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Effects of BKCa Activation and ROS Scavenging on Aging Vasculature

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ABSTRACT

Aging blood vessels undergo physiological changes that result in reduced vasodilation, reduced responsiveness to stimuli, and increased stiffness. Specifically, older vasculature exhibits both increased ROS damage and reduced function of the vasoregulatory large-conductance Ca^{2+}-activated K^+ (BKCa) channel. We investigated if MitoTEMPO (a mitochondrial ROS scavenger) or rottlerin (a BKCa channel activator) can beneficially modulate vascular function in aged mouse aortas. Results showed that while both drugs significantly reduced vascular contraction in an age-dependent manner, they also reduced nitric oxide (NO) - mediated vasodilation. This negative trade-off suggests that these drugs may not be effective therapeutics for aging or diseased vessels.

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INTRODUCTION

The Aging Population of the United States

Cardiovascular disease is one of the world's leading causes of death, contributing to 1 out of 4 deaths in the United States alone (Carvalho-de-Souza et al., 2012). Aging is a risk factor of cardiovascular disease that results in a variety of structural and functional changes in the vasculature, including reduced vasodilation, reduced response to stimuli, and increased stiffness. According to the United States Department of Commerce, the U.S. will experience a considerable growth in its aging population between 2012 and 2050. By 2050, it is predicted that 83.7 million people in the United States will be 65 or older, almost double the population recorded in 2012 (Ortman et al., 2014). Various types of vascular dysfunction are associated with natural aging as well as other common diseases like diabetes and hypertension. This high frequency highlights the importance of developing therapeutics to alleviate vascular dysfunction.

Oxidative Stress in Aging Vasculature

Aging is associated with numerous health complications including heart disease, cancer, stroke, chronic respiratory diseases, Alzheimer's disease, and diabetes (Ortman, et al., 2014). Since 1910, the CDC has reported heart disease as the leading cause of death among Americans 65 years or older, with the exception of 1918 - 1920 when the influenza epidemic took the lives of many Americans. In fact, from 2007 to 2009, 27.7% of elderly deaths in America were caused by heart disease (Ortman, et al., 2014). The effect of aging on vascular function has been a research focus in hopes of limiting the risk of heart disease and high blood pressure in the aging population. Vascular disease in patients 65 years or older is often associated with decreased endothelium-dependent relaxations induced by nitric oxide (Hongo, et al., 1998). Endothelial cells form the innermost layer of blood vessels and serve as regulators of vascular function. Nitric oxide (NO) is produced within the endothelial cells and works as a vasodilator through the activation of guanylyl cyclase in the vascular smooth muscle cells that compose the middle layers of blood vessels. The decreased NO response seen in aging also contributes to morphological changes in the inner lining of blood vessels, such as plaque and neointima formation (Creager, et al., 2003).

Research has identified a variety of key processes that are involved in decreased vascular function associated with aging. These include inflammation, endothelial dysfunction, and

increased oxidative stress. Oxidative stress is caused by an increase in reactive oxygen species (ROS), such as superoxide anions, which inactivate NO and form peroxynitrite (Creager, et al., 2003). This molecule has been shown to oxidize tetrahydrobiopterin (BH_4), a nitric oxide synthase (NOS) cofactor, resulting in dihydrobiopterin. In the presence of decreased BH_4, NOS can function to produce superoxide, rather than NO (Milstein, et al., 1999). In summary, aging vasculature is associated with decreased endothelium-derived nitric oxide levels (potentially through ROS generation), a vital driver of vasodilation.

In addition to overall decreases in NO levels, diseased vessels have also shown decreased responsiveness to NO. Previous studies have shown that in the presence of equal concentrations of SNP, a nitric oxide donor, diabetic vasculature exhibited a decreased vasodilatory response (Clements, et al., 2009). Similarly, aging vessels exhibit impaired endothelium-dependent vasodilation. This phenotype is attributed to the increase in oxidative stress production, which reduces the bioavailability of NO (Taddei, et al., 2001).

Large-Conductance Ca^{2+}-activated K^+ Channels

The role of large-conductance Ca^{2+}-activated K^+ (BKCa) channels in vascular smooth muscle function and tone has been widely studied (Ledoux, et al., 2008), (Hu, et al., 2012), (Clements, et al., 2015), (Ko, et al., 2008). These channels, which are highly expressed in vascular smooth muscle cells, are composed of two subunits. The smaller β1-subunit consists of two transmembrane domains and is not directly connected to the α-subunit. The larger α-subunit consists of eleven hydrophobic domains, the first seven of which are located in the plasma membrane. The pore through which potassium ions flow is located between the fifth and sixth hydrophobic domains. *In vivo*, the native BKCa channel of smooth muscle is formed by the association of four β1-subunits and four α-subunits that form a tetramer.

BKCa channels are regulated in various ways throughout the body. This is due to both the widespread distribution of the channel in tissues and the alternative splicing and associations of the subunits based on site. This channel can be activated through membrane depolarization, Ca^{2+} binding, and NO (Hu, et al., 2012), (Clements, et al., 2015). Upon activation, potassium ions flow out of the cell, leading to hyperpolarization of the cell membrane, closing of voltage-dependent Ca^{2+} channels, and subsequent vasorelaxation. Conversely, closing of the BKCa channel favors vasoconstriction (Hu, et al., 2012), (Ko, et al., 2008).

Because of their important role in regulating vascular tone and blood pressure, potential impairment of BKCa function has been explored in a variety of disorders that display vascular symptoms. As a result of these studies, malfunction of the BKCa channel has been connected to multiple conditions including hypertension, brain injury, coronary artery disease, and diabetes (Hu, et al., 2012), (Clements, et al., 2015), (Ko, et al., 2008).

The effectiveness of the BKCa channel in regulating vasoreactivity has been shown to be reduced in aging vasculature. In addition to the previously mentioned decrease in NO levels and subsequent BKCa inactivation, aging has also been linked to decreased expression of the channel itself. Specifically, studies showed abnormal expression and functionality of the BK channel was linked to decreased vascular responsiveness in aging rats (Carvalho-de-Souza et al., 2012). Further studies showed that this functional change can be attributed to reduced acetylcholine-induced vasorelaxation due to decreased BK activity in small mesenteric arteries (Matsumoto et al., 2012). Lastly, additional studies showed that aging led to increased production of ROS, strong inhibitors of BKCa channels in aging blood vessels (Taddei, et al., 2001). This offers a potential connection between aging vasculature, ROS, and BKCa channels that calls for further investigation.

Key Modulators of Vasomotion and BKCa Channel Function

In the experiments of this project, a range of vasodilators, vasoconstrictors, ROS scavengers, and BKCa channel-activators were used. These included the stable thromboxane mimetic U46619, sodium nitroprusside (SNP), MitoTEMPO, and rottlerin (a.k.a. mallotoxin).

Thromboxane (U46619)

U46619 is a thromboxane A_2 analogue that is known to cause vasoconstriction. Specifically, the compound acts to both activate voltage-gated cation channels and inhibit the ion flow through voltage-gated K^+ channels. This leads to depolarization of the membrane, release of Ca^{2+} stores to the cytoplasm, and subsequent contraction of the vascular smooth muscle through activation of myosin. The mechanism of this inhibition has been attributed to a special form of protein kinase C (PKC). When the thromboxane receptor is activated by U46619, this special form of PKC modifies the BKCa channel such that ions can no longer flow out of it, triggering vasoconstriction (Cogolludo, et al., 2003).

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Sodium nitroprusside (SNP)

Sodium nitroprusside is a well-characterized vasodilator which functions as a nitric oxide donor. The donated NO triggers the reduction of intracellular $[Ca^{2+}]$, leading to hyperpolarization of the membrane and subsequent relaxation of the vascular smooth muscle. Specifically, NO is the first messenger in a cascade of phosphorylation reactions. NO works by activating soluble guanylate cyclase to make cGMP, which then activates PKG. PKG phosphorylates amino acids on various cellular proteins, resulting in changes in function, subcellular localization, or regulatory features. The culmination of phosphorylation reactions ultimately leads to increased endothelial dilation (Francis, et al., 2010). Secondarily, NO also targets the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and the plasma membrane Ca^{2+} ATPase. By activating one or both of these ion transporters, the amount of intracellular Ca^{2+} is reduced and vasodilation occurs (Cogolludo, et al., 2001).

MitoTEMPO

MitoTEMPO, a well-characterized scavenger of ROS, functions as a mitochondria-targeted antioxidant. In experiments with cultured endothelial cells, treatment with MitoTEMPO was shown to decrease both mitochondrial and total cellular superoxide and increase the amount of bioavailable NO (Dikalova, et al., 2010). Similarly, through *in vivo* mouse studies, MitoTEMPO was shown to decrease vascular superoxide, increase vascular NO production, promote vessel relaxation, and alleviate hypertension (Dikalova, et al., 2010). Importantly, MitoTEMPO showed no effects on the blood pressure of healthy, non-hypertensive mice, indicating that the compound specifically targets the overproduction of ROS that occurs in the disease state (Dikalova, et al., 2010). For this reason, there is wide interest in using MitoTEMPO and other ROS scavengers to improve vessel function in a variety of diseases that cause vascular complications, including aging and diabetes.

Rottlerin

In recent years, rottlerin has been identified as an effective vasodilator. In experiments using BKCa channel knockout mice, it was shown that this vasodilation is achieved through highly potent activation of the BKCa channel (Cordeiro, et al., 2015). Specifically, the EC50 dosage (1.7 µM) of rottlerin was capable of decreasing the voltage-dependent activation of the

BKCa channel by approximately 70 mV (Zakharov, et al., 2005). This shift was considerably greater than any other BKCa channel activator previously characterized. Though highly potent, rottlerin is known to have multiple documented off-target effects (Clements, et al., 2015). Because of this fairly low specificity, rottlerin is not widely regarded as a viable BKCa channel activator for clinical use.

Our Hypothesis

The BKCa channel has diverse roles within tissues including the regulation of neurotransmitter release, neuronal excitability, and smooth muscle tone (Bentzen, et al., 2014). Within the vascular smooth muscle cells, BKCa channels are expressed on the plasma membrane and aid in the regulation of vascular relaxation (Clements, et al., 2015). ROS overproduction, which may help reduce nitric oxide levels, is widely exhibited in the aging population (Lu, et al., 2011). Additionally, ROS overproduction can also damage the normal dilatory signaling cascades and BKCa channels (Taddei, et al., 2001). This study aims to explore the effects of BKCa activation to combat vasoconstriction using aged mouse aortic rings. We hypothesize that activation of calcium-activated potassium channels using known BKCa activators will restore the vasodilatory function of blood vessels in aging mice. In addition, we predict that increasing nitric oxide sensitivity through scavenging of ROS in aging mice is will combat the decrease in BKCa function, revealing another therapeutic target to improve vascular function.

Improving BKCa channel function and subsequent vasodilation in aging mice opens possibilities for combating increased coronary artery disease, reduced angiogenesis, and peripheral vasculopathy in aging patients (Carvalho-de-Souza, et al., 2013). Our project aims to test this therapeutic strategy and explore its clinical applicability in treating vascular conditions among the aging population.

MATERIALS AND METHODS

These methods were adapted from their original use by Cordeiro et al. in 2015. Detailed protocols can be found in the Appendix.

Ethical Approvals for Animal Experiments

All protocols and methods for animal experiments were approved by the Rhode Island Institutional Animal Care and Use Committee, and all animals were treated in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication 85-23, revised 1985).

Mice

Both young (4-8 weeks) and old (1-2 years) FVB mice were used in this study. There were no obvious phenotypic differences observed between the two age groups, except older mice were generally larger. The littermates of either sex were used. Previous studies identified no sex-dependent differences of contractile and relaxation responses in these mice (Cordeiro et al., 2015).

Vascular Ring Studies

Mice were first heparinized and subsequently anesthetized with ketamine (80 mg/kg) and xylazine (5 mg/kg). All three drugs were administered via intraperitoneal injection. Once all pain response ceased as assessed by toe pinch, a sternotomy was performed and the heart and lungs were removed. Mouse aortas were then dissected and isolated from the chest cavity and placed in cold, oxygenated Krebs-Henseleit buffer. Vessels were cleaned of perivascular adipose tissue and cut into approximately 2.5 to 3 mm lengths. Care was taken to avoid any stretch of the vessel. Aortic rings were placed on 2 metal hooks (0.05 mm) and suspended in one of four perfused 15 mL tissue baths (Radnoti, Monrovia, CA) with oxygenation and temperature control. One end of the hook was attached using a stainless-steel wire to a force transducer (Kent Scientific Torrington, CT). All measurements were recorded on a data acquisition system (Powerlab, AD Instruments Colorado Springs, CO). Figure 1, below, illustrates the experimental setup. Vessels were allowed to equilibrate in the bath for 40 minutes while resting tension was slowly increased in 0.1 g increments to approximately 0.4 g, a level empirically determined to

provide optimal U46619 constriction in healthy mice. After equilibration, perfusion was stopped and vessel contraction and viability were tested with 60 mM potassium chloride for 10 minutes. Vessels were then washed for 20 minutes before beginning experiments using contractile agonists.
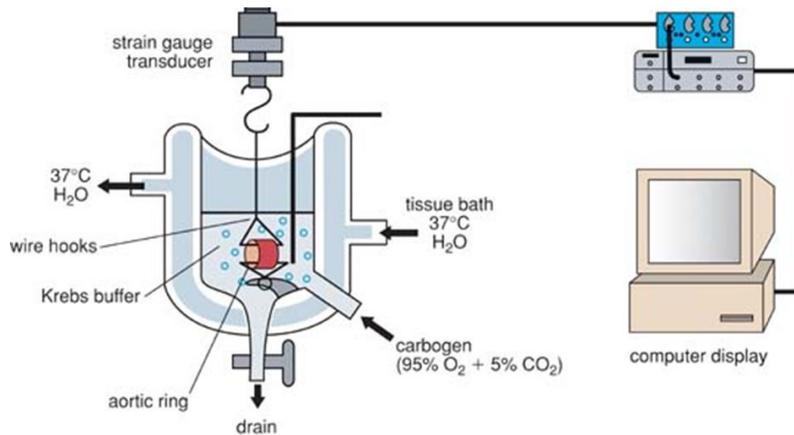

FIGURE 1: Diagram of the experimental setup. Baths have inlets and outlets for continuous perfusion with Krebs buffer as desired. Baths are jacketed to allow temperature regulation from heated water. Oxygenation is achieved through a gas port with adjustable gas valves (not pictured). Aortic rings are suspended between two hooks, the top of which is attached by a wire to a force transducer. Force readouts are displayed continuously on a computer program (www.radnoti.com).

Pretreatment Experiments

After washing, perfusion was stopped and resting tension was readjusted to approximately 0.4 g if necessary. Vessels were then treated with 1 μM rottlerin, 10 μM Mitotempo, or DMSO vehicle (control). After 30 minutes incubation, cumulative dose response treatments were applied. First, vessels were exposed to increasing doses of U46619, applied every 2 minutes. The doses were $1x10^{-9}$ M, $1x10^{-8}$ M, $3x10^{-8}$ M, $6x10^{-8}$ M, and $1x10^{-7}$ M. Constriction of vessels was measured. After the final dose, the contractile response was allowed to stabilize for ~10 minutes. Following, vessels were exposed to increasing doses of SNP, applied every 2 minutes. The doses were $1x10^{-9}$ M, $1x10^{-8}$ M, $1x10^{-7}$ M, $1x10^{-6}$ M, and $1x10^{-5}$ M. Relaxation of vessels was measured. Constriction responses were quantified as percent of the vessel's response to KCl. Relaxation responses were quantified as percent relaxation from maximum contraction.

Statistical Analysis

Contraction and relaxation curves were created from raw force readouts. Statistical significance between treatments was determined following 2-way repeated measures analysis of variance (ANOVA), Student-Newman-Keuls post-hoc analysis. The significance level (alpha) was set at 0.05.

RESULTS

Aged Vessels Exhibit Unstimulated, Spontaneous Contractions

When suspended in the tissue perfusion baths, aged vessels exhibited spontaneous contractions that were not seen in young vascular rings. Specifically, aged vessels demonstrated unstimulated, spontaneous contractions that occurred in rhythmic patterns. These contractions generally peaked at a magnitude of 125% of the resting tension and lasted for approximately ten seconds each for periods of up to ten minutes. Figure 2, below, shows a quantitative comparison between these spontaneous contractions in aged and young vessels. As illustrated in the figure, aged vessels exhibited approximately 2.5 spontaneous contractions per minute while young vessels exhibited fewer than 0.5 spontaneous contractions per minute. A Student's t-test concluded that this difference was statistically significant ($p < 0.05$).
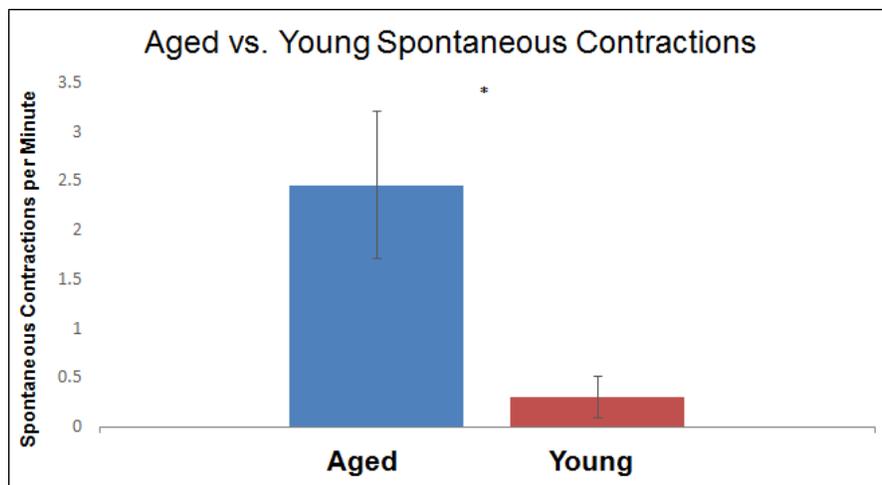

FIGURE 2: Frequency of unstimulated, spontaneous contractions in aged and young vessels. Compared to their young counterparts, aged vessels exhibited significantly more spontaneous contractions per minute ($p < 0.05$). Contractions were counted over ten minute periods starting at the initiation of reperfusion after viability testing with KCl.

Untreated Aged and Young Vessels Show Comparative Responsiveness to Contractile and Dilatory Agonists

Vehicle-treated (DMSO) aortic rings were exposed to increasing doses of the contractile agonist U46619, a stable analog of thromboxane (Figure 3A). Aged and young aortic rings exhibited similar responses to U46619. Similarly, to measure the relaxation responses of the vessels, aortic rings were exposed to increasing doses of the vasodilator SNP (Figure 3B). There

were also no differences in relaxation of aged and young aortas. Both age groups contracted to between 200% and 230% of their KCl responses and relaxed to between 65% and 85% of the magnitude of their maximum contraction.
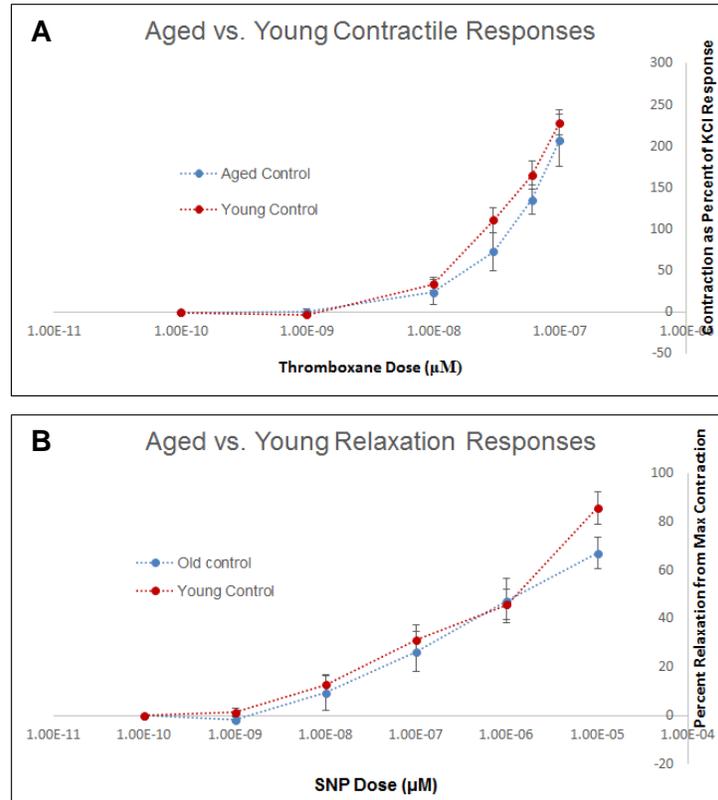

FIGURE 3: A) Contractile responses of vehicle-treated (DMSO) aged and young vessels. Contraction was measured as a percent of the baseline KCl-induced contraction in response to increasing doses of thromboxane. B) Relaxation responses of aged and young vehicle-treated vessels. Relaxation was measured as the percent of relaxation from the maximum level of U46619-induced contraction, with 100% relaxation being a return to baseline tension (n = 6 old, 15 young).

MitoTEMPO Reduces Contraction in Only Aged Vessels; Rottlerin Reduces Contractions in Only Young Vessels

To test the effects of ROS scavenging on vascular function, a subset of vessels were pretreated with MitoTEMPO (Figure 4A). In young vessels, the contractile response was comparable between the DMSO-treated controls and the vessels treated with MitoTEMPO (p = 0.16). In the aged vessels, however, there was a statistically significant (p < 0.05) reduction in the thromboxane-induced contractile response of those treated with MitoTEMPO.

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To test the effects of BKCa channel activation on vascular function, vessels were pretreated with the BKCa activator rottlerin (Figure 4B). Rottlerin had no effect on the contractile responses for the aged group. However, in the young vessels, there was a strong but non-significant trend ($p = 0.07$) for diminished thromboxane-induced contractile response of rottlerin-treated vessels.

FIGURE 4: A) Contractile responses of aged and young vessels treated with the ROS scavenger MitoTEMPO. Contraction was significantly reduced in aged vessels treated with the drug ($p < 0.05$. Two-Way RM ANOVA, SNK, minimum $n = 6$ per group). B) Contractile responses of aged and young vessels treated with the BKCa channel activator rottlerin. Contraction was reduced in young vessels treated with the drug, but this failed to reach significance ($p = 0.07$. Two-Way RM ANOVA, SNK, minimum $n = 6$ per group).

MitoTEMPO Reduces Relaxation in Aged Vessels. Rottlerin Does Not Affect Relaxation.

To test the effects of ROS scavenging on relaxation, a subset of vessels was pretreated with MitoTEMPO (Figure 5A). In young and aged vessels, the SNP-induced relaxation response was diminished in the presence of MitoTEMPO. While this pattern did not reach statistical significance in the young vessels, it did in the aged vessels ($p < 0.05$).

Similarly, a subset of vessels was pretreated with rottlerin to test the effects of BKCa activation on relaxation (Figure 5B). Rottlerin showed no statistically significant effects on the relaxation of either aged or young vessels.
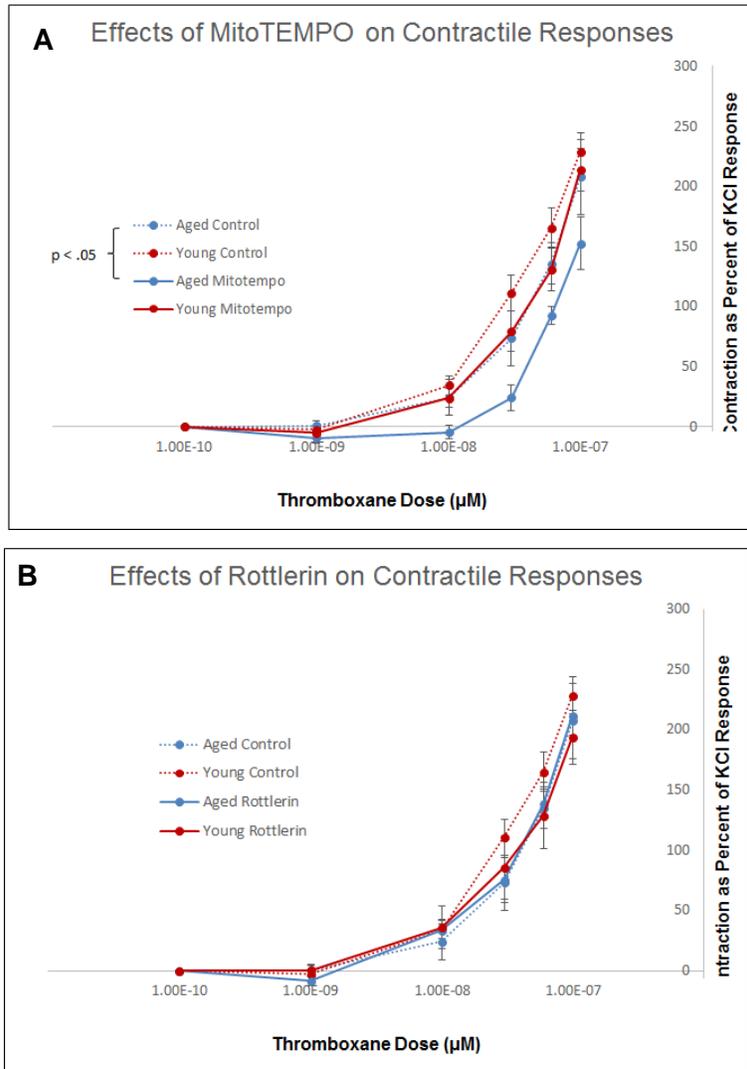

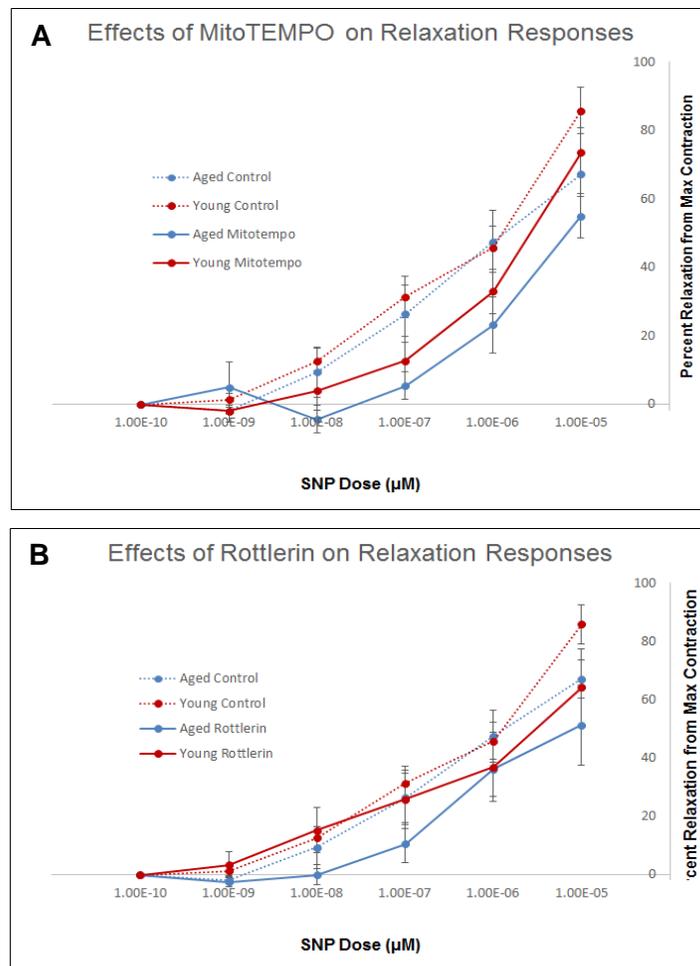**FIGURE 5**: A) Relaxation responses of aged and young vessels treated with and without the ROS scavenger MitoTEMPO. Relaxation was significantly reduced in aged vessels treated with the drug ($p < 0.05$. Two-Way RM ANOVA, SNK, minimum n = 6 per group). B) Relaxation responses of aged and young vessels treated with the BKCa channel activator rottlerin. No significant changes were observed for either age group.

DISCUSSION

Our study demonstrates that both ROS scavenging and BKCa channel activation play a role in modulating vascular function. Specifically, significant changes were seen when 1) young vessels were treated with the BKCa activator rottlerin, resulting in a decreased contractile response compared to the control and when 2) aged vessels were treated with MitoTEMPO, resulting in a decreased contractile response, compared to their controls. Moreover, we identified increased spontaneous contractions in aged vessels. This phenotype has been shown in previous research studies with BKCa channel knockout mice (Cordeiro, et al., 2015). Importantly, only aged vessels share this phenotype with BKCa channel knockout mice, allowing us to infer that the BKCa channels in aged vessels have decreased functionality or exhibit lower expression.

In this study, we found that BKCa activation with rottlerin decreased responsiveness to thromboxane, however this was only in young vessels. It has been shown in previous research that the aging vasculature has decreased BKCa functionality or decreased expression (Carvalho-de-Souza et al., 2012). The increased vasoconstriction response seen in aged vessels may also be attributed to the lack of expression and functionality of this vital channel. A probable reason that rottlerin was unable to reduce the vasoconstriction response of aged vessels is due to the decreased expression of this channel in aged vessels. Future studies will need to quantify the expression of the BKCa channel in aged vs. young vessels.

Scavenging mitochondrial ROS with MitoTEMPO also decreased the contractile response of aging vasculature to thromboxane. One possible explanation for the significant decrease in contraction in aging vasculature with MitoTEMPO is the increase in reactive oxygen species (ROS) found within aging vessels. ROS causes oxidative stress within cells by inactivating nitric oxide (NO), contributing to morphological changes, including plaque formation (Creager, et al., 2003). MitoTEMPO acts as a ROS scavenger, decreasing cellular and mitochondrial ROS and increasing the bioavailable NO (Dikalova, et al., 2010). Enhancing basal NO signaling may engage NO-dependent signaling pathways to counteract contractile responses. Another possibility is that elevated ROS can damage or modulate activity of proteins involved in vasoreactivity. ROS could damage smooth muscle dilatory signals such as PKG and myosin phosphatase, as well as pro-dilatory K^+ channels. In addition, ROS can impair specific ion channel functions (i.e. calcium channels), in some cases making them more leaky, which could

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potentially enhance contractile signaling by elevating intracellular Ca^{++}. Finally, ROS may be required for contractile signaling. Interestingly, acute MitoTEMPO treatment was able to reduce thromboxane-dependent constriction, indicating that ROS-dependent protein modifications are dynamic, or that ROS-dependent acute signaling is important. Nevertheless, our data demonstrates that ROS leads to a decrease in the contractile response.

Sodium nitroprusside (SNP) is a well-known vasodilator and stimulates dilation by donating nitric oxide, a physiological vasodilator (Cogolludo, et al., 2001). In the presence of SNP, young and aged aortic rings were expected to vasodilate. This vasodilatory response was expected to be increased in the presence of MitoTEMPO and rottlerin in aged and young mice, respectively. However, our results showed that there was no significant difference in the relaxation response in the presence of MitoTEMPO or rottlerin compared to controls in both aged and young vessels. In fact, aged mice treated with MitoTEMPO relaxed up to 85% from their maximum thromboxane response, but the relaxation response was delayed compared to controls. This means that higher doses of SNP were required to achieve the same relaxation response. This may indicate that, as with the contractile response, some level of ROS is required for proper vasodilation. Furthermore, aged and young vessels treated with rottlerin showed no significant increase in the relaxation response in the presence of SNP. Similarly to the response seen in aged vessels treated with MitoTEMPO, aged vessels treated with rottlerin exhibited non-significant trends for reduced relaxation responses compared to controls. One potential explanation for this lack of effect is that the BKCa channels present are already being maximally activated with NO. Alternatively, the decreased BKCa channel expression in aged vasculature may simply mean that there is not a sufficient number of channels to significantly alter vasomotion when activated. In either case, our data showed that BKCa channel activation with rottlerin was not successful in promoting relaxation in aged vessels.

Clinically, the goal in pharmacologically modulating vessel function is to decrease contraction and increase the SNP-dependent relaxation response. This combination of changes would effectively counteract the detrimental vascular symptoms that occur in aging, hypertension, heart disease, diabetes, and more. Though initially expected to decrease the contractile response in aged vessels and increase the relaxation response, MitoTEMPO and rottlerin produced unexpected results that limit their clinical applicability. MitoTEMPO decreased the contractile response in aged vessels, but this positive result was coupled with a

negative tradeoff in the form of decreased relaxation responses. As a result of the decreased relaxation response, MitoTEMPO and rottlerin are ineffective treatments to counteract the effects of aging.

Interestingly, aging vasculature exhibits many of the structural and functional changes that diabetic vasculature exhibits. Vascular disease in diabetic patients is often associated with decreased levels of nitric oxide and malformation of the BKCa channel (Zimmermann, et al., 1997). Activation of calcium-activated potassium channels using known BKCa activators is expected to restore the vasodilatory function of blood vessels in diabetic mice. Additionally, increasing nitric oxide sensitivity in diabetic mice would combat the decrease in BKCa function, revealing another therapeutic target to improve vascular function. A future direction of this research is to understand how BKCa activators and ROS scavengers will affect the functionality of diabetic vasculature. Future experiments completed at the Cardiovascular Research Center at Rhode Island Hospital will use the results gathered in these experiments as wild type measurements for their studies analyzing the impact BKCa activators and ROS scavengers have on diabetic vessels.

It is important to note that our study does have its limitations. First, these results are taken from experiments on mouse models. While the anatomy and physiology of a mouse is very similar to that of a human, these results may not translate accurately into the human system. Second, the aged mice used in this study were between one and two years of age. In humans, aging reaches a much greater magnitude and the aging processes are distinctly different and occur over a much longer time. Because of this, targeting certain aging processes and phenotypes in mice may not have the same effects in humans. Finally, the vessel of choice for these experiments was the aorta, which is a large conduit vessel. In reality, the major drivers of vascular resistance are the small arterioles. Therefore, the results found in isolated aortas may not mirror the actual physiological changes that would occur in the peripheral vasculature and in the environment of an entire cardiovascular system.

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APPENDIX

Aortic Ring Study Protocol

1. Prepare perfused tissue baths:
    a. Turn on water heater to 37°C.
    b. Open Oxygen tank and adjust flow rate to a slow bubble.
    c. Turn on vacuum and ensure waste beakers are empty.
    d. Turn on circulator to run 1-2 L of milli-Q H2O through baths.
    e. After wash is completed, run Krebs-Heinseleit solution through baths for 5 minutes and then turn off circulator.
2. Prepare data collection software.
    a. Open Lab Chart 7.
    b. Calibrate force transducer using 5 g weight.
3. Anesthetize the mouse with a combination of ketamine (80 mg/kg) and Xylazine (5 kg/mg) via IP injection.
4. Ensure disappearance of pain response by toe pinch.
5. Surgically dissect aorta from chest cavity of mouse.
6. Place aorta in ice-cold, oxygenated Krebs-Heinseleit buffer.
7. Remove perivascular adipose tissue (fat) from aorta using forceps and surgical scissors.
8. Use a razor blade to cut aorta into 2.5-3 mm lengths.
9. Place vascular rings on 2 metal hooks (d=0.05 mm).
10. Attach hooks to aortic ring set-up.
11. Turn on perfusion.
12. Equilibration period
    a. Every ten minutes, increase the tension applied to hooks manually by 0.1 g until 0.4 g of tension is applied to each ring.
13. Turn off perfusion and apply 60 mM of KCl to each bath. Allow to sit for 10 minutes while the contractile response of each ring is measured on Lab Chart 7.
14. Turn on perfusion and allow to wash for 10 minutes.
15. Turn off perfusion. Add the following:
    a. Ring 1: 10 uM MitoTEMPO

    b. Ring 2: 1 uM rottlerin

    c. Ring 3 and 4: Controls (DMSO)

16. Allow the treatments to sit for 10 minutes.

17. Add the following doses of U46619 (contractile agonist) to each bath every two minutes. Note the addition of each dose by inserting a "note" into Lab Chart 7.

    a. 1×10^{-9} M

    b. 1×10^{-8} M

    c. 3×10^{-8} M

    d. 6×10^{-8} M

    e. 1×10^{-7} M

18. Allow the final treatment to sit for 10 minutes.

19. Add the following doses of SNP (relaxation agonist) to each bath every two minutes. Not the addition of each dose by inserting a "note" into Lab Chart 7.

    a. 1×10^{-9} M

    b. 1×10^{-8} M

    c. 1×10^{-7} M

    d. 1×10^{-6} M

    e. 1×10^{-5} M

20. Allow the final treatment to sit for 10 minutes.