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The autophagy enhancer spermidine reverses arterial aging

Thomas J. LaRocca, Rachel A. Gioscia-Ryan, Christopher M. Hearon Jr., Douglas R. Seals *

Department of Integrative Physiology, University of Colorado, Boulder, CO 80309, USA

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ABSTRACT

Arterial aging, characterized by stiffening of large elastic arteries and the development of arterial endothelial dysfunction, increases cardiovascular disease (CVD) risk. We tested the hypothesis that spermidine, a nutrient associated with the anti-aging process autophagy, would improve arterial aging. Aortic pulse wave velocity (aPWV), a measure of arterial stiffness, was ~20% greater in old (O, 28 months) compared with young C57BL6 mice (Y, 4 months, $P < 0.05$). Arterial endothelium-dependent dilation (EDD), a measure of endothelial function, was ~25% lower in O ($P < 0.05$ vs. Y) due to reduced nitric oxide (NO) bioavailability. These impairments were associated with greater arterial oxidative stress (nitrotyrosine), superoxide production, and protein cross-linking (advanced glycation end-products, AGEs) in O (all $P < 0.05$). Spermidine supplementation normalized aPWV, restored NO-mediated EDD and reduced nitrotyrosine, superoxide, AGEs and collagen in O. These effects of spermidine were associated with enhanced arterial expression of autophagy markers, and in vitro experiments demonstrated that vascular protection by spermidine was autophagy-dependent. Our results indicate that spermidine exerts a potent anti-aging influence on arteries by increasing NO bioavailability, reducing oxidative stress, modifying structural factors and enhancing autophagy. Spermidine may be a promising nutraceutical treatment for arterial aging and prevention of age-associated CVD.

1. Introduction

Aging causes two key changes in arteries that significantly increase the risk of cardiovascular diseases (CVD): stiffening of the large elastic arteries (aorta and carotid arteries) and the development of vascular endothelial dysfunction (Lakatta and Levy, 2003; North and Sinclair, 2012). Arterial stiffening results from age-related changes in the arterial wall including increases in collagen deposition, reductions in elastin and cross-linking of these and other structural proteins via formation of advanced glycation end-products (AGEs) (O'Rourke and Hashimoto, 2007). Vascular endothelial dysfunction develops with age primarily due to reduced nitric oxide (NO) bioavailability, as reflected by impaired NO-mediated endothelium-dependent dilation (EDD) (Brandes et al., 2005; Lakatta, 2003).

Although the mechanisms underlying arterial aging are incompletely understood, the characteristics of age-associated vascular dysfunction are consistent with dysregulated cellular protein homeostasis, i.e., oxidative stress and increased molecular damage that ultimately impair cell and tissue function (Koga et al., 2010; Lakatta, 2003; Seals et al., 2011). Autophagy, the cellular process of recycling damaged biomolecules, is a major mechanism for protein homeostasis and defense against oxidative stress (Koga et al., 2010; Mizushima and Komatsu, 2011) and may, therefore, play an important role in arterial aging. Indeed, numerous longevity pathways exert their effects through autophagy (Rubinsztein et al., 2011), and recent work from our laboratory suggests that impaired autophagy contributes to arterial aging (LaRocca et al., 2012). Thus, therapeutic strategies aimed at improving protein quality control by enhancing autophagy may have the potential to prevent/reverse age-associated arterial dysfunction and CVD.

Because many known autophagy inducers have off-target effects (e.g., rapamycin) or uncertain translational promise (e.g., caloric restriction), there is considerable interest in natural food components or "nutraceuticals" that promote autophagy (Galluzzi and Kroemer, 2012; Sudarsanam and Johnson, 2010). Recently, the

Abbreviations: ACh, acetylcholine; AGEs, advanced glycation end-products; Atg3, autophagy-related protein 3; CVD, cardiovascular disease; EDD, endothelium-dependent dilation; EPR, electron paramagnetic resonance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L-NAME, *N*-G-nitro-L-arginine methyl ester; LC3-II, lipid-modified microtuble-associated protein light chain 3; NO, nitric oxide; SNP, sodium nitroprusside.

* Corresponding author at: Department of Integrative Physiology, University of Colorado, 354 UCB, Boulder, CO 80309, USA. Tel.: +1 303 492 5305; fax: +1 303 492 6778.
E-mail addresses: seals@colorado.edu, thomas.larocca@colorado.edu (D.R. Seals).

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polyamine spermidine has been identified as a potent and specific inducer of autophagy (Eisenberg et al., 2009; Madeo et al., 2010). Spermidine is a natural dietary compound found in high concentrations in Mediterranean and Asian diets (Binh, 2010; Soda et al., 2010). Supplementation with spermidine extends lifespan in yeast and flies by an autophagy-dependent mechanism and reduces oxidative stress (Eisenberg et al., 2009; Guo et al., 2011; Minois et al., 2012). The mechanism of action for spermidine involves enhanced transcription of autophagy-relevant proteins and is contingent on de-acetylation of histone H3 (Eisenberg et al., 2009; Morselli et al., 2011). However, the potential for spermidine to promote autophagy and exert anti-aging effects in arteries is entirely unknown.

Here, we tested the hypothesis that supplementation with spermidine would reduce arterial stiffness and improve vascular endothelial function in old mice. The results of these experiments provide the first evidence that spermidine may hold efficacy for treating age-associated arterial dysfunction by enhancing autophagy, reducing oxidative stress and increasing NO bioavailability.

2. Materials and methods

2.1. Animals

Young (4–6 months) and old (27–29 months; ~50% survival rate) male C57BL6 mice, an established model of aging and vascular dysfunction (Sindler et al., 2011; Sprott and Ramirez, 1997), were obtained from the National Institute on Aging rodent colony. Control animals received regular drinking water, whereas treated animals received water supplemented with 3 mM spermidine (Sigma–Aldrich, St. Louis, MO, USA), a previously reported dose (Eisenberg et al., 2009), for a period of 4 weeks. Spermidine supplemented water was replaced every 3 days, prepared from a 1 M aqueous stock solution (spermidine/HCl, pH 7.4) stored at −20 °C. Mice were housed in an animal care facility at the University of Colorado at Boulder on a 12:12 h light–dark cycle. All procedures conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 2011) and were approved by the University of Colorado at Boulder Animal Care and Use Committee.

2.2. Aortic pulse wave velocity (arterial stiffness)

Aortic pulse wave velocity was measured as previously described (Fleenor et al., 2012; Reddy et al., 2003). In brief: mice were anesthetized with 2% isoflurane and positioned supine on a heating board (37 °C) with limbs secured to ECG electrodes. Doppler probes were used to detect flow velocity signals at the transverse aortic arch and the abdominal aorta while simultaneously recording ECG (MouseDoppler acquisition system, Indus Instruments, Wester, TX, USA). Time elapsed between the ECG R-wave and the foot of the Doppler signal was determined for each site (Fig. 1), and pulse wave velocity was calculated as the distance between the two probes divided by the difference in time elapsed at each site.

2.3. Vascular endothelial function

EDD and endothelium-independent dilation were determined ex vivo in isolated carotid arteries as previously described (LaRocca et al., 2012; Rippe et al., 2010). Mice were anesthetized with isoflurane and sacrificed by exsanguination via cardiac puncture. Carotid arteries were dissected free of surrounding tissue, cleaned and cannulated onto glass micropipettes in myograph chambers (DMT, Aarhus, Denmark). Arteries were pressurized to 50 mmHg at 37 °C in physiological saline solution and allowed to equilibrate for 1 h. After preconstriction with phenylephrine (2 μM), NO-mediated EDD was determined by measuring increases in luminal diameter in response to acetylcholine (ACh, $1 \times 10^{-9} - 1 \times 10^{-4}$ M, Sigma–Aldrich) in the presence or absence of *N*-G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM, 30 min incubation to block NO production, Sigma–Aldrich) or the superoxide dismutase mimetic TEMPOL (1 mM, 60 min incubation to scavenge superoxide, Sigma–Aldrich). Endothelium-independent dilation was determined as dilation in response to the exogenous NO donor sodium nitroprusside (SNP, 1×10^{-10} to 1×10^{-4} M, Sigma–Aldrich), and is used as a measure of vascular smooth muscle sensitivity to NO. Dose–response data are presented on a percentage basis to account for differences in carotid artery diameter between young and old animals. NO-dependent dilation was determined from maximal EDD with or without L-NAME as: NO-dependent dilation (%) = max $\text{dilation}_{\text{ACh}}$ − max $\text{dilation}_{\text{ACh+L-NAME}}$.

2.4. Arterial superoxide production

Superoxide production was assessed by electron paramagnetic resonance (EPR) spectroscopy as previously described (Fleenor et al., 2012; LaRocca et al., 2012). Freshly dissected and cleaned 2 mm aortic segments were incubated for 60 min at 37 °C in Krebs–HEPES buffer with the superoxide-specific spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (0.5 mM; Enzo Life Sciences, Farmingdale, NY, USA). EPR signal amplitude was analyzed immediately on an MS300 X-band EPR spectrometer (Magnettech, Berlin, Germany) with the following settings: centerfield, 3350 G; sweep, 80 G; microwave modulation, 3000 mG; microwave attenuation, 7 dB. Data are expressed relative to the mean of the young control group.

2.5. Arterial protein expression

Measurements of protein expression were performed on cleaned mouse aortas (a representative large elastic artery) to provide sufficient tissue for analysis. Thoracic aortas were excised, cleaned of surrounding tissue and analyzed by standard Western blotting techniques as previously described (LaRocca et al., 2012; Rippe et al., 2010). Briefly: whole aortas were homogenized in radio-immunoprecipitation assay lysis buffer with protease and phosphatase inhibitors. 10 μg protein was loaded onto 4–12% polyacrylamide gels, separated by electrophoresis and transferred to nitrocellulose membranes (Criterion System; Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4 °C with primary antibodies: collagen-I (1:1000 dilution; Abcam, Cambridge, MA, USA), AGEs (1:2000; Abcam), nitrotyrosine (1:500; Abcam), lipid-modified microtubule-associated protein light chain 3 (LC3-II, 1:2000; Cell Signaling, Danvers, MA, USA), p62 adaptor protein (1:2000; MBL International, Woburn, MA, USA), acetylated (Lys9) histone H3 (1:500; Cell Signaling), autophagy-related protein Atg3 (1:1000; Cell Signaling). Proteins were visualized on a digital acquisition system (ChemiDoc-It; UVP, Upland, CA, USA) using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and ECL chemiluminescent substrate (Pierce, Rockford, IL, USA). Protein expression is presented normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000; Cell Signaling), and data expressed as a ratio of the mean of the young control group.

2.6. In vitro tissue culture experiments

Aortas were excised from young mice and perivascular fat removed from the arteries. Equal length segments of thoracic aorta were incubated in DMEM (with antibiotics, Sigma–Aldrich) in a humidified incubator at 37 °C and 5% CO_2 with or

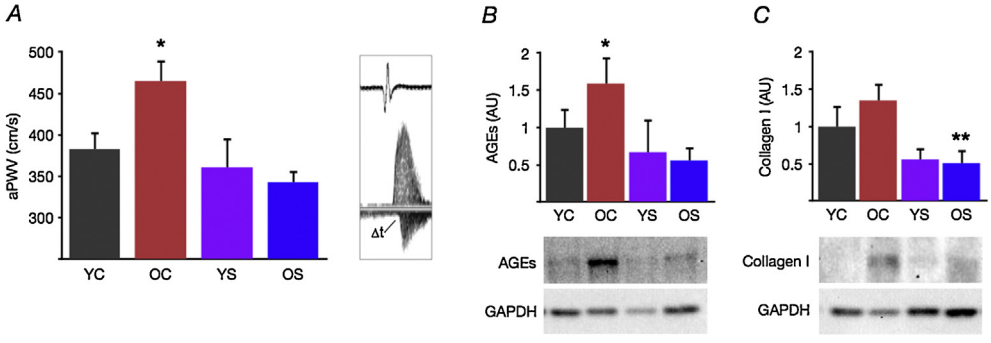

Fig. 1. Spermidine supplementation normalizes large elastic artery stiffness and structural proteins. (A) Aortic pulse wave velocity in young and old control (YC and OC) and young and old spermidine supplemented (YS and OS) mice. Representative ECG and Doppler flow signals at right, (B) advanced glycation end products (AGEs), and (C) protein expression of collagen I in whole aortas. Representative Western blot images below. Protein expression data expressed relative to GAPDH and normalized to YC mean value. Values are means ± SEM (n = 7–8 per group). *$P < 0.05$ vs. YC. **$P < 0.05$ vs. OC.