The Effect of Pyridoxamine on Large Artery Stiffening and Brain Oxidative Stress and Inflammation with Age

by

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Dr. Ashley Walker

As people age, large elastic arteries become stiffer and cerebral arteries become dysfunctional. This research sought to determine whether treatment with pyridoxamine, a form of vitamin B6, prevents age-related arterial stiffening by limiting the increases in advanced glycation end products (AGEs), and thereby ameliorates the downstream consequences in cerebral arteries and cerebral cortex by limiting increases in oxidative stress and inflammation. 20 old mice were treated with pyridoxamine for 4 months and pulse wave velocity (PWV), a measure of aortic stiffness, was performed at baseline and every other month. Tissue samples from old control, young control, and old pyridoxamine treated mice were analyzed for gene expression of pro- and antioxidant enzymes and inflammatory cytokines. Aorta samples from each group were analyzed for AGEs by immunofluorescence and for thickness of the arterial wall by Verhoeff Van Gieson staining. Aortic PWV was greater in old control versus young control arteries (306 ± 12 cm/s vs. 273 ± 16 cm/s, $p=0.005$) however, aortic PWV in pyridoxamine-treated mice did not increase over time ($p>0.05$). Pyridoxamine did not change AGEs (0.05 ± 0.02 AU) versus old and young control arteries (0.01 ± 0.003 AU

vs 0.1 ± 0.05 AU, p=0.10). There was no change to arterial wall structure between groups. In the cerebral cortex, SOD1 trended toward elevation in old pyridoxamine arteries as compared to old controls. IL-1β was significantly increased in old pyridoxamine versus young control (1.0 ± 0.29 AU vs. 3.2 ± 0.60 AU, p=0.03), the same was true in the MCA (1.0 ± 0.29 AU vs. 3.2 ± 0.60 AU, p=0.03). These results suggest that pyridoxamine may reduce arterial stiffening. While previous studies have indicated a reduction in AGEs with pyridoxamine treatment, this was not evident in the present study. Pyridoxamine may increase SOD1 expression, while increases in IL-1β is likely a result of the aging process and not modified by pyridoxamine treatment. Further studies are needed to identify the mechanism by which pyridoxamine prevents age-related arterial stiffening.

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Introduction

Aging is inevitable. Both nationally and globally, populations are aging rapidly. A recent report by the U.S. Census Bureau states that by 2035, there will be more adults over the age of 65 than children under age 18 for the first time in American history (Bureau, 2018). However, as a result of the aging population there is an increase in diseases associated with age. Concurrent projections suggest that 132 million people will have at least one type of cardiovascular disease (CVD) by 2035, an increase from 102.7 million people in 2015 (Khavjou, Olga, Phelps, Diane, & Leib, 2016). Trends also indicate that the incidence of chronic kidney disease, another disease associated with advancing age, will increase over the same time frame (Stevens, Viswanathan, & Weiner, 2010). Although some evidence suggests that the prevalence of dementia and related cognitive disorders within age groups is decreasing, the rapid expansion of the elderly population will spark an associated rise in neurocognitive disorders (Y.T. Wu et al., 2017). Thus, it is expected that, barring any major medical breakthrough, the dramatic rise in older individuals with these conditions will outpace the expansion in the support network of caregivers and healthcare providers and cause a crisis in elder care (Seals, Justice, & Larocca, 2016; Warshaw & Bragg, 2014).

Globally, improvements in healthcare, sanitation, and medical knowledge have rapidly improved median human lifespan, but this has triggered an increase in the development of aging diseases (Thorin-Trescases et al., 2018). Increasingly, the scientific community has begun to place greater emphasis on understanding and preventing the underlying mechanisms of aging; it is therefore imperative to understand the fundamental mechanisms of aging. This project examined a pharmaceutical agent,

pyridoxamine, and sought to elucidate the mechanism by which it alters large artery stiffening and thus potentially alleviates the downstream consequences such as diminished cerebral artery function, increases in vascular inflammation and oxidative stress, and neuroinflammation.

What is Aging?

At the cellular level, aging results from the accumulation of damaged macromolecules within a cell (McDonald, 2014). Once organisms reach adulthood, the rate of damaged macromolecule buildup begins to surpass the rate of breakdown, altering normal cellular function (McDonald, 2014). While aging itself is not considered a disease, it is a major risk factor for a variety of diseases ranging from cognitive disorders and cancer to CVD (Seals et al., 2016).

Innovative technologies and medical advances have successfully increased median lifespan in humans; however, most are reactive rather than preventative and do not address the underlying biochemical pathways that cause aging. Proposed mechanisms of vascular aging include mitochondrial dysfunction, inflammation, epigenetics, damage to telomeres, oxidative stress, and cellular senescence (Seals et al., 2016). These mechanisms likely feedback cyclically on one another – for example, increases in oxidative stress lead to increases in inflammation, which in turn causes oxidative stress to increase (Seals et al., 2016).

Cardiovascular Aging

Changes to the cardiovascular system have long been identified as potential causative agents in poor aging. In fact, stiffening in the large elastic arteries is a powerful indicator of future CVD (Scuteri et al., 2007). Arterial stiffness also serves as a predictor of future diagnosis of dementia in patients with existing mild cognitive impairment (MCI) (Rouch et al., 2018). A study of 375 patients with MCI over six years found that increased pulse wave velocity (PWV), a measure of arterial stiffness, was the only factor associated with a greater risk of dementia after controlling for age and other confounding variables (Rouch et al., 2018). This outcome suggests that arterial stiffening with age plays a role in the development and worsening of dementia and neurocognitive disorders.

As arteries throughout the body stiffen, there is a chronic increase in the resistance the heart must work against to pump blood into the aorta and throughout the body (Seals, Jablonski, & Donato, 2011). Increased total peripheral resistance (TPR) causes extra stress on the heart and cardiovascular system combined with dietary and lifestyle factors can eventually lead to heart attack, stroke, and other cardiovascular dysfunction (Seals et al., 2011). Cardiovascular damage is also linked to the development of cognitive disorders associated with aging such as Alzheimer's disease and dementia (Seals et al., 2011).

Pulse pressure (the difference between systolic and diastolic blood pressure) widens with age, increasing the differential pressure blood vessels are subject to (Thorin-Trescases et al., 2018). Research suggests that stiffened arteries transmit the pulse wave at greater pressure further into the vasculature, causing the walls of smaller

and smaller vessels to oscillate more and thus causing more mechanical damage (Thorin-Trescases et al., 2018). Blood vessels in the brain are smaller and more fragile than large elastic arteries such as the aorta and thus are more susceptible to damage from increased pulsatility and blood pressure (Thorin-Trescases et al., 2018). The pulse is transmitted with greater force into the vasculature which may be a cause of endothelial dysfunction (Thorin-Trescases et al., 2018).

Molecular Mechanisms of Cardiovascular Aging

With age, a variety of biochemical changes lead to reduced arterial compliance, the ability of a vessel to expand in response to increased pressure. Smooth muscle cells migrate into the tunica intima, the innermost layer of an artery, affecting the ability of substances to diffuse into the arterial wall and reducing the vessel's ability to dilate and constrict (Bilder, 2016). Decreased bioavailability of nitric oxide (NO), increased superoxide concentration, and formation of advanced glycation end products (AGEs) that cross-link collagen fibers in tunica adventitia all act to inhibit a vessel's dilatory capacity (Seals et al., 2011). Over time, evidence suggests there is also increased presence of collagen proteins and decreased elastin and thus more collagen and fewer elastic fibers in the arteries, further contributing to increased arterial stiffness (Henson, Walker, Reihl, Donato, & Lesniewski, 2014).

Oxidative stress is the result of oxidation-reduction reactions that form byproducts such reactive oxygen species (ROS) (McDonald, 2014)**.** One common ROS is superoxide, which is primarily produced by NADPH oxidase (NOX) and by leaks in the electron transport chain within the mitochondria during cellular respiration (Harrison, 2012). ROS and other reactive species serve important functions in normal

cellular activity; for example, immune cells use superoxide radicals to kill bacteria or foreign substances while other cell types use them for signaling purposes (Harrison, 2012). However, with age research suggests that arterial NOX activity increases (Ungvari, Kaley, de Cabo, Sonntag, & Csiszar, 2010). A localized buildup of these byproducts causes intercellular damage by reacting with proteins and other molecules in the cell, altering their structure and thus, impairing their function (McDonald, 2014). While there are antioxidant enzymes specifically designed to breakdown ROS such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, the rate of superoxide production eventually surpasses the rate at which SODs are able to break them down (Bilder, 2016).

AGEs are another significant contributor to arterial stiffness. AGEs are formed via the oxidation of proteins that then covalently bond to a sugar and lysine or arginine group, thus altering the structure of the protein (Bilder, 2016). In collagen fibers, AGEs go on to form cross-links with each other making an already stiff protein (collagen) all the more inflexible and decreasing arterial malleability (Bilder, 2016). Collagen is an important structural component of arteries as it provides shape and support, however, increased cross-linkage can be damaging (McDonald, 2014). When the artery stiffens, it decreases vascular compliance and causes degeneration of the collagen scaffold, indirectly impacting the cell's ability to communicate with its external environment (Bilder, 2016). Because AGEs are resistant to breakdown, they impact collagen fiber function in tissues throughout the body such as the kidney and cornea in addition to arteries (Bilder, 2016).

NO is a local vasodilator and an antioxidant (Bilder, 2016). NO in the vasculature is produced by endothelial nitric oxide synthase (eNOS). With increased age, there is decreased bioavailability of NO (Bilder, 2016). This is the result of reactions with ROS that inhibit the activity of NO, thus reducing the amount available for the cell to use (Harrison, 2012). Furthermore, with age there is a decrease in eNOS expression which means less NO is produced overall (Ungvari et al., 2010). With age, eNOS can become uncoupled due to a reduction in the available cofactor and can therefore produce superoxide rather than NO (Yang, Huang, Kaley, & Sun, 2009). NO acts as an inhibitor of adhesion molecules which signal inflammatory processes to occur, thereby halting inflammatory signaling (Harrison, 2012). Reduction of bioavailability of NO therefore indirectly increases inflammation (Harrison, 2012). All of these mechanisms and factors compound to cause cellular damage and decrease the function of the cells, and thus tissues and organs leading to external makers of the aging process.

Preventative and Pharmaceutical Measures

Currently, the most effective preventative measures against aging are diet and exercise (Seals et al., 2016). In physically active individuals, body systems that typically decline with age such as respiratory fitness or cardiovascular health are significantly improved as compared to those individuals leading sedentary lifestyles (Seals et al., 2016). Surprisingly, those who become physically active later in life after periods of inactivity as young adults have similar aging profiles to those who have remained active throughout life, suggesting that beginning exercise later could still be a valuable preventative measure (Seals et al., 2016).

Chronic aerobic exercise is also shown to reduce inflammation and oxidative stress and therefore leads to maintained or improved bioavailability of NO (Seals et al., 2016). Superoxides and other (ROS) are byproducts of metabolism that react with and inhibit cellular components. It therefore follows that with chronic exercise, less superoxide is available to interact with NO, and thus more is available to regulate local vasodilatory responses (Seals et al., 2016).

Diet also plays a prominent role in preventing or delaying negative aging processes. Restricted calorie diets are shown to increase lifespan and decrease age-related damage to several body systems including the cardiovascular system (Seals et al., 2016). Specifically, caloric restriction has been shown to reduce endothelial dysfunction and arterial stenosis (Seals et al., 2016). However, caloric reduction and exercise are not necessarily viable options for all individuals. For example, among older adults, calorie-restricted diets could lead to malnutrition and existing disabilities or

motor impairments may inhibit the ability to exercise (Seals et al., 2016). Thus, it is important to identify other potential preventative measures.

Pyridoxamine and Vascular Aging

The goal of this research was to identify possible pharmaceutical interventions to prevent the damaging effects of arterial stiffening in large elastic arteries. Pyridoxamine is a naturally-occurring form of the vitamin B6. It is hypothesized to prevent the formation of AGEs which are implicated in arterial stiffening (Chang, Liang, Tsai, Wu, & Hsu, 2009).

Chang et al. induced diabetes in a set of rat models and then treated half for eight weeks with pyridoxamine (Chang et al., 2009). The pyridoxamine-treated rodents displayed decreased wave reflection in the aorta, a marker of arterial stiffness, suggesting that their arteries were less stiff than those of the control rats (Chang et al., 2009). Additionally, pyridoxamine treatment inhibited the hypertrophy of the left ventricle typically seen in diabetes (Chang et al., 2009). More significantly, collagen cross-linking as a result of AGEs was significantly decreased in the treated group (Chang et al., 2009). Chang et al. (2009) hypothesized that pyridoxamine inhibits AGEs in three ways: stopping the formation of AGEs by getting rid of toxic oxidative byproducts, removal of metal ions, and inhibition of the Amadori intermediate breakdown.

In a similar study, aged rats treated with pyridoxamine were compared to young counterparts (E. T. Wu, Liang, Wu, & Chang, 2011). Treated rats showed significantly fewer AGEs after 5 months (E. T. Wu et al., 2011). Furthermore, the body weight of aged rats decreased 7.2% over the course of treatment (E. T. Wu et al., 2011). The

increase in TPR typically seen in aged arteries was reduced significantly in pyridoxamine-treated rats (E. T. Wu et al., 2011). Wu et al. (2011)posited that by reducing the development of AGEs, more NO was available and thus the dilation of the arteries was preserved.

Preliminary data from our lab suggests that pyridoxamine partially prevents age-related reductions in endothelial function in the middle cerebral artery (MCA) (*Figure 1*). However, the mechanism by which pyridoxamine preserves dilatory capacity is unknown.

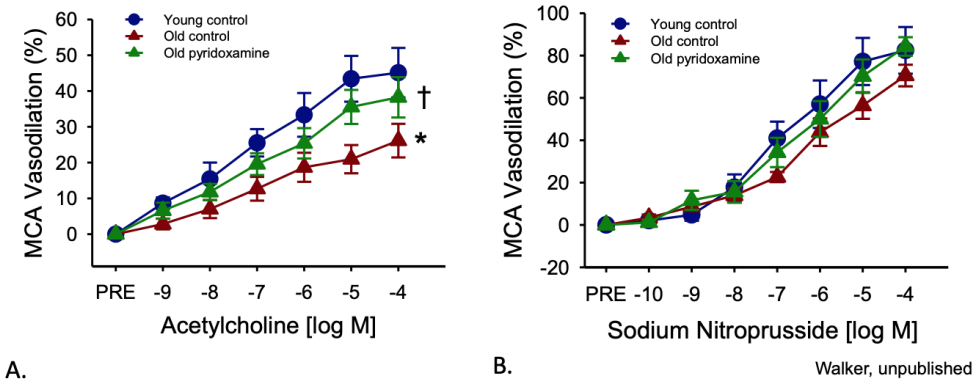


Figure 1. Pyridoxamine partially preserves endothelial function in MCAs of old treated animals

A. Dilation of MCAs in response to increased doses of acetylcholine, an endothelium dependent dilator via eNOS. Pyridoxamine improves vasodilation as compared to old control animals; B. Dilation in response to increased doses of sodium nitroprusside, an endothelium-independent dilator. This demonstrates that the decrease in dilation with age is due to endothelial dysfunction.

Therefore we examined the pyridoxamine's effect on aortic stiffness and morphology, MCA and brain tissue inflammation to establish this relationship. We posited that pyridoxamine inhibits large artery stiffening and prevents downstream

consequences such as endothelial dysfunction which preserves cognitive function (*Figure 2*).

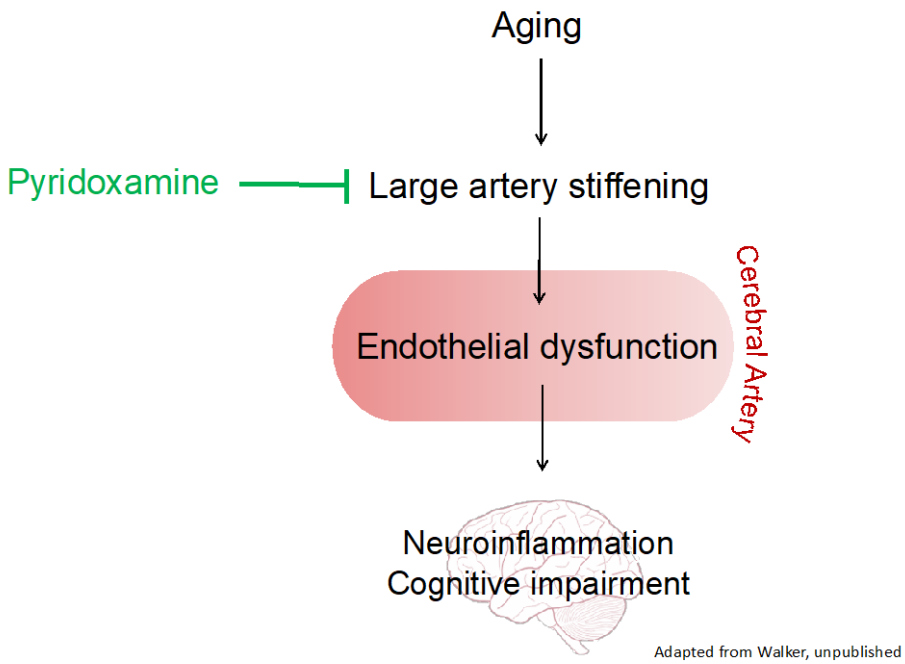


Figure 2. Proposed mechanism by which pyridoxamine mitigates cerebral arterial aging.

Pyridoxamine has been shown to inhibit large artery stiffness and therefore we hypothesize that it reduces endothelial function and the downstream development of cognitive impairment and cerebral inflammation with age.

We hypothesized that treatment with pyridoxamine will prevent age-related arterial stiffening and thereby preserve cerebral artery function by limiting the increase in oxidative stress and associated dysfunction, which will in turn prevent increases in neuroinflammation.

Methods

Model Organism: C57BL6 Mice

Young and old male C57BL6 mice were used as a model of naturally occurring arterial aging. Characteristics of young, old control, and old treated animals used in gene expression and immunofluorescence experiments appear in Table 1. *Figure 3* provides the breakdown of each group of mice and how they were used in the present study.

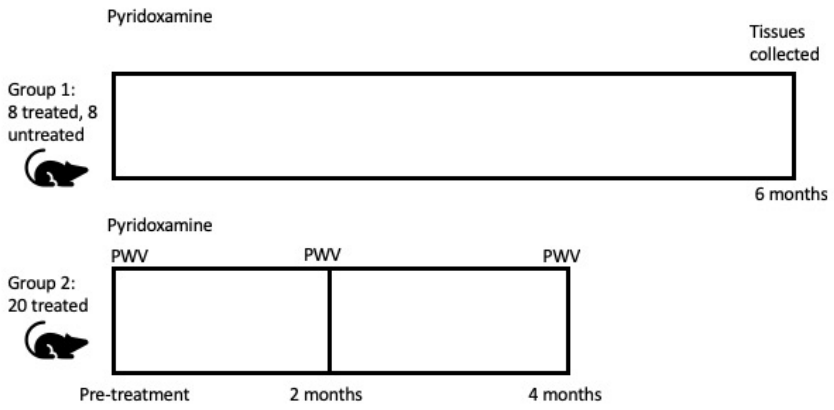


Figure 3. Schematic of animal treatment groups and their uses

Group 1 consisted of 8 treated and untreated middle-aged mice whose tissues were collected after 6 months of pyridoxamine treatment or vehicle control. Group 2 consisted of 20 pyridoxamine treated mice for whom PWV was measured prior to treatment and then every other month for 4 months.

20 mice were given pyridoxamine in water (1 mL pyridoxamine/384 mL water) *ad libitum* for 4 months. Mice treated with pyridoxamine for PWV were 21 months old at the start of treatment. Old control mice were 25 and 27 months old and young control mice were 4 months old when PWV was measured. Mice from whom tissues were

collected were 7.4 and 24.8 months old for young and old control, respectively (*Table 1*). Old pyridoxamine mice were 24.8 months old at tissue collection (*Table 1)*.

	Young Control	Old Control		Old Pyridoxamine	
		Pre	Post	Pre	Post
n	10		11		13
Age, months	7.4 ± 0.1		24.8 ±0.2*		24.8 ±0.2*
Body mass, g	28.7 ± 0.9	33.0 ±0.4*	32.0 ±0.4*	33.7 ±0.5*	31.5 ±0.6*

Table 1. Characteristics of Young and Old Animals from which Tissues were Collected, adapted from Walker

Masses and ages of young animals at time of testing and old animals pre and post treatment. * indicates significance as compared to young control ($p<0.05$).

Mice are a valuable model organism because the structure of their cerebral arteries mirrors that of humans – both humans and mice have a Circle of Willis which supplies blood to the brain (*Figure 4)*. Female mice were not used because they do not undergo a menopause similar to that of human women and thus have higher levels of estrogen throughout their lifespans. Estrogen has a significant protective effect against aging. Mouse weight was assessed weekly during the treatment period.

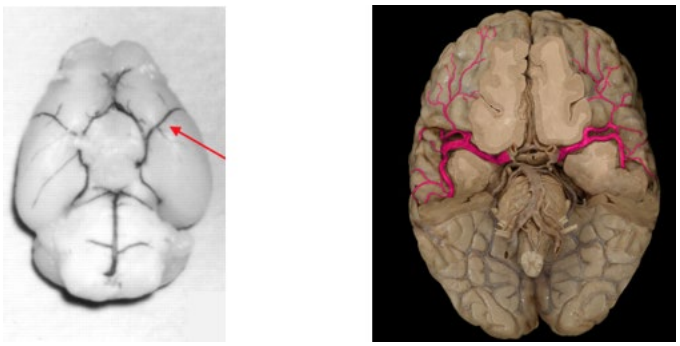


Figure 4. Mouse and Human Brains with MCA highlighted

The image on the left shows a mouse brain with the MCA indicated by a red arrow (Walker, unpublished). The image on the right illustrates a human brain with the MCA indicated by a pink highlight ("Anatomy & Physiology Revealed v3," 2017). The Circle of Willis (pathway of arterial circulation) is evident in both images.

PWV as a Measure of Aortic Stiffness

PWV of the aorta was measured using previously described methods (Walker et al., 2015). Mice were exposed to 2% inhaled isoflurane and oxygen until anesthetized and carefully secured to a surgical heating platform at 39˚C in a supine position (Walker et al., 2015). Anesthesia was maintained with a nose cone supplying a constant stream of 2% isoflurane and oxygen (Walker et al., 2015). Velocities of the transverse aortic arch and abdominal aorta were measured using 20 MHz Doppler probes (Indus Industries, Webster, TX, USA) and recorded simultaneously using Indus software (Indus Industries) (Walker et al., 2015) (*Figure 5*).

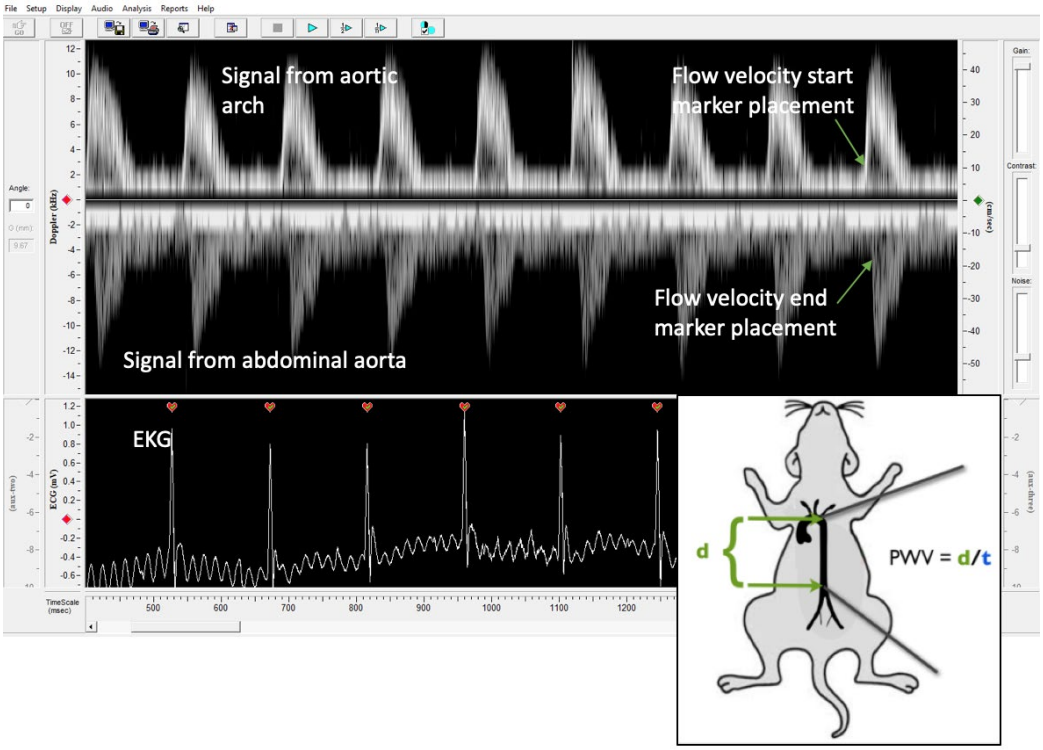


Figure 5. Experimental Pulse Wave Velocity Setup, adapted from Henson

Mouse is anesthetized and secured to a surgical platform with a nose cone (not pictured) providing a constant supply of oxygen and isoflurane, Doppler probes are placed on the arch of the aorta and abdominal aorta, respectively. Recordings of blood velocity and EKG over time are obtained. To analyze, markers are placed in the indicated locations and the distance between the probes is measured.

Once the data were collected, the distance between the probes was measured using calipers (Walker et al., 2015).

Immunofluorescence

Sections of thoracic aorta were frozen in optimal cutting temperature (OCT) and then sliced into 8 μm sections using a cryostat (Nikon, Minato, Tokyo, Japan) and adhered to a charged slide (Walker et al., 2015). For each animal, 10 slides were prepared with 4 sections of artery per slide. To begin staining, OCT was removed with

forceps and slides were rehydrated in acetone for 10 minutes. A PAP pen was used to draw a circular hydrophobic barrier around each artery and slides were incubated in a blocking buffer (0.1% Triton-X, 1% BSA, PBS) for 15 minutes. To quantify AGEs, three arterial samples on each slide were stained with a primary antibody anti-carboxymethyl lysine (AbCam 27684, AbCam, Cambridge, England, UK) at a concentration of 1:100 while the fourth served as a control and was incubated in only blocking buffer for 2 hours. All samples were subsequently incubated with Alexa Fluor 647 (AF-647) (Life Technologies, Grand Island, NY, USA) secondary antibody for 1 hour. Slides were then mounted with Prolong Gold with DAPI (Life Technologies) to stain cell nuclei and coverslips were applied and allowed to dry. Images of the artery were taken using a Zeiss LSM 880 Confocal Microscope SR-SIM (Zeiss, Oberkochen, Germany) with three filters applied to show autofluoresence of elastic lamina in the FIT-C channel, cell nuclei in the DAPI channel, and AGEs in the AF-647 channel (Walker et al., 2015). AGEs images were then obtained for each aortic section using only the AF-647 channel.

Verhoeff-Van Gieson Staining

Arterial samples were stained via Verhoeff-Van Gieson (VVG) stain to determine the structure of the arterial wall (Walker et al., 2015). Slides of aortas were obtained via the same cryostat procedure described above and submerged in 10% neutral buffered formalin for 10 minutes. Slides were then rinsed in tap water and RO water. Slides were placed in working elastic stain solution (Ferric chloride solution, Weigert's Iodine solution, Hematoxylin solution, dH_2O; Sigma-Aldrich, St. Louis, MO) and rinsed again in tap water and subsequently in RO water. Slides were then placed in

working ferric chloride solution to lighten the stain for 1.5 minutes, stained with Van Gieson solution, and dehydrated using a series of increasing ethanol concentrations to xylenes over the course of 18 minutes. Histological samples were mounted with Permount mounting medium (Electron Microscopy Sciences, Hatfield, PA) and coverslips were applied. Slides were allowed to dry and imaged using a Leica Microscope (Leica, Wetzlar, Germany) and Leica LAS software (Leica).

Real-time Gene Expression

Gene expression was examined by real-time PCR, a technique that amplifies DNA samples to a measurable amount, to quantify the amount of each gene of interest present in the tissue of interest. RNA was isolated from cerebral cortex samples of young, old control, and old pyridoxamine treated mice using the RNAeasy Mini kit (Qiagen, Hilden, Germany) (Walker et al., 2015). 500 μL of Qiazol was added to frozen cortex samples and allowed to thaw. Samples were then mixed using a homogenizer and loaded into the QiaShredder column. Samples were centrifuged and chloroform was added. Samples were then centrifuged for 15 minutes at 4˚C and the aqueous layer was transferred to a new tube. 1.5 volumes of 100% ethanol were added, then samples were added to the RNeasy mini column and centrifuged with a series of RW1 and RPE buffers. Water was added to the column and the RNA was collected in a new tube. MCAs were isolated using a slightly modified protocol to increase RNA yield. Arteries were flash-frozen in liquid nitrogen and crushed with a homogenizer tip before Qiazol was added and after thawing, samples were Sonicated for 2 minutes at 50% power. Columns from the Qiagen RNAeasy Micro Kit (Qiagen) were used in place of the RNAeasy Mini Kit (Qiagen) due to the small size of the arteries. All RNA samples were

Nanodropped to determine the amount (ng/μL) of nucleic acid. RNA was then converted to cDNA using the Qiagen Reverse Transcriptase Kit (Qiagen). Based on Nanodrop values, gDNA wipeout, water, and the calculated volume of RNA were added to a tube and incubated for 2 minutes at 42˚C. reverse transcriptase (RT) buffer, RT, and RT primer mix were added, and the samples were incubated at 42˚C for 15 minutes. The temperature was increased to 95˚C for 3 minutes then removed from heat. Samples of cDNA were diluted appropriately (80 μL dH_2O in cerebral cortex samples, 25 μL dH_2O in MCAs). The cDNA was then amplified with primers to bind to genes of interest and was analyzed for the expression of those genes in each experimental group. Cortex samples were examined for expression of reporter gene 18S and subsequently for expression of IL-1β, TNFα, NOX1, NOX2, SOD1, SOD2, and SOD3. MCA samples were studied for expression of reporter gene 18S and subsequent expression of NOX2, SOD1, and IL-1β.

Data Analysis

To analyze PWV data, Indus software (Indus Industries) was used to determine absolute pulse arrival times based on the foot of each waveform (Walker et al., 2015). Calculation of the aortic PVW was determined by the fraction of distance between the probes divided by the difference in absolute arrival times (Walker et al., 2015). Immunofluorescence and VVG data were analyzed using ImageJ software (NIH, Bethesda, MD). Immunofluorescence analysis for AGEs images in ImageJ (NIH) consisted of tracing the arterial structure in the composite image using the elastic lamina as a guide, applying this trace to the individual AGEs images, setting a threshold of 75

to eliminate background brightness, and measuring the mean gray value (Walker et al., 2015).VVG analysis consisted of examining the differences in thickness of the aorta between young, old control, and old treated mice using ImageJ software (NIH) by tracing the inner and outer walls of the artery and calculating the area for each section then averaging the results (Walker et al., 2015). This method was repeated twice for each artery image, once taking into account the elastic lamina only and once taking into account both the elastic lamina and tunic adventitia. Gene expression was determined by normalizing each gene to the expression of 18S and calculating the 2^ddCT. Data are reported as average ± SEM.

Statistics

Statistical analyses were conducted using SPSS and Prism. Group differences were compared by one-way ANOVA. A post-hoc Tukey's multiple comparisons test was run if ANOVA results were significant to determine differences between groups. Significance was set at $p<0.05$.

Results

PWV

Heart rate during PWV measurement was not different between groups (*Table 2*). PWV is significantly increased in old control mice as compared to middle-aged mice prior to pyridoxamine treatment and young control mice ($p=0.005$) (*Figure 6*). Pyridoxamine resulted in lowered PWV two months into treatment as compared to old control mice tested at four months but was not different after four months of treatment ($p > 0.05$) (*Figure 6*). PWV did not differ significantly over the course of pyridoxamine treatment in old mice ($p=0.11$).

Group	Heart Rate (bpm)
Pretreatment	442 ± 12
2 Months Treatment	416 ± 9
4 Months Treatment	440 ± 14
Old Control	455 ± 13
Young Control	405 ± 14

Table 2. Average Heart Rates of Mice During PWV

Heart rate was not different between groups during PWV testing. Data are reported as Average ± SEM.

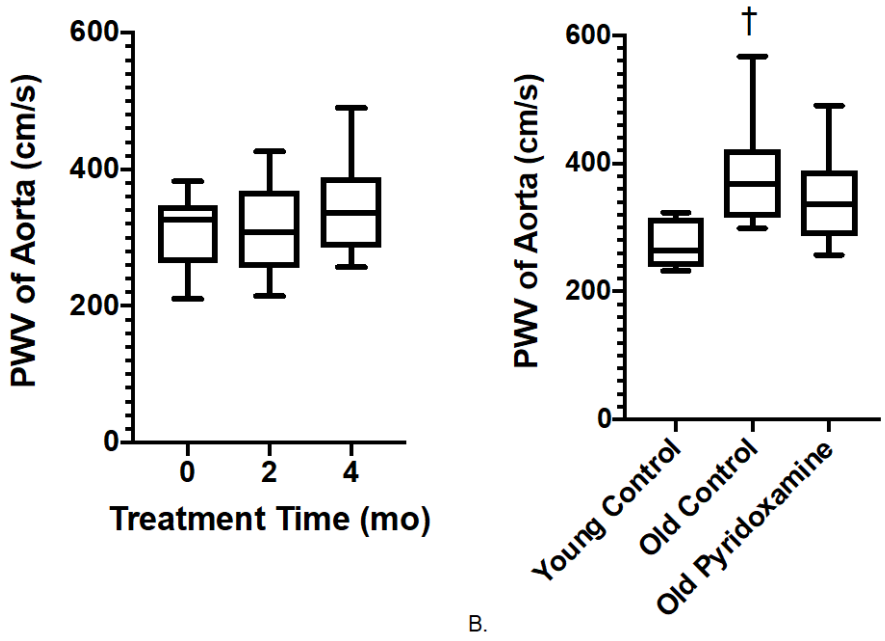


Figure 6. PWV of pyridoxamine treated animals versus treatment time and PWV comparison between young control, old control, and pyridoxamine treated groups

A. PWV (cm/s) over a treatment period of 4 months. There was no significant change in PWV over the course of 4 months of treatment. B. PWV at 4 months of pyridoxamine treatment course compared to young control and old control mice at the 4-month time point. Old control mice had significantly increased PWV as compared to young control and pretreatment middle-aged mice, † indicates significance versus young control ($p<0.05$).

AGEs Immunofluorescence

Young mice trended toward having fewer AGEs than old control mice in the aorta (p=0.08) (*Figure 7*). AGEs in the pyridoxamine treated animals were not different than old or young control mice (p=0.10) (*Figure 7*).

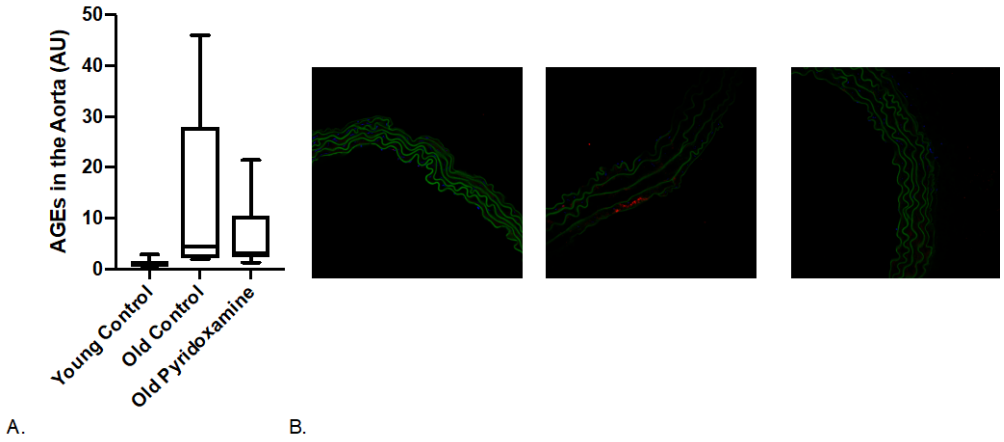


Figure 7. Pyridoxamine does not alter AGEs in pyridoxamine-treated aortas

A. AGEs in the aorta of young control, old control, and old pyridoxamine treated mice. AGEs quantified with ImageJ, no significant difference between groups; B. Aortas stained via immunofluorescence for AGEs. Green indicates elastin, blue indicates cell nuclei, and red indicates AGEs. Left to right: young untreated aorta, old untreated aorta, old pyridoxamine treated aorta.

VVG Staining

The overall thickness of the aortic wall was unchanged between young, old treated, and old control subjects (p=0.3) (*Figure 8*). Treatment also did not alter adventitial thickness between young, old control, and old treated groups (p=0.4) (*Figure 8*). However, young animals trended toward having thinner aortas.

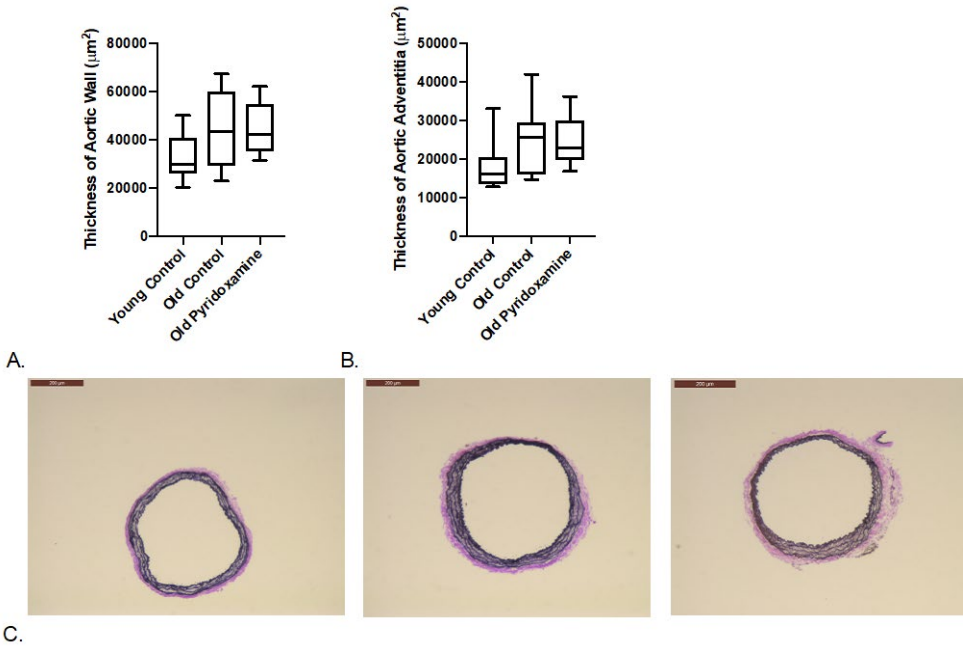


Figure 8. Pyridoxamine does not alter arterial wall structure in treated aortas

A. Area of the aortic wall in μm^2, no difference between young control, old control, and pyridoxamine treated arteries; B. Area of the aortic adventitia in μm^2, no difference between groups; C. Aortas stained with Verhoeff Van-Gieson stain for elastin and collagen. Pink indicates collagen, purple indicates elastin. Left to right: young untreated animal, old untreated animal, old pyridoxamine treated animal.

MCA Gene Expression

In the MCAs, gene expression of SOD1 was not different between groups ($p>0.05$). However, IL-1β was significantly higher in pyridoxamine treated animals than young control animals ($p=0.03$) (*Figure 9*). NOX2 trended toward being elevated in old control MCAs as compared to young control and old pyridoxamine animals ($p=0.092$) (*Figure 9*).

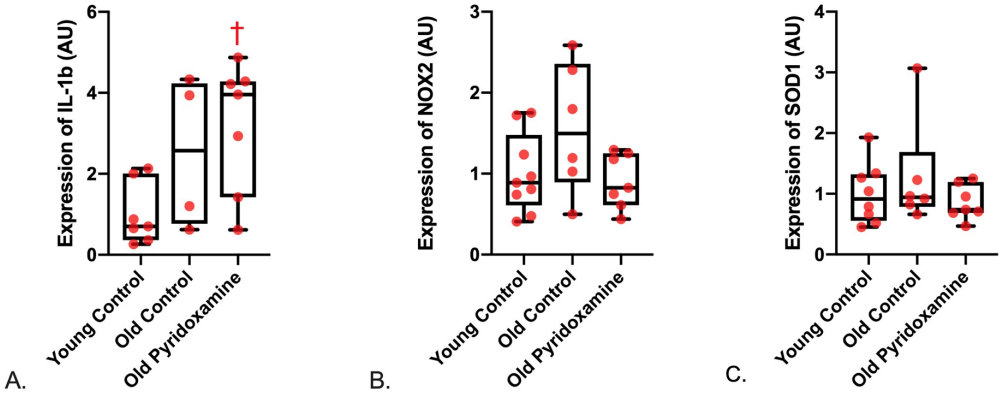
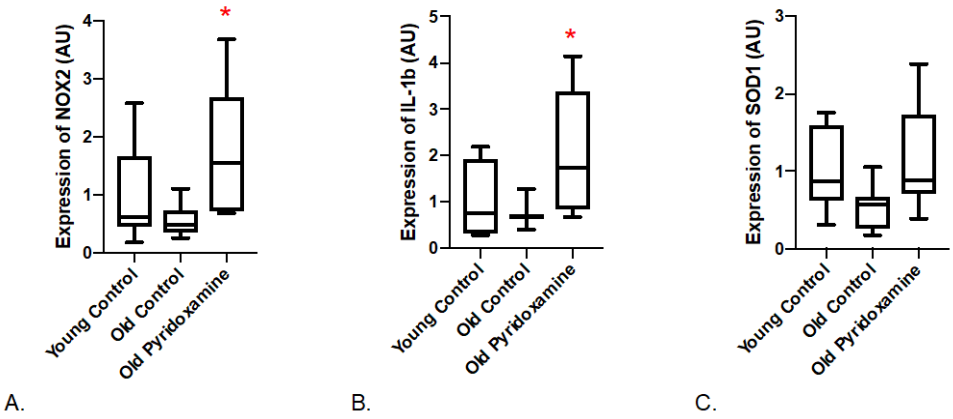


Figure 9. MCA expression of IL-1β (AU) is increased in old pyridoxamine vs. young control; Expression of NOX2 trends higher in old control vs. young control and old pyridoxamine. Expression of SOD1 is not changed between groups.

A. Gene expression of IL-1β is elevated in pyridoxamine as compared to young control animals. No difference between old control and pyridoxamine treated. No difference between young and old control animals. * indicates significance versus young control ($p<0.05$); B. Expression of NOX2 trends toward elevation in old control versus young control and old pyridoxamine MCAs. C. SOD1 is not different between the three groups.

Cerebral Cortex Gene Expression

In the cerebral cortex, SOD1 trended toward being reduced in pyridoxamine treated animals as compared to old control animals and thus similar to values seen in young controls ($p=0.1$) (*Figure 10*). NOX2 was significantly elevated in pyridoxamine treated mice as compared to old control animals ($p=0.04$) (*Figure 10*). IL-1β was significantly increased in old pyridoxamine as compared to old control mice ($p= 0.05$) (*Figure 10*). SOD2 and SOD3 were not different between groups ($p>0.05$). Expression of TNFα and NOX1 in the cerebral cortex was too low to quantify.



A. B. C.

Figure 10. Cerebral cortex expression of NOX2 is higher in old pyridoxamine vs. old control; Expression of IL-1β is significantly elevated in old pyridoxamine vs. old control; Expression of SOD1 trends higher in young control and old pyridoxamine.

A. Expression of IL-1β (AU) is significantly elevated in pyridoxamine treated as compared to old control mice; B. Expression of NOX2 (AU) is significantly elevated in pyridoxamine treated as compared to old control mice; C. Expression of SOD1 trends toward being higher in old pyridoxamine treated animals than old control animals; * indicates significance versus old control ($p<0.05$).

Discussion

In the present study we treated mice with pyridoxamine for four months to determine the mechanism by which pyridoxamine attenuates cerebral artery endothelial dysfunction in old mice and somewhat preserves dilatory response to a NO-mediated dilator (*Figure 1*). We found that pyridoxamine prevents the expected increase in PWV. However, contrary to our hypothesis, treated mice had increased expression of IL-1β and NOX2, both pro-oxidants, in the cerebral cortex. The trend toward greater expression of SOD1 in the cortex supported the hypothesis that pyridoxamine increases antioxidants. In the MCA, IL-1β was also significantly elevated and NOX2 trended toward being decreased, however, SOD1 was not affected by pyridoxamine treatment. Therefore, pyridoxamine may prevent age-related increases in large artery stiffness, but also increase pro-inflammatory and pro-oxidant factors in the brain.

Pyridoxamine attenuates PWV somewhat after 4 months of treatment

These data agree with previous literature stating that PWV increases with age (Di Lascio, Stea, Kusmic, Sicari, & Faita, 2014) – old control mice had significantly greater PWV than both young control and middle-aged mice prior to pyridoxamine treatment. However, pyridoxamine treated mice did not have increased PWV after 4 months of treatment, suggesting that pyridoxamine may attenuate arterial stiffening with age. We do not have the same time course of PWV measurements for the old control mice, thus it cannot be said with certainty that pyridoxamine prevents age-related increases in PWV. Nevertheless, our result concurs with prior studies suggesting

that pyridoxamine prevents arterial stiffening (Chang et al., 2009; E. T. Wu et al., 2011), but the mechanism remains unclear.

Pyridoxamine does not alter AGEs or arterial wall structure

These results suggest that pyridoxamine treatment does not prevent the formation of AGEs in large elastic arteries. While previous studies have indicated a reduction in AGEs with pyridoxamine treatment, this was not evident here. It is possible that our method for measuring AGEs (immunofluorescence) was not sensitive enough to detect differences with pyridoxamine treatment. Therefore future studies will measure AGEs via Western blot, similar to previous studies (E. T. Wu et al., 2011). As anticipated, treatment has no effect on the structure of the arterial wall in aged mice. These results suggest that the improvement in MCA vasodilation with pyridoxamine treatment and perhaps the age-related increase in PWV, were not due to a reduction of AGEs in the aorta.

Pyridoxamine's effect on oxidative stress and inflammation

Oxidative stress refers to the increase in reactive byproducts that alter cellular function. Antioxidant enzymes such as SOD1 breaks down ROS and helps maintain the balance between overproduction of damaging ROS and a sufficient quantity to maintain cellular immune function (Harrison, 2012). SOD1 also likely plays a role in mitigating cellular senescence (Harrison, 2012). With increased age, there is a reduction in mRNA expression of SOD1 in rodent brain tissue (Semsei, Rao, & Richardson, 1991). Furthermore, mice that are missing both copies of the SOD1 gene have accelerated aging profiles and decreased lifespans (Y. Zhang et al., 2017). Our data suggest that

young mice trend toward having more SOD1 in the cerebral cortex and are therefore better able to break down ROS. While there is no significant difference in old control and old treated animals, SOD1 trends toward restoration to young values in pyridoxamine-treated brains suggesting a possible role for pyridoxamine improving expression of SOD1. SOD2 and SOD3 were not affected by pyridoxamine treatment, likely because SOD1 is the only cytoplasmic form of the enzyme and thus perhaps more relevant to arterial function.

IL-1β is a pro-inflammatory cytokine released as a part of the body's response to inflammation. With age, there is an increase in systemic inflammation and therefore IL-1β is elevated (Csiszar, Ungvari, Koller, Edwards, & Kaley, 2003). The increase of IL-1β in cortex samples appears to be a result of pyridoxamine treatment. However, the increase seen in the MCAs of pyridoxamine-treated animals is likely a result of the aging process as it is similarly (although not significantly) elevated in old untreated arteries. In serum samples, Zhang et al. (2016) similarly found that injection pyridoxamine had little effect on the production of IL-1β. Further, they found that pyridoxamine did not alter the expression of IL-1β mRNA (P. Zhang et al., 2016). Thus, pyridoxamine appears to have little effect on the reduction of IL-1β expression.

NOX2 is an enzyme that produces superoxide, a ROS, that helps maintain immune function (Harrison, 2012). However, with age there is an increase in activity of NOX and a decrease in antioxidant enzymes to break ROS down (Ungvari et al., 2010). Cerebral cortex expression of NOX2 was increased in pyridoxamine treated as compared to old control animals, suggesting an increase in ROS. MCA expression of NOX2 trended toward being reduced to young control levels, which was in line with

expectations of reduced ROS. A study of the effect of pyridoxamine on high- and low-fat diet versus control diets in mice found that pyridoxamine treatment reduced NOX expression (Hagiwara et al., 2009). They concluded that the decrease in NOX activity was an effect of lowered fat accumulation rather than a direct effect of pyridoxamine (Hagiwara et al., 2009). However, that study did not take into account the effects of aging, so the increase in NOX2 in the MCAs and cerebral cortex may be a result of aging. Furthermore, it is possible that alterations to gene expression in the cerebral cortex are the result of a different pathway than in the MCAs.

In this study, we also measured the expression of TNFα, a pro-inflammatory cytokine, and NOX1 a pro-oxidant enzyme, in the cerebral cortex. However, the expression of TNFα and NOX1 were too low to be quantified.

Limitations

This study has a significant limitation in that it did not track PWV in old control mice with increasing age concurrently with treated mice. Thus, it is unclear whether pyridoxamine limits the increase in PWV as compared to old control animals over time. Another set of mice with both treatment and control groups should be studied longitudinally. We were unable to examine protein expression due to the small sizes of the arterial tissues. However, the amount of protein expressed is typically more physiologically important than gene expression. Group sizes were also limited, with larger groups might have made group differences more significant. Finally, levels of pyridoxamine in the blood should be measured to determine that levels of circulating pyridoxamine were increased.

Conclusion

The aim of this research was to determine the mechanism by which pyridoxamine preserves endothelial function with age and prevents arterial stiffening. Pyridoxamine appears to attenuate arterial stiffening and thereby limit increases in PWV, however, it did not affect the formation of AGEs. Expression of antioxidant enzymes appeared to increase, however, proinflammatory cytokines and enzymes also increased, suggesting pyridoxamine's role in oxidative stress may be multifactorial. Further studies are needed to identify the mechanism by which pyridoxamine prevents age-related arterial stiffening and preserves the vasodilatory response.

Future Directions

As previous studies have found correlations between arterial stiffness and dementia, future studies should examine cognitive function during and post-treatment with pyridoxamine in old animals. It is possible pyridoxamine prevents formation of AGEs in cerebral arteries or even brain tissue which we were unable to assess in this study. Thus, future studies should examine these tissues to determine whether or not AGEs are affected. This will provide insight into the interplay between arterial stiffening and cognitive decline and help elucidate the relationship between large artery stiffness, cerebral artery function, and cognitive function.

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