Trehalose inhibits solute carrier 2A (SLC2A) proteins to induce autophagy and prevent hepatic steatosis

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Trehalose is a naturally occurring disaccharide that has gained attention for its ability to induce cellular autophagy and mitigate diseases related to pathological protein aggregation. Despite decades of ubiquitous use as a nutraceutical, preservative, and humectant, its mechanism of action remains elusive. We showed that trehalose inhibited members of the SLC2A (also known as GLUT) family of glucose transporters. Trehalose-mediated inhibition of glucose transport induced AMPK (adenosine 5'-monophosphate–activated protein kinase)–dependent autophagy and regression of hepatic steatosis in vivo and a reduction in the accumulation of lipid droplets in primary murine hepatocyte cultures. Our data indicated that trehalose triggers beneficial cellular autophagy by inhibiting glucose transport.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the world (1). Indeed, more than 1 billion individuals worldwide have this highly morbid disease, which is characterized in its earliest stages by excessive hepatic fat deposition and in its latest stages by steatohepatitis, fibrosis, cirrhosis, liver failure, and progression to hepatocellular carcinoma (1). Unfortunately, few efficacious treatments exist.

We elucidated a role for the solute carrier 2A (SLC2A) family (also referenced as the glucose transporter or GLUT family) of hexose transporter homologs in mediating NAFLD (2). The GLUT family consists of fourteen 12-transmembrane domain–containing proteins that mediate uptake of hexose and other substrates across cellular membranes (3); thus, these carrier proteins are central regulators of cellular energetics. GLUT2 and GLUT8 are the most abundant hepatic GLUTs (2), both of which transport glucose and fructose (3). GLUT8 deletion in mice blunts hepatic hexose membrane hexose flux and prevents fructose-induced hepatic steatosis (2). Defects in cellular energetics can activate nutrient-sensing pathways that stimulate macroautophagy (hereafter, “autophagy”)—tightly regulated cellular “self-eating,” which occurs in response to amino acid or glucose starvation—to counteract acutely diminished energy substrates. The adenosine 5'-monophosphate (AMP)–activated protein kinase (AMPK) pathway is among the signaling cascades activated in this context (4), and this kinase stimulates autophagy by phosphorylating unc-52–like kinase-1 (ULK1) at Ser177 in response to increased ratio of cellular AMP to adenosine 5'-triphosphate (ATP) (5, 6).

Sugars that are not transported can act as competitive inhibitors for the GLUT transporters (7). For example, the disaccharide maltose inhibits uptake of a glucose analog by GLUT2 with a Kᵣ (inhibition constant) of ~100 mM (7). Thus, exogenous sugars may impair cellular energetics and induce autophagy. One particular disaccharide, trehalose, is a glucose–glucose disaccharide linked by an α-(1,1)-glycoside bond. Trehalose is ubiquitous to diverse organisms, including bacteria, yeast, fungi, insects, and invertebrates, but trehalose is absent in vertebrate animals (8). Trehalose has garnered attention for its ability to mitigate protein aggregate accumulation by stimulating cellular autophagy in several cell types. In particular, trehalose has reduced toxic protein aggregates in murine models of the neurological diseases Huntington’s disease (9), amyotrophic lateral sclerosis (10), and prion disease (11) and in the pancreatic β cells of a mouse model of type 2 diabetes (12). The mechanism by which trehalose activating autophagy, however, is not fully described. Because autophagic insufficiency may underlie the pathogenesis of NAFLD (13), mechanistic insights into how trehalose induces hepatic autophagy may aid in the development of novel NAFLD therapeutics.

Therefore, we tested the hypothesis that trehalose induces hepatocyte autophagy by inhibiting hexose uptake. Moreover, because the efficacy of trehalose in treating or preventing NAFLD in preclinical models has not been addressed, we tested whether this autophagy–inducing agent mitigates hepatic steatosis. Our data indicated that trehalose induces hepatocyte autophagy and mitigates hepatic lipid droplet accumulation—at least in part—by inhibiting hexose uptake and subsequently activating the AMPK to ULK1 pathway.

RESULTS

Trehalase and the trehalose receptor Tre1 share homology with SLC2A family members

We performed phylogenetic analysis with Clustal Omega multiple sequence alignment to examine evolutionary relationships among SLC2A members and trehalose-specific enzymes—trehalase and the Drosophila trehalose receptor Tre1. Phylogenetic analysis revealed evolutionary commonality between trehalase, Tre1, and the mouse SLC2A family member SLC2A8, with more distant common evolutionary branches connecting SLC2A2 and SLC3A to Tre1 and trehalase (Fig. 1A). SLC2A1 and SLC2A4 were the most distant, lacking any shared evolutionary branches. We quantified domain-specific homology using pairwise alignment between Tre1 and SLC2A1 using the crystal structure data for SLC2A1 (14). Regions of highest conservation occurred between residues 55 to 79 of Tre1 and residues 257 to 281 of SLC2A1 and between residues 46 to 85 of Tre1 and residues 297 to 332 of SLC2A8 (Fig. 1B). In both SLC2A1 and SLC2A8, residues 257 to 281
and residues 297 to 332 (GLUT8) include a conserved region that corresponds to the pore-forming α-helical seventh transmembrane domain, which confers SLC2A family member substrate specificity (15).

Trehalose inhibits hexose uptake in multiple cell types

We examined whether trehalose modulated SLC2A-mediated uptake of [1H]2-deoxy-D-glucose ([1H]2-DG), a glucose analog that is transported but not metabolized. We stably transfected 293 cells that also stably expressed GLUT1 short hairpin RNA (shRNA) to knock down the endogenous GLUT1 transporter (16) with empty vector or with plasmids expressing one of the four class I GLUTs (GLUT1 to GLUT4), which are ubiquitous (3), or GLUT8, which is abundant in hepatocytes (2). We also tested the effect of trehalose on GLUT8-mediated 2-DG uptake, because blocking GLUT8 prevents the development of hepatic steatosis (2). We assayed uptake of 2-DG in each cell line over a range of substrate concentrations to determine the half-maximal inhibitory concentration (IC50) in each GLUT isoform–enriched system.

Trehalose inhibited [3H]2-DG uptake in each of the class I GLUT–overexpressing and GLUT8-overexpressing 293 lines (Fig. 1C).

We also evaluated the effects of trehalose on [1H]2-DG uptake and [14C]fructose uptake in cultured primary murine hepatocytes and the human hepatocyte cell line HepG2. Because GLUT8 is abundant in hepatocytes and the overexpression study indicated an IC50 of 126 mM, we exposed the hepatocyte cells to 100 mM trehalose. In primary hepatocyte cultures, 100 mM trehalose inhibited 2-DG uptake without significantly affecting [14C]fructose uptake (Fig. 1D). Therefore, for all subsequent experiments involving primary hepatocytes, we used 100 mM trehalose. In HepG2 hepatocytes, 100 mM trehalose significantly blunted both [1H]2-DG and [14C]fructose uptake when compared with vehicle-treated cultures (Fig. 1E). The inhibition of fructose uptake may reflect greater dependence of HepG2 cells on GLUT8-mediated fructose uptake in comparison with primary hepatocytes (2).

Trehalose rapidly induces AMPK and ULK1 activation in cultured primary hepatocytes

Preventing glucose uptake into hepatocytes is predicted to induce a starvation-like state and activate cellular autophagy. We therefore determined whether trehalose depleted hepatocyte intracellular ATP by incubating primary hepatocyte cultures with or without trehalose for 30 min before lysis and then performing an enzymatic-fluorimetric ATP concentration assay (17, 18). ATP concentration in trehalose-treated

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**Fig. 1.** Trehalose inhibits glucose transport through SLC2A transporters. (A) Common evolutionary ancestry for trehalase, Tre1, and SLC2A family members by ClustalW2 phylogenetic analysis. (B) Tre1 pairwise alignment with the substrate-selecting seventh transmembrane domains in SLC2A1 and SLC2A8. (C) 2-DG uptake in response to increasing trehalose concentrations in 293 cells expressing the indicated GLUT protein. Data are expressed as the means percent uptake relative to control cultures not treated with trehalose ± SEM from n = 3 independent experiments, n = 3 replicates per experiment. (D) [1H]2-DG and [14C]fructose uptake in primary hepatocytes. Data are expressed as the means uptake relative to trehalose-untreated control groups ± SEM from n = 3 independent experiments, n = 3 replicates per experiment. (E) [1H]2-DG and [14C]fructose uptake in HepG2 cells. Data are shown as means uptake relative to trehalose-untreated control groups ± SEM from n = 3 independent experiments, n = 3 replicates per experiment. ***P < 0.001 versus vehicle-treated by two-tailed t test. ns, not significant versus vehicle-treated.
**Fig. 2.** Trehalose decreases hepatocyte ATP and activates AMPK and autophagic flux. (A) ATP quantification in hepatocytes treated with or without 100 mM trehalose for 30 min. Data are shown as means fold of control (growth media–treated) ATP ± SEM for *n* = 3 independent experiments, *n* = 3 replicates per experiment. *P* < 0.05 by two-tailed *t* test. (B) Left: Immuno blot demonstrating AMPK (Thr172) phosphorylation in trehalose-treated hepatocytes (0 to 4 hours). Right: Immuno blot quantification of phosphorylated AMPK (pAMPK) (Thr172) normalized to AMPKα band density. Data are shown as means ± SEM for *n* = 3 independent experiments, *n* = 2 to 3 replicates per experiment. **P < 0.01 and ***P < 0.001 versus untreated control (“0 hour” data) [one-way analysis of variance (ANOVA) and Sidak’s post hoc analysis]. pAMPK, phosphorylated AMPK. A.U., arbitrary units. (C) Left: Immunoblot depicting AMPK and ACC1 phosphorylation in HBSS-starved hepatocytes, followed by refeeding with un supplemented HBSS (denoted as “−” group on blot and “control” on graph) or HBSS containing 25 mM glucose in the presence or absence of 100 mM trehalose for 30 min. Right: Quantification of AMPK and ACC1 phosphorylation at Thr172 and Ser79 normalized to AMPKα and ACC1 band density, respectively. Data are shown as means ± SEM for *n* = 3 independent experiments, *n* = 2 replicates per experiment. In the glucose-treated group, *P < 0.05 and ***P < 0.001 versus controls (one-way ANOVA and Sidak’s post hoc analysis). In the glucose + trehalose–treated group, ***P < 0.001 versus cells treated with glucose alone (one-way ANOVA and Sidak’s post hoc analysis). **P < 0.01 and ***P < 0.001 versus control group (one-way ANOVA and Sidak’s post hoc analysis). (D) Left: Immunoblot depicting ULK1 phosphorylation (Ser317 and Ser757) and LC3B-II accumulation after 1-hour incubation without (control) or with 100 mM trehalose. Right: Quantification of AMPK and ULK1 phosphorylation and LC3B-II band density normalized to AMPKα, ULK1, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), respectively. Data are shown as means ± SEM for *n* = 3 independent experiments, *n* = 2 replicates per experiment; **P < 0.01 versus control and ***P < 0.001 versus control by two-tailed *t* test. pULK1, phosphorylated ULK1. (E) LC3B-I and LC3B-II immunoblotting in hepatocytes after treatment with or without 100 mM trehalose and 100 nM bafA1 for 1 hour. Control groups were treated with dimethyl sulfoxide (DMSO). Right: LC3B-II band density, normalized to GAPDH density. Data are shown as means ± SEM for *n* = 5 independent experiments, *n* = 1 to 3 replicates per experiment; ***P < 0.001 versus control groups. ###P < 0.001 between bracketed groups by one-way ANOVA and Sidak’s post hoc analysis for multiple comparisons.
hepatocyte cultures was 20% lower when compared with untreated cultures (Fig. 2A). Reduced ATP concentration should increase the AMP/ATP ratio, leading to the phosphorylation at Thr172 and activation of AMPK, an energy-sensing kinase that stimulates autophagy (19). We monitored phosphorylated Thr172 of AMPK by Western blotting in primary hepatocyte cultures exposed to vehicle or trehalose for up to 4 hours. Trehalose stimulated an increase in AMPK (Thr172) phosphorylation within 30 min, and this increase was sustained throughout the 4-hour time course (Fig. 2B). In contrast, AMPK activation was at basal levels in cells that had not been exposed to trehalose (0 in Fig. 2B) and were analyzed after 4 hours.

To test whether trehalose blocked glucose-mediated suppression of AMPK activity, we deprived primary hepatocytes of glucose and then replaced the starvation solution with unsupplemented Hanks’ balanced salt solution (HBSS; denoted as “control,” which contains 5.6 mM glucose) or with HBSS containing 25 mM glucose (denoted as “glucose”) in the presence or absence of trehalose (denoted as “glucose + trehalose”) for 30 min. For these experiments, we monitored not only AMPK activation as phosphorylation at Thr172 but also AMPK activity as measured by phosphorylation of its substrate acetyl-coenzyme A (CoA) carboxylase 1 (ACC1) at Ser79. Compared to control hepatocytes, glucose refeeding suppressed phosphorylation of AMPK and ACC1 (Fig. 2C). In contrast, hepatocytes subjected to glucose refeeding in the presence of trehalose exhibited marked ACC1 (Ser79) and AMPK (Thr172) phosphorylation when compared with control cultures or when compared with cultures subjected to 25 mM glucose refeeding alone (Fig. 2C), suggesting that glucose refeeding in the presence of trehalose does not simply recapitulate the fed state.

AMPK activates autophagy by phosphorylating the autophagy-promoting protein ULK1 at Ser317 (6). To determine whether trehalose activates ULK1 and autophagy, we treated primary hepatocytes with or without trehalose and then monitored ULK1 phosphorylation and the abundance of the autophagosome marker protein LC3B-II (12) by Western blot. Consistent with induction of autophagy, trehalose significantly enhanced AMPK (Thr172) and ULK1 (Ser317) phosphorylation when compared with untreated primary hepatocyte cultures was 20% lower when compared with untreated cultures (Fig. 2A). Reduced ATP concentration should increase the AMP/ATP ratio, leading to the phosphorylation at Thr172 and activation of AMPK, an energy-sensing kinase that stimulates autophagy (19). We monitored phosphorylated Thr172 of AMPK by Western blotting in primary hepatocyte cultures exposed to vehicle or trehalose for up to 4 hours. Trehalose stimulated an increase in AMPK (Thr172) phosphorylation within 30 min, and this increase was sustained throughout the 4-hour time course (Fig. 2B). In contrast, AMPK activation was at basal levels in cells that had not been exposed to trehalose (0 in Fig. 2B) and were analyzed after 4 hours.

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Fig. 3. Orally administered trehalose rapidly accumulates in peripheral circulation and induces hepatic autophagy. (A) Liquid chromatography–mass spectrometry analysis of serum isolated from mice administered trehalose (3 g/kg) by gavage and analyzed after 0.5, 1, 2, or 4 hours. Serum analyzed at the “0 hour” trehalose treatment time point was derived from mice 30 min after gavage with 0.9% NaCl. n = 5 mice per treatment group. (B) Left: Immunoblot analysis of crude liver lysates from mice administered trehalose (3 g/kg) and analyzed after 0.5, 1, 2, or 4 hours. Liver lysates analyzed at the “0 hour” trehalose treatment time point were derived from mice 30 min after gavage with 0.9% NaCl. Right: Quantification of LC3B-II/actin band density ratio. Data are shown as means ± SEM for n = 5 independent mice per treatment group; ***P < 0.001 versus leupeptin–0-hour treatment group (one-way ANOVA and Sidak’s post hoc analysis). (C) Left: Immunoblot analysis of LC3B-II in liver lysates from mice treated with or without leupeptin (40 mg/kg) 1 hour before oral gavage with trehalose (3 g/kg). Right: Quantification of LC3B-II/actin band density ratio. Data are shown as means ± SEM for n = 3 to 4 independent mice per treatment group; ***P < 0.001 and ****P < 0.0001 versus saline-treated controls (two-tailed t test). ###P < 0.001 between trehalose-treated groups treated with leupeptin versus leupeptin-untreated group (denoted by brackets) by two-tailed t test.
hepatocytes (Fig. 2D). Consistent with a nutrient-deprivation response, we also observed that ULK1 phosphorylation at Ser\textsuperscript{757}—a site phosphorylated by the mammalian target of rapamycin complex 1 (mTORC1) (6)—was significantly lower in trehalose-treated cultures after 1 hour (Fig. 2D).

These changes in the autophagy-activating AMPK signaling pathway correlated with LC3B-II accumulation. To directly test whether LC3B-II accumulation was indeed due to trehalose induction of autophagic flux—and not secondary to defects in LC3B-II degradation—we incubated primary hepatocytes with trehalose for 1 hour in the presence or absence of bafilomycin A1 (BafA1), a V-ATPase (vacuolar-type H\textsuperscript{+} adenosine triphosphatase) inhibitor (I2) that prevents LC3B-II degradation in autophagosomes. Trehalose-treated hepatocytes exhibited increased LC3B-II when compared with untreated cultures (Fig. 2E), and BafA1 further enhanced LC3B-II accumulation (Fig. 2E).

Our data indicated that trehalose acted as a glucose transport inhibitor that induced hepatocyte autophagic flux. Therefore, we evaluated whether other structurally unrelated glucose transport inhibitors could induce autophagy. The HIV protease inhibitors are a class of compounds that inhibit SLC2A family members with distinct isoform specificity (20). The HIV protease inhibitor lopinavir is a class I SLC2A glucose transport inhibitor (IC\textsubscript{50} range, 4 to 32 \mu M for GLUT1, GLUT2, GLUT3, and GLUT4) (I6). To test whether lopinavir blocked glucose uptake in hepatocytes, we incubated primary hepatocyte cultures with solvent (DMSO) or with lopinavir (40 \mu M) 5 min before performing 2-DG uptake measurements. Lopinavir reduced 2-DG uptake in primary hepatocytes by about 50% when compared with solvent-treated cultures (fig. S1A). Moreover, within 15 min, we detected AMPK (Thr\textsuperscript{172}) and ULK1 (Ser\textsuperscript{317}) phosphorylation and LC3B-II accumulation in lopinavir-treated primary hepatocytes (fig. S1B). In contrast, the disaccharide sucrose (100 mM) did not induce AMPK signaling or LC3B-II accumulation at any time point through 4 hours of incubation in primary hepatocytes (fig. S2).

**Trehalose activates AMPK signaling and autophagic flux in mouse liver**

We assessed the effect of trehalose on AMPK signaling and autophagic flux in mice. To first determine whether orally delivered trehalose was detectable in peripheral venous circulation, we administered by saline gavage (3 g/kg) for 0.5 to 4 hours to wild-type mice, collected peripheral blood, and performed liquid chromatography–mass spectrometry analysis on separated serum. Within 30 min, we detected trehalose in the serum of the mice (Fig. 3A). To ascertain whether AMPK and ULK1 were activated in response to trehalose administration, we excised liver from the mice and immunoblotted for AMPK (Thr\textsuperscript{172}) and ULK1 (Ser\textsuperscript{317}) phosphorylation. Livers from mice administered trehalose exhibited increased AMPK Thr\textsuperscript{172} within 30 min, increasing ULK1 Ser\textsuperscript{317} phosphorylation that plateaued after 2 hours, and a peak of LC3B-II accumulation after 1 hour (Fig. 3B). Because LC3B-II could accumulate in liver tissue due to increased autophagic flux or due to decreased LC3B-II degradation, mice were administered saline or trehalose (3 g/kg) 1 hour after intraperitoneal injection with saline or leupeptin (40 mg/kg) to block LC3B-II degradation in autophagosomes (I2, I7). Consistent with an increase in autophagic flux, leupeptin enhanced the accumulation of LC3B-II in the trehalose-treated animals (Fig. 3C).

**Trehalose reduces triglyceride accumulation in cultured hepatocytes**

Autophagic insufficiency may underlie the pathogenesis of NASH (22), a disorder of excessive hepatic fat accumulation that ranges in phenotype from simple steatosis to steatohepatitis, cirrhosis, and liver failure (I). The accumulation of triglycerides in cultured hepatocytes serves as an in vitro model of fatty liver disease and can be induced by exposing the cells to the monosaccharide fructose (2, 23). We tested whether trehalose mitigates hepatocyte triglyceride accumulation in this cell culture model by incubating serum-deprived primary hepatocytes in the presence or absence of fructose (5 mM for 48 hours) with or without coadministration of trehalose. We extracted the lipids and performed enzymatic-colorimetric triglyceride analysis. Fructose significantly induced triglyceride accumulation in the primary hepatocyte cultures, and trehalose blocked the accumulation of triglyceride (Fig. 4A). To quantify key fructose-responsive regulatory factors, we quantified the abundance of transcripts encoding carbohydrate response element binding protein (ChREBP) and glycerol 3-phosphate acyltransferase (GPAT). Trehalose partially blocked the fructose-mediated increase in ChREBP and GPAT mRNA abundance (Fig. 4B). Trehalose also significantly reduced fatty acid–induced triglyceride accumulation in primary hepatocytes exposed to bovine serum albumin (BSA)–conjugated oleic, palmitic, stearic, and linoleic acids at a ratio of 1:1:1:1 for a total of 500 \mu M fatty acid for 48 hours (Fig. 4C).

To determine whether the protective effects of trehalose on hepatocyte triglyceride accumulation extended to other models of hepatic triglyceride accumulation, we evaluated the effect of trehalose on hepatocytes from mice genetically deficient in hepatic microsomal triglyceride transfer protein (MTTP), hepatocytes from mice expressing knock-in hypomorphic alleles of the gene encoding the autophagy-promoting complex protein ATG16L1 [ATG16L1\textsuperscript{hm} (24)], and HepG2 cells. MTTP-knockout mice develop hepatic steatosis in the absence of dietary stress (25). Trehalose reduced the accumulation of triglycerides in cultured hepatocytes from MTTP-knockout mice compared with the abundance of triglycerides in the untreated MTTP-knockout hepatocytes (Fig. 4D).

Compared to hepatocytes from wild-type mice, the hepatocytes from the ATG16L1\textsuperscript{hm} mice exhibit increased triglyceride accumulation even in growth medium (Fig. 4E). However, exposure to the BSA-conjugated fatty acids induced a significant increase in triglyceride accumulation that trehalose did not reduce (Fig. 4E), indicating that the ability of trehalose to prevent triglyceride accumulation required autophagy, which was compromised in the cells from the ATG16L1\textsuperscript{hm} mice. HepG2 cells are a cell line derived from a human hepatocellular carcinoma. Similar to the primary hepatocyte response to trehalose, HepG2 cultures exposed to trehalose exhibited significantly greater LC3B-II accumulation, and this response was enhanced by BafA1 (fig. S3A). Furthermore, BSA-conjugated fatty acids induced triglyceride accumulation in the HepG2 cells, and trehalose reduced this response (fig. S3B).

**Feeding mice trehalose prevents NAFLD and dyslipidemia**

High-fructose diet (HFrD; 60% of calories from fructose) induces hepatic lipid droplet accumulation and dyslipidemia in mice (2, 26–28) and thus is a model of NAFLD. The data in the cultured hepatocytes suggested that trehalose may be liver-protective. To investigate this possibility, we fed mice 3% trehalose water ad libitum 48 hours before placing the mice on HFrD for 10 days. Biochemical analysis of plasma from fasted mice revealed significantly reduced circulating triglycerides and cholesterol without significant changes in free fatty acids (Fig. 5A) in trehalose-treated mice fed HFrD. Histological analysis and quantitation of lipid vesicles in liver tissue by Oil Red O neutral lipid staining revealed marked steatosis in HFrD-fed mice, which was significantly reduced by trehalose (Fig. 5B). Biochemical analysis of liver tissue showed that liver triglycerides, cholesterol, and free fatty acids were increased in the HFrD-fed mice and that each was significantly reduced in livers from the trehalose-treated mice fed HFrD (Fig. 5C). Consistent with histological and biochemical improvements, animals fed HFrD and trehalose exhibited significant reductions in transcripts encoding ACC1, stearyl-CoA desaturase-1 (SCD1), GPAT, ...
and peroxisome proliferator–activated receptor γ (PPARγ) (Fig. 5D), compared with their abundance in animals fed HFD alone.

**Overexpression of either GLUT8 or AMPK reverses the beneficial effects of trehalose on hepatic steatosis**

The data from the hepatocyte cultures and mouse model of NALFD suggested that trehalose inhibits hepatocyte hexose transport, activates AMPK and ULK1, and subsequently stimulates autophagic flux. To confirm that inhibition of hexose transport was essential to this mechanism of trehalose action, we tested whether trehalose-induced autophagy was reversed by expression of a SLC2A family member in primary hepatocytes. We transfected primary hepatocytes with adenovirus encoding green fluorescent protein (GFP), GLUT8, or GLUT2, and then exposed the cells to trehalose for 1 hour. Adenoviral overexpression of GLUT8, but not GLUT2, abolished trehalose-stimulated LC3B-II accumulation (Fig. 6A). GLUT8 overexpression also blunted the reduction in fructose-induced triglyceride accumulation in response to trehalose (Fig. 6B).

To test that trehalose required AMPK signaling to induce hepatocyte autophagy and mitigate fructose-induced triglyceride accumulation, we expressed GFP or kinase-dead AMPK (KD-AMPK) in the primary hepatocytes and exposed the cells to trehalose. Trehalose treatment resulted in robust ULK1 (Ser177) phosphorylation in GFP-expressing hepatocytes but not in the KD-AMPK–expressing hepatocytes (Fig. 6C). Moreover, KD-AMPK expression attenuated LC3B-II accumulation in cells exposed to trehalose (Fig. 6D). Hepatocytes expressing KD-AMPK also exhibited significantly less effective trehalose-mediated reduction in triglyceride accumulation in response to fructose (Fig. 6E).

**DISCUSSION**

The data herein provide evidence that trehalose inhibits glucose transport to induce hepatic autophagy and prevent hepatic steatosis in an SLC2A- and AMPK-dependent manner (Fig. 6F). Trehalose may therefore create a functionally “starved” state that activates AMPK signaling in the hepatocyte, even in the presence of abundant glucose. The beneficial effects of hepatic AMPK activation in increasing fat oxidation and insulin sensitivity are well documented (29).

Although the data support a role for AMPK signaling hepatocyte autophagy and limiting triglyceride accumulation, we also found decreased ULK1 Ser177 phosphorylation, which is a target of mTORC1. Thus, we cannot rule out the possibility that trehalose also results in inhibition of mTORC1 signaling and that mTORC1 inhibition is required for the full effects of trehalose in promoting autophagy. There is precedent for AMPK–mTORC1 crosstalk. In response to starvation, mTORC1–dependent sites on ULK1 and ULK2 are rapidly dephosphorylated (30). Moreover, AMPK phosphorylates the mTORC1 subunit raptor to inhibit mTORC1 activity (30). Therefore, trehalose-activated autophagy may involve mTORC1 suppression—perhaps through crosstalk from the AMPK pathway. Future mechanistic work should address the specific role of mTORC1 in trehalose-induced autophagy.

Compared with the rapid LC3B-II accumulation in hepatocytes within minutes of trehalose exposure that we found, prior reports describing the effects of trehalose reported a protracted (24 hours) time course required for trehalose to induce LC3B-II accumulation in COS-7 cells (9). The timing of the autophagic response to apparent starvation is likely a tissue-specific phenomenon. Physiologically, a rapid response of hepatocytes to nutrient stress is reasonable, given an organism’s requirement for rapid hepatic adaptation to “starvation” to quickly mobilize energy stores and avert hypoglycemia. The observed differences could also reveal distinct acute (minutes) and longer-term (hours to days) mechanisms of trehalose action that remain to be fully elucidated. For example, Rubinsztein and colleagues...
demonstrated LC3B-II accumulation 24 hours after trehalose exposure in COS-7 cells. This LC3B-II accumulation was recapitulated by inducing trehalose synthase expression in T-REx 293 cell lines, which have been engineered to synthesize intracellular trehalose in response to tetracycline (9). Trehalose access to the cytoplasm through fluid-phase endocytosis, pinocytosis, or other transport mechanisms may underlie any distinctions between acute and longer-term cellular actions of trehalose. Despite these intriguing differences, our work suggested that trehalose—or potentially other glucose transport modulators—may be useful clinically to prevent or treat diseases of autophagic insufficiency (31), including NAFLD.

Fig. 5. Trehalose reduces HFrD-induced hepatic steatosis in vivo. (A) Eight-week-old mice were fed chow, 10-day 60% fructose diet, or 10-day 60% fructose diet initiated 2 days after initiating 3% trehalose fed ad libitum in drinking water. Fasting plasma triglycerides, cholesterol, and free fatty acids (FFAs) were quantified. Data are shown as means ± SEM for n = 6 to 12 independent mice per treatment group. **P < 0.01 (one-way ANOVA and Sidak’s post hoc analysis). (B) Left: Oil Red O staining in frozen liver sections from mice described above. Scale bar, 200 µm. Right: Blinded-observer quantification of staining red staining density (minimum three random fields from three cryosections obtained from three different mice per group) by ImageJ (version 1.47) densitometry software. Data are shown as means ± SEM for n = 9 to 12 mice per treatment group. ****P < 0.0001 (one-way ANOVA and Sidak’s post hoc analysis). (C) Hepatic TG, cholesterol, and FFA quantification in 8-week-old mice fed chow, 10-day 60% fructose diet, or 10-day 60% fructose diet initiated 2 days after initiating 3% trehalose fed ad libitum in drinking water. Data are shown as means ± SEM for n = 9 to 12 mice per treatment group. ****P < 0.0001, **P < 0.01, and *P < 0.05 (one-way ANOVA and Sidak’s post hoc analysis). (D) Left to right: qRT-PCR analysis of ACC1, SCD1, GPAT, and PPARγ mRNA in liver tissue mRNA extracted from mice fed 10-day HFrD with or without 3% trehalose water. Target abundances are normalized to α-actin expression within each sample. Data are shown as means ± SEM for n = 9 to 12 mice per treatment group. ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05 (one-way ANOVA and Sidak’s post hoc analysis).
MATERIALS AND METHODS

Mouse models
Male and female wild-type C57BL/6J mice (Jackson Laboratory) were used for all experiments. ATG16L1HM (24) mice were obtained from H. W. Virgin’s laboratory (Washington University School of Medicine). Hepatic MTTP–deficient mice (25) were obtained from N. Davidson’s laboratory (Washington University School of Medicine). All experiments were performed in accordance with the Washington University Animal Studies Committee.

Cell cultures
Methods used to generate the human embryonic kidney (HEK) 293 cell lines selectively overexpressing human GLUT1, GLUT2, GLUT3, GLUT4, or GLUT8 with concomitant GLUT1

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**Fig. 6.** GLUT8 and AMPK mediate trehalose-induced autophagy and protection from triglyceride accumulation. (A) LC3B-II immunoblots in hepatocyte lysates after adenoviral GFP, GLUT8, or GLUT2 expression. (B) Quantitative triglyceride measurements in hepatocytes treated with or without 5 mM fructose in the presence or absence of 100 mM trehalose after adenoviral expression of GFP, GLUT8, or GLUT2. Data are shown as means ± SEM for n = 3 independent experiments, n = 2 replicates per experiment. ****P < 0.0001, ***P < 0.001, and *P < 0.05 (one-way ANOVA and Sidak’s post hoc analysis). (C) Top: Immunoblot depicting pULK1 (Ser317) in trehalose-treated hepatocytes expressing a kinase-dead AMPK mutant (KD-AMPK). Bottom: pULK1 band density quantification, normalized to ULK1 band density. Data are shown as means ± SEM for n = 3 independent experiments, n = 1 to 3 replicates per experiment. ****P < 0.0001 between bracketed groups (two-tailed t test). (D) Top: Immunoblot of LC3B-II accumulation in 100 mM trehalose–treated hepatocytes (1 hour) expressing KD-AMPK. Bottom: Quantification of LC3B-II band normalized to GAPDH. *P < 0.05; ns, not significantly different versus growth media group (two-tailed t test). (E) Quantitative triglyceride measurements in hepatocytes treated with or without 5 mM fructose in the presence or absence of 100 mM trehalose (48 hours) after adenoviral expression of GFP or KD-AMPK. Data are shown as means ± SEM for n = 3 independent experiments, n = 3 replicates per experiment. ****P < 0.0001, ***P < 0.001, and *P < 0.05 (one-way ANOVA and Sidak’s post hoc analysis). (F) Proposed model depicting trehalose blockade of GLUT2 and GLUT8, resulting in AMPK and ULK1 activation, and enhanced autophagic flux. Glc, glucose; AAs, amino acids.
shRNA overexpression were recently described (16). Briefly, HEK 293 cells obtained from the American Type Culture Collection were forced to stably express GLUT1-directed shRNA after lentiviral transfection (obtained from the RNAi Core laboratory at Washington University School of Medicine) to knock down endogenous “background” glucose transport through GLUT1 in subsequent studies in which GLUT2, GLUT3, GLUT4, and GLUT8 were overexpressed and studied. After puromycin selection, cells were stably transfected to express codon-optimized human GLUT2, GLUT3, GLUT4, or GLUT8 DNA in the pcDNA3.1(−)hygro plasmid (Life Technologies). GLUT1 overexpression was carried out in wild-type 293 cells not expressing GLUT1 shRNA. After hygromycin selection, colonies were grown to near confluence in 4-cm tissue culture dishes, and the highest-expressing colonies for each of the GLUT isoforms were selected using [3H]2-DG uptake and qRT-PCR. Absolute GLUT mRNA quantity for 293 cells expressing GLUT1, GLUT2, GLUT3, GLUT4, and GLUT8 is shown in table S1.

HepG2 cultures were obtained from the American Type Culture Collection. Cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (4.5 g/liter) and 10% fetal bovine serum (FBS) (regular growth media) through passage 15.

Primary murine hepatocytes obtained from wild-type, ATG16L1<sup>HM</sup> knock-in mice and MTTP-knockout mice were isolated precisely as described (2). Briefly, mice were cannulated through the portal vein under anesthesia with methapone and pentobarbital, and the liver was perfused with oxygenated calcium- and magnesium-free HBSS (pH 7.4) (Invitrogen) for 5 min, followed by perfusion of oxygenated DMEM (Sigma-Aldrich) containing 0.05% type IV collagenase (Sigma-Aldrich) containing 0.05% type IV collagenase (Sigma-Aldrich) for 5 to 10 min. The perfusate was drained through an incision in the inferior vena cava. After perfusion, the liver was removed and submerged into ice-cold DMEM, and the cells were released gently. The resultant cell suspension was then filtered through a 45-μm nylon mesh, pelleted on ice for 30 min, and then resuspended in regular growth media. Cells were plated onto tissue culture plates coated with type I collagen from Sigma-Aldrich and incubated at 37°C under 5% CO2 in regular growth media. After 1 hour of attachment, cultures were washed in regular growth media, and the cell monolayers were maintained overnight in regular growth media before assay. Adenoviral constructs (KD-AMPK, GLUT2, GLUT8) were purchased from Applied Biological Materials.

Statistics
All data were analyzed using GraphPad Prism 6.0. P < 0.05 was defined as statistically significant after post hoc correction for multiple comparisons. Specific statistical tests applied are noted in the figure legends.

Sequence alignment and phylogeny
Accession numbers for sequences aligned using ClustalW2 were NP_067456.1 for trehalase, BAA95533.1 for Tre1, EDL30474.1 for GLUT1, NP_112474.2 for GLUT2, and NP_062361.1 for GLUT8. The following program-default parameters were used: output format—Clustal without numbers; no input sequence dealignment; MBED-like clustering iteration and MBED-like clustering guide tree; 0 combined guide tree iterations; “default” maximum guide tree and “default” maximum HMM iterations were used; aligned order output.

[3H]2-DG uptake
HEK 293 cells overexpressing human GLUT1, GLUT2, GLUT3, GLUT4, or GLUT8 were grown in minimum essential medium (MEM) supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). 2-DG (50 μM, 1 μCi/ml; obtained from American Radiolabeled Chemicals) uptake was determined at 37°C according to Tordjman et al. (32) with the following modifications. To adhere the cells to tissue culture plates, dishes were first pretreated with polyethyleneimine (25 μg/ml) (FLUKA, catalog no. P3143) in 150 mM NaCl for 20 min followed by the removal of the solution by vacuum. Cells were then plated at MEM (2 × 10<sup>5</sup> cells/ml) supplemented with 10% FBS, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) (normal growth medium) in 12-well plates and incubated overnight at 37°C. After 30-min incubation at 37°C in glucose-free Hepes-buffed saline (pH 7.3), in the presence or absence of 0 to 500 mM trehalose, 2-DG uptake was measured in glucose-free Hepes (pH 7.3) for 6 min at 37°C. Cultures were washed extensively in phosphate-buffered saline and lysed in 0.1 N NaOH, 1% SDS lysis buffer, and 80% of each lysate was subjected to liquid scintillation counting (Ultima Gold, Thermo Fisher). Protein concentration was determined in the remaining lysate, and total protein per well was used to normalize total 6-min 2-DG uptake in each well. Uptake data are plotted as percent uptake relative to trehalose-unexposed HEK 293 cultures.

Primary hepatocytes and HepG2 cells were plated at 5 × 10<sup>5</sup> cells per well in 12-well plate and maintained at 37°C overnight in regular growth media until 2-DG uptake was assayed. On the day of assay, cultures were incubated (30 min at 37°C) in glucose-free Hepes-buffed saline (pH 7.3), in the presence or absence of 100 mM trehalose or 40 μM lopinavir (NIH AIDS Reagent Program, Germantown, MD). Cultures were then incubated with [3H]2-DG (1 μCi/ml) (50 μM) in glucose-free Hepes (pH 7.3), at 37°C for 6 min in the presence or absence of 100 mM trehalose or 40 μM lopinavir before washing, lysis, and analysis as described above. DMSO (vehicle) was used as the vehicle control in 2-DG uptake assays in which lopinavir was used to inhibit 2-DG uptake.

[3H]2-DG uptake was performed as described (2). Briefly, primary hepatocyte and HepG2 cultures were plated at 5 × 10<sup>5</sup> cells per well in 12-well plates and maintained at 37°C overnight in regular growth media until assay. On the day of assay, cultures were incubated (30 min at 37°C) in glucose-free Hepes-buffed saline (pH 7.3), in the presence or absence of 100 mM trehalose. Cultures were then incubated with [3H]2-DG (1 μCi/ml) (4 μM) (American Radiolabeled Chemicals) for 1 min, before washing, lysis, and analysis as described above for [3H]2-DG uptake.

Hepatocyte ATP measurement
ATP concentrations in cultured hepatocytes were measured as described previously (17, 18). Briefly, primary hepatocytes grown in six-well plates in DMEM containing glucose (4.5 g/liter) and 10% FBS were treated in the presence or absence of 100 mM trehalose for 30 min. Cells were trypsinized, and the cell pellet was washed in phosphate-buffered saline before homogenization in 0.1 ml of 0.1 N NaOH and protein denaturation by incubation at 80°C for 20 min. The homogenate was neutralized with 50 μl of neutralization buffer [0.15 N HCl, 0.1 M tris-HCl (pH 6.6)] to produce a final 34 mM tris-HCl (pH 8.1) cell extract.

The enzymatic-fluorimetric ATP assay was carried out using 20 μl of cell extract. Tris-HCl (34 mM) (pH 8.1) was used as a “blank” reaction to quantify background fluorescence. ATP standards and cell extracts were added to 50 μl of ATP reagent [60 mM tris-HCl (pH 8.1), 0.03% BSA, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 450 μg/ml glucose, 100 mM nicotinamide adenine dinucleotide phosphate, hexokinase (3 μg/ml) without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and glucose-6-phosphate dehydrogenase (1 μg/ml) without (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>). The reaction was carried out at 60°C for 20 min and terminated by adding 10 μl of 0.5 N NaOH. Wavelength emission (340 nm) was subsequently measured on a Packard BF 10000 FluoroCount microtiter plate fluorometer after addition of 1 ml of 6 N NaOH, 10 mM imidazole, and 0.01% H<sub>2</sub>O<sub>2</sub> to each reaction. ATP concentrations in each experiment were normalized to total protein [determined by bicinechonic acid assay (BCA assay; Thermo Scientific)] in the neutralized cell extract and were calculated as (nmol ATP)/(mg total protein)<sup>−1</sup>. Data from three
Immuno blotting

Immuno blotting was performed as recently described (33). Samples for immuno blot analysis were prepared as follows: cultures for HepG2 and primary hepatocytes were seeded at 1 × 10⁶ cells per well, grown in six-well plates, and placed on ice after experimental manipulations. Each sample was lysed in 125 μl of ice-cold RIPA (radioimmunoprecipitation assay) [50 mM tris-HCl (pH 8.0) with 150 mM sodium chloride, 1% IGEPAL CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate]. Cell debris was cleared from each lysate by microcentrifugation [16,200 relative centrifugal force (RCF)] before protein concentration determination by BCA assay (Thermo Fisher) to normalize protein loading. Samples were diluted 1:1 in 2x Laemmli Sample Buffer (Bio-Rad). Ten to 25 μg of protein were resolved by 10% bis-acrylamide SDS–polyacrylamide gel electrophoresis and transferred to 0.2-μm-pore nitrocellulose membranes (VWR) by semi-dry electrophoresis (Bio-Rad). Non-specific antibody binding to nitrocellulose membranes was blocked by 60-min incubation in 5% milk in tris-buffered saline–Tween 20 [TBST; 0.05 M tris-HCl, 0.138 M NaCl, 0.0027 M KCl, Tween 20 (pH 7.4)]. After antibody blocking, primary antibodies were diluted in TBST containing 5% BSA (Sigma–Aldrich) overnight at 4°C. Membranes were washed in TBST and incubated (1 hour at room temperature) with horseradish peroxidase–linked anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG (both obtained from Cell Signaling Technology) diluted in TBST containing 5% milk. After TBST washing, immunoreactive bands were visualized by incubating each membrane with chemiluminescent horseradish peroxidase substrate (Clarity ECL, Bio-Rad) and exposing to autoradiographic film (VWR).

The following primary antibodies were from Cell Signaling Technology: AMPKα (#5831), phospho-AMPK (T172) (#2535), phospho-ACC1 (S79) (#11818), ACC1 (#30767), phospho-ULK1 (S317) (#2535), phospho-ULK1 (S757) (#6888), ULK1 (#8054), actin (#4970), and GAPDH (#5174). LC3B antibody was obtained from Novus Biologicals (#NB-100-2200).

For autophagic flux assays, primary hepatocytes and HepG2 cell lines were maintained in regular growth media before experimentation. Cultures were then incubated in the presence or absence of DMSO or 100 mM BafA1 (Cayman Chemical) with or without 100 mM trehalose for 1 hour. Cultures were lysed and subjected to immuno blot analysis as described above.

Lopinavir effects on autophagic flux were tested in primary hepatocytes grown in regular growth media overnight after isolation. DMSO or 40 μM lopinavir was added to regular growth media and added to cultures for 15 min before lysis and immuno blotting as described above.

For HBSS starvation-refeed assays, primary murine hepatocytes were incubated in HBSS (5.3 mM KCl, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 137.9 mM NaCl, 0.34 mM Na₂HPO₄, 5.56 mM α-glucose; Thermo Fisher) before addition of HBSS alone or HBSS supplemented to a final glucose concentration of 25 mM in the presence or absence of 100 mM trehalose. Cultures were lysed in ice-cold RIPA before protein determination and immuno blot analysis as described above.

Gas chromatography–mass spectrometry

trehalose quantification

[¹³C₁₂]Trehalose (Omicron Biochemicals) was used as an extraction and derivatization internal standard for samples. Internal standard (5 nmol) was added to excess 200 μl of isopropanol/CH₃CN/H₂O, and the sample was vortexed and centrifuged to achieve phase separation. The supernatant was dried at room temperature overnight under nitrogen gas after addition of MSTFA with 10% pyridine in CH₃CN. Derivatized samples were analyzed on an Agilent 7890A gas chromatograph interfaced to an Agilent 5975C mass spectrometer. The gas chromatography column used for this study was an HP-5ms (30 m, 0.25-mm internal diameter, 0.25-μm film coating; F.J. Cobert). A linear temperature gradient was used. The initial temperature of 80°C was held for 2 min and increased to 300°C at 10°C/min. The temperature was held at 300°C for 2 min. The samples were run by electron ionization, and the source temperature, electron energy, and emission current were 200°C, 70 eV and 300 μA, respectively. The injector and transfer line temperatures were 250°C.

Real-Time qRT-PCR

qRT-PCR was performed with primer sequences and method precisely as described (2). Briefly, RNA was isolated from either 1 × 10⁶ cells (for primary hepatocyte experiments) or from 100 mg of dissected liver tissue (derived from in vivo chow-, HFrD-, and trehalose-treated animals) homogenized in TRIzol reagent (Invitrogen) by manufacturer’s protocol.

One thousand nanograms of RNA per sample was reverse-transcribed to complementary DNA (cDNA) using the QuantiTec QIAGEN Reverse Transcription Kit (QIAGEN). cDNA was subjected to real-time qRT-PCR using the SYBR Green Master Mix reagent (Applied Biosystems). Primers for real-time qRT-PCR (listed 5’ to 3’) are as follows (2, 28, 34, 35): ACC1, TGTCGCACTGACTGTAACCA (sense) and TGTCGCACTGACTGTAACCA (antisense); and GAPDH, ChREBP, CCGAGCTGTATATGGAGACG (sense) and GAAGTCACCATTCATAGCTCC (antisense); and PPARγ, CACACTCCCGCATC (sense) and GTGACCTTGATGAGGATGAC (antisense); and SCD1, CCGGAGACCTTAGATCGA (sense) and TAGCCGTAAAGATTTCTGCA (antisense).

Oil Red O staining

Oil Red O staining was performed precisely as described (2). Briefly, 10-μm frozen sections from dissected mouse livers were fixed for 5 min (−20°C) and washed in phosphate-buffered saline. Sections were stained in 0.3% Oil Red O in 60% isopropanol (15 min) followed by extensive distilled water washes until the effluvium cleared. Sections were mounted in 54% aqueous glycerol mounting media before imaging. For staining density quantification, high-powered (40× objective) photomicrographs of stained sections were obtained while each slide’s treatment group was blinded to the research assistant. Within ImageJ software (version 1.47), pixel densities from three to five random fields within three distinct cryosection photomicrographs obtained from at least three different mice per group were quantified. Photomicrograph data were unblinded before calculating density mean and SEM, as presented in Fig. 5.

Analysis of AMPK signaling and autophagic flux in mouse liver

For in vivo autophagic flux assays, 8-week-old wild-type mice were injected intraperitoneally with leupeptin hemisulfate (40 mg/kg) (21) (Sigma–Aldrich) or with saline 1 hour before orogastric gavage with saline or with trehalose (3 g/kg) in saline. Mice were sacrificed 2 hours after orogastric gavage, and livers were rapidly dissected and snap-frozen in liquid nitrogen before homogenization in ice-cold RIPA lysis buffer and immunoblot analysis. Throughout the experiment, mice were given ad libitum access to standard rodent chow and water.

For in vivo signaling assays, 8-week-old wild-type mice were subjected to orogastric gavage with trehalose (3 g/kg) dissolved in saline. Mice were sacrificed 0.5 to 4 hours after gavage, and livers were rapidly dissected and snap-frozen in liquid nitrogen before homogenization in ice-cold RIPA lysis buffer and immunoblot analysis. Control mice were gavaged with saline alone
and sacrificed 0.5 hour after gavage. They are labeled as “0 hour” trehalose exposure.

Plasma and liver biochemical analyses

In vivo plasma and liver biochemistries were performed precisely as described previously (2, 28). Briefly, 100 mg of dissected liver tissue was homogenized on ice in 2 ml of 2:1 chloroform/methanol (both obtained from Sigma-Aldrich). Residual tissue debris was pelleted by centrifugation at 16,200 RCF, and 20 μl of supernatant was dried in chloroform-resistant microcentrifuge tubes (Thermo Fisher) before addition of 200 μl of Infinity triglyceride assay reagent (1 hour at room temperature) and 490-nm optical density quantification by 96-well format plate reader (BioTek). Glyceral standard solutions (5 mM) were treated identically in parallel to obtain substrate specificities and effects of transport inhibitors. Biochem. J. 290, 701–706 (1993).


RESEARCH ARTICLE

RESEARCH ARTICLE

22. R. Singh, A. M. Cuervo, Autophagy in the cellular energetic balance.


Submitted 10 May 2015
Accepted 3 February 2016
Final Publication 23 February 2016
10.1126/scisignal.aac5472

Trehalose inhibits solute carrier 2A (SLC2A) proteins to induce autophagy and prevent hepatic steatosis

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DOI: 10.1126/scisignal.aac5472

A sugary inhibitor of liver disease

The accumulation of lipids in hepatocytes that occurs in nonalcoholic fatty liver disease (NAFLD) can result in liver failure or liver cancer. Trehalose is a ubiquitous sugar that is present in the food consumed by animals. DeBosch et al. determined that trehalose blocked glucose uptake into cells by inhibiting glucose transporters in the plasma membrane, which induced a “starvation”-like response that activated autophagy even in the presence of adequate nutrients and glucose. Furthermore, providing trehalose to mice that are a model of NAFLD prevented lipid accumulation in the liver. As noted by Mardones et al. in the associated Focus, trehalose, which has been previously under investigation to treat neurodegenerative diseases characterized by toxic protein aggregates, may be a “silver bullet” for treating diseases resulting from inadequate cellular degradative metabolism.