Original Communication

Naturally occurring rare sugars are free radical scavengers and can ameliorate endoplasmic reticulum stress

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Abstract: Because of potential use of naturally occurring rare sugars as sweeteners, their effect on superoxide (SO), hydroxyl and peroxyl radicals and endoplasmic reticulum (ER) stress was examined in human coronary artery endothelial cells. SO generation was measured using the superoxide-reactive probe 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-A]pyrazin-3-one hydrochloride chemiluminescence. Phycoerythrin fluorescence based assay was used to monitor scavenging activity of sugars in the presence of hydroxyl or peroxyl radical generators [$CuSO_4$ and azobis (2 amidinopropane) hydrochloride respectively]. Measurements were made in relative light units (RLU). ER stress was measured with an ER stress-sensitive secreted alkaline phosphatase (SAP) assay and by Western blot analysis of the expression and phosphorylation of key proteins in the unfolded protein response, namely CHOP47, eIF2α and JNK1. D-Glucose (27.5 mM) increased SO generation (5536 ± 283 vs. 2963 ± 205 RLU in controls; $p < 0.0007$) and decreased SAP secretion (73411 ± 3971 vs. 101749 ± 7652 RLU in controls; $p < 0.005$) indicating ER stress. Treatment of cells with 5.5 or 27.5 mM of D-allulose, D-allose, D-sorbose and D-tagatose reduced SO generation (all $p < 0.05$). This could not be attributed to inhibition of cellular uptake of dextrose by the rare sugars tested. In a cell free system, all four rare sugars had significantly more SO, hydroxyl and peroxyl radical scavenging activity compared to dextrose (all $p < 0.01$). Treatment of cells with rare sugars reduced ER stress. However, unlike other three rare sugars, D-sorbose did not inhibit tunicamycin-induced eIF2α phosphorylation. Naturally occurring rare sugars are free radical scavengers and can reduce ER stress.

Keywords: free radical scavengers, oxidative stress, rare sugars, endoplasmic reticulum stress, unfolded protein response, sugar substitutes, sweetener

Introduction

Oxidative stress and endoplasmic reticulum (ER) stress have been implicated in a variety of disease states and are suspected to be an important contributor to diabetes-related complications [1, 2]. In addition reactive oxygen species have important signaling function in various cellular compartments [3] Oxidative stress occurs when the production of free radicals exceed the antioxidant defense capacity of the cells. The ER is the cellular site where secreted and membrane-bound proteins acquire their tertiary structure with proper folding. When misfolded protein levels exceed the capacity of clearing mechanisms, ER stress occurs with launching of unfolded protein response (UPR). This response includes transcriptional and translational changes to inhibit further protein synthesis [1].

Various signals generated by nutrients, hormones and inflammatory cytokines are known to initiate oxidative and ER stress. A common source of such stress is increased plasma concentrations of D-glucose and free fatty acids that is often observed in uncontrolled diabetes [2]. Indeed, hyperglycemia-induced oxidative and ER stress has been shown to have a potential role in mediating metabolic damage to endothelial cells, macrophages and smooth muscle cells [4–8] thereby driving the increased incidence of premature atherosclerosis in people with diabetes.

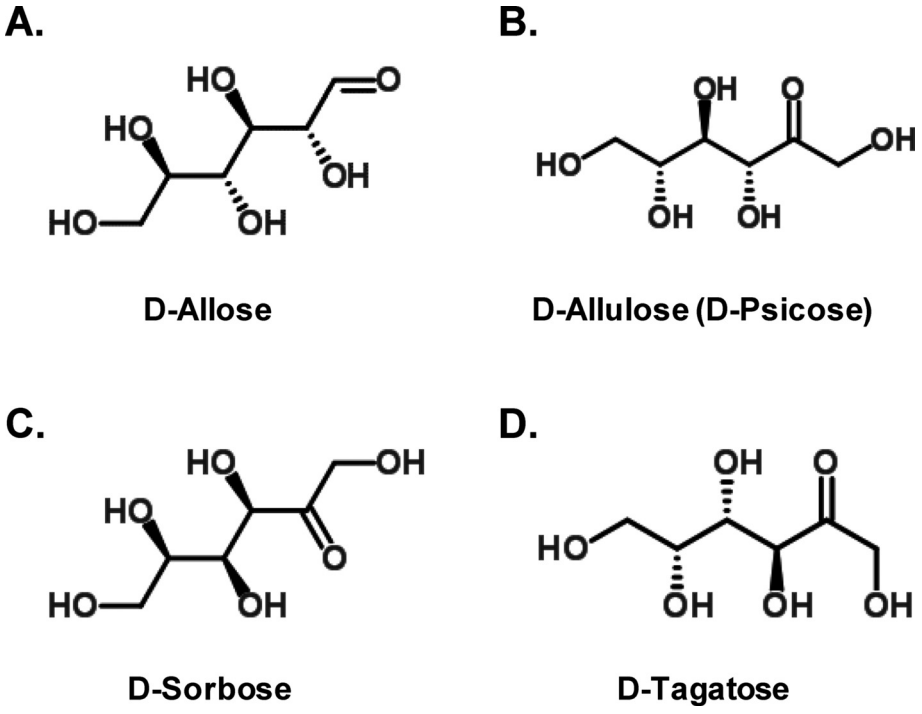

Figure 1. The chemical structure of the rare sugars studied.

With the recent increase in awareness of health consequences associated with excess consumption of simple table sugar (sucrose), there is renewed interest in using naturally occurring rare sugars as alternative sweeteners [9]. Examples of the latter include D-allose, D-allulose (D-psicose), D-sorbose, and D-tagatose (Figure 1) [10]. The potential advantages of this category of sweeteners include palatability, lack of significant calories, and possibly antioxidative [11, 12] and anticancer effects [13, 14]. Because of the pivotal role of oxidative and ER stresses in the pathogenesis of various diseases, the effect of these naturally occurring rare sugars on free radical scavenging and ER stress were examined in human coronary artery endothelial cells (HCAEC).

Materials and methods

Materials

D-Glucose, 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), mannose, galactose, fructose, 4-phenylbutyrate, mammalian protein extraction reagent (MPER) and protease inhibitor and phosphatase inhibitor cocktail, and tunicamycin were purchased from Thermo-Fisher Scientific (Pittsburg, PA). Porphyridium cruentum β-3-phycoerythrin (β-3-PE) was purchased from Sigma Chemical Co. (St. Louis, MO). D-Allose, D-allulose, D-sorbose, and D-tagatose were kindly provided by Matsutani Chemical Industry Co. Ltd. (Hyogo, Japan). The superoxide anion probe 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-A] pyrazin-3-one hydrochloride (MCLA) was purchased from Invitrogen (Carlsbad, CA). The chemiluminescent secreted alkaline phosphatase substrate disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD) was purchased from CloneTech (Mountain View, CA). [^{3}H]-2-deoxyglucose was purchased from Perkin Elmer (Waltham, MA). Dulbecco's modified Eagles medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from BioWhittaker, Inc. (Walkersville, MD). Vascular cell growth medium and its supplements were purchased from American Type Culture Collection (Manassas, VA). Primary antibodies to β-actin (PA1-21167), C/EBP homologous protein 47 (CHOP47) (JM3421100) and phosphorylated CHOP47 (PA5-35669) were purchased from Thermo-Fisher Scientific while antibodies to the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (#9722) and phosphorylated eIF2α (#3398) were purchased from Cell Signaling, Inc. (Danvers, MA). Primary antibodies to total (sc-136205) and phosphorylated (sc-293136) c-*jun* N-terminal kinase 1 (JNK1) were purchased from Santa Cruz Biotechnology (Dallas, TX). Secondary antibodies (1030-05 and 4010-05) were purchased from Southern Biotech (Birmingham, AL). All other reagents were from Sigma Chemical Company or Thermo-Fischer Scientific.

Cell Culture

HCAEC were maintained in vascular endothelial cell growth medium containing 0.2% bovine brain extract, 5 ng/ml recombinant human epidermal growth factor,

10 mM glutamine, 1 μg/ml hydrocortisone hemisuccinate, 0.75 units/ml heparin sulfate, 50 μg/ml ascorbic acid, 2% fetal bovine serum, 10 units/ml penicillin and 10 μg/ml streptomycin. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2. Cells between passages 3 and 5 were used in all experiments.

Superoxide generation

Superoxide (SO) generation was measured using MCLA chemiluminescence as previously described [8]. HCAEC were treated with 5.5 or 27.5 mM D-glucose and various other test sugars and MCLA was added to a final concentration of 1 μmol/L in Hank's balanced salt solution (HBSS) containing 1.26 mM $CaCl_2$, 5.37 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2 \cdots 6\ H_2O$, 0.41 mM $MgSO_4 \cdots 7\ H_2O$, 136.7 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, and 5.5 mM D-glucose. Additional experiments were carried out after adding 100 μM of H_2O_2 in cell-free HBSS. Superoxide-induced chemiluminescence was measured with a luminometer and is expressed in relative light units (RLU). Sugar concentrations were chosen to simulate the common physiologic range (5.5–27.5 mM) of blood glucose concentrations in non-diabetic and diabetic people. The choice of the concentrations was also based on our previous observations of the effects of dextrose in endothelial cell cultures (7, 8).

Hydoxyl and peroxyl radical scavenging activity measurements

The fluorescence of phycoerythrin was monitored in phosphate buffer in the presence of various concentrations of sugars incubated at 37°C with or without a hydroxyl radical generator ($CuSO_4$) and a peroxyl radical generator (2,2-azobis(2-amidinopropane) hydrochloride (AAPH)) using a previously published protocol [15]. Phosphate buffer (0.075 M) pH 7.0 was made from 0.075 M NaH_2PO_4 and 0.075 M K_2HPO_4 and passed through a 10 ml Chelex 100 resin column (Na-form, BioRad, Richmond, CA) to remove all metal ions. All reagents and compounds were made up in this buffer. The final reaction mixture contained 1.65×10^{-8} M β-3-PE, 0.075 M phosphate buffer or various sugars and either 5 μM $CuSO_4$ or 4 mM AAPH. The following compounds were tested: D-glucose, D-allulose, D-allose, D-sorbose and D-tagatose. The solutions were incubated with β-3-PE in 37°C bath 10 min before adding 5 μM CuSO4 to start the reaction (Time 0). Fluorescence was measured at 565 nm with excitations at 540 nm using a Perkin-Elmer 650-10S fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT). The fluorescence of phycoerythrin is a very sensitive measure of the chemical integrity of the molecule and in the presence of hydroxyl or peroxyl free-radical generators, the change in fluorescence over time is an index of free radical damage of this high molecular weight protein. The inter-assay variability in these measurements was less than 10%.

Transient transfection and measurement of secreted alkaline phosphatase activity

HCAEC in six-well plates were transfected with two μg of the plasmid pSEAP2.Control (expressing secreted alkaline phosphatase (SAP)) using Lipofectamine. After 24-hours, the cells were treated with test substrates and 24-hours later the medium was collected and assayed for SAP activity [7, 8, 16]. Briefly, one-ml of medium was transferred from the cells to a microcentrifuge tube and any floating cells in the samples were removed by centrifugation for five-minutes at 10,000 $\times g$ at 4°C. SAP dilution buffer was added to 25-μl of each sample and incubated at 65°C for 30-minutes. After addition of 100-μl of CSPD the samples were incubated at room temperature for 30-minutes and luminescence was measured with a Turner Biosystems luminometer (Sunnyvale, CA) and is expressed in relative light units (RLU) or percent change from control.

Immunoblotting

HCAEC were treated as described in each figure and protein samples were prepared by lysing the cells in MPER and quantified using the bicinchoninic acid (BCA) assay [17]. Samples consisting of 50 μg of protein were separated by electrophoresis on 10% sodium dodecylsulfate (SDS)-polyacrylamide gels [18] and transferred to Immobilon-P (Millipore, Billerica, MA) transfer membrane [19]. The membranes were blocked in 10% newborn calf serum (NCS) in Tris-buffered saline (50 mM tris-[hydroxymethyl]aminomethanehydrochloride (Tris-Cl) (pH 8.0), 150 mM NaCl, TBS) containing 0.1% Tween 20 (TBST) for two hours at room temperature and incubated with the primary antibody overnight in 10% NCS in TBS at 4°C according to the manufacturer's directions. The membranes were washed four times in TBST, then incubated with the appropriate secondary antibody (diluted 1:4000 in 10% NCS in TBS), washed four more times in TBST, five minutes each, then washed twice with TBS prior to visualizing by enhanced chemiluminescence (ECL) (Rockford, IL) and autoradiography. The amount of immune-reactive material was then quantified using NIH Image J.

Glucose uptake assays

Cells in 12-well dishes were allowed to grow to 90% confluence prior to treatment and addition of

[^{3}H]-2-deoxyglucose. Assays were performed as described previously [20]. Briefly, the cells were washed three times with 1 ml of HBSS (without the glucose), then incubated in 1 ml of media containing 1% bovine serum albumin (BSA) for 2-hours at 37°C. The cells were then washed two times with 1 ml of warm HBSS, after which 1 ml of warm HBSS containing 1 μCi [^{3}H]-2-deoxyglucose (31.3 nM; specific activity 8.0 Ci/mM) was added along with the sugars to be tested (5.5 mM and 27.5 mM each). As an internal control 20 μM cytochalasin B (a known pharmacologic inhibitor of glucose transport) was also tested. After 20 minutes, the media was removed by aspiration and the cells were washed three times with 1 ml of HBSS, then 400 μl of 1% sodium dodecylsulfate (SDS) was added to each well and placed on a shaker at room temperature for 10 minutes. The cell lysate was transferred to 4 ml of scintillation fluid and counted on a Perkin Elmer Tricarb scintillation counter.

Statistics

Measurements are reported as the mean ± standard deviation. Analysis of variance (ANOVA) followed by a Bonferroni correction for post-hoc analysis, and the Students t-test for independent variables were performed with Statistica for Windows (Statsoft Inc., Tulsa, OK).

The normal distribution of the data was assessed with the Shapiro-Wilk test while equality of variance was tested using Leven's test. Statistical significance was defined as a two-tailed $p < 0.05$.

Results

The effect of select rare sugars on dextrose induced superoxide anion generation

As has been shown in previous publications, addition of 27.5 mM D-glucose significantly increased SO anion generation (5536 ± 283 RLU) relative to cells exposed to 5.5 mM D-glucose (2963 ± 205 RLU) ($p < 0.0007$) (Figure 2). Whereas the addition of 5.5 mM or 27.5 mM fructose, galactose, or mannose could not reduce dextrose induced SO generation, the addition of 5.5 mM or 27.5 mM D-allulose, D-allose, D-sorbose, and D-tagatose resulted in a dose-dependent decreases in dextrose induced SO generation (relative to cells treated with 27.5 mM D-glucose, $p < 0.02$, $p < 0.02$, $p < 0.02$ and $p < 0.0004$ for 5.5 mM D-allulose, D-allose, D-sorbose, and D-tagatose respectively, and $p < 0.0006$, $p < 0.0009$, $p < 0.0005$ and $p < 0.0005$ for 27.5 mM D-allulose, D-allose, D-sorbose, and D-tagatose respectively) (Figure 2).

Free radical scavenging effect of select rare sugars

In a cell free assay using H_2O_2 as SO generating source, the addition of 5.5 mM or 27.5 mM D-allulose, D-allose, D- sorbose, or D-tagatose suppressed SO anion levels in a

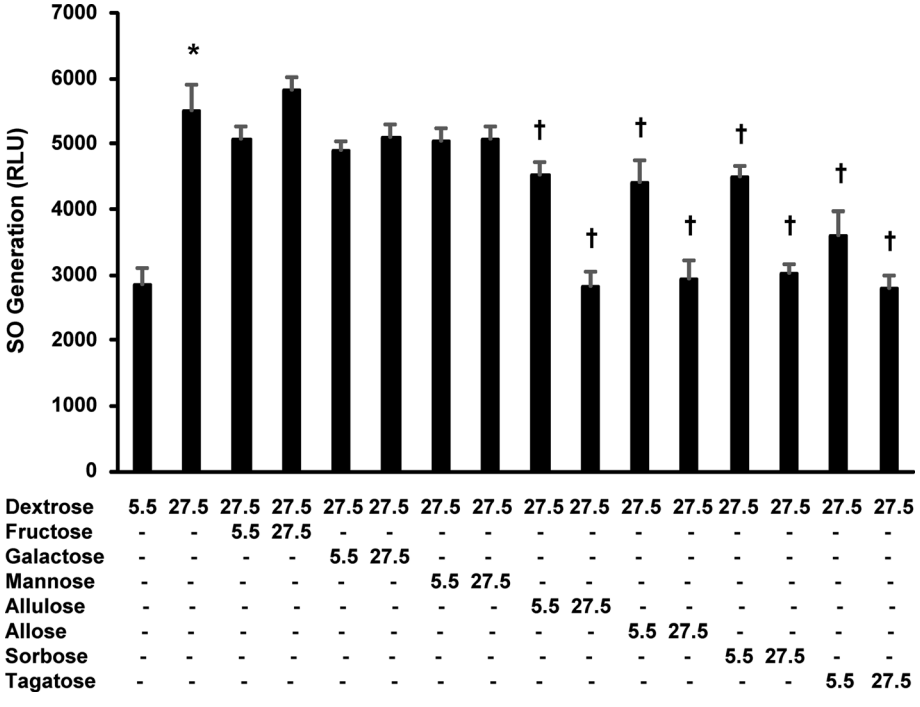

Figure 2. The effect of select rare sugars on superoxide anion generation in human coronary artery endothelial cells. Cells were treated with 5.5 mM D-glucose (dextrose) or 27.5 mM dextrose and with and without 5.5 mM or 27.5 mM fructose, galactose, mannose, D-allulose, D-allose, D-sorbose, and D-tagatose. The error bars are the ± SD; N = 6; *, $p < 0.0007$ relative to cells treated with 5.5 mM dextrose. †, $p < 0.02$, $p < 0.0006$, $p < 0.02$, $p < 0.0009$, $p < 0.02$, $p < 0.0005$, $p < 0.004$, and $p < 0.0005$, respectively, relative to cells treated with 27.5 mM dextrose.

Table 1. Free radical scavenging activity of dextrose and select rare sugars. Controls are H_2O_2, $CuSO_4$ and 2,2′ azobis(2-amidinopropane) hydrochloride (AAPH) alone as a source of superoxide, hydroxyl and peroxy radicals respectively, in the absence of any sugar. The experiments are done in cell-free solutions. The mean ± SD measurements for H_2O_2, $CuSO_4$ and AAPH controls in the absence of any sugars were 3994 ± 421 RLU, 0.418 ± 0.034 (fluorescence at 565 nm) and 0.408 0.025 (fluorescence at 565 nm) respectively. N = 6.

Sugars	Superoxide percent of control (mean ± SD)	Hydroxyl radical percent of control (mean ± SD)	Peroxyl radical percent of control (mean ± SD)
Dextrose (5.5 mM)	103.5 ± 8.5	100.8 ± 2.2	71.3 ± 5.3[e]
Dextrose (27.5 mM)	100.2 ± 11.4	280.4 ± 4.8[b,f]	52.5 ± 4.0[f]
Allulose (5.5 mM)	61.8 ± 8.9[a,e]	61.3 ± 5.9[a,e]	45.9 ± 5.1[b,f]
Allulose (27.5 mM)	29.1 ± 14.6[c,f]	19.5 ± 8.9[d,f]	16.1 ± 3.3[d,f]
Allose (5.5 mM)	62.0 ± 15.1[a,e]	67.0 ± 6.7[a,e]	54.0 ± 3.5[b,f]
Allose (27.5 mM)	30.1 ± 8.8[c,f]	34.1 ± 9.2[d,f]	36.5 ± 4.4[d,f]
Sorbose (5.5 mM)	64.2 ± 9.3[a,e]	49.8 ± 6.9[a,f]	57.3 ± 5.3[a,e]
Sorbose (27.5 mM)	41.4 ± 12.3[d,f]	20.3 ± 9.0[d,f]	45.3 ± 4.9[d,f]
Tagatose (5.5 mM)	43.4 ± 11.3[b,f]	41.0 ± 9.0[b,f]	50.4 ± 3.6[b,f]
Tagatose (27.5 mM)	28.8 ± 3.6[d,f]	15.5 ± 6.1[d,f]	24.3 ± 3.6[d,f]

[a]$p < 0.01$ compared with 5.5 mM dextrose.
[b]$p < 0.001$compared with 5.5 mM dextrose.
[c]$p < 0.01$ compared with 27.5 mM dextrose.
[d]$p < 0.001$ compared with 27.5 mM dextrose.
[e]$p < 0.01$ compared with controls.
[f]$p < 0.001$ compared with controls.

dose-dependent manner (Table 1). SO anion levels were reduced significantly more by rare sugars compared to dextrose. Addition of galactose, mannose, and 5.5 mM fructose had no effect on SO anion generation, while addition of 27.5 mM fructose further enhanced it ($p < 0.05$ relative to H_2O_2 treatment) (Data not included in Table 1).

All four rare sugars had significantly more hydroxyl and peroxyl radical scavenging activity compared to dextrose (Table 1). It is noteworthy that, 25 mM D-glucose, but not 5 mM D-glucose solution could significantly potentiate $CuSO_4$-induced free radical damage. In contrast, D-allulose, D-allose, D-sorbose and D-tagatose had an antioxidant effect in this assay (Table 1). In the presence of AAPH, 25 mM D-glucose, and to a lesser extent, 5 mM D-glucose, had statistically significant peroxyl radical quenching effect (71.3 ± 5.3% and 52.5 ± 4.0% of control; $p < 0.006$ and $p < 0.006$, respectively). The corresponding peroxyl radical quenching effect expressed as percentage of controls for 5 mM D-allulose, D-allose, D-sorbose and D-tagatose were 45.9 ± 5.1%, 54.0 ± 3.5%, 57.3 ± 5.3%, 50.4 ± 3.6%, respectively ($p < 0.0007$, $p < 0.0005$, $p < 0.002$, and $p < 0.004$, respectively) (Table 1).

The effect of various sugars on endoplasmic reticulum (ER) stress

Treatment of HCAEC with 27.5 mM D-glucose caused significant ER stress as measured by decreased SAP activity relative to cells treated with 5.5 mM D-glucose ($p < 0.005$) (Figure 3). Addition of 5.5 mM fructose, galactose or mannose had no effect on SAP activity, while addition of 27.5 mM fructose further decreased SAP activity ($p < 0.03$) (Figure 3A). It is noteworthy that the high osmolarity of culture media in experiments where 27.5 mM galactose or mannose was added to 27.5 mM D-glucose did not alter SAP activity compared to cells treated with 27.5 mM D-glucose alone.

Addition of D-allulose increased SAP activity in a dose-dependent manner ($p < 0.01$ and $p < 0.003$ in cells treated with 5.5 and 27.5 mM D-allulose, respectively) (Figure 3A). Treatment with 10 μM 4-phenylbutyrate,a pharmacologic ER stress inhibitor, or treatment with 27.5 mM D-allose, and 5.5 mM or 27.5 mM D-sorbose or D-tagatose increased SAP activity relative to cells exposed to 27.5 mM D-glucose alone ($p < 0.02$, $p < 0.02$, $p < 0.03$, and $p < 0.005$, respectively) (Figure 3B).

Treatment with a pharmacologic ER stress inducer (0.1 μM tunicamycin) decreased SAP activity from 127656 ± 6175 RLU in control cells to 85371 ± 4996 RLU in tunicamycin-treated cells ($p < 0.0008$, relative to control cells) (Figure 4A). While treatment with fructose, galactose, and mannose had no effect on SAP activity in tunicamycin-treated cells, addition of allulose increased SAP activity in a dose-dependent manner (101511 ± 4205 and 127641 ± 3535 RLU in cells treated with 5.5 and 27.5 mM D-allulose, respectively; $p < 0.01$ and $p < 0.0003$, respectively, relative to cells treated with tunicamycin) (Figure 4A). Treatment with 5.5 mM D-allose had no effect on SAP activity in tunicamycin-treated cells, however addition of 27.5 mM D-allose increased SAP activity significantly ($p < 0.008$) (Figure 4B). Treatment with either 5.5 mM or 27.5 mM

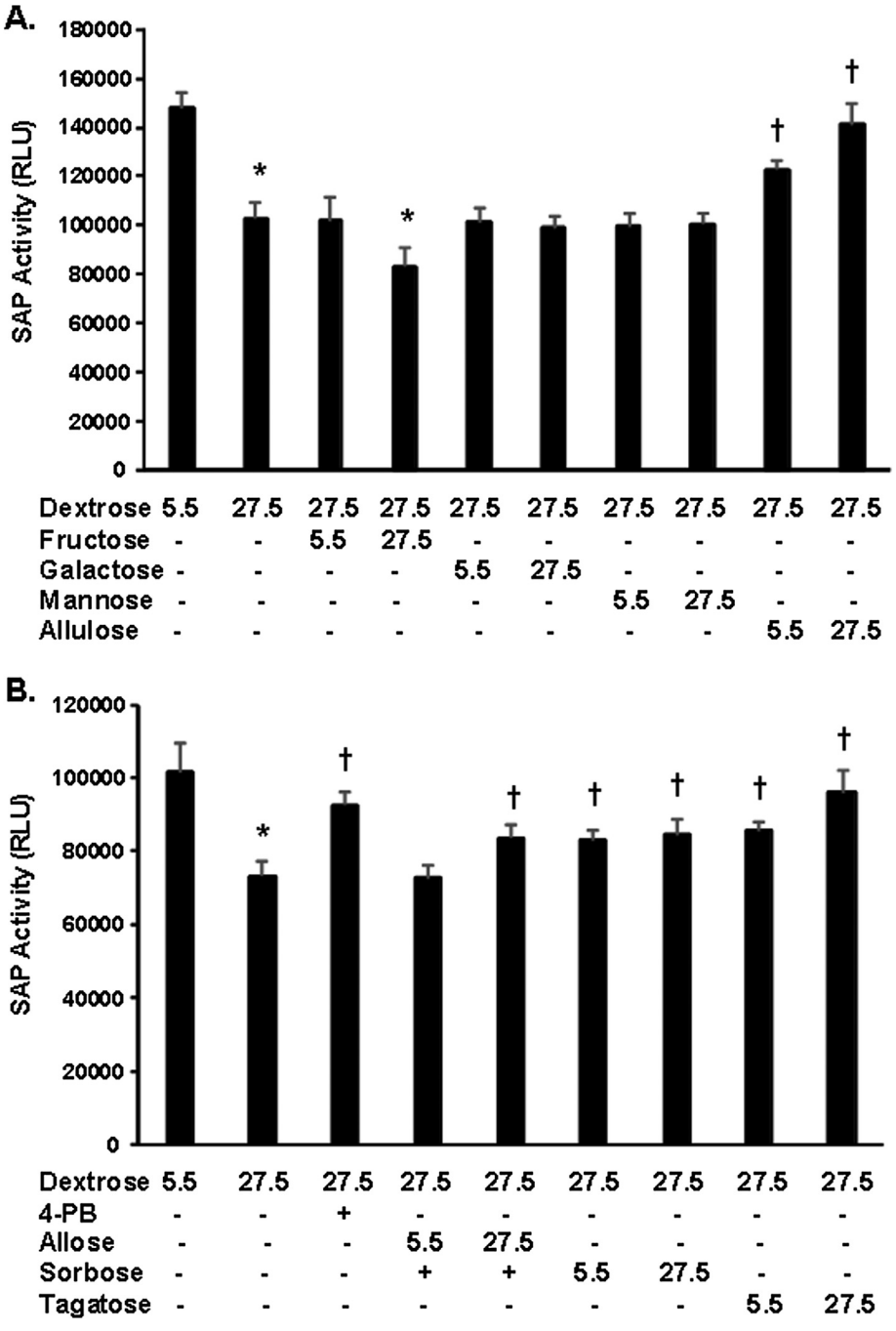

Figure 3. The effect of select rare sugars on secreted alkaline phosphatase (SAP) activity in human coronary artery endothelial cells. **A.** Cells were exposed to either 5.5 mM or 27.5 mM D-glucose (dextrose), with or without 5.5 mM or 27.5 mM fructose, galactose, mannose, or D-allulose, and 24-hours later SAP was measured in relative light units (RLU). The error bars are the ± SD; N = 6; *, $p < 0.0007$ relative to cells treated with 5.5 mM dextrose. †, $p < 0.01$ and $p < 0.003$, respectively, relative to cells treated with 27.5 mM dextrose. **B.** Human coronary artery endothelial cells were exposed to either 5.5 mM or 27.5 mM dextrose, with or without 10 μM 4-phenylbutyrate (4-PB) or 5.5 mM or 27.5 mM D-allose, D-sorbose, or D-tagatose, and 24-hours later SAP was measured. The error bars are the ± SD; N = 6; *, $p < 0.005$ relative to cells treated with 5.5 mM dextrose. †, $p < 0.003$, $p < 0.002$, $p < 0.03$, $p < 0.01$, and $p < 0.005$, respectively, relative to cells exposed to 27.5 mM dextrose.

D-sorbose or D-tagatose significantly increased SAP activity relative to cells treated with tunicamycin ($p < 0.009$, $p < 0.0003$, $p < 0.0005$, and $p < 0.0003$, respectively) (Figure 4B). As expected, treatment with the ER stress inhibitor 4-phenylbutyrate increased SAP activity to a level similar to control cells (96741 ± 4599 RLU) ($p < 0.0004$, relative to cells treated with tunicamycin) (Figure 4B).

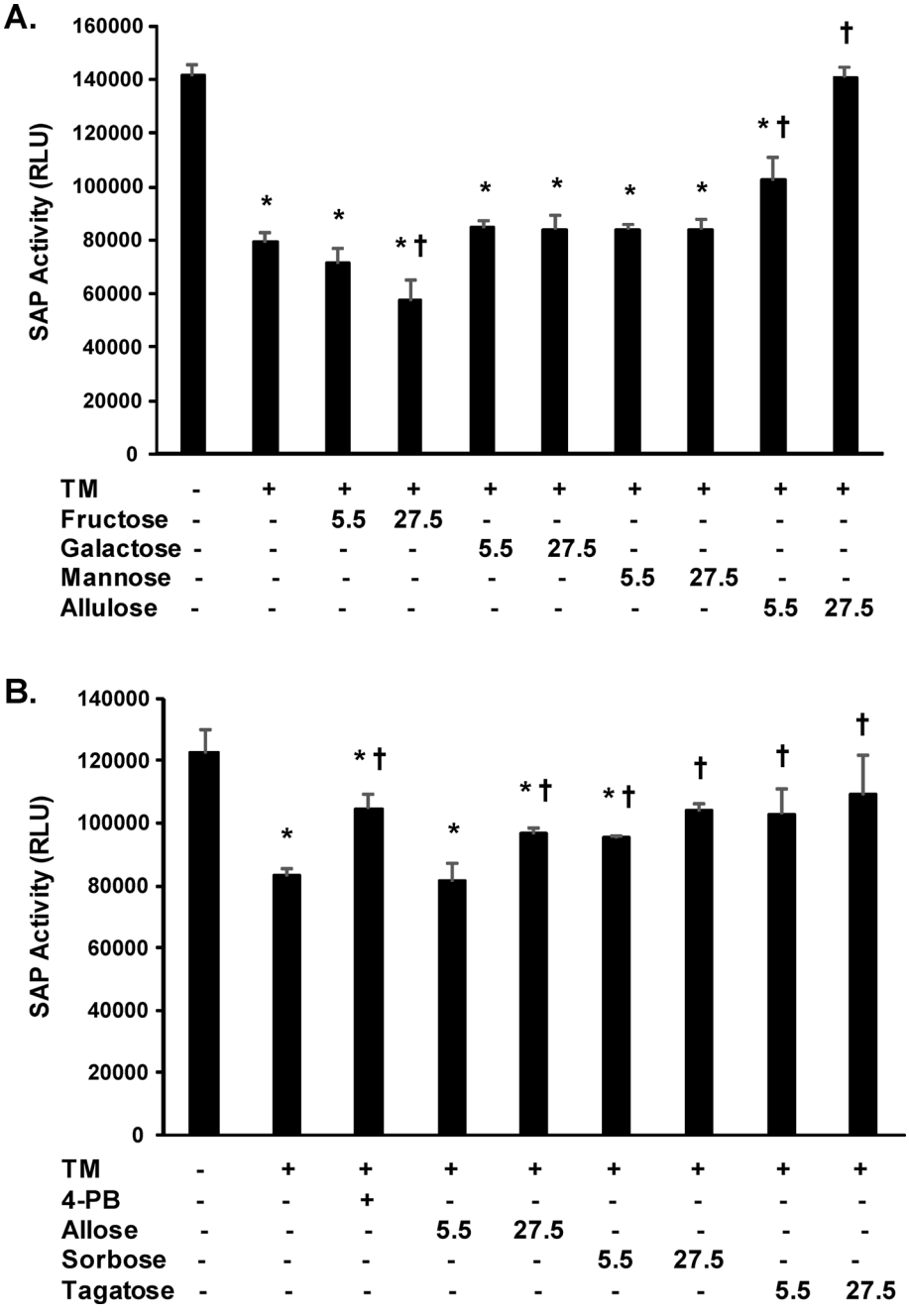

Figure 4. The effect of select rare sugars on secreted alkaline phosphatase (SAP) activity in human coronary artery endothelial cells treated with tunicamycin (TM). **A.** SAP in relative light units (RLU) following treatment with either solvent (dimethylsulfoxide) (-) or 0.1 μM TM (+), along with 5.5 mM or 27.5 mM fructose, galactose, mannose, and D-allulose. The error bars are the ± SD; N = 6; *, $p < 0.0008$, $p < 0.0005$, $p < 0.002$, $p < 0.0005$, $p < 0.0004$, $p < 0.0006$, $p < 0.0008$, and $p < 0.0004$, respectively, relative to control cells. †, $p < 0.01$ and $p < 0.0003$, respectively, relative to cells treated with 0.1 μM TM. **B**. SAP activity following treatment with either solvent (-) or 0.1 μM TM (+) and exposed to 10 μM 4-phenylbutyrate (4-PB), or 5.5 mM or 27.5 mM D-allose, D-sorbose, or D-tagatose. The error bars are the ± SD; N = 6; *, $p < 0.0005$, $p < 0.0009$, $p < 0.002$, $p < 0.02$, and $p < 0.05$, respectively, relative to control cells. †, $p < 0.0004$, $p < 0.008$, $p < 0.001$, $p < 0.0003$, $p < 0.0005$, and $p < 0.0003$, respectively, relative to cells treated with 0.1 μM TM.

The effect of rare sugars on ER stress markers in HCAEC

HCAEC were treated with solvent, 0.1 μM tunicamycin, or tunicamycin plus 10 μM 4-phenylbutyrate, 27.5 mM of D-allose, D-allulose, D-sorbose, or D-tagatose for 24-hours and CHOP47, eIF2α, and JNK1 expression and phosphorylation were measured along with β-actin levels (Figure 5). The dextrose concentration in the medium was 5.5 mM. Representative Western blots are shown in panels A, C, and E and are quantified in panels B, D, and F of Figure 5. Twenty four hour treatment with tunicamycin had no effect on CHOP47, eIF2α, and JNK1 expression relative to β-actin levels, therefore phospho-CHOP47, phospoh-eIF2α and phospho-JNK1 levels were normalized to the total expression of their respective parent moieties. While treatment with tunicamycin increased CHOP47 phosphorylation by 191% ($p < 0.0005$, relative to control cells) (A and B), increased eIF2α phosphorylation by 168% ($p < 0.05$, relative to control cells) (Figure 5C and D) and increased phospo-JNK1 levels by 184% ($p < 0.0007$, relative to control cells) (Figure 5E and F), treatment with 4-phenylbutyrate, D-allose, D-allulose, and D-tagatose decreased CHOP47 phosphorylation, eIF2α phosphorylation and phospo-JNK1

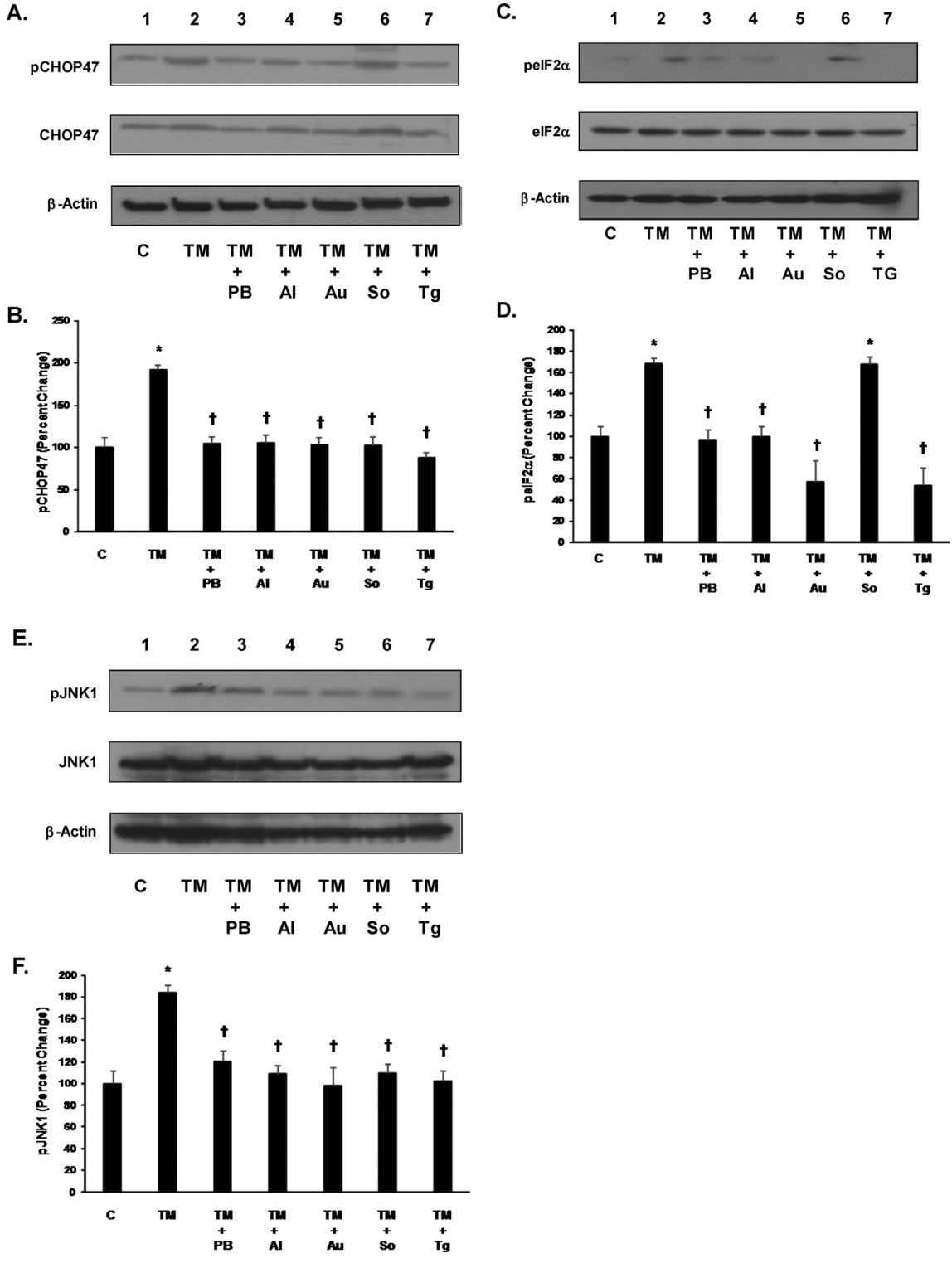

Figure 5. The effect of select rare sugars on CHOP, eIF2α, and JNK1 expression and phosphorylation in tunicamycin (TM)-treated human coronary artery endothelial cells. Cells were treated with solvent, 0.1 μM TM, 10 μM 4-phenylbutyrate (PB), 27.5 mM D-allose (Al), 27.5 mM D-allulose (Au), 27.5 mM D-sorbose (So), or 27.5 mM D-tagatose (Tg) and ER stress markers were measured by Western blot (A, C, E) and quantified (B, D, F). Addition of TM induced eIF2α phosphorylation which was inhibited by addition of PB, D-allose, D-allulose, and D-tagatose. **B.** N = 3; *, $p < 0.0005$ relative to control cells; †, $p < 0.0004$, $p < 0.0005$, $p < 0.0009$, $p < 0.0005$, and $p < 0.0001$, respectively, relative to cells treated with 0.1 μM TM. **D.** N = 3; *$p < 0.0005$ and $p < 0.0003$, respectively, relative to control cells; †, $p < 0.0004$, $p < 0.0005$, $p < 0.0001$, and $p < 0.0001$, respectively, relative to cells treated with 0.1 μM TM. **F.** N = 3; *, $p < 0.0007$ relative to control cells; †, $p < 0.003$, $p < 0.0007$, $p < 0.002$, $p < 0.0008$, and $p < 0.0006$, respectively, relative to cells treated with 0.1 μM TM. The error bars are ± SD.

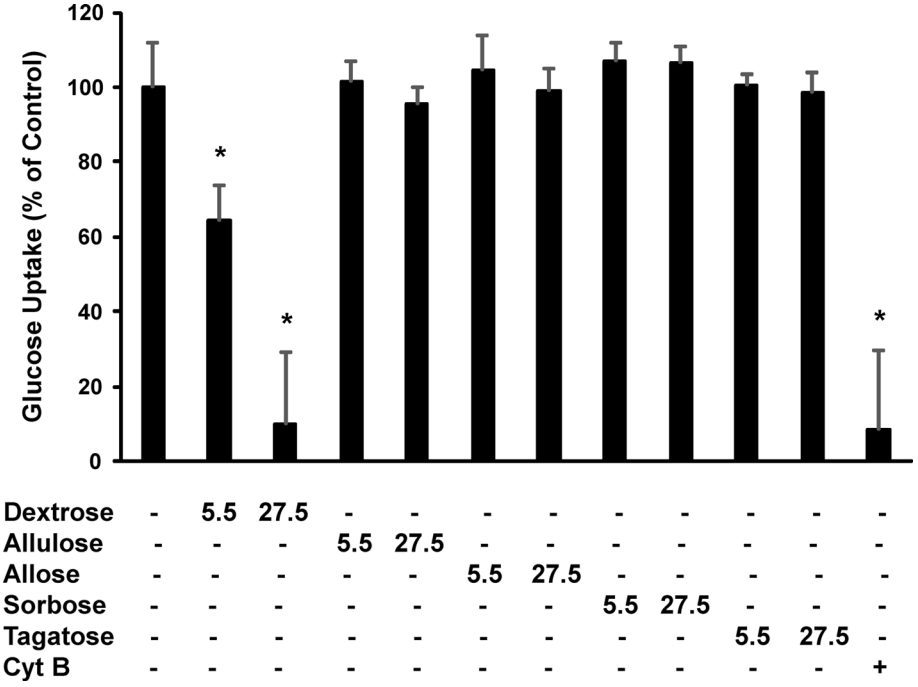

Figure 6. The effect of select rare sugars on dextrose transport by human coronary artery endothelial cells. Cells were exposed to either 5.5 mM or 27.5 mM dextrose, D-allulose, D-allose, D-sorbose, and D-tagatose in the presence of [^{3}H]-2-deoxyglucose and intracellular [^{3}H]-2-deoxyglucose was measured 20 minutes later. Negative control cells were incubated with 20 μM cytochalasin B (Cyt B). Exposure of the cells to D-allulose, D-allose, D-sorbose, and D-tagatose had no effect on [^{3}H]-2-deoxyglucose uptake. The error bars are the ±SD. N = 3; *, $p < 0.01$, $p < 0.0002$, and $p < 0.0002$, respectively, relative to control experiments in the absence of unlabeled sugars.

to levels observed in control cells. Though treatment with D-sorbose inhibited tunicamycin-induced CHOP47 phosphorylation and JNK1 phosphorylation, it did not inhibit eIF2α phosphorylation. Similar observation were made in HepG2 cells (data not shown).

The effect of rare sugars on cellular transport of [^{3}H]-2-deoxyglucose

Glucose uptake assays were performed to determine whether or not each of the rare sugars affected entry of glucose into the cell. HCAEC were exposed to 5.5 mM and 27.5 mM glucose, D-allulose, D-allose, D-sorbose, and D-tagatose in the presence of [^{3}H]-2-deoxyglucose for 20 minutes and the cells were lysed and the amount of intracellular [^{3}H]-2-deoxyglucose was measured (Figure 6). Addition of 5.5 mM and 27.5 mM dextrose inhibited glucose uptake 35.7% ($p < 0.01$) and 90.0% ($p < 0.0002$), respectively relative to control cells (in the absence of unlabeled dextrose). Likewise, treatment with cytochalasin B (as internal control) suppressed [^{3}H]-2-deoxyglucose uptake 91.5% relative to control cells ($p < 0.0002$) (Figure 6). Addition of D-allulose, D-allose, D-sorbose, and D-tagatose had no effect on [^{3}H]-2-deoxyglucose uptake relative to the control cells.

Discussion

There are multiple biochemical pathways of glucotoxicity in various tissues. These include oxidative stress, non-enzymatic glycation of proteins, activation of protein kinase C and aldose reductase-mediated increase in intracellular accumulation of sorbitol and depletion of myoinositol [2]. Another recently appreciated cellular event that contributes to glucotoxicity is D-glucose - induced ER stress [2]. Previous studies have shown that monosaccharides under certain conditions can have either pro-oxidant or antioxidant activities [15]. Dextrose in high concentrations can promote free radical generation in various cell lines [7, 8] and this property may well be a mediator of diabetes –related complications. The present study demonstrates that unlike common monosaccharides notably D-glucose, several naturally occurring rare sugars have free radical scavenging activity and can ameliorate ER stress. The latter favorable effect was demonstrable both in D-glucose –induced ER stress as well as tunicamycin- induced ER stress (Figures 3–5). The changes could not be attributed to alterations in the osmolality of the culture media as the addition of 5.5-27.5 mM mannose or galactose to HCAEC culture media did not alter SO generation or ER stress as measured by SAP activity measurements. The generalizability of the observations in endothelial cells to other tissues was demonstrated with experiments carried out in hepatoma cell line HepG2 (data not shown).

This is the first report that rare sugars ameliorate ER stress, however the precise mechanisms remain unknown. This favorable effect could not be attributed to competitive inhibition of D-glucose transport (Figure 6). The precise transport carriers of the rare sugars are not known but our results show that they do not compete for the glucose carriers. In addition, the effectiveness of the rare sugars was demonstrated in tunicamycin treated cells as well. It is

noteworthy that the ER stress reducing effects of the tested sugars could not be attributed to their antioxidant effects as antioxidants in modest concentrations generally do not alter ER stress while in high concentrations they aggravate ER stress [21–23].

Unlike the other rare sugars tested, treatment with D-sorbose inhibited tunicamycin-induced CHOP47 phosphorylation and JNK1 phosphorylation but it did not inhibit eIF2α phosphorylation. Of the three branches of the UPR [protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6)], eIF2α phosphorylation and changes in CHOP47 expression are both mediated by the PERK axis [1]. Our results suggest that D-sorbose may suppress the UPR by inhibiting the IRE1 and/or ATF6 axes, but not PERK. Future studies of the effect of D-sorbose on X box-binding protein 1 (XBP1) mRNA splicing (a measure of IRE1 activity) and ATF6 cleavage would shed further light on the changes in IRE1 pathway.

Some rare sugars are low-energy sweeteners since they do not undergo substantial metabolism. About 15% to 25% of orally administered D-allulose appears in the urine suggesting that it is absorbed by the small intestine while unabsorbed portion undergoes fermentation in the large intestine. Approximately 98% of intravenously administered D-allulose is excreted in the urine within 6 hours [24–26]. Some rare sugars may also have anti-inflammatory effects. One study showed that D-allulose inhibits dextrose induced monocyte chemoattractant protein-1 (MCP-1) mRNA and protein expression in endothelial cells. This effect was mediated in part by p38-mitogen-activated protein kinase (p38-MAPK) suggesting that this rare sugar may also modify inflammatory stress [27]. It is currently not clear whether or not these rare sugars have effects on nuclear factor κ-B (NF-κB) activity, a pro-inflammatory transcription factor that is important in promoting endothelial dysfunction [28, 29]. It is also not clear whether or not these rare sugars have any effect on nuclear factor erythroid 2-related factor 2 (Nrf-2) expression and/or activity. Nrf-2 has been shown to promote expression of genes involved in reducing oxidative stress [30].

Overall these observations indicate that rare sugars tested are potent free radical scavengers. All four rare sugars had significantly more hydroxyl and peroxyl radical scavenging activity compared to dextrose (Table 1). It is noteworthy that, 25 mM D-glucose, but not 5 mM D-glucose solution can significantly potentiate $CuSO_4$-induced free radical damage. In contrast D-allulose, D-allose, D-sorbose and D-tagatose had an antioxidant effect in this assay (Table 1). Moreover, unlike D-glucose, several naturally occurring rare sugars ameliorate dextrose and tunicamycin induced ER stress. However, the molecular mechanisms of reducing ER stress maybe sugar specific.

The antioxidant activity and ER stress ameliorating property of these naturally occurring rare sugars may contribute to their favorable biological effects. D-Allulose and D-tagatose have been most intensively tested in both animals and humans for their beneficial effects in type 2 diabetes and obesity, and both have been approved as generally regarded safe (GRAS) by the United States Federal Drug Agency (FDA) [9]. However, it is still too early to draw conclusions about the clinical relevance of the antioxidant activity and ER stress ameliorating property of these rare sugars. Nevertheless, their potent free radical scavenging properties may well be useful in prolonging the shelf life of food prepared with these sugars.

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Declaration of Conflict of interest
Two of the co-authors are employees of Matsutani Chemical Industry CO., LTD. that provided the rare sugars tested.

Author contribution
Arshag D. Mooradian designed the experiments, participated in data analysis and interpretation, and wrote the majority of the manuscript.
Michael J. Haas helped design the experiments, participated in data analysis and interpretation, and assisted in writing the manuscript.
Luisa Onstead-Haas carried out many of the experiments described in the manuscript.
Yuma Tani, Tetsuo Iida and Masaaki Tokuda provided important discussions assisting in data interpretation and editing of the manuscript.

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