Available online at www.sciencedirect.com

ScienceDirect

Journal of Nutritional Biochemistry 45 (2017) 15–23

Journal of Nutritional Biochemistry

# Trehalose supplementation reduces hepatic endoplasmic reticulum stress and inflammatory signaling in old mice☆

Michael J. Pagliassotti[a,*], Andrea L. Estrada[a], William M. Hudson[a], Yuren Wei[a], Dong Wang[a], Douglas R. Seals[b], Melanie L. Zigler[b], Thomas J. LaRocca[b]

[a]Department of Food Science and Human Nutrition, Colorado State University, Fort Collins, CO 80523-1571, USA
[b]Department of Integrative Physiology, University of Colorado, Boulder, CO 80309, USA

Received 23 November 2016; received in revised form 12 January 2017; accepted 17 February 2017

**Abstract**

The accumulation of damaged proteins can perturb cellular homeostasis and provoke aging and cellular damage. Quality control systems, such as the unfolded protein response (UPR), inflammatory signaling and protein degradation, mitigate the residence time of damaged proteins. In the present study, we have examined the UPR and inflammatory signaling in the liver of young (~6 months) and old (~28 months) mice ($n$=8/group), and the ability of trehalose, a compound linked to increased protein stability and autophagy, to counteract age-induced effects on these systems. When used, trehalose was provided for 4 weeks in the drinking water immediately prior to sacrifice ($n$=7/group). Livers from old mice were characterized by activation of the UPR, increased inflammatory signaling and indices of liver injury. Trehalose treatment reduced the activation of the UPR and inflammatory signaling, and reduced liver injury. Reductions in proteins involved in autophagy and proteasome activity observed in old mice were restored following trehalose treatment. The autophagy marker, LC3B-II, was increased in old mice treated with trehalose. Metabolomics analyses demonstrated that reductions in hexosamine biosynthetic pathway metabolites and nicotinamide in old mice were restored following trehalose treatment. Trehalose appears to be an effective intervention to reduce age-associated liver injury and mitigate the need for activation of quality control systems that respond to disruption of proteostasis.


*Keywords:* Aging; Inflammation; Unfolded protein response; Liver; Hexosamine biosynthetic pathway; Proteostasis

## 1. Introduction

Trehalose is a nonreducing disaccharide composed of two glucose molecules connected by an alpha, alpha-1,1 linkage [1]. Trehalose displays many remarkable qualities including the ability to stabilize proteins and activate autophagy [1–3]. For example, oral administration of trehalose reduced polyglutamine aggregates in the cerebrum and liver in a transgenic mouse model of Huntington disease *via* mechanisms that involved stabilization of partially unfolded polyglutamine-containing protein [2]. Similarly, oral administration of trehalose ameliorated dopaminergic and tau pathology in parkin deleted/tau overexpressing mice [3]. More recently, trehalose induced hepatocyte autophagy and mitigated hepatocyte steatosis in response to a high-fructose diet [4]. Results such as these suggest that trehalose may be a relevant therapeutic agent in chronic disease and age-associated pathologies linked to impairments in protein homeostasis (proteostasis).

Impairments in proteostasis are a common feature of aging, and collapse of proteostasis has been postulated as a fundamental mechanism leading to protein and cellular damage not only in aging, but also in metabolic diseases, such as obesity and nonalcoholic fatty liver disease [5,6]. Proteostasis is monitored and maintained by complex quality control systems that include the cytosolic heat shock response, mitochondrial unfolded protein response and endoplasmic reticulum unfolded protein response (ER UPR) [6]. The ER UPR interacts with insulin signaling and inflammatory signaling pathways that are also characterized by impaired regulation in aging and metabolic diseases [7,8]. Therefore, it has been postulated that the ER UPR can initiate inflammation, and the coupling of the ER UPR and inflammatory signaling may be a fundamental component of age- and disease-associated cellular stress and inflammation [7,9].

Older age has been associated with several changes in the liver and hepatic sinusoid including thickening of the liver sinusoidal endothelial cell, deposition of perisinusoidal basal lamina and collagen, increased numbers of activated Kupffer cells, reductions in protein chaperone function and increased stress-induced activation of the ER UPR, and increased inflammation [10–14]. Recent studies have suggested that genetic and pharmacologic approaches that reduce inflammation or enhance protein degradation improve life-span and age-associated liver damage [13,14]. Therefore, one aim of this study was to examine the ER UPR and inflammatory signaling in the liver of old and young mice, and

☆ Funding sources: This work was supported by NIH grant DK072017 and the Lillian Fountain Smith Endowment (MJP), NIH MERIT award AG013038 (DRS) and a pre-doctoral NRSA Fellowship, AG0039210 (TJL).
* Corresponding author. Tel.: +1 970 491 1390, fax: +1 970 491 3875.
*E-mail address:* michael.pagliassotti@colostate.edu (M.J. Pagliassotti).

http://dx.doi.org/10.1016/j.jnutbio.2017.02.022
0955-2863/

to determine whether trehalose can mitigate age-associated changes in these signaling pathways.

It has been suggested that the metabolism of trehalose is similar to that of glucose, given that trehalose is thought to be rapidly hydrolyzed to glucose by the enzyme trehalase. Trehalase has been found in humans and most animals at the brush border of the intestinal mucosa, as well as in the kidney, liver and circulation [15–17]. Thus, trehalose metabolism does not appear to be consistent with its ability to improve proteostasis or activate degradative pathways. Therefore, the second aim of this study was to examine the metabolite profile in the liver of old and young mice in the absence and presence of trehalose supplementation.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed on frozen livers from male C57BL/6 mice, an established model of aging [18,19]. These animals were obtained from the National Institute on Aging rodent colony and housed in an animal care facility at the University of Colorado at Boulder on a 12:12-h light–dark cycle. The mice were group housed and fed *ad libitum* until time of sacrifice. The mice were divided into four groups: young control, young+trehalose, old control and old+trehalose. The young mice were 5–6 months old at the time of sacrifice and the old mice were 27–28 months old ($n$=8/group). The treatment groups received 2% trehalose in their drinking water for 4 weeks leading up to sacrifice ($n$=7/group). Mice were anesthetized using isoflurane and killed by exsanguination *via* cardiac puncture. All procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85–23, revised 2011) and were approved by the University of Colorado at Boulder Animal Care and Use Committee [18].

### 2.2. Plasma analyses

Plasma glucose and triglycerides were determined enzymatically using reagent kits (Sigma, St. Louis, MO, USA). Plasma aspartate aminotransferase and alanine aminotransferase were analyzed using kits (Abcam, Cambridge, MA, USA). Plasma IL-1β and IL-6 were measured by bead assay (eBioscience, San Diego, CA, USA) and IL-18 by ELISA (RayBiotech, Norcross, GA, USA).

### 2.3. Liver triglycerides

Liver lipid was extracted using the procedure of Bligh and Dyer [20]. Triglyceride concentration was determined using a kit (Sigma).

### 2.4. Liver caspase-1 and proteasome activity

Liver caspase-1 and proteasome activity were determined fluorometrically (BioVision, Milpitas, CA, USA). The caspase-1/ICE fluorometric assay kit is based on detection of cleavage of substrate YVAD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). The proteasome activity assay takes advantage of the chymotrypsin-like activity of the 20S proteasome assembly by utilizing an AMC-tagged peptide substrate which releases free, highly fluorescent AMC in the presence of proteolytic activity. Proteasome activity was differentiated from other protease activity using the proteasome inhibitor MG-132, which suppresses all proteolytic activity due to proteasomes.

### 2.5. Polysome fractionation

Liver tissue was pulverized in liquid nitrogen and lysed with NP40-lysis buffer [10 mM Tris–HCl at pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet-P40, 40 mM dithiothreitol, 500 U/ml RNAsin and 1% (wt/vol) deoxycholate] [21]. Nuclei were removed *via* centrifugation (12,000*g*, 10 s, 4 °C) and the supernatant was supplemented with 500 μl of 2× extraction buffer (0.2 M Tris–HCl at pH 7.5, 0.3 M NaCl), 150 μg/ml cycloheximide, 650 μg/ml heparin and 10 mM phenyl-methyl-sulfonyl fluoride, and centrifuged (12,000*g*, 5 min, 4 °C) to remove mitochondria and membrane debris [21]. The supernatant was layered onto a 10-ml linear sucrose gradient (15%–40% wt/vol) and centrifuged (Beckman SW41Ti) for 2 h at 38,000 rpm at 4 °C. Fractions were collected and digested with 100 μg proteinase K in 1% SDS and 10 mM EDTA for 30 min at 37 °C. RNA was recovered using phenol–chloroform–isoamyl alcohol followed by ethanol precipitation.

### 2.6. RNA isolation and analysis

Total RNA was extracted with Trizol reagent using the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) or as described above. For real-time PCR, reverse transcription was performed using 0.5 μg of DNase-treated RNA, Superscript II RnaseH- and random hexamers. PCR reactions were performed in 96-well plates using transcribed cDNA and IQ-SYBR green master mix (Bio Rad, Hercules, CA, USA). PCR efficiency was between 90% and 105% for all primer and probe sets and linear over 5 orders of magnitude. The specificity of products generated for each set of primers was examined for each fragment using a melting curve and gel electrophoresis. Reactions were run in triplicate and data calculated as the change in cycle threshold (ΔCT) for the target gene relative to the ΔCT for $\beta_2$-microglobulin and cyclophilin (control genes) according to the procedures of Muller et al. [22]. Results were similar regardless of the control gene; therefore, data in the results section are reported using $\beta_2$-microglobulin. Mouse primer sequences (5′–3′) were as follows: *CHOP*: forward CGCTCTCCAGATTCCAGTCAG, reverse GTTCTCCTGCTCCTT CTCCTTC; *GADD34*: forward GAGATTCCTCTAAAAGCTCGG, reverse TCTCTCCTGGTAGA CAACGC; *GRP78*: forward GAGGCGTATTTGGGAAAGAAGG, reverse GCTGCTGTAGGCT CATTGATG; *Caspase-1*: forward AGATGGCACATTTCCAGGAC, reverse GATCCTCCAGCAG CAACTTC; *Col1a1*: forward CCGCCGATGTCGCTATCC, reverse TCTTGAGGTTGCCAGTCTGC; *IL-1α*: forward CACGGGGACTGCCCTCTAT, reverse TGTCGGGGTGGCTCCACT; *IL-1β*: forward TCTTTGAAGTTGACGGACCC, reverse TGAGTGATACTGCCTGCCTG; *IL-33*: forward AGCTCTCCACCGGGGCTCAC, reverse CCTGCGGTGCTGCTGAACT; *NLRP3*: forward AGCCT TCCAGGATCCTCTTC, reverse CTTGGGCAGCAGTTTCTTTC; *SREBP-1c*: forward TGGTGG GCACTGAAGCAAAG, reverse CACTTCGTAGGGTCAGGTTCTC; *αSMA*: forward GCACCACT GAACCCTAAGG, reverse CCAGAGTCCAGCACAATACC; *TGFβ1*: forward TGGACA CACAGTACAGCAAGG, reverse GTAGTAGACGATGGGCAGTGG; *XBP1s*: forward GTCTGCT GAGTCCGCAGCAGG, reverse GATTAGCAGACTCTGGGGAAG.

### 2.7. Western blot analysis

Western blot analysis was performed as described in detail previously [23,24]. Membranes were incubated with antibodies against Beclin-1 (Cell Signaling, Danvers, MA, USA; #3495), p62 (Cell Signaling; #5114), α-tubulin or β-actin (Cell Signaling; #3873 or #8457), LC3B (Novus, Littleton, CO, USA; #NB600-1384), AMPK (AMP-activated protein kinase; Cell Signaling; total=#5831; phosphorylated=#4188), eIF-2α (eukaryotic initiation factor-2α; Santa Cruz Biotechnology, Santa Cruz, CA, USA; #sc133132) and phosphorylated eIF-2α (Abcam; #Ab32157). Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Bio-Sciences, Marlborough, MA, USA) and an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). Density was quantified using a UVP Bioimaging system (Upland, CA, USA).

### 2.8. Metabolomic analysis

Metabolites were extracted from liver tissue, which was flushed of blood prior to removal, with 2:1 chloroform/methanol (vol:vol) followed by centrifugation (13,000*g*, 15 min, 4 °C). An internal standard, $^{13}$C-glucose, was added to the aqueous extract and the extract was dried. The dried extract was resuspended in methanol to reduce glycogen and was centrifuged, and the supernatant was dried. This dried extract was resuspended in pyridine containing methoxyamine hydrochloride, incubated at 60 °C, sonicated and incubated again at 60 °C. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane was added and the samples were incubated at 60 °C. Samples were then centrifuged (3000*g*) for 5 min and cooled to room temperature, and a portion was transferred to a glass insert in a GC–MS autosampler vial. Metabolites were detected using a Trace GC Ultra coupled to a Thermo ISQ Mass Spectrometer (Thermo Scientific). Samples were injected in a 1:10 split ratio twice in discrete randomized blocks. Separation was achieved using a 30 m TG-5MS column (Thermo Scientific; 0.25 mm i.d., 0.25-μm film thickness) with a 1.2-ml/min helium gas flow rate. The program consisted of 80 °C for 30 s, a ramp of 15 °C/min to 330 °C and an 8-min hold. Masses between 50 and 650 $m/z$ were scanned at 5 scans/s after electron impact ionization. The ionization source was cleaned and retuned and the injection liner replaced between injection replicates.

### 2.9. Data analysis

#### 2.9.1. Metabolomic analysis

For each sample, molecular features as defined by retention and mass ($m/z$) were generated using XCMS software in R. Outlier injections were detected based on total signal and PC1 of principle component analysis, and the mean area of the chromatographic peak was calculated among replicate injections. Features were grouped based on a novel clustering tool, RAMClustR, which groups features into spectra based on coelution and covariance across the full experiment. Compounds were annotated based on spectral matching to in-house, NISTv12, Golm, Metlin and Massbank metabolite databases. The peak areas for each feature in a spectrum were condensed *via* the weighted mean of all features in a spectrum into a single value for each compound. Analysis of variance was conducted on each compound using the analysis of variance function in R, and $P$ values were adjusted for false positives using the Bonferroni–Hochberg method in the p.adjust function in R. PCA was conducted on mean-centered and pareto variance-scaled data using the pcaMethods package in R. Trehalose, lactic acid and pyruvic acid were quantified absolutely by external calibration using a 7-point calibration curve with a fixed concentration of $^{13}$C-labeled glucose as internal standard.

#### 2.9.2. Data presentation and statistics

Results are presented as means±standard deviation. Comparisons among groups were determined using analysis of variance. Significance was determined using $P$<.05.