Intracellular trehalose improves osmotolerance but not desiccation tolerance in mammalian cells

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Abstract Trehalose has been shown to play a role in osmotolerance or desiccation tolerance in some microorganisms, anhydrobiotic invertebrates and resurrection plants. To test whether trehalose could improve stress responses of higher eukaryotes, a mouse cell line was genetically engineered to express bacterial trehalose synthase genes. We report that the resulting levels of intracellular trehalose (~80 mM) are able to confer increased resistance to the partial dehydration resulting from hypertonic stress, but do not enable survival of complete desiccation due to air drying.

Key words: Anhydrobiosis; Desiccation; Mammalian cell; Osmotolerance; Trehalose

1. Introduction

Trehalose (α-D-glucopyranosyl-1,1-α′-D-glucopyranoside) is a non-reducing disaccharide of glucose which is involved in several physiological stress responses, including osmotolerance [1,2] and anhydrobiosis [3,4]. Both of these phenomena are responses to loss of cellular water, either through hypertonicity of the environment or through desiccation, respectively. The chief role of trehalose in osmotolerance is as a compatible solute, acting to counterbalance extracellular osmotic pressure [5,6] and to stabilise biomolecules by preferential exclusion within the cell [7]. In contrast, in anhydrobiosis trehalose has been proposed to act as a water replacement molecule [8], as a means of avoiding damaging phase transitions in biomembranes [9] and as a glass former, including biological molecules in a protective matrix [10]. Since the early stages of desiccation can involve exposure to hypertonic environments, trehalose may also play a role as a compatible solute in anhydrobiosis.

Given the involvement of intracellular trehalose in the cellular response to reduced water activity in some organisms, it may be possible to engineer other cell types sensitive to water loss to produce trehalose and thereby increase their resistance to water stress. Accordingly, improved desiccation tolerance has been demonstrated in Gram-negative bacteria with high intracellular trehalose concentrations [11–13] and in transgenic plants expressing bacterial trehalose synthase genes [14].

A similar approach may be applicable to mammalian cells, which are generally regarded as very sensitive to dehydration. They are unable to synthesise trehalose and, because trehalose will not cross intact cell membranes, they cannot easily be loaded with it. A genetic engineering approach in which mammalian cells are made to express foreign trehalose synthase genes has been proposed as a route towards desiccation-tolerant mammalian cells [15]. Indeed, recently, human fibroblasts infected with an adenoviral vector transiently expressing the *Escherichia coli otsA* and *otsB* trehalose synthase genes, and accumulating 1–1.5 nmol trehalose per 10^6 cells (0.3–0.5 pg/cell), have been reported to survive complete desiccation for up to 5 days [16], although these data have been the subject of some debate [17,18]. We now report genetically engineered mammalian cell lines which accumulate up to ~40 pg/cell (~80 mM) intracellular trehalose and show that, although they exhibit improved resistance to the partial dehydration resulting from hypertonic shock, they are unable to survive complete desiccation.

2. Materials and methods

2.1. DNA constructs

The *E. coli otsA* and *otsB* genes were amplified by PCR from plasmid pFF15 [19] using the following primers: *otsA* 5′ end: 5′-GGGTCGACCATGGGTCGTTTAGTCGTAGTA-3′; *otsA* 3′ end: 5′-GGGGTACCTACGCAAGCTTTGGAAAGG-3′; *otsB* 5′ end: 5′-GGACTAGTGCTAGCCATGGCAGAACCGTTAACCGAAACC-3′; *otsB* 3′ end: 5′-GGAATTCTTAGATACTACGACTAAACG-3′.

The *otsA* gene was placed under the control of the *Drosophila melanogaster* hsp70 promoter from pF1 [20] with an SV40 polyadenylation signal. The *otsB* gene was driven by a constitutive SV40 early promoter with an SV40 polyadenylation signal.

2.2. Cell lines and transfection

The thymidine kinase-deficient mouse fibroblastoid cell line, $LMTK^-$ (ATCC CCL-1.3), was routinely grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 10 mM glutamine, 500 U/ml penicillin and 0.5 mg/ml streptomycin ('supplemented DMEM'). Cell lines were transfected by incubating for 12 h in the presence of DNA-calcium phosphate precipitate (Profection kit, Promega, Madison, WI, USA), then washed with phosphate-buffered saline (PBS; pH 7.4), exposed to 15% glycerol in HEPES-buffered saline (pH 7.1) for 2 min, and washed again. After 24 h transfectants were selected with 1 mg/ml G418 (Geneticin, Sigma-Aldrich, Poole, UK) and/or 250 μg/ml zeocin (Invitrogen, Groningen, The Netherlands) depending on the constructs used. Heat shock was carried out on attached cells in a water bath at 42°C for 1 h. Lines synthesising trehalose after heat shock were chosen, and one cell line, designated L-Tre1, was used for detailed study.

2.3. Intracellular trehalose determination

Between 10^6 and 10^7 cells were washed with PBS (pH 7.4), trypsi-

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nised, counted, centrifuged and resuspended in 1 ml 70% ethanol. To this, 0.1 mg sucrose was added as internal reference. Trehalose extraction was carried out at 70°C for 1 h, samples were centrifuged at 18 000×*g* for 5 min and the supernatant collected. The sample volume was then doubled with water and 250 μl of chloroform was added. After 5 min centrifugation at 18 000×*g* the aqueous phase was collected and dried in glass vials. Samples were derivatised at 55°C for 2 h with 100 μl of an equal mix of pyridine and *N*,*O*-bis(trimethylsilyl)-trifluoroacetimide plus 1% trimethylchlorosilane (Pierce, Rockford, IL, USA). Gas chromatography was carried out on a Perkin Elmer 8410 fitted with a 30 m×0.25 mm DB-5 column (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas with a flow rate of 50 ml/min. Injector and detector were set at 320°C and column temperature was set at 230°C for the first 3 min, and then increased at 3°C/min up to 290°C. Trehalose concentrations were calculated against the sucrose standard with reference to cell counts. Cell volumes were assumed to be 1.53 pl and were calculated on the basis of an average measured isotonic cell diameter of 14.31 μm for the L-Tre1 cell line.

2.4. Hypertonic shock

For cells in suspension, ~10^6 cells were tested after centrifugation at 335×*g* for 5 min and replacement of the medium with supplemented DMEM containing either 0.5 M NaCl, 1 M sorbitol or 1 M trehalose. The resulting final osmolarities, as measured with a 13/13DR-Autocal freezing point osmometer (Roebling, Berlin, Germany), were 1208, 1298 and 1420 mOsm respectively, compared to 298 mOsm for the supplemented DMEM alone (isotonic). For attached cells, hypertonic solutions were added directly to sub-confluent cells after removal of isotonic medium. All cells were incubated in the hypertonic media for 2 h at 37°C after which the medium was diluted 1:2 with isotonic supplemented DMEM, incubated for 15 min, and then completely replaced with supplemented DMEM.

2.5. Desiccation

To test for desiccation tolerance, cells were grown to near confluence attached to collagen-coated coverslips. One set of slides was dipped in a 1 M trehalose solution prior to drying. Coverslips were air-dried in a laminar airflow cabinet at ambient temperature (22–24°C) for 1 h and were then immediately rehydrated in supplemented DMEM and tested for cell viability.

2.6. Cell viability assays

Dye exclusion viability assays were performed, 6 h after hypertonic shock, in 0.2% trypan blue. Cells were counted with a haemocytometer and a total of 20 counts per sample were carried out. Differences were taken as significant for $P<0.05$ in a standard Student's *t*-test. Long-term viability was assayed by the ability to form colonies after 5 days in culture. Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA) assays were carried out 30 min after hypertonic shock following the manufacturer's protocol, where cells were incubated at room temperature for 30 min in 2 μM calcein AM and 4 μM ethidium homodimer-1. Fluorescence was viewed simultaneously using a 450–490 nm excitation filter and a 505 nm longpass filter set.

3. Results

3.1. Trehalose induction profile

Transfection of the LMTK$^-$ cell line with a heat shock-inducible *otsA* gene, which encodes the *E. coli* trehalose-6-phosphate synthase, together with a constitutively expressed *otsB* gene, encoding the *E. coli* trehalose-6-phosphate phosphatase, resulted in a cell line L-Tre1 which produces significant amounts of trehalose upon heat shock (Fig. 1). Intracellular trehalose concentration increases steadily after heat shock, reaching its maximum of ~40 pg/cell (~80 mM) after 30 h and declining rapidly after this. This decline is attributed to the limited period that the trehalose synthase genes are active after heat shock: once the respective mRNA and enzyme pools decline, intracellular trehalose will be diluted out by the continued growth of the cell population. Non-induced

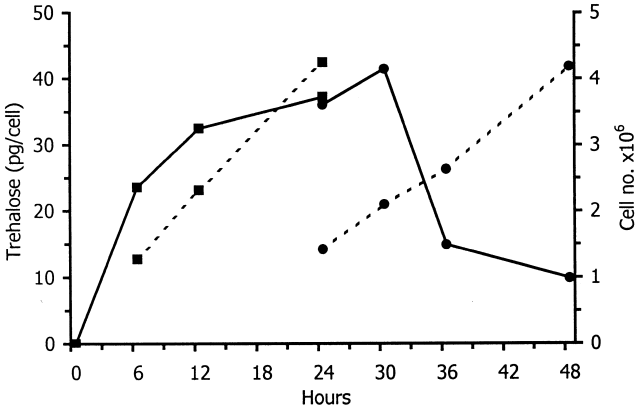

Fig. 1. Intracellular trehalose accumulation profile in L-Tre1 cells after heat shock at 42°C for 1 h. The experiment was set up with two distinct sets of flasks with relatively high or low initial cell density after heat shock to allow maximal cell growth throughout. High initial density flasks were harvested for cell counting and trehalose estimation during the first 24 h period (filled squares); low initial density flasks were harvested during the second 24 h (filled circles). Trehalose concentration is shown as a solid line; cell number as a broken line.

L-Tre1 and the control LMTK$^-$, with or without heat shock, produced no detectable trehalose (<0.2 pg/cell). The 30 h time point was chosen for subsequent experiments on osmotolerance and desiccation tolerance.

3.2. Osmotolerance

L-Tre1 cells were induced to accumulate trehalose by heat shock and, together with control cells lacking intracellular trehalose, subsequently subjected to hypertonic conditions for 2 h, either in suspension or while attached to the culture surface (Fig. 2). Addition of 0.5 M NaCl to cells in suspension not containing trehalose resulted in a survival rate of less than 5%, as measured by membrane integrity after 6 h. L-Tre1 cells containing trehalose, however, showed a 14% survival rate. This difference was more marked when the challenge was carried out on attached cells, where ~70% of the trehalose-containing cells survived compared to <8% of control cells (Fig. 2A). Although these results demonstrate improved osmotolerance in trehalose-containing cells, the greater improvement seen in attached cells was unexpected. These experiments were therefore repeated using sorbitol and trehalose to increase the medium osmolarity. On addition of 1 M sorbitol, cells not containing trehalose, either in suspension or attached, showed survival rates of <12%, while at least 25% of those containing trehalose survived. There was no significant difference in survival of attached cells and cells in suspension (Fig. 2B). Addition of 1 M trehalose resulted in <2% survival of cells not containing trehalose and 6–7% survival of those producing trehalose. Again, no difference was observed in survival rates of cells in suspension or attached cells (Fig. 2C); only cells stressed with NaCl showed this distinction. Nevertheless, overall, for the hypertonic conditions tested, cells accumulating trehalose showed significantly ($P<0.05$) improved survival rates compared to the LMTK$^-$ parental cell line, with or with out heat shock, or the non-induced L-Tre1.

3.3. Desiccation tolerance

Survival of complete desiccation was tested on subconfluent

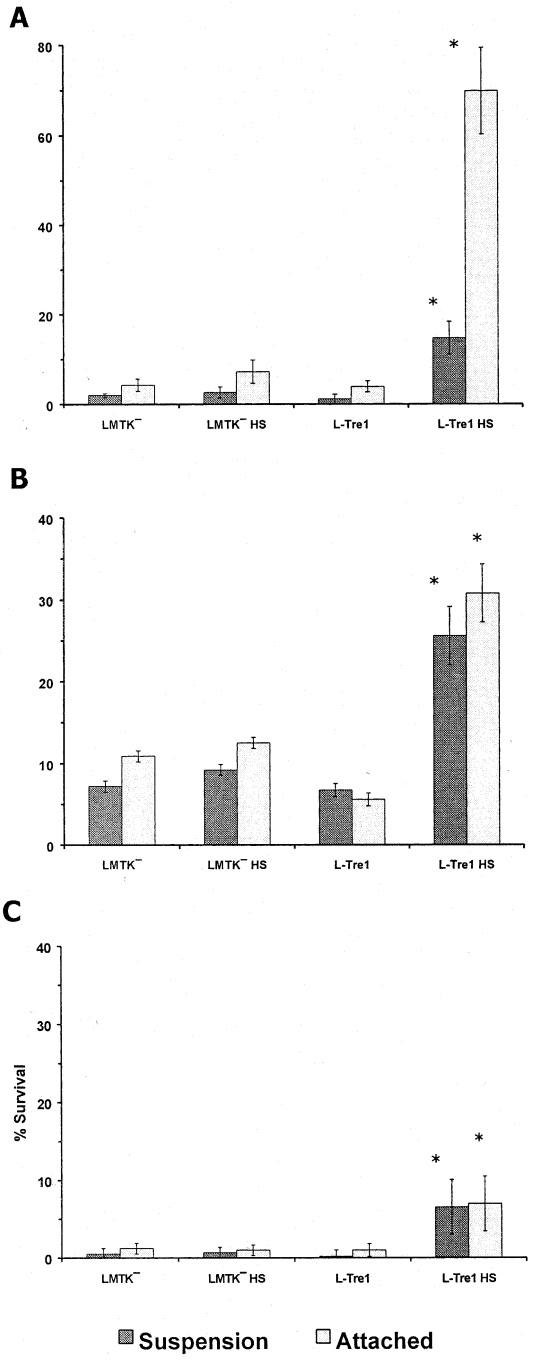

Fig. 2. Survival of LMTK⁻ and L-Tre1 cells with or without heat shock (HS) after 2 h in medium with added (A) 0.5 M NaCl, (B) 1 M sorbitol, or (C) 1 M trehalose, and tested either in suspension and assayed 6 h after plating, or while still attached and assayed 6 h later. Error bars represent ±S.D. and the asterisk represents *t*-test significant difference ($P < 0.05$) of heat-shocked (HS) L-Tre1 cells against all other cells within the same assay.

L-Tre1 cells containing ~40 pg/cell trehalose and grown on collagen-coated coverslips. Coverslips were dried by exposure to laminar airflow either with or without extracellular trehalose. No viable cells could be recovered after drying as measured by a trypan blue dye exclusion assay 6 h after rehydration. Replica slides left to incubate in isotonic medium for 5 days after rehydration showed no viable cells or colony formation. The same was observed for control cells not producing trehalose, while non-dried cells continued to grow normally on the coverslip. This complete lack of cell viability after desiccation was further confirmed by a Live/Dead Viability/Cytotoxicity cell stain performed within 30 min of rehydration. All cells appeared with a red nucleus resulting from staining with ethidium homodimer-1, indicating a permeabilised (i.e. damaged) plasma membrane, and only a very faint yellow cytoplasmic stain due to calcein AM, indicating loss of intracellular esterase activity.

4. Discussion

The L-Tre1 cell line has been shown to produce up to 40 pg/cell trehalose, the highest levels reported to date within a mammalian cell. Taking into account measured cell diameters and assuming equal distribution within the cell, this is equivalent to ~80 mM trehalose; for a mammalian cell dry weight of 250 pg [21], this corresponds to 16% dry weight. When induced to accumulate trehalose the L-Tre1 cell line showed a generally improved resistance to the partial dehydration resulting from medium supplemented with 0.5 M NaCl, 1 M sorbitol or 1 M trehalose. Previous studies indicate that under these conditions at least 55% of cellular water is lost [22]. Some mammalian cells compensate for water loss due to hypertonic environments by accumulating compatible solutes such as sorbitol, inositol, betaine, taurine, and glycerophosphorylcholine [23]. It was therefore expected that biosynthesis of intracellular trehalose, a common compatible solute in microorganisms and some eukaryotes, would provide protection against hypertonic stress. The higher survival rates obtained for attached cells when challenged with 0.5 M NaCl may be due to a protective effect resulting from their attachment to the culture surface, but why this is not apparent when challenged with sorbitol or trehalose needs to be further explored.

L-Tre1 cells containing trehalose could not survive the complete removal of water resulting from air-drying. Despite containing in the order of 100 times more trehalose than achieved by means of adenoviral vectors expressing *otsA* and *otsB* genes, this result does not support that of Guo et al. [16], who claim over 50% viability of human fibroblasts after 1 day in the desiccated state.

Assuming that intracellular trehalose is necessary for a high degree of desiccation tolerance, it is not easy to predict what concentration is required in mammalian cells. The concentrations found in anhydrobiotic organisms vary greatly. While some tardigrades are apparently able to survive drying with trehalose levels of less than 2% dry weight [24], *Saccharomyces cerevisiae* can accumulate up to 375 mM (~20%) dry weight as trehalose [3,25]. Some evidence suggesting that high trehalose concentrations may be required in mammalian cells comes from osmotolerant kidney cells which under extreme hypertonic conditions accumulate up to 244 mM sorbitol [26]. If trehalose were able to provide protection against complete desiccation it may be required at similar levels, or above. Recently, mammalian cells loaded with up to 0.4 M trehalose by controlled poration have been reported to show improved cryotolerance [27] and drying of these cells deserves attention. However, further work should also be aimed at discerning what other factors may play a role in desiccation tolerance. For example, it may be that trehalose also needs to be present in other cellular compartments, such as mitochondria, as well as the cytoplasm. In addition, other features commonly

shared by anhydrobiotic organisms may also be essential to achieve viable dry mammalian cells. These include extracellular protective walls and accumulation of other compatible solutes such as glycerol. A closer look at the molecular biology of desiccation-resistant organisms may provide the answer, and, given that the ability to dry mammalian cells in a viable form would have clear biotechnological applications for tissue engineering and long-term cell banks, is worth pursuing.

Intracellular trehalose accumulation, in addition to improved osmotolerance and desiccation tolerance, has also been associated with improved cryotolerance, thermotolerance and barotolerance. With ~80 mM intracellular trehalose in the L-Tre1 cell line we did not observe protection from these stresses (unpublished data). These effects may become apparent only at higher intracellular trehalose concentrations.

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