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Hesperidin reverses perivascular adipose-mediated aortic stiffness with aging

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Abstract

We tested the hypothesis that hesperidin would reverse age-related aortic stiffness, perivascular adipose (PVAT) mediated-arterial stiffening and PVAT advanced glycation end-products (AGE) accumulation. Aortic pulse wave velocity (aPWV) and intrinsic mechanical stiffness, two measures of arterial stiffness, were assessed in C57BL/6 mice that were young (6 months), old (27–29 months), or old treated with hesperidin for 4 weeks. Old compared with young mice had increased aPWV (444 ± 10 vs. 358 ± 8 cm/s, $P < 0.05$) and mechanical stiffness (6506 ± 369 vs. 3664 ± 414 kPa, $P < 0.05$). In old mice hesperidin reduced both aPWV (331 ± 38 cm/s) and mechanical stiffness (4445 ± 667 kPa) to levels not different from young. Aortic segments from old animals cultured with (+) PVAT had greater mechanical stiffness compared to young (+) PVAT (6454 ± 323 vs. 3575 ± 440 kPa, $P < 0.05$) that was ameliorated in arteries from old hesperidin treated cultured (+) PVAT (2639 ± 258 kPa). Hesperidin also reversed the aging-related PVAT AGE accumulation (all, $P < 0.05$). A 4-week treatment with the AGE inhibitor aminoguanidine reversed both the age-related increase in aPWV (390 ± 7 cm/s) and mechanical stiffness (3396 ± 1072 kPa), as well as mechanical stiffness in arteries cultured (+) PVAT (3292 ± 716 kPa) (all, $P < 0.05$) to values not different from young. In conclusion, hesperidin ameliorates the age-related increase in aortic stiffness and the PVAT-mediated effects on arterial stiffening. Hesperidin also reversed PVAT AGE accumulation, where PVAT AGE were shown to promote aortic stiffness with aging.

Keywords

Advanced glycation end-products; Artery; Fat; Age

1. Introduction

Aging is the major risk factor for cardiovascular disease (CVD) risk with nearly 70% of adults older than 60 years of age having at least one form of CVD (Writing Group et al., 2016). As such, aging has detrimental effects on arteries and arterial function leading to increased CVD risk (Lakatta and Levy, 2003). A key consequence of arterial aging is aortic stiffness, which is an independent predictor of cardiovascular events as assessed by aortic

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Conflict of interest

pulse wave velocity (aPWV) (Mitchell et al., 2010). Notably, aortic perivascular adipose tissue (PVAT) is associated with increased arterial stiffening and incident CVD (Britton et al., 2013a; Britton et al., 2013b), and has also been shown to promote aortic stiffness in animal models of aging and disease (Du et al., 2015; Fleenor et al., 2014). Age-related aortic stiffness is due, in part, to accumulation of advanced glycation end products (AGE). Importantly, AGE activates the nuclear factor-kappa B (NF- κ B) signaling and increases oxidative stress (Schmidt et al., 1994; Yan et al., 1994) that, in turn, contributes to arterial stiffness (Wells-Knecht et al., 1996). It is unknown, however, if age-related increases of AGE within PVAT contributes to the stiffening process.

Developing novel interventions to reduce aortic stiffness and thereby attenuating CVD risk are clinically relevant. Hesperidin is a flavonoid naturally occurring in many fruits and vegetables (Kakadiya et al., 2010; Wilmsen et al., 2005). Importantly, increased dietary hesperidin intake from fruits and vegetables is associated with reduced CVD events and CVD-related mortality (Cassidy et al., 2012), suggesting the potential of hesperidin to reduce aortic stiffness and CVD risk with aging. Notably, hesperidin has been shown to improve endothelial function, a key contributor to arterial stiffness, and reduce AGE accumulation in experimental models of disease (Rizza et al., 2011; Shi et al., 2012). Currently, however, it is unknown if hesperidin reverses age-related aortic stiffness and PVAT AGE accumulation, or if AGE within PVAT promotes aortic stiffening.

The purpose of this investigation was to determine the effects of hesperidin on age-related aortic stiffness and PVAT AGE accumulation, and to also assess the effects of AGE within PVAT on aortic stiffening. We, therefore, hypothesized age-related aortic stiffness mediated by PVAT and AGE accumulation within this fat depot would be reversed by a 4-week hesperidin intervention. In addition, we hypothesized the AGE inhibitor, aminoguanidine (AMG), would reverse age-related aortic stiffness mediated by PVAT.

2. Materials and methods

2.1. Animals

Young (6 months) and old (27–29 months) C57BL/6 mice were acquired from The Jackson Laboratory or the National Institute on Aging aged rodent colony at Charles River Laboratories, respectively. All mice were housed in a 12:12 h light:dark cycle animal facility on the University of Kentucky campus having ad libitum access to diet and water. Four groups of animals were studied: young control (N = 5), old control (N = 4), old hesperidin treated (N = 4) and old aminoguanidine (AGE inhibitor; AMG) treated (N = 3). Control mice were given a normal, low-fat (10%) rodent diet throughout the study. The low-fat diet was also enriched with hesperidin (0.5%; Research Diets), which was administered to old mice for four-weeks. The AGE inhibitor AMG was provided ad libitum in water (0.1%) for four-weeks. Following control or treatment conditions, all mice were euthanized by cardiac exsanguination while anesthetized with isoflurane. The University of Kentucky Animal Care and Use Committee approved all protocols prior to study.

2.2. Aortic pulse wave velocity (aPWV)

Aortic stiffness was assessed by aPWV as previously described (Du et al., 2015; Fleenor et al., 2014). In short, anesthetized (isoflurane) mice were placed supine on a heated electrocardiograph (ECG) board with the limbs secured. Non-invasive Doppler probes (Indus Instruments) were placed at the transverse aortic arch and abdominal aorta. The difference in time for pressure wave to reach the aortic arch and abdominal aorta velocities relative to the R-wave of the ECG was calculated. The distance between each Doppler probe was divided by the difference in the aortic pressure wave times to calculate aPWV, which is presented as centimeters/second (cm/s).

2.3. Intrinsic mechanical properties testing

Mechanical testing was performed as described previously (Du et al., 2015; Fleenor et al., 2012). Segments of the descending thoracic aorta were isolated and cleaned of the surrounding perivascular adipose. The vessels (~2 mm in length) were placed on two 200 μ m pins in a preheated (37 °C) myograph well (Danish Myo Technology, 620 M) containing calcium and magnesium-free PBS. The aortic segments were acclimated, which was followed by a pre-stretch protocol where the myograph pins were displaced ~1 mm for 3 min and returned to resting state for 30 s. This procedure was repeated twice. After pre-stretching, tension was relieved from the vessels and the myograph force recordings were set to zero. The vessels were then stretched to a tension of 1mN for 3 min. Each subsequent stretch lasted for 3 min and the force measurement was taken at the end of 3 min period until the yield point was reached (force decreased due to mechanical failure of artery). Stress-strain curves were derived for each sample and the elastic modulus, an index of mechanical stiffness, was calculated. One-dimensional stress (t) was calculated as: $t = \lambda L / 2HD$, and strain (λ) calculated as: $\lambda = d/d(i)$ with L = one-dimensional load applied, H = wall thickness, D = length of vessel, d = change in diameter, $d(i)$ = initial diameter. Histological sections were used to assess the wall thickness and diameter in frozen segments taken adjacent to the mechanically tested aortic segment. Images were acquired with a Nikon 80i microscope and analyzed with ImageJ (NIH). Arterial length was measured under a dissecting microscope using calipers. The slope of the stress-strain curve was used to determine the elastic modulus, which was defined as the greatest linear fit.

2.4. Perivascular adipose tissue (PVAT) culture studies

Additional descending thoracic aortic segments were isolated and cultured as described (Du et al., 2015; Fleenor et al., 2014). Arterial segments from all groups were cultured in DMEM containing antibiotics either in the presence or absence of the adjacent PVAT for 72 h at 37 °C and 5% CO₂ with daily changes of the culture media. All aortic segments were ~2 mm in length. Arterial segments cultured with PVAT had the equivalent length of fat (~2 mm) that was immediately adjacent to the artery. Following the 72-h culture, PVAT was removed from all segments and intrinsic mechanical properties testing was performed as described above. Our previous findings indicate aortic stiffness is not influenced by PVAT in young control mice (Du et al., 2015; Fleenor et al., 2014). We, therefore, did not acquire data on aortic segments from young mice without PVAT.

2.5. Western blot

PVAT protein expression of AGE was assessed in samples by western blotting (Du et al., 2015; Fleenor et al., 2012). Briefly, PVAT was isolated from the aorta, frozen in liquid nitrogen and stored at -80°C . Samples were homogenized using RIPA lysis buffer that contained protease and phosphatase inhibitors (Roche), and a phosphatase inhibitor cocktail (Sigma). Using a polyacrylamide gel (4–20%) equal amounts of protein (30 $\mu\text{g}/\text{lane}$) were loaded, separated by electrophoresis and transferred to a PVDF membrane (BIO-RAD). An antibody specific for AGE (1:1000, Abcam ab23722) was applied to the membranes for an overnight incubation at 4°C . Membranes were exposed using ECL. ImageJ (NIH) was used to determine the density of each band, which was normalized to each respective internal Beta-actin (1:8000, Santa Cruz, sc-47,778) control. The normalized values are expressed relative to the young control group.

2.6. Immunohistochemistry

Immunohistochemistry was performed following standard procedures (Du et al., 2015; Fleenor et al., 2014). Segments of aorta, 2 mm in length, without the surrounding adipose tissue, were frozen in optimal cutting temperature compound (Tissue-Tek) by placing the samples in liquid nitrogen. Aortas were stored in a -80°C freezer, sectioned (8 μm thickness) and fixed with acetone. Slides were stained with the Dako EnVision+ System-HRP-DAB kit (Agilent, USA) as directed by manufacturer's instructions. Primary antibodies for nitrotyrosine (1:100, abcam 61,392), nuclear factor kappa b (NF- κB , 1:100, abcam 19,870), were separately applied to individual slides and incubated at 4°C overnight. Secondary antibody, labeled polymer was applied for 30 min at room temperature, and diaminobenzidine (DAB) was used for 2 min to visualize the staining. Ethanol, 50% to 100%, was used to dehydrate slides, which were cleared with 100% xylene. Slides were subsequently coverslipped. Digital images using a 40 $\times$ objective were captured with a Nikon 80i microscope. Densitometry analysis was performed on the medial layer of 3 tissue cross sections for each sample. The mean density was determined from a pre-optimized red-green-blue color model. All measurements were processed in ImageJ software (NIH). The data are presented as relative density in arbitrary units (AU).

2.7. Statistics

Data are presented as mean $\pm$ S.E.M. GraphPad Prism (GraphPad Software, Inc.) was used to analyze all data, and a one-way analysis of variance (ANOVA) was utilized for all statistical comparisons with Bonferroni posthoc analyses. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of hesperidin on aortic stiffness

Arterial stiffness assessed by aPWV (Fig. 1A) ($P < 0.05$) and ex vivo mechanical properties testing (Fig. 1B) ($P < 0.05$) were both greater in old compared with young control animals. The four-week hesperidin and AMG treatment in old mice reversed both aPWV and intrinsic mechanical stiffness to young control values (Fig. 1A and B) ($P < 0.05$, both). Heart rate

acquired during aPWV was not different between groups (Young: 463 ± 19 ; Old: 451 ± 8 ; Old hesperidin: 441 ± 32 ; Old AMG: 485 ± 22 bpm; $P > 0.05$).

3.2. Influence of hesperidin on PVAT-mediated aortic stiffness and AGE accumulation

Mechanical stiffness was assessed in aortic segments from young cultured with (+) PVAT, as well as old, old hesperidin and old AMG treated mice, which were cultured in the presence (+) or absence (–) of PVAT. Compared with arterial segments from young (+) PVAT, old (+) PVAT had greater intrinsic mechanical stiffness (Fig. 1C) ($P < 0.05$), which was reversed in segments from old (–) PVAT (Fig. 1C) ($P < 0.05$). Compared with old (+) PVAT, old animals treated with hesperidin and AMG had reduced intrinsic mechanical stiffness in aortic segments cultured (+) or (–) PVAT (Fig. 1C) ($P < 0.05$, both). Hesperidin also ameliorated the age-related increase in PVAT AGE accumulation (Fig. 1D) ($P < 0.05$).

3.3. Influence of PVAT on aortic inflammation and oxidative stress

NF- κ B p65, a key pro-inflammatory transcription factor, and nitrotyrosine, a marker of oxidative stress were assessed in aortic segments from young, old, old hesperidin and old AMG treated mice, which were cultured in the presence (+) of PVAT, and old mice cultured without (–) PVAT. Segments from mice treated with hesperidin and AMG cultured without PVAT were not assessed as there were no arterial stiffness changes in these samples. Compared with young (+) PVAT, old aortic segments (+) PVAT had greater NF- κ B p65 (Fig. 2A) ($P < 0.05$) and nitrotyrosine (Fig. 2B) ($P < 0.05$) expression, which was reversed in old (–) PVAT aortic segment. Aortic segments from Old Hes (+) PVAT and AMG (+) PVAT had reductions in both NF- κ B p65 and nitrotyrosine compared to Old (+) PVAT (Fig. 2A and B) ($P < 0.05$).

4. Discussion

Elucidating novel therapeutic interventions and identifying potential mechanisms of action that decrease age-related aortic stiffness and, therefore, reduce CVD risk are clinically significant. As such, our findings provide initial evidence for hesperidin to reverse: 1) aortic stiffness associated with aging; 2) PVAT mediated arterial stiffness and AGE accumulation; and, 3) PVAT-mediated aortic inflammation and oxidative stress. Additionally, our data reveals a novel effect of PVAT AGE accumulation to promote aortic stiffness mediated by this fat depot. Thus, these findings provide important preclinical evidence for hesperidin to reduce age-related aortic stiffness and PVAT AGE accumulation, while elucidating a novel role of AGE in PVAT to promote arterial stiffness with aging.

Aortic stiffness, assessed by aPWV, is an independent predictor of CVD-related events and mortality (Mitchell et al., 2010). Our findings are in support of previous investigations demonstrating increased aPWV in preclinical models of aging (Fleenor et al., 2014; Fleenor et al., 2012; Stepan et al., 2012). We, however, extend these findings demonstrating an effect of hesperidin to reverse the age-related increase in aPWV. In addition to reducing aPWV, the age-related increase in intrinsic mechanical stiffness was also reversed with hesperidin. The greater mechanical stiffness with aging indicates unfavorable structural remodeling within the artery, which is also an important mediator of aortic stiffening (Du et

al., 2015; Fleenor et al., 2014). Hesperidin has also been shown to reduce blood pressure in healthy adults (Morand et al., 2011). However, blood pressure was not assessed in the current study, but could be another potential mechanism by which hesperidin de-stiffens arteries. Our preclinical findings, therefore, suggest hesperidin is a novel, translatable intervention that may be effective in reducing aortic stiffness and CVD risk in older adults.

Our previous findings have shown an effect of PVAT to promote aortic stiffness in animal models of aging and disease (Du et al., 2015; Fleenor et al., 2014). Herein, our data demonstrate for the first time an effect of hesperidin to reverse the PVAT-mediated aortic stiffness observed with aging. Importantly, hesperidin was also shown to reduce the aging-related PVAT AGE accumulation. This is the first investigation, to our knowledge, to demonstrate greater PVAT AGE accumulation, and to elucidate a novel target of hesperidin within PVAT to reduce aortic stiffness. In support of our finding, hesperidin has also been shown to reduce retinal AGE accumulation in an animal model of diabetes (Shi et al., 2012), which our findings now extend to adipose tissue, specifically PVAT.

The increased PVAT AGE accumulation may, in part, be due to greater oxidative stress within this adipose depot as we have previously demonstrated increased superoxide signaling within PVAT (Fleenor et al., 2014). Oxidative stress is an important contributor to AGE formation (Prasad et al., 2012), and the collective findings in PVAT are consistent, which suggests oxidative stress promotes AGE formation with advancing age. Notably, hesperidin reduces oxidative stress, in part, by increasing superoxide dismutase (SOD) expression (Kamel et al., 2014). Our prior experiments showed an effect of TEMPOL, a SOD mimetic, to reduce the PVAT-mediated aortic stiffness with aging (Fleenor et al., 2014). Although largely unknown, it is plausible that hesperidin reduces PVAT AGE accumulation by reducing oxidative stress via increases in antioxidant expression and/or activity.

An additional finding from this work demonstrates an effect of AGE inhibition to reverse age-related aortic stiffness, which has been shown previously and is supported by others (Steppan et al., 2012). The current findings, however, provide novel insight for PVAT AGE accumulation to promote arterial stiffening in the context of aging. AGE binds to the receptor of AGE (RAGE) that, in turn, promotes pro-inflammatory intracellular signaling cascades. Importantly, we have shown greater pro-inflammatory cytokine secretion from PVAT with aging (Fleenor et al., 2014) but the signaling mechanism(s) by which this occurs is unknown. The current data and prior findings suggest PVAT AGE accumulation promotes pro-inflammatory signaling and cytokine release that may contribute to age-related aortic stiffening, which is reversed by hesperidin. This is consistent with the literature as hesperidin-induced reductions in AGE are associated with decreases in pro-inflammatory cytokine secretion (Shi et al., 2012). Moreover, our data reveals that AGE inhibition in PVAT of old animals attenuates both pro-inflammatory NF- κ B expression and oxidative stress in the aorta that is associated with a concomitant reduction in arterial stiffness. Hesperidin reduces PVAT AGE accumulation in addition to reversing PVAT NF- κ B expression and oxidative stress. Thus, our findings suggest that hesperidin via reductions in PVAT AGE accumulation may reduce aortic inflammation and oxidative stress. Currently, our data are associative and additional studies are required to provide a causative relation.

5. Conclusion

In summary, our findings provide novel evidence for hesperidin to reverse age-related aortic stiffness, PVAT-mediated arterial stiffening and PVAT AGE accumulation. Moreover, greater AGE abundance in PVAT promotes aortic stiffness, which is reduced with hesperidin and is associated with de-stiffening effects of this novel citrus-derived compound. Thus, these preclinical data provide insight for hesperidin as a novel, potentially translatable intervention to de-stiffen the aorta and reduce CVD risk in older adults.

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Fig. 1.

Effects of hesperidin on age-related aortic stiffness, perivascular adipose-mediated arterial stiffness and PVAT advanced glycation end-products accumulation. Arterial stiffness assessed by (A) aortic pulse wave velocity (aPWV) and (B) intrinsic mechanical stiffness from young, old, old mice treated with hesperidin (Hes) and old mice treated with aminoguanidine (AMG). (C) Isolated aortic segments from young, old, old treated with Hes and old mice treated with Hes AMG were cultured for 72 h in the presence (+) or absence (-) of PVAT followed by mechanical testing. (D) Advanced glycation end-products (AGE) accumulation in PVAT from young, old and old mice treated with Hes. N = 3–5/group; Values are mean $\pm$ S.E.M. *P < 0.05 vs. all.

Fig. 2.

Inflammation and oxidative stress protein expressions. Protein expression of (A) NF- κ B p65 and (B) nitrotyrosine in aortic segments from young, old, old mice treated with hesperidin (Hes) and old mice treated with aminoguanidine (AMG) cultured in presence (+) of PVAT and old control mice in the absence (-) of PVAT. N = 3–5/group; Values are mean $\pm$ S.E.M. *P < 0.05 vs. all. Scale bars = 50μm.