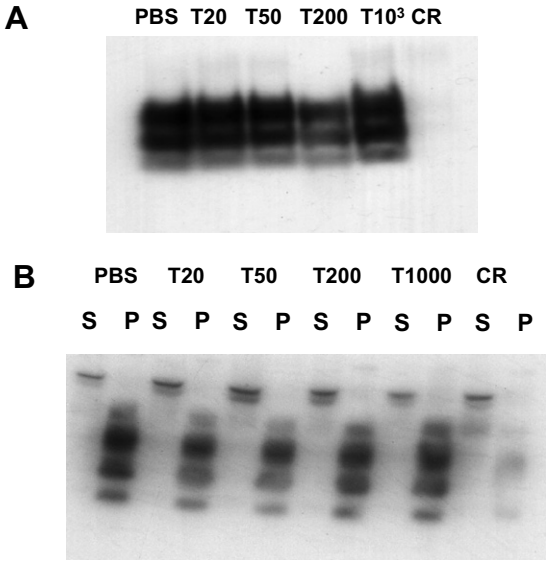

Fig. 2. Trehalose treatment does not modify proteinase K resistance and detergent insolubility of PrP^{Sc} (A) ScN2a cells were cultured for 6 passages in the presence of 50 μM Trehalose (TreH). Cells were lysed and equal amounts of total proteins content were treated with proteinase K for 30 min at 37 °C. (B) ScN2a cells were treated for 6 days with increasing concentrations of trehalose (ranging from 20 to 1000 μM). Cells were lysed and the lysates were centrifuged at 14000 rpm for 45 min at 4 °C and the pellets (P) and the supernatants (S) were isolated. PrP^{Sc} was detected by Western blot with SAF Mix antibodies.

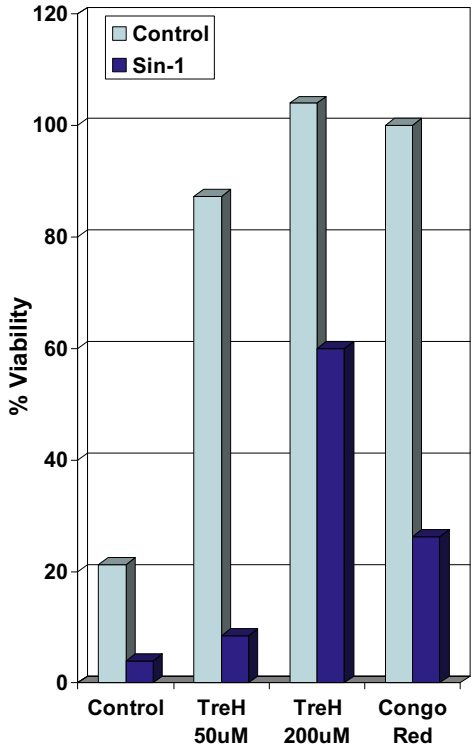

Fig. 3. Trehalose protects prion infected cells against SIN-1 induced oxidative damage ScN2a cells were treated for 3–4 days with different concentrations of trehalose prior to oxidative insult. Oxidative stress was induced by a 3 h treatment of SIN-1 as described in Experimental methods section. Cell viability was assessed by CellTiter 96® AQ_{ueous} Assay (Promega). Data are expressed as a percentage of cell survival compared to the Congo red treated control cells. They are representative of three separate experiments ($n = 5$ for each experiment).

red, as previously demonstrated, completely inhibited PrP^{Sc} production.

In addition to protease resistance, another hallmark property of the scrapie prion protein is its insolubility in non-denaturing detergents [26]. ScN2a cells were treated with different concentrations of trehalose or Congo red (5 μg/ml). Insolubility of PrP^{Sc} in prion-infected cells was evaluated by sedimentation after high-speed centrifugation of the cell detergent lysate. Following trehalose incubation, the insolubility of the molecule was not modified compared with control cells, with most of the PrP^{Sc} being mostly detergent insoluble (Fig. 2B). As expected, Congo red treatment cured the cells from PrP^{Sc}. Despite interfering with the size and localization of PrP^{Sc} aggregates, trehalose treatment did not modify intrinsic PrP^{Sc} biochemical properties.

Questions have often arisen whether the large amyloid fibrils observed in the brain of patients affected by prion diseases were the prime cause of the disease. When PrP^{Sc} aggregates from scrapie-infected hamster brains were size fractionated and analyzed with respect to their infectivity and their converting activity, it was apparent that the smaller PrP oligomers (14–28) molecules) were the most efficient initiators of prion diseases [27]. Even by dispersing PrP^{Sc} aggregates, trehalose does not modify the abnormal biochemical properties of PrP^{Sc} which suggests that, as described in Silveira et al. study, incomplete attempts to destabilize PrP^{Sc} aggregates for therapeutic purposes might increase the production of the pathological aggregates, and therefore enhance disease associated symptoms.

Trehalose treatment protects prion-infected cells from SIN-1 induced oxidative stress

The oxidant SIN-1 (3-morpholinosydnonimine) induced in neuronal cells a rapid and reproducible cell death that was higher in prion-infected cells [13]. To study whether trehalose could protect mammalian prion-infected cells against SIN-1 induced cell death, ScN2a cells were challenged with SIN-1. As shown in Fig. 3, trehalose produced a dose-dependent increase in cell viability in SIN-1 challenged cells, as compared with control cells (60% viability in the presence of 200 μM Trehalose versus 5%). Even in the absence of SIN-1, cell viability was increased in the presence of trehalose suggesting that this sugar has an overall protective effect on prion-infected ScN2a cells. Trehalose protects prion-infected cells against induced oxidative stress despite the presence of PrP^{Sc}, whereas with Congo red cells are cleared from abnormal PrP^{Sc}.

In order to test whether this effect was due to a stimulation of cell proliferation rather than a real protective effect, we measured BrdU incorporation in proliferating non-treated and trehalose-treated cells. Counting of the BrdU-labelled nuclei showed that trehalose treatment did not enhance cell proliferation (Table 1). This suggested that the trehalose effect on ScN2a cell viability observed with SIN-1 challenged ScN2a cells was not due to an increase of

Table 1
Effect on trehalose on cell proliferation

Cell treatment	% BrdU incorporation
Control	55.4
Congo red	52
Glucose 50 μM	49
Trehalose 50 μM	49.3
Trehalose 200 μM	44.7

ScN2a cells were cultured for 3–4 days in the presence of trehalose, glucose or Congo red at the indicated concentrations. The next day, BrdU (Roche Applied Science) was added to cells. Cells were fixed and processed according to the manufacturer's instructions. BrdU positive cells were visualized by immunofluorescence and counted. Data are expressed as percentage of BrdU positive cells compared to total number of cells. It can be seen that trehalose treatment had no effect on cell proliferation.

cell proliferation but rather to a real inhibition of SIN-1 induced cell death.

Because prion-infection impairs the cellular response to oxidative stress, it has been proposed that PrP^{c} could function as a specific stress sensor leading to resistance of cells to oxidative stress [28]. Conversion into the abnormal PrP^{Sc} form would impair its ability to sense the oxidative stress of the cells. A role for trehalose in protection against oxidative damage in mammalian cells has not been proposed previously in the literature. Our data are the first evidence for such a protective role of trehalose. In order to explain this effect on prion-infected mammalian cells, one has to refer to data published in other living organisms. In yeast, trehalose has been proposed to protect proteins from oxidative damage by inhibiting the covalent modification of proteins by oxygen radicals [10]. The capacity of trehalose to reduce oxidative stress-induced modifications of proteins and its ability to prevent protein aggregation most likely involve different biochemical mechanisms. On the other hand, proteins which have been damaged by free radicals have a high tendency to denature and to aggregate, and therefore both mechanisms may be important in protecting cells against oxidative stress.

The results presented in this study provide the first evidence that trehalose is very effective in impairing aggregation of PrP^{Sc}, and in protecting prion-infected cells from induced oxidative stress. Trehalose has been approved by the FDA (US Food and drug Administration) and toxicological studies both in animal and humans indicate that trehalose is very well tolerated. Given its ability to inhibit aggregation and to pass the blood–brain barrier, trehalose has been proposed as a potential powerful therapeutic agent for the treatment of Alzheimer and Huntington diseases.

It is tempting to speculate that trehalose could be tested for the treatment of prion diseases. However, because in prion diseases the most infectious units are smaller than the large amyloid fibrils often observed in infected tissues [27], our data raise a large safety concern whether trehalose could be efficient for *in vivo* treatments, or on the contrary whether such a treatment could diminish the size of PrP^{Sc} aggregates, resulting in an unintended increase in infectivity of the prion particles. It will be essential to determine whether consumption of trehalose could enhance prion diseases pathologies, considering that trehalose is widely used in the food industry and that the daily predicted intake of trehalose from all proposed uses, except chewing-gum, is estimated to be 10 g/day for an adult in the USA.

However, the fact that trehalose is able to disperse PrP^{Sc} aggregates and protects neuronal cells from induced oxidative stress should be taken in consideration. Consequently, the use of trehalose as a potential therapeutic agent for prion diseases will require methodical studies in several different models of prion-infected animals which are presently under investigation, and its effect on prion propagation will be carefully monitored.

Experimental methods

Cells, buffers, and reagents. N2a and ScN2a cells used in this paper correspond, respectively, to PrP over expressing N2a cells (N2a#58) and prion strain 22L-infected N2a#58 cells (N2a#58-22L) described previously [29]. The infected status of the cells was regularly assessed by proteinase K digestion analysis to confirm the presence of PrP^{Sc}. Cell culture conditions and monoclonal antibodies used to improve PrP detection were described in Beranger et al. [30]. Other antibodies were from Santa Cruz Biotechnology Inc. (USA). All other reagents were from Sigma (France).

Immunofluorescence microscopy. ScN2a cells were fixed in 4% paraformaldehyde in PBS for 30 min, washed 3 times with PBS and permeabilised in 0.5% Triton X-100–PBS for 5 min. The cells were then treated for 5 min in 3 M guanidine thiocyanate and further incubated overnight at 4 °C with primary antibodies diluted in 5% milk in PBS. After three washes in PBS, cells were further incubated with Alexa-555 anti-mouse antibodies (dilution 1/10000) for 1 h at room temperature. Nuclei were stained with Hoechst 33286. Cells were washed and mounted in FluorSave reagent (Calbiochem). Images were collected and processed using a Leica microscope.

Proteinase K digestion, assay of detergent insolubility, and Western blotting. Cells were washed in PBS and lysed for 20 min at 4 °C in Triton/DOC lysis buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris pH 7.5 and protease inhibitors). Post nuclear supernatants were collected and assayed for total protein content with a BCA Protein Assay kit (Pierce). For detection of protease resistant PrP proteins, equivalent volumes of samples were digested with 16 μg of proteinase K per mg of protein at 37 °C for 30 min, and the digestion stopped by incubation with Pefablock protease inhibitor (1 mM) (Roche Diagnostics) for 5 min on ice. The samples were centrifuged at 14000 rpm for 45 min at 4 °C and the pellets re-suspended in 25 μl of SDS loading buffer and boiled for 5 min. Proteins were analyzed by Western blotting according to Beranger et al. [30].

For the detergent insolubility assay, post-nuclear supernatants were centrifuged at 20,000*g* for 1 h at 4 °C to separate detergent-soluble (S) and detergent-insoluble (P) proteins. Insoluble fractions were dissolved in SDS loading buffer. Proteins from soluble fractions were methanol precipitated, and proteins from both fractions were immunoblotted.

Cell viability assay. ScN2a cells were cultured for 3–4 days in the presence of Trehalose, Glucose (used as control) or Congo red (known to inhibit PrP^{Sc} formation in ScN2a cells) at the concentrations indicated in the figure legends, and then plated in a 96 wells plate at 6000 cells/well. The next day, the medium was changed for DMEM medium without sodium pyruvate and serum. Twenty-four hours later, the cells were challenged for 3 h with 500 μM 3-morpholinosydnonimine (SIN-1) (Invitrogen Life Technologies) in PBS with Ca^{2+} and Mg^{2+}. The medium was then changed and the cells were incubated for an additional 24 h in DMEM without sodium pyruvate and in the presence of 5% foetal calf serum without the drugs. Cell viability was assessed with the CellTiter 96® AQ_{ueous} Assay (Promega) [32].

BrdU incorporation assay. ScN2a cells were cultured for 3–4 days in the presence of Trehalose, Glucose or Congo red at the concentrations indicated in the table, then plated in 12 wells plates at 2×10^5 cells/well and further cultured in the presence of the different compounds. The following day, BrdU (Roche Applied Science) was added and the cells incubated for 45 min. Cells were fixed and processed. BrdU positive cells were visualized by immunofluorescence and counted.

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