Spring 1-1-2015

The efficacy of oral trehalose therapy to reverse age-associated vascular dysfunction in humans

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THE EFFICACY OF ORAL TREHALOSE THERAPY TO REVERSE AGE-ASSOCIATED VASCULAR DYSFUNCTION IN HUMANS

by

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A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Integrative Physiology
2015
This dissertation entitled:
The efficacy of oral trehalose therapy to reverse age-associated vascular dysfunction in humans
written by Rachelle Elizabeth Kaplon
has been approved for the Department of Integrative Physiology

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Date______________

The final copy of this thesis has been examined by the signatories, and we
Find that both the content and the form meet acceptable presentation standards
Of scholarly work in the above mentioned discipline.

IRB protocol # 12-0116
Kaplon, Rachelle Elizabeth (Ph.D., Integrative Physiology)

The efficacy of oral trehalose therapy to reverse age-associated vascular dysfunction in humans
Thesis directed by Professor Douglas R. Seals

Cardiovascular diseases (CVD) remain the leading cause of mortality in modern societies. Age is the major risk factor for CVD due largely to adverse changes to arteries. Two key features of arterial aging that increase CVD risk are the development of vascular endothelial dysfunction, characterized by reduced nitric oxide (NO)-mediated endothelium-dependent dilation (EDD), and large elastic artery stiffness. These adverse functional changes are driven by increased oxidative stress and inflammation.

Trehalose is a naturally occurring disaccharide with antioxidant and anti-inflammatory properties that protects against aging in lower organisms. Moreover, oral trehalose treatment improves NO-mediated EDD and reduces arterial stiffness in old mice. However, the efficacy of trehalose to reverse arterial aging in humans is unknown. The goal of this dissertation was to test the hypothesis that oral trehalose supplementation would improve resistance and/or conduit artery NO-mediated EDD in healthy MA/O adults, and that improvements would be related to reduced inflammation and oxidative stress. A secondary hypothesis was that trehalose supplementation would decrease large elastic artery stiffness in this same population.

Thirty-two men and postmenopausal women aged 50-77 years consumed 100g/day of trehalose or maltose (energy equivalent control disaccharide without the reported health benefits of trehalose) for 12 weeks (randomized, double-blind). Resistance artery NO-mediated EDD increased with trehalose in subjects remaining weight-stable, and this was associated with evidence of decreased endothelial cell inflammation. Contrasting, trehalose did not modify conduit artery EDD or oxidative stress. These findings indicate that trehalose may be a novel therapy for reducing vascular inflammation and improving resistance but not conduit artery EDD in MA/O adults able to maintain stable body mass.
In a secondary protocol of the parent investigation, large elastic artery stiffness was assessed in 31 healthy adults 50-77 years before and after 12 weeks of oral trehalose or maltose supplementation (100g/day). Trehalose was not effective for reversing arterial stiffness in this population.

These studies indicate that although trehalose has heterogeneous effects on different aspects of arterial aging and its caloric content poses challenges, this may be an effective intervention for the primary prevention of CVD by reversing resistance artery EDD in healthy MA/O adults.
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Research Articles:

Kaplon RE, Hill SD, Bispham NS, Santos-Parker JR, Nowlan MJ, Stauber LL, LaRocca TJ, Chonchol M, and Seals DR. Twelve weeks of oral trehalose supplementation does not improve large elastic artery stiffness or wave reflection in healthy middle-aged and older adults. *In preparation.*


Hill SD, McNamara MN, Seals DR, and Kaplon RE. Endothelial cell senescence with age is associated with oxidative stress-mediated suppression of endothelial function in healthy adults. *In Preparation.*


**Abstracts:**


CHAPTER II

Introduction

Cardiovascular diseases (CVD) remain the leading cause of mortality in modern societies (80, 97, 133). Advancing age is the major risk factor for CVD due in a large part to adverse changes to arteries (80, 118). Two key features of arterial aging that increase CVD risk in middle-aged and older (MA/O) adults are the development of vascular endothelial dysfunction, as assessed by endothelium independent dilation (EDD), and stiffening of the large elastic arteries (73, 113, 183).

Impaired EDD with advancing age is primarily mediated by reduced bioavailability of the vascular-protective vasodilator, nitric oxide (NO) (73, 113, 183). The mechanisms underlying age-associated large elastic artery stiffening involve both structural and functional changes. The former include increased expression of the load-bearing protein collagen and its cross-linking by advanced glycation end products, as well as the fragmentation and degradation of the elasticity-conferring protein elastin (51, 62, 144). Functional changes that contribute to arterial stiffening involve factors that increase vascular smooth muscle tone, including increased vasoconstrictor and decreased vasodilator signaling (51, 92, 144). Because NO is a key endothelium-derived vasodilator, NO bioavailability is an important regulator of large elastic artery stiffness with age (140, 184, 185).

Oxidative stress and chronic low-grade inflammation play a fundamental role in mediating the above functional changes in arteries with advancing age. Substantial cross-talk between pro-inflammatory and -oxidant singling causes these pathways to mutually upregulate one another with age in a feed-forward fashion (37). Oxidative stress can be defined as increased bioactivity of reactive oxygen species (ROS) relative to antioxidant defenses (77). Excess ROS decreases NO bioavailability both directly, by reacting with NO to form damaging
reactive nitrogen species, and indirectly by oxidizing the enzyme (endothelial nitric oxide synthase) and co-factors necessary for NO synthesis (8, 11). Oxidative stress also induces structural changes in the arterial wall, including increased collagen deposition, decreased elastin and augmented advanced glycation end product formation (51, 53), and promotes the induction of adverse pro-inflammatory signaling (38, 51).

Advancing age results in suppression of the adaptive immune system and consequent up-regulation of the innate immune system, which generates a phenotype of chronic low-grade inflammation known as “inflammaging” (136). This phenotype presents as an increase in circulating levels of inflammatory mediators and elevated pro-inflammatory gene and protein expression in the vascular wall (40, 142). Age-associated pro-inflammatory signaling stimulates endothelial activation (increased expression of adhesion molecules), cellular oxidant production, accumulation of cell damage, as well as changes in autocrine and paracrine signaling (33, 84, 148). Together, these changes disrupt cellular homeostasis and inhibit EDD (142, 178), and are associated with arterial remodeling and increased arterial stiffness with age (107, 187).

Due to the central role of oxidative stress and chronic low-grade inflammation in the development of vascular dysfunction with age, these processes are key therapeutic targets for improving arterial function in MA/O adults. The naturally occurring disaccharide trehalose reduces oxidative stress and inflammation in numerous in vitro and in vivo models of age-associated disease states (6, 39, 132, 138). Moreover, trehalose improves EDD and decreases large elastic artery stiffness in old mice (83, 84). However, the ability of trehalose to reverse age-associated vascular dysfunction in humans is unknown.

Accordingly, the goal of this dissertation was to test the hypothesis that oral supplementation with the disaccharide trehalose would improve resistance and/or conduit artery EDD in MA/O adults, and that improvements would be related to increased NO bioavailability and reduced inflammation and oxidative stress. A secondary hypothesis was that oral trehalose
supplementation would reduce large elastic artery stiffness in healthy MA/O adults free from CVD.
Specific Aims

**Specific Aim 1 (Chapter IV):** To determine if 12 weeks of oral trehalose supplementation improves resistance and/or conduit artery EDD in healthy MA/O adults

**Specific Aim 2 (Chapter IV):** To determine if improvements in EDD with trehalose treatment are mediated by improved NO bioavailability

**Specific Aim 3 (Chapter IV):** To determine if improved EDD with trehalose treatment is associated with reduced systemic and/or vascular oxidative stress and inflammation.

**Specific Aim 4 (Chapter V):** To determine if 12 weeks of oral trehalose supplementation decreases large elastic artery stiffness in healthy MA/O adults.
Clinical relevance of age-associated vascular dysfunction

CVD remain the leading cause of mortality in modern societies (99, 181). Advancing age is the major risk factor for CVD, as over 90% of all incident deaths from CVD occur in adults over the age of 55 (99, 133). The increased risk for CVD with age results largely from adverse changes to arteries that make the arterial system more susceptible to disease (80, 118). This section highlights the increasing healthcare burden posed by the unprecedented number of older adults and the contribution of arterial aging to the development of CVD in this population.

Current and future demographics of aging and cardiovascular diseases

This year, over 500 million adults will be 65 years of age or older and this number is expected to triple by 2050 (102). Without effective intervention, this marked increase in the number of older adults will result in an unprecedented rise in the prevalence of CVD and associated health care burden. Specifically, by 2030 over 40% of the population is expected to have CVD and associated annual health care costs are projected to pass 800 billion dollars in the United States alone (65). As such, establishing effective strategies that can delay, minimize, or prevent the development of CVD with aging is among the highest biomedical research priorities (102).

Increased CVD risk with age results in part from age-associated changes to the structure and function of arteries. Two key changes to arteries that increase the risk of CVD with aging are vascular endothelial dysfunction and increased stiffness of the large elastic arteries.
Vascular endothelial dysfunction with age

The vascular endothelium is a monolayer of cells anchored to a basal lamina that lines the lumen of blood vessels. Once thought to primarily act as a physical barrier, the vascular endothelium is now recognized as a dynamic organ with functions that extend far beyond regulating vascular permeability (21, 48). Rather, the endothelium plays an essential role in the control of fibrinolysis, thrombosis, angiogenesis, immune function, metabolism and vascular tone. Together, these functions maintain blood fluidity and tissue perfusion and are imperative for the preservation of physiological function and health (21, 48, 137).

The term vascular endothelial dysfunction describes any changes in these fundamental roles of the vascular endothelium that impairs physiological function. With advancing age, the endothelium develops a pro-thrombotic, pro-inflammatory, vasoconstrictor phenotype that facilitates the development of CVD (142, 183). Many of these changes are mediated by a loss in the bioavailability of the vasodilatory and vascular protective molecule, nitric oxide (NO) (175). NO stimulates relaxation of vascular smooth muscle cells and inhibits platelet aggregation, release of the potent vasoconstrictor endothelin-1 and the expression of, and leukocyte binding to, endothelial adhesion molecules (a fundamental process in leukocyte trafficking across the endothelium during inflammatory processes) (48, 171). As such, a loss in NO bioavailability with age has a broad impact on endothelial function.

Endothelial cell production of NO

NO is a highly reactive signaling molecule that is produced in the vasculature primarily through enzymatic formation by nitric oxide synthase (NOS) enzymes. The NOS family of enzymes contains three isoforms that include endothelial, neuronal and inducible NOS. Endothelial NOS and, to a lesser extent in the vascular endothelium, neuronal NOS are constitutively expressed and produce relatively small, transient levels of NO in response to receptor-mediated and physical stimuli (164). In contrast, inducible NOS is often up-regulated
in settings of inflammation and produces high levels of NO that can disrupt endothelial homeostasis (174). In healthy individuals, endothelial NOS is thought to be the primary producer of endothelial-derived NO (174).

Endothelial NOS catalyzes the production of NO from the oxidation of L-arginine to L-citrulline in the presence of oxygen and requires numerous cofactors and prosthetic groups including calcium calmodulin, tetrahydrobiopterin (BH$_4$), heme, nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and zinc. NO production by endothelial NOS is regulated by transcriptional control and post-translational modifications. Post-translational modifications known to modulate endothelial NOS activity include phosphorylation, S-nitrosylation and acetylation. Acetylation and S-nitrosylation have inhibitory effects on endothelial NOS activity whereas phosphorylation both activates and inhibits the enzyme depending on the amino acid phosphorylated. Protein-protein interactions are also potent regulators of endothelial NOS activity. Such interactions include endothelial NOS inhibition through binding to the scaffolding protein caveolin-1 located in invaginations of the cell membrane. Heat shock protein 90 and calcium-dependent calmodulin binding are potent up-regulators of endothelial NOS activity (48, 55). A number of neural-hormonal mediators and physical stimuli (such as shear stress exerted by blood flow on the vascular endothelium) can stimulate NO production through both transcriptional and post-translational regulation of endothelial NOS. Many known activators of endothelial NOS work in part by increasing intracellular calcium concentrations (171).

**Clinical assessment and relevance of endothelial dysfunction with age**

Clinically, endothelial dysfunction is primarily characterized by assessing endothelium-dependent dilation (EDD). EDD refers to techniques that measure vasodilation in response to stimuli that act on the vascular endothelium to produce NO and induce vasodilation. The best characterized and most widely used methods for assessing EDD include a) assessment of
brachial artery dilation in response to an increase in blood flow produced by temporary forearm ischemia (flow-mediated dilation, FMD) and b) changes in forearm blood flow in response to the pharmacological stimulus acetylcholine (142). Importantly, individuals who demonstrate impaired coronary artery EDD also demonstrate reduced EDD in peripheral arteries (4) suggesting that impaired EDD is a systemic event that can be observed through non- or minimally-invasive methods in peripheral vasculature.

FMD and forearm blood flow to acetylcholine are usually compared to an endothelium-independent vasodilatory stimulus. Endothelium-independent vasodilation is most commonly assessed as the change in brachial artery diameter or forearm blood flow in response to a nitric oxide donor. This allows for the examination of smooth muscle cell sensitivity to NO and helps to establish whether impairments in EDD are due to changes in NO bioavailability or smooth muscle cell responsiveness to NO (142).

Substantial clinical evidence implicates a loss of NO bioavailability in age-associated vascular dysfunction and increased CVD risk. FMD declines progressively after the age of 40 in healthy males and 55 in healthy females (17). Similarly, FMD is roughly 50% lower in healthy middle-aged and older adults without major CVD risk factors compared with young controls (29, 34, 43, 45, 46, 127). Furthermore, peak forearm blood flow to acetylcholine (and synthetic analogues) declines progressively throughout life (60, 156) in healthy adults and is reduced by roughly 50% percent in middle-aged and older individuals free from CVD and major CVD risk factors vs. young controls (28, 33, 60, 84, 119). These findings correspond with the limited number of studies that have investigated EDD (dilation in response to acetylcholine) in the coronary arteries, which have demonstrated an inverse relation between coronary artery EDD and age (176, 188, 192).

The majority of studies assessing the effect of age on endothelium-independent dilation have not observed age group differences in healthy adults in the forearm vasculature or the brachial artery (28, 29, 33, 34, 43, 45, 46, 60, 84, 127). Declines in endothelium-independent
dilation with age have been observed in select studies but these changes are smaller than those observed with EDD (119, 154, 156). In addition, the subjects in these studies tended to be at elevated risk for CVD due to inclusion of current smokers (154, 156). As such, these studies suggest that impairment in EDD with advancing age cannot be completely explained by changes in smooth muscle cell sensitivity to NO. Furthermore, inhibition of EDD by the endothelial NOS inhibitor \( N^\bigcirc \)-monomethyl-L-arginine (L-NMMA) declines progressively with age in healthy adults (155). This finding suggests that impaired EDD with age is due in part to decreased NO production. Indeed, circulating and urinary concentrations of nitrite and nitrate, products of NO metabolism that can also be recycled back to NO under certain conditions (101), decline with age providing further evidence for decreased NO bioavailability in middle-aged and older adults (30, 103, 163).

Of importance, both FMD and peak forearm blood flow to acetylcholine are independently predictive of future CVD events in adults without a history of CVD. These measures of EDD also improve risk stratification for first major CVD event when added to the Framingham risk score (95, 189, 190). Thus, evidence to date demonstrates that endothelial dysfunction with advancing age as assessed by EDD results in part from decreased NO bioavailability and is an important intermediate phenotype in the development of CVD.

**Large elastic artery stiffness with age**

In healthy individuals, the aorta dampens oscillations in blood pressure that occur between ventricular contractions by stretching to store stroke volume (the blood ejected by the left ventricle). These vessels then recoil during diastole to propel the stored blood forward through circulation (120) and provide more continuous blood flow to peripheral tissues (98). Ejection of blood from the left ventricle during systole creates a pressure or pulse wave. This pressure wave is propagated along the artery wall at a velocity that is dependent on the material stiffness and thickness of the artery. Material stiffness is defined as the capacity of a material to
resist distention and is an intrinsic property (dependent on the material composition) of the arterial wall. At a given pressure, a thicker artery will distend less than a thinner artery that has the same material stiffness (62).

As the incident/forward moving pressure waves created by the ejection of blood from the heart encounter impedance (resistance to the forward transmission of the pressure wave), reflected pressure waves are produced. Impedance can be generated by a change in arterial diameter and stiffness, bifurcations and arterial lesions. As blood flows away from the heart, arteries decrease in diameter and increase in stiffness causing a gradual increase in impedance along the arterial tree (62).

Together, these structural properties of arteries are important determinants of the velocity at which the pressure wave travels and the subsequent timing at which the reflected waves return to the heart. This timing has important consequences on cardiovascular health. In young healthy individuals, reflected pressure waves return to the heart during diastole (113). With aging, increased stiffening of the large elastic arteries causes faster transmission of both the forward and reflected pressure waves. This results in the return of reflected waves to the heart during systole (74). Early wave reflection and decreased ability of the aorta to stretch to accommodate stroke volume increases systolic blood pressure and pulse pressure and decreases diastolic blood pressure (120). The resultant rise in systolic pressure increases afterload (the pressure against which the heart ejects blood) which, over time, promotes the development of left ventricular hypertrophy and heart failure (100). Augmented pulse pressure with age promotes end organ damage by transmitting excessive pulsatility into the microcirculation of peripheral organs including the heart, kidney and brain. Transmission of high pulsatile stress to the microcirculation increases myogenic tone, promotes vascular remodeling and induces changes in gene expression that increase vascular resistance to protect downstream vessels from pulsatile damage. However, an increase in vascular resistance can lead to decreased blood flow and subsequent ischemic tissue damage (110, 135). Furthermore,
with regard to the coronary circulation, arterial stiffening and earlier return of reflected pressure waves decreases diastolic blood pressure. This results in a decrease in coronary perfusion which can limit oxygen delivery to the heart and impair contractile function (120). Thus, arterial stiffening with age promotes microvascular remodeling and ischemic damage of the heart, kidney and brain, emphasizing the clinical importance of this aging phenotype.

The arterial wall is composed of three distinct layers: the intima, media, and adventitia. The intima is the inner most layer and contains endothelial cells anchored to a basal lamina. The media, or middle layer, is composed of smooth muscle cells, elastin, collagen, and ground substance. The outermost layer of the vessel wall is the adventitia. This layer is the most heterogeneous containing extracellular matrix, progenitor and immune cells, fibroblasts and adrenergic nerves (152). The media and adventitia predominantly determine the stiffness and resilience (the ability to return to initial dimensions after distention) of the artery. Collagen and elastin are the two primary structural components of the arterial wall. Elastin confers the properties of stretch and resilience to arteries whereas collagen is much stiffer and prevents over distention at high pressures (62, 196).

The mechanisms by which large elastic arteries stiffen with age involve both structural and functional changes (80, 118, 184). Structural changes include increases in collagen proteins and/or their cross-linking and fragmentation, degradation, and calcification of elastin (51, 62, 144). Functional changes that contribute to stiffening involve factors that increase vascular smooth muscle tone, including renin-angiotensin and sympathetic nervous system activity and vasoactive factors released from the endothelium such as NO and endothelin-1 (31, 106, 182).

**Clinical assessment and relevance of arterial stiffening with age**

The speed at which the arterial pulse wave travels can be measured non-invasively using applanation tonometry to record tracings of arterial pressure waves at different locations.
along the arterial tree. Specifically, using the R-wave of an ECG recording, the time delay (transit time) between the foot of pressure waves at different arterial locations can be determined. Pulse wave velocity (PWV) is calculated as the distance between measurement sites divided by transit time of the arterial pulse wave (112).

Aortic (carotid to femoral) PWV, a measurement of the transit time of the arterial pressure wave through the aorta, has been shown to increase with advancing age in healthy adults and be independently predictive of future cardiovascular events (7, 111, 113, 153, 166). Specifically, in nonsmoking, normotensive adults without CVD, aortic PWV doubles between the ages of 20 and 90 (166). In contrast, previous studies have demonstrated that PWV through peripheral arteries does not change or only increases modestly with advancing age (7, 113). These findings suggest that structural changes with advancing age primarily occur in the large elastic arteries. Similarly, aortic but not peripheral PWV has been shown to be an independent predictor of CVD incident in healthy adults without a history of CVD (111). A one standard deviation increase in aortic PWV is associated with a 48% increase in the risk for a first major CVD event when controlling for traditional CVD risk factors (111). Furthermore, elevated aortic PWV is associated with overall and CVD-related mortality in a generally healthy population (153). These data suggest that stiffening of the aorta with age, as measured by aortic PWV, is an important clinical antecedent to the development of CVD in otherwise healthy adults.

**Mechanisms of age-associated vascular dysfunction**

Vascular homeostasis depends on tight regulation of oxidative and inflammatory signaling. Increasing evidence suggests that aging is associated with up-regulation of oxidant and inflammatory pathways that promote the development of age-associated vascular dysfunction characterized by the phenotypes described above. Substantial crosstalk between inflammatory and oxidant signaling has been established providing evidence for synergistic effects of these pathways with advancing age.
Oxidative stress

Oxidative stress can be defined as increased bioactivity of reactive oxygen species (ROS) relative to antioxidant defenses (77). With advancing age, oxidative stress occurs in the vasculature primarily due to maladaptive increases in superoxide production by mitochondria and NADPH oxidase (34, 130, 165). Superoxide reduces NO bioavailability by reacting with NO to form peroxynitrite. Peroxynitrite, in turn oxidizes tetrahydrobiopterin, an essential cofactor for NO synthesis by endothelial NOS (56, 147). Tetrahydrobiopterin is required for stabilizing the active dimer of endothelial NOS and reduced bioavailability of tetrahydrobiopterin leads to the “uncoupling” or dissociation of the endothelial NOS dimer into two monomers (48). Uncoupled endothelial NOS produces superoxide rather than NO in a vicious cycle that further reduces NO bioavailability (81, 87).

Expression of NADPH oxidase and nitrotyrosine abundance, a marker of peroxynitrite-mediated oxidative damage, are elevated in endothelial cells obtained from healthy middle-aged and older adults compared to young controls and endothelial expression of nitrotyrosine is inversely related to FMD (34). Aortic tissue from old vs. young mice demonstrates elevated levels of superoxide production in addition to increased NADPH oxidase expression and nitrotyrosine abundance (54, 82, 148). Accordingly, preclinical studies have demonstrated that 3 weeks of treatment with the superoxide dismutase mimetic, TEMPOL restores NO-mediated EDD in old mice (53) and clinical studies have demonstrated that acute supra-physiological infusion of the potent antioxidant ascorbic acid (vitamin C) improves NO-mediated EDD in middle-aged and older adults (45, 155). *These findings demonstrate that superoxide is a fundamental mechanism underlying the suppression of NO-mediated EDD with age.*

Interestingly, endothelial NOS activation via phosphorylation at serine 1177 is elevated in biopsied endothelial cells from middle-aged and older adults vs. young controls. This finding suggests that endothelial NOS activity may be up-regulated with age in humans to compensate
for decreased NO bioavailability in the presence of oxidative stress. Importantly, compensatory activation of endothelial NOS with age is not able to restore NO bioavailability but may function to produce increased superoxide secondary to endothelial NOS uncoupling as described above (35).

The oxidative stress-mediated decrease in NO bioavailability with age has both direct and indirect effects on vascular smooth muscle cell tone. In addition to being an important vasodilator, NO also inhibits release of the potent endothelium-derived vasoconstrictor endothelin-1(10). Endothelin-1-mediated vasoconstriction is augmented in older experimental animals and humans compared with young controls (35, 169). Furthermore, the expression of endothelin-1 is greater in endothelial cells biopsied from the brachial artery of middle-aged and older adults vs. young controls and is positively related to nitrotyrosine abundance and inversely related to EDD (35). In old animals, blockade of the receptor for endothelin-1 restores EDD to levels observed in young mice (35). These data suggest that aging is associated with an increase in oxidative stress and vasoconstrictor phenotype mediated by decreased NO bioavailability and an attendant increase in endothelin-1 signaling.

Preclinical studies suggest that the age-associated increases in vascular superoxide production is also associated with changes in the structural properties of arteries with age, including increased collagen deposition, decreased elastin, and augmented advanced glycation end product formation (cross links formed between reducing sugars and protein amino groups on collagen which contribute to arterial stiffness with age) (51). Indeed, age-associated increases in aortic PWV and adventitial collagen are reversed in old mice following 3 weeks of TEMPOL treatment (53). Mice deficient in the mitochondrial antioxidant enzyme, manganese superoxide dismutase, demonstrate increased mitochondrial oxidative stress and prematurely elevated aortic PWV with age. These mice also demonstrate increased vascular smooth muscle cell collagen 1 and decreased elastin expression compared with wild type mice (194). These findings demonstrate that oxidative stress is an important factor mediating arterial
stiffness with age in preclinical models, in part, through the regulation of the structural properties of arteries.

Previous clinical studies have also demonstrated associations between vascular oxidative stress and arterial stiffness. In adults varying in CVD risk, vascular superoxide generation (assessed in biopsied saphenous veins) is inversely related to aortic compliance, a measure of arterial distention relative to pressure that provides an indirect measure of vascular stiffness (higher compliance being associated with lower stiffness) (26). Furthermore, in healthy adults aortic PWV is positively related to circulating cystine, a marker of systemic oxidative stress (125). Intravenous infusion of supra-physiological doses of ascorbic acid (vitamin C) improves carotid artery compliance in postmenopausal women but not similarly aged men (44, 115). PWV measurements in men also are unaffected by acute ascorbic acid infusion suggesting that sex differences may exist in the contribution of oxidative stress to vascular stiffness with age in humans (44).

In summary, increased superoxide production with age decreases NO bioavailability and promotes a vasoconstrictor phenotype in the aged vasculature. ROS also induce structural changes in the arterial wall that, in combination with oxidative stress-induced increases in vascular smooth muscle tone, contributes to large elastic artery stiffening with age.

Inflammation

Aging results in suppression of the adaptive immune system and consequent up-regulation of the innate immune system which generates a phenotype of chronic low-grade inflammation known as “inflammaging” (136). This phenotype presents as an increase in circulating levels of inflammatory mediators and elevated pro-inflammatory gene and protein expression in aged tissues (40, 142). Indeed, pro-inflammatory protein expression is elevated in the aorta of old mice and biopsied human endothelial cells from middle-aged and older adults compared with young controls (33, 84, 148). Increased inflammation in the vascular wall has
numerous adverse effects including endothelial activation (increased expression of cellular adhesion molecules), immune cell infiltration, and changes in autocrine and paracrine signaling (23).

Activation of the nuclear factor kB (NFkB) pathway is thought to play a primary role in the development of this age-associated pro-inflammatory phenotype. NFkB controls transcription of adhesion molecules, cytokines, chemokines, and the pro-oxidant enzyme NADPH oxidase (23, 38). Activation of NFkB can be stimulated by circulating pro-inflammatory mediators including the acute phase protein, C-reactive protein (172) and cytokines, as well as by ROS suggesting an important point of communication between cellular oxidative and inflammatory signaling (38). Indeed, NFkB expression is greater in endothelial cells from healthy middle-aged and older adults compared with young controls (33). NFkB activation is associated with oxidative stress in both human and animal models (34, 89, 165) and inhibition of NFkB activity in experimental animals and humans decreases vascular inflammation and oxidative stress (in part through modulation of NADPH oxidase expression and activity) (89, 128, 178) (89). Furthermore, inhibition of NFkB signaling improves NO-mediated EDD in experimental animal models and both obese and healthy middle-aged and older adults (89, 128, 178). Together these findings support a central role for inflammatory signaling through NFkB in the regulation of vascular oxidative stress and endothelial dysfunction with age.

Inflammation may also have direct effects in modulating arterial stiffness with age. In preclinical studies, interventions which decrease arterial stiffness are associated with reduced expression of inflammatory mediators in the arterial wall (52, 90, 148). In addition, previous studies have demonstrated associations between inflammatory markers and clinical measures of arterial stiffness. Specifically, circulating levels of C-reactive protein are an independent predictor of aortic PWV in healthy adults (187) and men over the age of 45 (107). Furthermore, aortic PWV is elevated in inflammatory disease states and normalized to levels observed in healthy controls by chronic anti-inflammatory treatment (104). While these findings provide
strong evidence for an association between inflammation and large elastic artery stiffness with age, cause and effect data is sparse.

**Therapeutic strategies.**

Due to the central role of oxidative stress and chronic low-grade inflammation in the development of vascular dysfunction with age, these processes are key therapeutic targets for improving arterial function in MA/O adults. Preclinical evidence suggests that the naturally occurring disaccharide α,α-trehalose (referred to from here on as trehalose) is a novel therapy to reduce oxidative and inflammatory signaling and improve arterial function with age.

**The therapeutic potential of trehalose**

Trehalose is a naturally occurring disaccharide of glucose with a 1,1 glycosidic linkage. Due to its 1,1 glycosidic bond, trehalose is not a reducing sugar (it does not donate electrons to weak oxidizing agents and does not promote the formation of advanced glycation end products in vivo) (41, 116, 131). Trehalose is found in many foods, including honey (~2%), baker’s yeasts (15-20%) and mushrooms (5-17%) (41, 131). Although mammals do not produce trehalose, microorganisms and insects utilize the carbohydrate for energy production and can synthesize it as a metabolic intermediate and/or product. In lower organisms, trehalose stabilizes lipid membranes and proteins under conditions of stress, prevents protein aggregation, protects against oxidative stress through scavenging free radicals (41, 72) and extends longevity in the nematode (67). As such, trehalose has an important cytoprotective role in lower organisms.

While mammals are not able to produce trehalose, treatment of mammalian cells with trehalose protects against pro-oxidant and pro-inflammatory stressors including proteasome inhibition and exposure to hemolysate and endotoxin (39, 49, 64, 151). Importantly, in the context of vascular aging, trehalose has been shown to specifically inhibit NFkB signaling in this
context (39, 64). Furthermore, trehalose blunts inflammatory signaling in response to endotoxin exposure \textit{in vivo} (6, 109) and protects against inflammation and oxidative stress in mouse models of Parkinson’s disease (132, 138). Together, these findings suggest that trehalose can protect against oxidative stress and inflammation in mammals.

Trehalose also improves physiological function in a number of pro-oxidant and inflammatory age-associated diseases. Previous studies have shown that trehalose inhibits bone loss in a mouse model of menopause (121), protects against subarachnoid hemorrhage (39) and metabolic stress (5, 93), and decreases protein aggregates and improves function in mouse models of Parkinson’s disease (132, 138, 186).

The mechanisms by which trehalose protects against oxidative stress and inflammation are incompletely understood but may be mediated through the ability of trehalose to up-regulate autophagy (15, 93, 132, 139, 186, 193). Autophagy is a vital stress response pathway by which damaged cellular constituents are degraded by the lysosome (69). Autophagy declines with age and this is decline is being increasingly recognized as a key mediator of age associated dysfunction and disease secondary to the accumulation of cell damage, and subsequent stimulation of oxidative and inflammatory signaling (20, 24, 69, 134). In addition to activating autophagy, trehalose may protect against oxidative stress and inflammation through directly scavenging free radicals (9, 39) and/or acting as a chemical chaperone (41, 72, 191).

Consistent with evidence for the ability of trehalose to preserve function in pro-oxidant and inflammatory age-related diseases, recent preclinical findings demonstrate that four weeks of trehalose in drinking water reduces aortic PWV (83) and completely restores acetylcholine-stimulated EDD in old (26-28 months) mice, while having no effect in young (4-6 months) animals (84). These improvements were associated with increased NO bioavailability, reduced aortic collagen expression, decreased arterial superoxide production, normalization of vascular inflammation, and restoration of markers of vascular autophagy (83, 84). As such, trehalose is
a promising nutraceutical for the inhibition of oxidative stress and inflammation with age, and the reversal of age-associated vascular dysfunction.

Importantly from a therapeutic standpoint, trehalose has been investigated extensively for safety, toxicity and carcinogenesis because of its widespread use in foods, cosmetics, and pharmaceuticals. Trehalose is well tolerated in acute oral doses up to 75 g and oral ingestion of trehalose produces blunted insulinemic and glycemic responses in comparison to glucose (105, 131, 167, 168). In 1994, the development of an enzyme based production method allowed for the widespread manufacture of trehalose (42). Furthermore, trehalose received the Food and Drug Administration’s designation “generally recognized as safe” in 2000. Together, the safety profile, low cost, and high availability to trehalose make it a feasible and attractive agent for clinical use (131).

Conclusions and future directions

A rapid increase in the number of older adults in the United States forecasts an unprecedented rise in the prevalence of CVD and associated health care burden. A key antecedent to the development of CVD with age is the development of arterial dysfunction. Oxidative stress and chronic low-grade inflammation are central mechanisms underlying the development of vascular dysfunction with age and, as such, these processes represent key therapeutic targets for the reversal of arterial aging in MA/O adults. Trehalose has been identified as a safe, naturally occurring disaccharide with antioxidant and anti-inflammatory properties that reverses age-associated vascular dysfunction in mice. The low-risk safety profile, high availability, and low cost of trehalose make this disaccharide an attractive therapy for humans. However, the efficacy of trehalose to improve vascular function in healthy MA/O adults is completely unknown.
CHAPTER IV

Oral trehalose supplementation improves resistance but not conduit artery endothelial function
in middle-aged and older adults who remain weight-stable

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Abstract

I tested the hypothesis that oral supplementation with the disaccharide trehalose would improve resistance and/or conduit artery endothelium-dependent dilation (EDD) in middle-aged and older (MA/O) adults, and that improvements would be related to increased nitric oxide (NO) bioavailability and reduced inflammation and oxidative stress. Thirty-two healthy men and postmenopausal women aged 50-77 years consumed 100g/day of trehalose (n=15) or maltose (n=17) for 12 weeks in a randomized, double-blind study. Conduit artery EDD, as assessed by brachial artery flow-mediated dilation (FMD), was unchanged in both groups (all P>0.1) and the absence of a treatment effect was not explained by the ~1 kg increase in body weight observed. Resistance artery EDD, as measured by forearm blood flow to brachial artery infusion of acetylcholine (FBF$_{ACh}$), also did not change significantly with trehalose treatment in the overall study population (P>0.1). However, in subjects who remained weight-stable (Δmass<2.3 kg), FBF$_{ACh}$ increased ~30% with trehalose (13.32±0.95 vs. 10.50±1.11 AUC, P<0.05) whereas there was no effect of maltose (P>0.1). This improvement in FBF$_{ACh}$ was abolished when endothelial NO production was inhibited (P>0.1 vs. FBF$_{ACh}$ at baseline), demonstrating that improved FBF$_{ACh}$ with trehalose was NO-dependent. In the weight-stable subgroup, trehalose treatment was associated with a reduction in arterial endothelial cell expression of the pro-inflammatory chemokine, monocyte chemoattractant protein-1 (0.75±0.08 vs. 1.0±0.05 AU, P<0.05). In contrast, neither trehalose nor maltose influenced markers of systemic inflammation (C-reactive protein and interleukin-6) or oxidative stress (circulating oxidized LDL) (all P>0.1). The FBF$_{ACh}$ response to co-infusion of the antioxidant, vitamin C, did not change in the weight-stable subgroup following 12 weeks of supplementation (P>0.1 for effect of time and group by time interaction), suggesting no change in oxidative stress-mediated suppression of resistance artery EDD. In the weight-stable cohort, endothelium-independent dilation, assessed by FBF to brachial artery infusion of sodium nitroprusside, increased ~30% with trehalose (155±13 vs. 116±12 AUC, P<0.05) but not maltose (P>0.1) demonstrating a beneficial effect of trehalose on
resistance artery smooth muscle sensitivity to NO. Together, these findings indicate that trehalose may be a novel therapy for reducing arterial inflammation and improving resistance but not conduit artery EDD in MA/O adults who are able to remain weight-stable.
Introduction

Endothelial dysfunction, as indicated by impaired endothelium-dependent dilation (EDD), occurs with primary aging and is a key antecedent to the development of cardiovascular diseases (80, 143). Impairment of EDD with age is mediated largely by a decrease in bioavailability of the vascular-protective and vasodilatory molecule, nitric oxide (NO) (11, 175), secondary to the development of systemic and vascular oxidative stress and inflammation (37, 142). As such, therapies that inhibit oxidative and inflammatory signaling with age may have the potential to improve NO-mediated EDD, and reduce CVD risk in healthy middle-aged and older (MA/O) adults (143).

The naturally occurring disaccharide α,α-trehalose (referred to as trehalose) is cytoprotective in lower organisms (9, 41, 67, 72, 131) and reduces oxidative stress and inflammation in numerous in vitro and in vivo models of age-related diseases (6, 39, 132, 138). Trehalose also preserves vascular function in pro-inflammatory age-related disease models (39, 138). As such, trehalose is emerging as a novel therapy to inhibit oxidative stress and inflammation and restore vascular function in diseases of aging.

Although the exact mechanisms underlying the potential anti-oxidant and –inflammatory effects of trehalose are unknown, trehalose may act through the activation of autophagy (15, 93, 132, 139, 186, 193), a cellular stress response pathway by which damaged cytoplasmic components are recycled through lysosomal degradation (20, 25). Indeed, autophagy declines with age and inhibition of autophagy increases cell damage and stimulates oxidative stress and inflammation (68, 69, 91, 134).

Recent preclinical findings by LaRocca et al. demonstrate that four weeks of trehalose in drinking water completely restores carotid artery EDD in old mice to levels observed in young, while having no effect in young animals. This improvement was associated with increased NO bioavailability, reduced arterial superoxide production, normalization of arterial inflammatory proteins, and restoration of vascular autophagy (84). These finding provide strong evidence
that trehalose may be an effective strategy for improving age-associated endothelial function. However, the efficacy of trehalose to reverse age-associated endothelial dysfunction in MA/O adults is unknown.

The aim of the present study was to translate these preclinical findings to humans. I hypothesized that oral trehalose supplementation would improve resistance and/or conduit artery EDD in healthy MA/O adults, and that improvements in EDD would be related to increased NO bioavailability and reduced oxidative stress and inflammation. To test this hypothesis, I conducted a randomized, double blind, parallel group study in which the diets of 32 MA/O adults free of CVD were supplemented with 100g/day of trehalose or maltose for 12 weeks. Importantly, this dose is roughly equivalent to the amount of trehalose that improved endothelial function in old mice on a g/kg body mass/day basis (84). In the present investigation, resistance artery EDD (forearm blood flow to acetylcholine, \( FBF_{ACh} \)) and conduit artery EDD (flow-mediated dilation, FMD) were assessed. Both of these measures are independently predictive of incident CVD and, consequently, represent clinically relevant indices of endothelial function. The proportion of resistance artery EDD mediated by NO was determined by measuring \( FBF_{ACh} \) in the absence vs. presence of N'-monomethyl-L-arginine (L-NMMA), an inhibitor of the NO producing enzyme, endothelial NO synthase. Similarly, oxidative stress-mediated suppression of EDD was examined by measuring \( FBF_{ACh} \) in the absence vs. presence of the potent antioxidant, vitamin C. Circulating oxidized low density lipoprotein (LDL) was measured to characterize systemic oxidative stress (79, 122). The acute phase protein, C-reactive protein (CRP) and the pro-inflammatory cytokine, interleukin-6 (IL-6) were assessed to characterize systemic inflammation (50, 57). Lastly, endothelial cells were biopsied from the brachial artery before and after the intervention period to determine expression of the pro-inflammatory chemokine, monocyte chemoattractant protein-1 (MCP-1) (33), and p62/sequestosome 1 (P62), a marker of impaired autophagy (114).
Methods

Subjects. Thirty-two men and postmenopausal women aged 50-77 years were studied. All subjects were non-smoking adults free from clinical disease as assessed by medical history, physical examination, blood chemistries, electrocardiogram, resting blood pressure, and cardiovascular responses to a graded exercise test. Subjects refrained from all cardiovascular acting medications for 24 hours prior to testing and all other medications for 12 hours. All procedures were approved by the Institutional Review Board at the University of Colorado Boulder. The nature, risks and benefits of all study procedures were explained to volunteers and their written informed consent was obtained before participation in the study.

Procedures. All testing was performed at the Clinical Translational Research Center (CTRC) at the University of Colorado Boulder following a 12-hour fast from food and caffeine and 24-hour abstention from exercise and alcohol. All women were post-menopausal as confirmed by absence of menstruation for >1 year and follicular stimulating hormone levels>40 IU/L (22).

Trehalose administration. Subjects were randomized to consume trehalose (100g/day) or maltose (100g/day) for 12 weeks in a double blind fashion. Food grade trehalose and maltose were purchased from Hayashibara (Okayama, Japan). Maltose was chosen as a control condition because 1) like trehalose, maltose is a disaccharide of glucose, 2) maltose does not have the same antioxidant and anti-inflammatory properties and/or beneficial physiological effects as trehalose when administered at similar concentrations (39, 67, 139, 149, 158) and 3) maltose provides an isocaloric control condition. To replicate the preclinical study by LaRocca et al. that demonstrated improved EDD in old mice following trehalose treatment, trehalose and maltose were administered orally dissolved in 12 ounces of water. Subjects were provided with individual containers for each day containing either 100g of trehalose or maltose. Subjects were also given a graduated water bottle and were instructed to mix one container of sugar with 12 ounces of water each day. Subjects were allowed to consume the intervention
drinks at their own pace over the course of the day. Adherence was documented by having subjects return empty and unused containers every two weeks during the 12-week intervention period. Every two weeks subjects also received in-person nutrition counseling by a dietitian at the Boulder CTRC to promote stability of diet and body weight throughout the intervention period.

Subject characteristics and circulating factors. Arterial blood pressure (BP) was measured in triplicate over the brachial artery during supine rest (Noninvasive Hemodynamics Workstation, Cardiovascular Engineering Inc.) at baseline and after the 12-week intervention. Waist and hip circumferences were measured by anthropometry, and percentage body fat was measured by dual-energy X-ray absorptiometry (DEXA, DXA-GE Lunar; software version 5.60.003) at these same time points. Aerobic fitness was assessed at baseline and after the intervention by indirect calorimetry during incremental treadmill exercise (Balke protocol) (28, 178). Total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, and fasting glucose were measured using standard assays at the University of Colorado CTRC Core Laboratory at baseline and after 4 and 12 weeks of trehalose and maltose supplementation. At these same time points, plasma oxidized LDL and IL-6 and were assessed by ELISA (oxidized LDL: ALPCO; IL-6: R&D systems) and high-sensitivity CRP was measured by immunoturbidimetry, as described previously (177, 178). All blood samples were drawn from an intravenous catheter placed in the left antecubital fossa.

Dietary analysis. Stability of dietary intake was assessed using 3-day diet records at baseline and during the last week of the intervention period (126). Dietary intake was assessed using Nutrition Data System for Research. Prior to starting the intervention, subjects met with a bionutritionist at the Boulder CTRC. All subjects were instructed to maintain their current caloric intake and were given suggestions on how to reduce carbohydrate intake by roughly 400 kcals, (the caloric content of the study drinks) based on their baseline diet record.
Resistance artery EDD and endothelium-independent dilation. Resistance artery EDD and endothelium independent dilation were assessed at baseline and after the 12 week intervention period using strain gauge venous occlusion plethysmography (AI6 Arterial Inflow System, D.E. Hokanson Inc., Bellevue, Washington) as described previously (28, 36, 71, 179). Briefly, a mercury-silastic strain gauge was placed around the forearm and an inflatable cuff was placed at the wrist and upper arm. The upper arm cuff cycled between 0 and 60 mmHg to occlude venous outflow during 7-second cycles. Wrist cuffs were inflated to 250 mmHg for the duration of all forearm blood flow (FBF) measures to exclude hand circulation. FBF was calculated during the last 1.5 minutes of each infusion period. FBF values are expressed as the area under the dose-response curve (AUC). In all subjects, the brachial artery of the non-dominant arm was catheterized for infusions. Forearm volume was measured by water displacement and drug infusion rates were normalized per 100 ml of forearm tissue.

Resistance artery EDD was determined by measuring FBF to increasing intra-arterial doses of ACh (FBF_{ACh}, 1, 2, 4, and 8 µg/100 mL forearm volume/min, 3.5-4 minutes per dose) and endothelium-independent dilation was assessed by measuring FBF to increasing doses of intra-arterial sodium nitroprusside (FBF_{SNP}; 0.5, 1, and 2 µg/100 mL forearm volume/min 3.5-4 minutes per dose). To determine the contribution of NO to resistance artery EDD, FBF_{ACh} was measured in the absence and presence (co-infusion) of the endothelial NO synthase inhibitor, L-NMMA (5 mg/min during a 10-minute loading dose followed by a 1 mg/min maintenance dose). Oxidative stress-mediated suppression of resistance artery EDD was assessed by measuring FBF_{ACh} in the absence and presence of the antioxidant vitamin C (25 mg/min during a 10-minute loading dose at an infusion rate of 2.5 ml/min followed by a 0.5 ml/min maintenance dose).

Conduit artery EDD. Conduit artery EDD was assessed by brachial artery flow-mediated dilation (FMD) as described previously (45, 71, 178). Briefly, the change in brachial artery diameter following five minutes of forearm blood flow occlusion was measured by duplex ultrasonography (Xario, Toshiba; multi-frequency linear-array transducer). FMD measurements
were reported as absolute and percent change in accordance with recent guidelines (32, 63). Because of the non-invasive nature of this measure, FMD was assessed at baseline and after 4 and 12 weeks of trehalose and maltose supplementation.

**Arterial endothelial cell protein expression.** Arterial endothelial cells were biopsied from the brachial artery at baseline and after the 12 week intervention as previously described (34, 36, 71, 84). Cells were recovered by centrifugation, fixed, plated on slides and stored at -80°C. Slides were later incubated with a primary antibody for the pro-inflammatory chemokine, MCP-1 (1:250 Santa Cruz Biotechnology) or the autophagy marker, p62 (1:500, MBL International) and a complementary fluorescent secondary antibody, AF647 (1:100, Abcam). Cells were also incubated with a primary antibody for vascular endothelial cadherin (VE-cad, 1:500 Abcam) and complementary fluorescent secondary antibody, AF488 (1:200, Abcam) for positive identification of endothelial cell phenotype. DAPI was used to confirm nuclear integrity. Slides were viewed using a fluorescence microscope (Eclipse Ni-U; Nikon) and whole-cell fluorescence was assessed using Metamorph Software (Universal Imaging). All protein expression data are reported as human endothelial cell intensity relative to human umbilical vein endothelial cell (HUVEC) intensity, normalized for baseline expression. Normalization to HUVEC protein expression provides a control to account for potential variations in staining intensity between staining sessions. Slides from baseline and week 12 of the intervention were stained on the same day for each subject.

**Data analysis.** Statistical analyses were performed in SPSS (IBM SPSS Statistics 22). The Shapiro-Wilk test was used to assess normality and non-normal variables were log-transformed. Group differences at baseline were assessed using independent Student’s t tests for between-group contrasts. Repeated measures ANOVA was used to determine group (maltose vs. trehalose) by time interactions for all clinical characteristics and primary outcome measures. In the case of a significant interaction or significant overall effect of time, a Student’s t-test for within-group contrast was performed with Bonferroni correction. The linear relation
between variables of interest was assessed using Pearson product-moment correlation analyses. Multiple linear regression was used to evaluate the independent relation between group randomization (coded, bivariate variable) and the change in primary outcomes across the intervention period. Statistical significance was set at P<0.05.

Results

Subject enrollment. One hundred ten subjects were consented for the study. Forty-five subjects did not meet inclusion criteria and 30 subjects opted out of the study prior to randomization due to the time commitment (n=16), study restrictions (n=1) and invasive testing procedures (n=5). An additional eight subjects never responded to scheduling requests. Nineteen subjects were assigned to the maltose group and 18 subjects to the trehalose group. Two subjects were excluded from the maltose group due to the development of side effects (n=1) and an adverse event related to a testing procedure (n=1). Three subjects were excluded from the trehalose group due to the development of side effects (Figure 1).

Side effects. The side effects reported include minor to moderate gastrointestinal discomfort (transient bloating, flatulence, and loose stools; maltose: n=0; trehalose: n=4) and changes in energy levels (maltose: n=1; trehalose n=2). These side effects were expected as they are characteristic of disaccharide consumption in general (131).
Subject characteristics. There were no group differences in age, gender, body mass, percent body fat, waist to hip ratio, systolic BP, diastolic BP, heart rate, maximal oxygen consumption, fasting glucose or total, HDL or LDL cholesterol between maltose and trehalose groups at baseline (all P>0.05, Table 1). There was a significant effect of time on body mass (F[2,27]=6.58, P<0.01) and this response did not differ between groups (P>0.1). In both the maltose and trehalose groups, body mass increased significantly from baseline to week 4 (maltose: 72.8±3.4 vs. 72.1±3.4 kg, P<0.05; trehalose: 74.1±4.8 vs. 73.4±4.7 kg, P<0.05) but not from week 4 to week 12 (both P>0.05). Importantly, percent body fat did not change significantly across the intervention (F[1,29]=2.49, P>0.1). Moreover, no subject characteristics changed differentially across the intervention period in the maltose and trehalose treated groups (all P>0.1, Table 1).
Dietary intake. Total energy intake and the relative intake of carbohydrates, fats, and protein did not differ between the maltose and trehalose groups at baseline (all $P>0.1$). There was a strong trend towards a significant effect of time on energy intake in both groups ($F[1,26]=3.83$, $P=0.1$). Relative carbohydrate intake was higher at week 12 vs. baseline in the maltose group (57±2 vs. 49±1 % total calories, $P<0.001$) and there was also a strong trend for carbohydrate intake to be elevated after 12 weeks of trehalose (55±2 vs. 50±3 %, $P=0.1$). In contrast, neither relative protein nor fat intake changed significantly across the intervention period ($P>0.1$, Table 2).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Maltose Baseline</th>
<th>Maltose Week 4</th>
<th>Maltose Week 12</th>
<th>Trehalose Baseline</th>
<th>Trehalose Week 4</th>
<th>Trehalose Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (men/women)</td>
<td>17 (8/9)</td>
<td>---</td>
<td>---</td>
<td>15 (7/8)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Age (years)</td>
<td>63±2</td>
<td>---</td>
<td>---</td>
<td>64±2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>72.1±3.4</td>
<td>72.8±3.4*</td>
<td>73.0±3.3*</td>
<td>73.4±4.7</td>
<td>74.1±4.8*</td>
<td>74.4±4.8*</td>
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<tr>
<td>Fat (%)</td>
<td>30.3±1.9</td>
<td>31.5±2.0</td>
<td>31.2±1.9</td>
<td>31.2±1.9</td>
<td>---</td>
<td>31.4±1.9</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>0.83±0.02</td>
<td>0.84±0.02</td>
<td>0.84±0.02</td>
<td>0.84±0.02</td>
<td>---</td>
<td>0.84±0.03</td>
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<tr>
<td>Systolic BP (mmHg)</td>
<td>125±4</td>
<td>126±4</td>
<td>129±4</td>
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<td>130±4</td>
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<td>Diastolic BP (mmHg)</td>
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<td>Heart rate (beats/min)</td>
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<td>57±1</td>
<td>57±2</td>
<td>57±2</td>
<td>56±2</td>
<td>58±2</td>
</tr>
<tr>
<td>VO2 max (ml/kg/min)</td>
<td>31.2±1.7</td>
<td>30.9±1.7</td>
<td>27.4±2.4</td>
<td>28.9±1.7</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>179±10</td>
<td>178±10</td>
<td>177±10</td>
<td>180±8</td>
<td>180±8</td>
<td>177±8</td>
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<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>59±3</td>
<td>58±2</td>
<td>56±2</td>
<td>58±6</td>
<td>58±6</td>
<td>55±5</td>
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<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>102±8</td>
<td>101±7</td>
<td>102±7</td>
<td>105±6</td>
<td>104±6</td>
<td>103±7</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>85±2</td>
<td>90±4</td>
<td>92±6</td>
<td>85±1</td>
<td>84±2</td>
<td>83±1</td>
</tr>
</tbody>
</table>

*Data are mean ± SE; *$P<0.05$ vs. baseline of same group. BP blood pressure, VO2 max maximal oxygen consumption, HDL high-density lipoprotein, LDL low-density lipoprotein.
Circulating factors. CRP and IL-6 were log transformed to meet normality assumptions. There were no baseline group differences in oxidized LDL, CRP, or IL-6 and these factors did not change differentially across the intervention period in the maltose and trehalose treated groups (all P>0.1, Table 3).

Because body mass can independently influence circulating levels of oxidized LDL, CRP, and IL-6 (14, 126, 159, 160, 195), and weight gain was observed during the intervention period in both groups, I determined the relation between the change in body mass and the change in these circulating factors from baseline to week 4 and from baseline to week 12. The only significant relation observed was a positive correlation between the change in body mass and the change in oxidized LDL after 12 weeks of supplementation (r=0.39, P<0.05). To determine if the association between weight gain and increased oxidized LDL might be masking a possible treatment effect, I used multiple linear regression to determine if there was an independent relation between group randomization (coded, bivariate variable) and the change in oxidized LDL from baseline to week 12, while holding the change in body mass constant. In this model, group randomization was not an independent predictor of the change in oxidized LDL (r=0.18, P>0.1), suggesting that trehalose did not have an independent effect on this circulating marker of oxidative stress.

### Table 2: Dietary intake

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Maltose Baseline</th>
<th>Maltose Week 12</th>
<th>Trehalose Baseline</th>
<th>Trehalose Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily energy intake (kcal)</td>
<td>1719±143</td>
<td>2050±141</td>
<td>1874±175</td>
<td>1977±166</td>
</tr>
<tr>
<td>Daily relative carbohydrate intake (% total kcal)</td>
<td>49±1</td>
<td>57±2‡</td>
<td>50±3</td>
<td>55±2</td>
</tr>
<tr>
<td>Daily relative protein intake (% total kcal)</td>
<td>16±1</td>
<td>14±1</td>
<td>15±1</td>
<td>15±1</td>
</tr>
<tr>
<td>Daily relative fat intake (% total kcal)</td>
<td>35±2</td>
<td>29±1.5</td>
<td>35±2</td>
<td>30±2</td>
</tr>
</tbody>
</table>

Data are mean ± SE; ‡P<0.001 vs. baseline of same group. kcal kilocalories.
Resistance artery EDD. To achieve a normal distribution, FBF values were log transformed; area under the dose-response curve (AUC) was calculated from the transformed values. FBF\textsubscript{ACh} (AUC) did not differ between groups at baseline (P>0.1) and did not change differentially across the intervention period with maltose and trehalose supplementation (F[1,23]=2.03, P>0.1).

Because changes in body mass also independently influence EDD (126), I assessed the relation between the change in body mass and the change in FBF\textsubscript{ACh} across the intervention period. The change in body mass from baseline to week 12 was inversely associated with the change in FBF\textsubscript{ACh} (r=-0.41, P<0.05). To determine if this relation was masking a treatment effect, I used multiple linear regression to assess the relation between group randomization and the change in FBF\textsubscript{ACh} while controlling for the change in body mass. In this model, there was a strong trend for group randomization to be an independent predictor of the change in FBF\textsubscript{ACh} from baseline to week 12 (P=0.1), suggesting a treatment effect when body mass is held constant. To further isolate the effect of trehalose treatment on resistance artery EDD, I analyzed the change in FBF\textsubscript{ACh} in a subgroup of subjects who maintained their body mass within 2.3 kg (~5 lbs.; maltose: n=11, trehalose: n=10). In this subgroup, FBF\textsubscript{ACh} changed differentially with maltose and trehalose treatment (F[1,19]=5.01, P<0.05). FBF\textsubscript{ACh} was ~30% greater after
12 weeks of trehalose (13.32±0.95 vs. 10.50±1.11 AUC, P<0.05), whereas there was no change with maltose supplementation (P>0.1, Figure 2).

**Resistance artery NO bioavailability.** In the cohort of subjects who remained weight-stable (Δmass<2.3 kg), the increase in FBF\textsubscript{ACh} following trehalose supplementation (as expressed as AUC, Figure 3) was abolished when endothelial NO production was inhibited by co-infusion of L-NMMA (P>0.1 vs. baseline FBF\textsubscript{ACh} in the absence of L-NMMA), demonstrating that improvements in FBF\textsubscript{ACh} were mediated by increased NO bioavailability.

**Oxidative stress-mediated suppression of resistance artery EDD.** In subjects who had a change in body mass <2.3 kg, the response of FBF\textsubscript{ACh} to co-infusion of the antioxidant vitamin C did not change across the intervention period (F[1,18]=2.19, P>0.1) and group randomization did not influence this response (F[1,18]=1.17, P>0.1). Co-infusion of vitamin C improved FBF\textsubscript{ACh} in both groups at baseline (maltose base: 12.77±1.05 vs. 10.14±1.20 AUC; trehalose base: 12.70±0.74 vs. 10.50±1.11 AUC, both P<0.01) and there was a strong trend for vitamin C to improve FBF\textsubscript{ACh} in both groups following the intervention period (maltose post: 12.22±1.14 vs. 10.68±1.00 AUC; trehalose post: 14.21±0.82 vs. 13.32±0.95 AUC, both P=0.1, Figure 4).
Figure 2. Forearm blood flow responses to acetylcholine (ACh) at baseline (closed circles) and following 12 weeks (open circles) of maltose and trehalose supplementation in all subjects (A) and in the subset of subjects who maintained body mass within 2.3 kg (B). FAV, forearm volume. Values are mean ± SE; *P<0.05 vs. baseline.
Resistance artery endothelium-independent dilation. FBF_{SNP}, a measure of smooth muscle sensitivity to nitric oxide, did not differ between groups at baseline (P>0.1). In all subjects, there was a trend for FBF_{SNP} to change differentially following 12 weeks of maltose and trehalose treatment (F[1,23]=3.18, P=0.1).

The change in body mass from baseline to week 12 was inversely related to the change in FBF_{SNP} (r=-0.40, P<0.05). Moreover, group randomization was an independent predictor of the change in FBF_{SNP} when controlling for the change in body mass from baseline to week 12 using multiple linear regression (r=0.39, P<0.05), indicating an independent effect of trehalose treatment on smooth muscle sensitivity to nitric oxide. In the subset of subjects who maintained body mass within 2.3 kg, FBF_{SNP} changed differentially across the intervention period in the maltose and trehalose treated groups (F[1,19]=5.87, P<0.05). FBF_{SNP} increased ~30% after 12
weeks of trehalose (155±13 vs. 116±12 AUC, P<0.05) whereas there was no change following 12 weeks of maltose supplementation (P>0.1, Figure 5).

**Conduit artery EDD.** Brachial artery FMD was similar between groups at baseline (P>0.1). FMD did not change differentially across the intervention in the trehalose and maltose treated groups when FMD was expressed as either percent or absolute change (both P>0.1, Figure 6).
In contrast to resistance artery EDD, the change in FMD at 4 and 12 weeks was not related to the change in body mass at these time points (all P>0.1). Moreover, group randomization was not an independent predictor of the change in FMD from baseline to week 4 or baseline to week 12 when holding body mass constant using multiple linear regression (all P>0.1).

**Arterial endothelial cell protein expression.** To gain insight into potential mechanisms underlying changes in resistance artery EDD in the weight-stable subgroup, biopsied arterial endothelial cells from these subjects were analyzed for expression of the pro-inflammatory chemokine, MCP-1 (33), and P62, a marker of impaired autophagy (114). In subjects who maintained body weight within 2.3 kg, there was a strong trend for arterial endothelial cell MCP-1 expression to change differentially across the intervention in the maltose and trehalose treated groups (F[1,18]=2.8, P=0.1). Arterial endothelial MCP-1 expression...
decreased after 12 weeks of trehalose (0.75±0.08 vs. 1.0±0.05 AU, P<0.05) whereas there was no change with maltose supplementation (P>0.1, Figure 7). In the weight-stable cohort, arterial endothelial p62 expression did not change across the intervention period in either the trehalose and maltose groups (P>0.1).

**Figure 7.** Arterial endothelial cell monocyte chemoattractant protein-1 (MCP-1) expression at baseline (base) and following 12 weeks of maltose and trehalose supplementation in the subset of subjects who maintained body mass within 2.3 kg. Values are mean ± SE; *P<0.05 vs. baseline of same group. Below: representative images of arterial endothelial cell MCP-1 expression.

**Discussion**

This is the first study to examine the potential therapeutic effects of trehalose on any type of physiological function with age in humans. The present findings provide novel evidence that 12 weeks of oral trehalose supplementation improves resistance artery EDD through
increasing NO bioavailability in MA/O who remain weight-stable. This improvement was associated with decreased arterial endothelial cell expression of the pro-inflammatory chemokine, MCP-1, suggesting a possible role of reduced vascular inflammation. Improvements in resistance artery endothelium-independent dilation were also observed with trehalose treatment in the weight-stable subgroup, indicating increased vascular smooth muscle sensitivity to NO. In contrast, trehalose supplementation had no effect on conduit artery EDD. Together, these findings demonstrate that oral supplementation with trehalose has heterogeneous effects on different types of arteries, and that trehalose treatment may be a novel strategy to decrease vascular inflammation and improve resistance artery EDD in healthy MA/O adults who are able to remain weight-stable. Because resistance artery EDD is an independent predictor of incident CVD, these findings have important clinical implications for the primary prevention of CVD with advancing age.

_Trehalose supplementation and endothelial function._ Resistance and conduit artery EDD are impaired with advancing age and are independent predictors of incident CVD (95, 189, 190). As such, resistance and conduit artery endothelial dysfunction represent an intermediate phenotype in CVD progression (80, 118). In the present study, resistance artery EDD, as assessed by FBF to intra-arterial ACh, improved ~30% following 12 weeks of oral trehalose supplementation in subjects who maintained their body mass within 2.3 kg. This effect was abolished by inhibition of endothelial NO production, indicating that the improvement in resistance artery EDD following trehalose treatment was NO-mediated. In contrast, maltose had no effect on resistance artery EDD or NO bioavailability in this same cohort. These findings are consistent with recent reports that trehalose has vascular-protective effects in age-related disease states including Parkinson’s disease (138) and subarachnoid hemorrhage (39). Moreover, these findings extend previous preclinical observations that oral trehalose supplementation improves NO-mediated EDD in response to the endothelium-dependent
dilator, ACh, in old mice, and suggests that trehalose may be a viable therapy for the improvement of resistance artery EDD in MA/O adults (84).

In contrast to findings with resistance artery EDD, no changes were observed in conduit artery EDD, as assessed by brachial artery FMD, with trehalose supplementation, even when accounting for changes in body mass. This was not anticipated as a previous study in mice by LaRocca et al. found improvements in EDD in the carotid artery with trehalose treatment (84). This inconsistency could be due to the stimulus used to induce EDD (pharmacological vs. mechanical) (47) in the mouse and humans investigations. Specifically, improved EDD stimulated by ACh was observed in both mice and humans in different arterial beds, whereas there were no improvements in EDD stimulated by increased shear stress in MA/O adults. As such, it is possible that improvements in EDD with trehalose may be specific to non-mechanical stimuli and this will need to be delineated in future investigations.

The discrepancy between findings on resistance and conduit artery EDD following trehalose supplementation is not surprising considering the heterogeneity in the structure, flow dynamics and microenvironment of these two types of arteries (1, 2, 173). While both resistance and conduit artery EDD predict incident CVD events (95, 189, 190), these measures are not related in MA/O adults free from CVD (47, 96). Additionally, with advancing age impairments in resistance artery EDD are observed over two decades before impairments in conduit artery EDD, demonstrating differential effects of aging on the function of these arteries (17, 156). As such, the different response observed for resistance and conduit artery EDD in the present study is in line with previous findings demonstrating dissociation between these two measures with age.

The effect of oral trehalose supplementation on arterial endothelial cell and systemic inflammation. Aging results in suppression of the adaptive immune system and consequent up-regulation of the innate immune system, which generates a phenotype of chronic low-grade inflammation known as “inflammaging” (136). This phenotype presents as an increase in
circulating levels of inflammatory mediators and elevated pro-inflammatory gene and protein expression in the vascular wall (40, 142). Age-associated pro-inflammatory signaling stimulates endothelial activation, accumulation of cell damage, as well as changes in autocrine and paracrine signaling (33, 84, 148) that disrupt cellular homeostasis and inhibit EDD (142, 178).

The age-associated increase in vascular inflammation is a key mechanism of impaired endothelial function with aging, as short term treatment with the anti-inflammatory agent, salsalate, reverses endothelial cell inflammation and improves EDD in MA/O adults, while having no effect in young adults (142, 178). In the present study, 12 weeks of oral trehalose supplementation resulted in a decrease in arterial endothelial cell expression of the pro-inflammatory chemokine, MCP-1, in subjects who remained weight-stable. MA/O adults with age-associated vascular dysfunction demonstrate increased MCP-1 expression in biopsied human endothelial cells compared with young adults (33), implicating MCP-1 in the pro-inflammatory phenotypic shift contributing to endothelial dysfunction with age (33, 38, 180). As such, decreased arterial endothelial cell expression of MCP-1 with trehalose treatment may be a mechanism of improved resistance artery EDD in the weight-stable cohort. The finding that trehalose decreases a marker of arterial endothelial cell inflammation is consistent with previous reports that trehalose protects against pro-inflammatory challenges in mammalian cells (39, 64, 151) and in vivo (6, 109, 138). Furthermore, this result extends to humans previous findings from old mice that trehalose protects against age-associated vascular inflammation (84).

In the present investigation, no changes in the circulating pro-inflammatory markers CRP and IL-6 were observed with trehalose treatment, despite evidence for decreased vascular inflammation with this intervention. This is in line with previous cross sectional studies demonstrating impaired EDD and elevated endothelial but not circulating markers of inflammation in MA/O vs. young adults (33, 127). These previous studies highlight that the expression of vascular and circulating inflammatory markers do not always correspond during healthy aging. Moreover, improvements in EDD with short-term salsalate treatment in MA/O
adults is associated with decreased endothelial cell but not circulating markers of inflammation (128, 178). As such, the findings from the present investigation are in line with previous studies in MA/O adults demonstrating improved EDD with changes in vascular but not systemic inflammatory signaling.

The effect of trehalose supplementation on systemic oxidative stress and oxidative stress-linked suppression of endothelial function. Oxidative stress develops with advancing age and inhibits NO-mediated EDD through the direct scavenging of NO and by damaging the enzymes and cofactors required for NO synthesis (8, 11, 45, 155, 177). In lower organisms and ex vivo models, trehalose seems to protect against reactive oxygen species (9, 39) and oral trehalose supplementation decreases markers of oxidative stress in a mouse model of Parkinson’s disease (132). In the preclinical study previously conducted by LaRocca et al., four weeks of trehalose supplementation reversed age-associated increases in aortic superoxide production and abolished the oxidative stress-mediated suppression of EDD observed in old control mice (84). In contrast, in the present investigation there was no change in circulating oxidized LDL, a marker of systemic oxidative stress, with either trehalose or maltose supplementation. Furthermore, the FBF\textsubscript{ACh} response to co-infusion of the antioxidant, vitamin C, a measure of oxidative-stress mediated suppression of FBF\textsubscript{ACh}, did not change with maltose or trehalose treatment in the weight-stable cohort. These observations suggest that a reduction in oxidative stress was not a key mechanism underlying the improvement in resistance artery EDD observed with trehalose supplementation in the weight-stable subgroup, in contrast to previous findings in mice. These discordant observations may be due to differences in the absorption, metabolism and/or clearance of trehalose in mice and humans (162).

Oral trehalose supplementation and endothelial cell autophagy. Trehalose has been reported to activate autophagy in vivo (15, 93, 132, 193) and this is believed to be a key mechanism underlying the anti-oxidant and anti-inflammatory properties, as well as the beneficial functional effects of the disaccharide. In old mice, improved EDD with trehalose
supplementation is associated with increases in whole artery (aortic) expression of autophagy markers (84), suggesting that autophagy could be a mechanism involved in improved vascular function with trehalose treatment in the weight-stable subgroup. To gain insight into whether oral trehalose supplementation up-regulates endothelial cell autophagy in weight-stable adults, I measured expression of the autophagy marker p62 in biopsied arterial endothelial cells. p62 is an adaptor protein that targets cellular constituents for degradation by autophagy. When autophagy is active, p62 is degraded by the lysosome along with the cellular constituents that it binds to, making p62 a reliable static marker of the dynamic process of autophagy (i.e. both synthesis and degradation of autophagy mediators) (75, 114). In the present investigation, arterial endothelial p62 expression did not change across the intervention period in weight-stable subjects supplemented with either maltose or trehalose. This finding suggests that increased endothelial cell autophagy was not a mechanism underlying improvements in resistance artery EDD with trehalose treatment in the weight-stable cohort. Because endothelial cell samples were collected ≥12 hours after the consumption of any trehalose (to maintain the validity of vascular assessments), there may have been transient increases in endothelial autophagic flux that were not captured in the endothelial cell biopsies.

*Oral trehalose supplementation and endothelium independent dilation.* Endothelium independent dilation is an important bioassay of smooth muscle sensitivity to nitric oxide. In the present study, trehalose improved resistance artery endothelium-independent dilation in weight-stable subjects by ~30%. This increase in smooth muscle sensitivity to nitric oxide may have been an indirect effect of improved NO bioavailability with trehalose treatment, as NO is an important regulator of endothelium-derived vasoconstrictor factors (48, 61, 171). Alternatively, this finding could be due to decreased vascular inflammation and a consequent increase in the expression of cGMP-dependent protein kinase, a key downstream mediator of NO-stimulated vasodilation in vascular smooth muscle that is down-regulated by inflammatory signaling (13, 94, 146).
The finding that trehalose improves endothelium-independent dilation in humans is inconsistent with previous findings in mice (84). This may be due to fundamental differences between these experimental models. Specifically, endothelium-independent dilation is not impaired with aging in C57BL/6 mice (36, 82, 88). As such, it may not have been possible to detect improvements in this outcome. In contrast, findings in humans are less consistent with some studies reporting modest impairments in endothelium-independent dilation with aging, especially in individuals with CVD risk factors (142). In agreement with these reports, there was a trend for impaired endothelium-independent dilation to be lower in subjects randomized to trehalose treatment at baseline and this may partially explain the conflicting observations in mice and humans.

Limitations. The primary limitations of the present study are related to the challenges associated with a nutritional intervention. I chose to administer trehalose at a body weight equivalent dose to that which improved EDD in old mice to optimize the chances of seeing a therapeutic effect of trehalose on endothelial function. However, there is a relatively high caloric content associated with 100g/day of trehalose (~400 kcals/day) and despite nutrition counselling every two weeks, not all subjects were able to maintain their body weight over the 12 week intervention period or keep the macronutrient composition of their diet constant. The dose of trehalose administered also resulted in some transient gastrointestinal discomfort, as has been reported previously with the consumption of disaccharides (131). Due to the weight gain, gastrointestinal side effects, and trend for changes in dietary macronutrient composition observed with 100g/day of oral trehalose supplementation, future investigations with alternative doses and methods of trehalose administration are warranted.

In the present study, 12 weeks of trehalose supplementation in weight-stable subjects was associated with decreased arterial endothelial cell MCP-1 expression. Although I cannot directly prove that reduced vascular inflammation is a causal mechanism underlying improved arterial function with trehalose supplementation in the present study, previous findings that
MCP-1 is a key factor involved in the pro-inflammatory phenotypic shift that promotes endothelial dysfunction with age (142, 180) supports a possible role.

Lastly, due to the low number of cells recovered from the endothelial cell biopsies (~400-800/biopsy), the biochemical assays available to analyze these samples is restricted to immunofluorescence. Using this technique, I was limited to measuring the expression of select proteins and, consequently, was not able to fully characterize the complex process of inflammation, which involves numerous pro- and anti-inflammatory mediators. However, as discussed above, MCP-1 is a key contributor to arterial inflammation with age (33). Furthermore, MCP-1 expression is regulated by the pro-inflammatory transcription factor, nuclear factor kappa b (NFκB) and, as such, indirectly reflects increased activation of this pivotal inflammatory pathway (12, 78). Because NFκB regulates the expression of over 150 genes (78), many of which augment inflammatory signaling, it is likely that the decrease in arterial endothelial cell MCP-1 expression reflects a more extensive change in endothelial inflammation.

Conclusions. Oral trehalose supplementation for 12 weeks decreases a marker of arterial endothelial cell inflammation and improves NO-mediated EDD and smooth muscle sensitivity to NO in resistance arteries of MA/O adults who remain weight-stable. These findings provide novel evidence that trehalose may be an effective intervention for the primary prevention of CVD by reversing resistance artery endothelial dysfunction in healthy MA/O adults. However, the caloric content associated with trehalose supplementation poses a challenge for the generalizability of this intervention.
Twelve weeks of oral trehalose supplementation does not improve large elastic artery stiffness or wave reflection in healthy middle-aged and older adults

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Abstract

Large elastic arteries become stiffer with age and this is a key antecedent to the development of cardiovascular diseases (CVD). Recently, the disaccharide trehalose has been reported to be an effective therapy for reducing arterial stiffness in old mice, but the effects in humans are unknown. I tested the hypothesis that trehalose supplementation in middle-aged and older (MA/O) adults would improve regional and local arterial stiffness, as determined by aortic pulse wave velocity (aPWV) and carotid β-stiffness index, respectively, and increase carotid artery compliance (CC). I further hypothesized that decreased arterial stiffness would be associated with a reduction in wave reflection, a hemodynamic consequence of arterial stiffening with age, as measured by carotid augmentation index (AIx). Thirty-one healthy men and postmenopausal women 50-77 years consumed trehalose or maltose (100g/day) for 12 weeks in in a randomized, double blind, parallel group study. In contrast to my hypotheses, aPWV, β-stiffness index, CC and AIx did not change differentially across the intervention period in the trehalose and maltose groups (all P>0.1). These findings suggest that 100g/day of trehalose for 12 weeks is not an effective therapy for reducing large elastic artery stiffness in healthy MA/O adults.
Introduction

Large elastic arteries become stiffer with age in the absence of disease (113, 166). The resulting physiological sequelae from this phenotypic change include augmented systolic blood pressure and pulse pressure, microvascular remodeling, decreased coronary perfusion, and end organ ischemic tissue damage (110, 135). Consistent with these observations, large elastic artery stiffness is an independent predictor of incident CVD in healthy middle-aged and older (MA/O) adults (111, 153). As such, the identification of therapies that reduce age-associated arterial stiffness is an important biomedical priority.

The mechanisms by which large elastic arteries stiffen with age involve both structural, and functional changes (118). Structural changes are characterized by increased expression of the load-bearing protein collagen and its cross-linking by advanced glycation end products, as well as the fragmentation and degradation of the elasticity-conferring protein, elastin (51, 62, 144). Functional changes include increased smooth muscle tone, which, in turn, is influenced by endothelial release of vasodilator and vasoconstrictor factors (51, 92, 144). Specifically, the key endothelium-derived vasodilator, nitric oxide (NO) has been shown to directly modulate local arterial stiffness (140, 185). In addition, endothelium-dependent dilation (EDD), a functional bioassay of nitric oxide bioavailability, is inversely associated with large elastic artery stiffness in healthy middle-aged adults (108).

The autophagy promoting disaccharide, trehalose, has emerged as a novel anti-aging therapy in invertebrates and mice (39, 42, 67, 121). Moreover, oral supplementation with trehalose reverses arterial stiffness in old mice in part through a reduction in arterial collagen expression (83). In a separate study, LaRocca et al. demonstrated that four weeks of oral trehalose restores NO bioavailability and EDD to levels observed in young animals (84). Together, these findings suggest that trehalose may be a novel therapy for reducing large elastic artery stiffness in MA/O humans by modulating both the structural and functional determinants of arterial stiffness with age.
Accordingly, I tested the hypothesis that oral trehalose supplementation would improve large elastic artery stiffness in healthy MA/O adults free from CVD. To do this, 23 men and postmenopausal women were randomized to consume 100g/day of trehalose of maltose for 12 weeks. This dose was chosen in order to replicate the dose used in the previous mouse studies assessing the effects of trehalose on vascular aging on a g/kg body mass/day basis. Before and after the intervention period, regional arterial stiffness along the entirety of the aorta was determined by the gold standard method of aortic pulse wave velocity (aPWV) (85, 86). Local stiffness of the carotid artery was determined ~2 cm proximal to the carotid bulb using two separate indices: carotid compliance (CC), a measure of arterial distention relative to pressure that provides an indirect measure of vascular stiffness (higher compliance being associated with lower stiffness), and β-stiffness index, a less blood pressure dependent estimate of local stiffness corrected for distending pressure (19). I also assessed wave reflection by carotid augmentation index (AIx) to characterize stiffness-associated hemodynamic changes (92).

Methods

Subjects. Twenty-three men and postmenopausal women aged 50-77 years were studied. All subjects were non-smoking adults free from clinical disease as assessed by medical history, physical examination, blood chemistries, electrocardiogram, resting blood pressure, and cardiovascular responses to a graded exercise test. Subjects refrained from all cardiovascular acting medications for 24 hours prior to testing and all other medications for 12 hours. All procedures were approved by the Institutional Review Board at the University of Colorado at Boulder. The nature, risks and benefits of all study procedures were explained to volunteers and their written informed consent was obtained before participation in the study.

Procedures. All testing was performed at the Clinical Translational Research Center (CTRC) at the University of Colorado at Boulder following a 12-hour fast from food and caffeine
and 24-hour abstention from exercise and alcohol. All women were post-menopausal as confirmed by absence of menstruation for >1 year and follicular stimulating hormone levels>40 IU/L (22).

**Trehalose administration.** Subjects were randomized to consume 100g/day of trehalose or maltose for 12 weeks in a double blind fashion. Food grade trehalose and maltose were purchased from Hayashibara (Okayama, Japan). Maltose was chosen as a control condition because 1) like trehalose, maltose is a disaccharide of glucose, 2) maltose does not have the same beneficial physiological effects as trehalose when administered at similar concentrations (39, 67, 139, 149, 158) and 3) maltose provides an isocaloric control condition. To replicate as faithfully as possible the previous preclinical studies demonstrating improved arterial function with trehalose in old mice, trehalose and maltose were administered orally dissolved in 12 ounces of water. Subjects were provided with individual containers for each day containing either 100g of trehalose or maltose. Subjects were also given a graduated water bottle and were instructed to mix one container of sugar with 12 ounces of water each day. Subjects were allowed to consume the intervention drinks at their own pace over the course of the day. Adherence was documented by having subjects return empty and unused containers every two weeks during the 12-week intervention period. Every two weeks subjects also received in-person nutrition counseling by a dietitian at the Boulder CTRC to promote stability of diet and body weight throughout the intervention period.

**Subject characteristics and circulating factors.** Arterial blood pressure (BP) was measured in triplicate over the brachial artery during supine rest (Noninvasive Hemodynamics Workstation, Cardiovascular Engineering Inc.) at baseline and after the 12-week intervention. Waist and hip circumferences were measured by anthropometry, and percent body fat was measured by dual-energy X-ray absorptiometry (DEXA, DXA-GE Lunar; software version 5.60.003) at these same time points. Aerobic fitness was assessed at baseline and after the intervention by indirect calorimetry during incremental treadmill exercise (Balke protocol) (28,
Total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, and fasting glucose were measured using standard assays at the University of Colorado CTRC Core Laboratory at baseline and after 12 weeks of trehalose or maltose supplementation. All blood samples were drawn from an intravenous catheter placed in the left antecubital fossa.

**Regional Arterial stiffness.** Regional arterial stiffness of the aorta was determined by aPWV using transcutaneous applanation tonometry of the carotid and femoral arteries with simultaneous ECG recording (Noninvasive Hemodynamics Workstation, Cardiovascular Engineering Inc.) as described previously (111, 113). Briefly, the time delay (transit time) between the foot of the carotid and femoral pressure waves was determined using the R-wave of the ECG recording as a timing reference. aPWV was calculated as the distance between measurement sites divided by transit time of the arterial pulse wave.

**Local arterial stiffness.** CC and β-stiffness index were determined by high-resolution ultrasonography (Xario, Toshiba; multi-frequency linear-array transducer) and subsequent applanation tonometry of the right common carotid artery ~2 cm proximal to the carotid bulb (Noninvasive Hemodynamics Workstation, Cardiovascular Engineering Inc.). CC was calculated as described previously (157, 170) using the equation:

\[
CC = \pi DD^2 \left[ \frac{\Delta D/DD}{2*PP} \right]
\]

where DD = diastolic diameter; ΔD = the change in diameter across the cardiac cycle; and PP = carotid artery pulse pressure. β-stiffness index was calculated as described previously (66, 157) using the equation:

\[
\beta = \frac{\ln(SBP/DBP)}{\Delta D*DD}
\]

where SBP = systolic blood pressure and DBP = diastolic blood pressure.
Wave reflection. Alx was measured using transcutaneous applanation tonometry of the right common carotid artery (Noninvasive Hemodynamics Workstation, Cardiovascular Engineering Inc.). Alx was calculated as the ratio of the forward wave amplitude relative to total pulse pressure as described previously (18, 113, 145) and as illustrated below (schematic modified from (86)):

Data analysis. Statistical analyses were performed in SPSS (IBM SPSS Statistics 22). Group differences at baseline were assessed using independent Student’s t tests for between-group contrasts. Repeated measures ANOVA was used to determine group (maltose vs. trehalose) by time interactions for all clinical characteristics and primary outcome measures. In the case of a significant interaction or a significant effect of time, a Student’s t-test for within-group contrast was performed with Bonferroni correction. Statistical significance was set at P< 0.05.

Results.

Subject characteristics. There were no group differences in age, gender, body mass, percent body fat, waist to hip ratio, systolic BP, diastolic BP, pulse pressure, heart rate, maximal oxygen consumption, fasting glucose or total, HDL or LDL cholesterol between maltose and
trehalose groups at baseline (all P>0.05). There was a significant effect of time on body mass (F[1,29]=12.14, P<0.01) and this response did not differ between groups (P>0.1). Body mass was significantly elevated at week 12 in both the maltose (72.2±3.3 vs. 71.3±3.3 kg, P<0.05) and trehalose (73.6±4.5 vs. 72.6±4.4 kg, p<0.05) groups. Importantly, there was no change in percent body fat across the intervention despite the observed increase in mass (P>0.1). Moreover, no subject characteristics changed differentially between groups with time (all P>0.05, Table 1).

Table 1: Subject Characteristics

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<th>Trehalose Baseline</th>
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Data are mean ± SE; * P<0.05 vs. baseline of same group. BP blood pressure; VO2 max maximal oxygen consumption, HDL high-density lipoprotein, LDL low-density lipoprotein.

**Regional Arterial Stiffness.** Regional arterial stiffness as assessed by aPWV was not different between maltose and trehalose groups at baseline (P>0.05). There was no effect of
time on aPWV (P>0.1) and aPWV did not change differentially across the intervention period in the trehalose and maltose groups (F[1,27]=0.31, P>0.1, Figure 1).

**Local Arterial Stiffness.** There were no baseline group differences in either CC or β-stiffness index (both P>0.1). CC changed significantly across the intervention period (F[1,26]=9.21, P<0.01) and this response did not differ between groups (CC: F[1,26]=0.78, P>0.1). However, the change in CC with time was driven primarily by a decline in CC with maltose supplementation (0.078±0.006 vs. 0.088±0.009 mm²/mmHg x 10⁻¹, P<0.05) as there was not a significant within-group change in CC with trehalose (P>0.05). This decline in CC with maltose treatment was likely due to the trend for increased PP observed in this group (Table 1), as no differences in β-stiffness index, a less blood pressure dependent measure of arterial stiffness, was observed with either trehalose or maltose supplementation (both P>0.1, Figure 2).

**Wave reflection.** Wave reflection as determined by carotid augmentation index did not differ between maltose and trehalose groups at baseline (P>0.05). AIx did not change across the intervention period (F[1,29]=0.13, P>0.1) and this response was not different between groups (F[1,29]=0.00, P>0.1, Figure 3).
Figure 1. Aortic pulse wave velocity (aPWV) at baseline (base) and following 12 weeks of maltose and trehalose supplementation. Values are mean ± SE.

Figure 2. Carotid compliance (left) and β-stiffness index (right) at baseline (base) and following 12 weeks of maltose and trehalose supplementation. Values are mean ± SE. *P<0.05 vs. baseline.
Discussion

Trehalose is a naturally occurring disaccharide that protects against vascular aging, including arterial stiffening, in mice. This study is the first to assess the efficacy of trehalose to reverse large elastic artery stiffening in healthy MA/O adults. In the present study, I used the gold-standard measure of aortic pulse wave velocity to characterize regional stiffness of the aorta, as well as $\beta$-stiffness index and carotid compliance to determine local stiffness in the common carotid artery, a highly disease-susceptible section of the arterial tree. In addition, wave reflection, a key hemodynamic consequence of arterial stiffening that contributes to elevated systolic blood pressure with age, was also assessed. 12 weeks of oral trehalose supplementation (100g/day) was not effective in decreasing regional or local arterial stiffness and had no effect on wave reflection in the present cohort. As such, my findings suggest that
12 weeks of oral trehalose treatment (100g/day) is not an effective therapy for reducing arterial stiffness in healthy MA/O adults.

_Trehalose and arterial stiffness._ Trehalose is a naturally occurring disaccharide of glucose with a 1,1 glycosidic linkage. Importantly, due to its 1,1 glycosidic bond, trehalose is not a reducing sugar, meaning that it does not promote the formation of advanced glycation end products in vivo, a known contributor to arterial stiffening with age (41, 116, 131). Moreover, trehalose protects against age-related diseases in mammals, suggesting this disaccharide may be a novel anti-aging therapy. In animal models, trehalose decreases protein aggregates and improves function in mouse models of Parkinson’s disease (132, 138, 186), protects against subarachnoid hemorrhage (39) and metabolic stress (5, 93), and inhibits bone loss during menopause (121).

In accordance with mounting evidence for an anti-aging effect of trehalose, it has recently been demonstrated that trehalose reverses age-associated vascular dysfunction in mice. Indeed, four weeks of oral trehalose supplementation (2% in drinking water) reduced aPWV in old mice to levels observed in young. This was associated with reductions in collagen expression, suggesting that oral trehalose treatment promotes beneficial structural changes within the arterial wall in mice (83). In a separate study, oral trehalose supplementation restored endothelial function in old mice by increasing nitric oxide bioavailability (84), a key regulator of the functional component of arterial stiffness with aging (140, 185). Together, these preclinical studies provide strong support for the efficacy of trehalose to reverse arterial stiffness with age in humans.

Despite promising preclinical evidence, trehalose had no effect on measures of regional and local arterial stiffness or wave reflection in healthy MA/O adults in the current investigation. Recently, this same lack of translation has been observed with two additional nutraceuticals, curcumin and sodium nitrite, which were effective for reducing arterial stiffness in old mice but had no effect in healthy MA/O humans (unpublished data from the Integrative Physiology of
\textit{Aging Laboratory}). These findings are in agreement with a growing body of literature demonstrating an inability of numerous nutrient-based interventions to reverse arterial stiffness in MA/O adults, despite promising results in preclinical models and/or in MA/O adults with cardiometabolic diseases. Such interventions include vitamin D (59), oral antioxidants (44, 124, 129, 197), polyunsaturated fatty acids, folic acid, α-lipoic acid, soy, garlic (124) and a combination of resveratrol, tea extract, pomegranate extract, quercetin, acetyl-l-carnitine, lipoic acid, curcumin, sesamin, cinnamon bark extract, and fish oil (150). While there are some conflicting reports supporting the efficacy of nutrient-based supplements for the reduction of arterial stiffness in healthy MA/O adults, these investigations have either a) only assessed indirect measures of arterial stiffness that are more dependent on smooth muscle cell tone than arterial remodeling (3, 117, 123) or b) incorporated interventions lasting multiple years (76). One exception to this is an investigation showing improved aPWV with isoflavone supplementation (161). As such, the majority of current literature supports the conclusion that short term, nutrient based interventions have, at best, a minimal therapeutic effect on age-associated large elastic artery stiffness in humans.

In contrast to nutrient-based strategies, a number of lifestyle interventions, including aerobic exercise (141), sodium restriction (58, 70), and weight loss (27) are effective in reversing age-associated arterial stiffening in healthy MA/O adults. These findings underscore the importance of lifestyle-based strategies in the primary prevention of vascular aging and CVD.

\textit{Limitations.} It is possible that the lack of efficacy observed in the present study could be due to trehalose not being administered long enough to induce arterial remodeling and subsequently improve arterial stiffness. Indeed, in a previous preclinical study showing reduced arterial stiffness in old mice with trehalose treatment, mice were supplemented for four weeks (83), which, when considering the differences in the lifespan between mice and humans, is ~10 times longer than a 12 week intervention in humans when normalized to average lifespan (16,
As such, it is possible that a longer treatment duration is needed to see improvements in arterial stiffness in humans. However, I chose a 12 week intervention for the present investigation because 12 weeks is a sufficient amount of time to observe changes in arterial stiffness with lifestyle interventions (27, 58, 70, 141). Furthermore, intervention periods longer than 12 weeks increase attrition and introduce a number of additional confounds including changes in lifestyle patterns (diet and exercise) and health status (new diagnoses, changes in medications, etc.).

The dose of trehalose chosen for this clinical study is a body weight equivalent dose to that which improved arterial function in old mice (83, 84). In contrast to findings in mice, there was a small (~1 kg) increase in body weight in MA/O adults. Because of the relatively small magnitude of this change, the healthy non-obese cohort studied in the present investigation, and the absence of any trend toward reduced arterial stiffness, I do not think that the increase in body mass explains the lack of efficacy of the trehalose intervention.

It is possible that 100 g/day of oral trehalose did not result in high enough bioavailability of this agent to induce functional changes and/or structural remodeling of the arterial wall. However, due to the caloric content associated with trehalose, administering an oral dose >100g/day for 12 weeks is not feasible as it is unlikely that MA/O adults would be able to remain weight-stable without modifying physical activity levels or undergoing drastic dietary changes.

**Conclusions.** Oral trehalose supplementation for 12 weeks (100g/day) is not an effective therapy for reducing large elastic artery stiffness or wave reflection in MA/O adults free of clinical disease. Due to substantial preclinical evidence supporting the therapeutic efficacy of trehalose for the reversal of arterial aging, future investigations using longer intervention durations may be warranted.
CHAPTER VI

Conclusions

The goal of this dissertation was to determine the efficacy of oral trehalose therapy to reverse age-associated vascular dysfunction in humans. Specifically, I tested the hypothesis that oral supplementation with the disaccharide trehalose would improve resistance and/or conduit artery endothelium-dependent dilation (EDD) in middle-aged and older (MA/O) adults, and that improvements would be related to increased nitric oxide (NO) bioavailability and reduced inflammation and oxidative stress. A secondary hypothesis was that 12 weeks of oral trehalose supplementation would reduce large elastic artery stiffness in this same population.

Trehalose treatment improved resistance artery EDD by ~30% in a healthy MA/O cohort who were able to maintain body mass within 2.3 kg, and this was mediated by an increase in NO signaling and associated with reduced arterial endothelial cell expression of the pro-inflammatory chemokine, MCP-1. In contrast, there was no improvement in conduit artery EDD. There was also no change in markers of oxidative stress, suggesting this was not a mechanism of improved resistance artery EDD with trehalose treatment in the weight-stable subgroup. Together, these findings indicate that trehalose has dissimilar effects on different types of arteries and may be a novel therapy for reducing vascular inflammation and increasing resistance but not conduit artery NO-mediated EDD in individuals able to remain weight-stable.

In contrast to previous findings in mice, oral trehalose supplementation did not reduce large elastic artery stiffness in healthy MA/O adults. This finding demonstrates that 100g/day of oral trehalose for 12 weeks is not an effective therapy for reversing large elastic artery stiffness with aging in humans.
Together, the findings from this dissertation demonstrate that oral trehalose supplementation has heterogeneous effects on different aspects of arterial aging. Trehalose may be a novel therapy for the primary prevention of cardiovascular diseases (CVD) secondary to improving resistance artery endothelial function, an independent predictor of incident CVD. However, the caloric content associated with trehalose supplementation poses a challenge for the generalizability of this intervention.


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