Dietary rapamycin supplementation reverses age-related vascular dysfunction and oxidative stress, while modulating nutrient-sensing, cell cycle, and senescence pathways

Lisa A. Lesniewski, Douglas R. Seals, Ashley E. Walker, Grant D. Henson, Mark W. Blimline, Daniel W. Trott, Gary C. Bosshardt, Thomas J. LaRocca, Brooke R. Lawson, Melanie C. Zigler and Anthony J. Donato

1Division of Geriatrics, Department of Internal Medicine, Salt Lake City, UT, USA
2Veteran’s Affairs Medical Center-Salt Lake City, Geriatrics Research Education and Clinical Center, Salt Lake City, UT, USA
3Department of Exercise and Sports Science, University of Utah, Salt Lake City, UT, USA
4Department of Integrative Physiology, University of Colorado Boulder, Boulder, CO, USA
5Department of Biochemistry, University of Utah, Salt Lake City, UT, USA

Summary

Inhibition of mammalian target of rapamycin, mTOR, extends lifespan and reduces age-related disease. It is not known what role mTOR plays in the arterial aging phenotype or if mTOR inhibition by dietary rapamycin ameliorates age-related arterial dysfunction. To explore this, young (3.8 ± 0.6 months) and old (30.3 ± 0.2 months) male B6D2F1 mice were fed a rapamycin supplemented or control diet for 6–8 weeks. Although there were few other notable changes in animal characteristics after rapamycin treatment, we found that glucose tolerance improved in old mice, but was impaired in young mice, after rapamycin supplementation (both P < 0.05). Aging increased mTOR activation in arteries evidenced by elevated 56K phosphorylation (P < 0.01), and this was reversed after rapamycin treatment in old mice (P < 0.05). Aging was also associated with impaired endothelium-dependent dilation (EDD) in the carotid artery (P < 0.05). Rapamycin improved EDD in old mice (P < 0.05). Superoxide production and NADPH oxidase expression were higher in arteries from old compared to young mice (P < 0.05), and rapamycin normalized these (P < 0.05) to levels not different from young mice. Scavenging superoxide improved carotid EDD in untreated (P < 0.05), but not rapamycin-treated, old mice. While aging increased large artery stiffness evidenced by increased aortic pulse-wave velocity (PWV) (P < 0.01), rapamycin treatment reduced aortic PWV (P < 0.05) and collagen content (P < 0.05) in old mice. Aortic adenosine monophosphate-activated protein kinase (AMPK) phosphorylation and expression of the cell cycle-related proteins PTEN and p27kip were increased with rapamycin treatment in old mice (all P < 0.05). Lastly, aging resulted in augmentation of the arterial senescence marker, p19 (P < 0.05), and this was ameliorated by rapamycin treatment (P < 0.05). These results demonstrate beneficial effects of rapamycin treatment on arterial function in old mice and suggest these improvements are associated with reduced oxidative stress, AMPK activation and increased expression of proteins involved in the control of the cell cycle.

Key words: aging; AMPK; arterial stiffness; endothelial function; mTOR; oxidative stress; rapamycin.

Introduction

Aging is a primary risk factor for cardiovascular diseases (CVDs) (D’Agostino et al., 2008), the leading cause of death in the USA (Mozaffarian et al., 2015). CVDs are primarily diseases of the arteries and are associated with reduced endothelial function and augmented large artery stiffening (Yeboah et al., 2007; Mitchell et al., 2010). It is well established that both of these characteristics are observed even in healthy older adults (Mitchell et al., 2004; Seals et al., 2010) and that they are independent predictors of future CVD diagnosis (Yeboah et al., 2007; Rossi et al., 2008). Although the initiating events of arterial aging are still unknown, several critical factors have been established, including reduced nitric oxide (NO) bioavailability, increased arterial oxidative stress, and increased arterial fibrosis.

Oxidative stress, marked by excess superoxide, is known to be an important contributor to age-associated arterial dysfunction as a result of the scavenging of NO by superoxide. This results in blunting of endothelial-mediated vasodilation and augmentation of large artery stiffness (Donato et al., 2013). There is a shift in redox balance in arteries and endothelial cells from old mice and humans that results from enhanced superoxide production, due to augmented activity of cytosolic enzymes such as NADPH oxidases (NOX), and inadequate upregulation of cellular antioxidant defenses (Donato et al., 2007, 2013). Excessive superoxide-associated oxidative stress leads to stiffening of large elastic arteries (Fleenor et al., 2014) with aging by producing changes in the major structural proteins (collagen and elastin) (Henson et al., 2014). This fibrotic state is exacerbated by age-related glucose intolerance contributing to postprandial hyperglycemia, which increases endothelial oxidative stress and enhances advanced glycation end product-associated cross-linking of collagen (Avendano et al., 1999; Goldin et al., 2006). Collectively, these mechanisms play an important role in age-related arterial dysfunction.

Nutrient-sensing molecular pathways, such as adenosine monophosphate-activated protein kinase (AMPK), sirtuins, and mammalian target of rapamycin (mTOR) are closely related to overall metabolic function (Howell & Manning, 2011; Medvedik et al., 2007), as well as in the arterial aging phenotype (Lesniewski et al., 2012; Donato et al., 2013). Although studies examining the direct effects of AMPK and sirtuin-1 activation have revealed selective beneficial effects on arterial
endothelial function in aged mice (Lesniewski et al., 2012; Gano et al., 2014), less is known about the effects of mTOR inhibition on age-associated arterial dysfunction.

Importantly, inhibition of mTOR by dietary rapamycin has been recently demonstrated to delay age-associated diseases and extend lifespan in mice (Harrison et al., 2009; Wilkinson et al., 2012). Moreover, lifespan extension was also seen when dietary treatment was started in middle age (Harrison et al., 2009). While the mechanisms underlying the beneficial effects of rapamycin treatment and mTOR inhibition are incompletely understood, it is clear that signaling through the mTOR pathway modulates numerous cell cycle proteins, which may have beneficial effects in a variety of tissues. Furthermore, in conditions such as diabetic kidney disease, mTOR inhibition has been demonstrated to decrease NOx expression and reduce superoxide/reactive oxygen species (ROS) generation (Eid et al., 2013). Taken together, these studies suggest that dietary rapamycin has substantial promise to counteract mechanisms responsible for arterial aging. However, the impact of mTOR inhibition by dietary rapamycin on arterial function in aged animals remains unknown.

In this study, we used an established mouse model of arterial aging to assess the role of mTOR activity in endothelial dysfunction and large artery stiffening with advancing age. We hypothesized that age-associated increases in arterial mTOR activation would be associated with impairments in vascular function (i.e. reduced endothelium-dependent dilation and increased large artery stiffness), increased oxidative stress and reduced NO, increased cellular oxidant enzymes, decreased AMPK activation, and increased extracellular matrix fibrosis in the large arteries. To investigate the role of mTOR activity further, we hypothesized that dietary inhibition of mTOR with rapamycin in old mice would ameliorate this arterial aging phenotype.

Results

Animal characteristics

Body mass, tissue mass, and mean arterial pressures for young and old untreated and rapamycin-treated mice can be found in Table 1 (N = 6–15/group). Because rapamycin is clinically used as an immunosuppressant, we assessed the complete blood count (CBC) and differential counts in peripheral blood collected at sacrifice from old untreated and rapamycin-treated mice. We found that rapamycin did not impact total white blood cell, differential, hemoglobin, hematocrit, or platelet counts (Table S1, Supporting information). Spleen mass, measured at sacrifice, was reduced with advancing age and after rapamycin treatment in young mice, but increased after rapamycin in old mice (Table 1). To determine whether these changes in mass were the result of tissue congestion, the dry weight of the spleen was calculated and expressed as a percent of tissue wet weight. Percent of dry weight of the spleen was reduced by aging (young: 14.3 ± 1.2% vs. old: 10.9 ± 0.9%, P < 0.05) and rapamycin treatment in young mice (young rap: 11.0 ± 0.8%, P < 0.05), suggesting tissue congestion in these groups. However, rapamycin increased dry weight of the spleens from old mice (old rap: 14.0 ± 0.9%, P < 0.05 vs. old untreated) and may be indicative of reduced congestion or increased cellularity in this tissue.

Glucose tolerance was impaired in old untreated compared to young untreated mice (P < 0.05), evidenced by a greater area under the curve for glucose (AUCglyc) during the GTT (Table 1). Rapamycin treatment improved glucose tolerance, that is resulted in a reduction in the AUCglyc (P < 0.01) in old mice (Table 1). In contrast, rapamycin impaired glucose tolerance in young mice such that there was an increased AUCglyc (P < 0.05) during the GTT. Blood glucose was lower, and plasma insulin was higher in the old compared to young untreated mice (Table 1). Although HOMA-IR% was not different, HOMA-B% was higher in old compared to young untreated mice (Table 1). However, blood glucose, plasma insulin, HOMA-IR%, and HOMA-B% were not impacted by rapamycin treatment in the young or old mice (Table 1). GTT responses are provided in Fig. S1 (Supporting information).

Enhanced arterial mTOR activation with aging is reversed after rapamycin treatment

Although total protein expression of mTOR target S6K, assessed by Western blotting, did not differ with aging or rapamycin treatment, phosphorylation of S6K, a marker of activation, was higher in aorta of untreated old compared to young untreated mice (P < 0.01) (Fig. 1). Dietary rapamycin

Table 1 Age, body and tissue mass, blood pressure and blood glucose, plasma insulin, and homeostatic model assessment of beta-cell function (HOMA-B) and insulin resistance (HOMA-IR) in untreated and rapamycin-treated young and old mice

|                      | Young | Old       | Young Rap | Old Rap       | N's   |
|----------------------|-------|-----------|-----------|---------------|------|
| Age (months)         | 3.8 ± 0.6 | 30.3 ± 0.2† | 4.0 ± 0.0 | 30.3 ± 0.2† | 9–15 |
| Body mass (g)        | 32.5 ± 1.6 | 35.6 ± 1.4 | 29.3 ± 0.4 | 36.9 ± 1.3† | 8–15 |
| Gastrocnemius mass (mg) | 243 ± 23 | 150 ± 8† | 170 ± 2† | 139 ± 5† | 7–12 |
| Heart mass (mg)      | 157 ± 10 | 202 ± 7† | 134 ± 4† | 209 ± 6† | 7–12 |
| Liver mass (g)       | 1.61 ± 0.06 | 1.97 ± 0.07† | 1.21 ± 0.04† | 1.87 ± 0.08† | 7–12 |
| Spleen mass (mg)     | 82 ± 5 | 75 ± 13† | 64 ± 21 | 116 ± 21†‡ | 6–8 |
| Epididymal WAT mass (mg) | 679 ± 99 | 327 ± 73 | 456 ± 23 | 576 ± 102 | 7–12 |
| Mean arterial pressure (mmHg) | 109 ± 3 | 101 ± 5 | 101 ± 4 | 95 ± 3 | 7–8 |
| AUCglyc (%fasted)    | 11210 ± 365 | 12410 ± 388‡ | 14620 ± 1127† | 11241 ± 333‡ | 8–12 |
| Fed blood glucose (mg dl⁻¹) | 142 ± 3 | 125 ± 6† | 134 ± 3 | 115 ± 9† | 8–13 |
| Fed plasma insulin (µU mL⁻¹) | 23.8 ± 4.3 | 47.3 ± 7.7† | 30.8 ± 2.9 | 39.0 ± 5.1 | 7–10 |
| HOMA-IR (%)          | 4.4 ± 0.5 | 5.5 ± 0.6 | 4.2 ± 0.4 | 5.1 ± 0.7 | 7–10 |
| HOMA-B (%)           | 110.8 ± 10.9 | 175.5 ± 20.4‡ | 117.5 ± 8.4 | 180.6 ± 7.9† | 7–10 |

Rap, rapamycin treated, WAT, white adipose tissue.
N = 6–15/group.
Data are means ± SEM, P < 0.05.
†Denotes difference from young.
‡Denotes difference from untreated old.

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treatment reduced S6K activation relative to both young ($P < 0.05$) and old untreated ($P < 0.01$) mice (Fig. 1).

**mTOR inhibition in old mice by dietary rapamycin ameliorates oxidative stress-mediated endothelial dysfunction**

To determine the impact of aging and rapamycin treatment on endothelial function and NO bioavailability, carotid arteries were excised and cannulated in the stage of an inverted microscope and dose responses to acetylcholine (ACH), an endothelium-dependent dilator (EDD), and sodium nitroprusside, an endothelium-independent vasodilator, were performed. EDD to ACH was reduced in carotid arteries of old compared to young mice ($P < 0.05$). This was the result of reduced NO bioavailability as evidenced by the loss of the age-associated differences in dilation after nitric oxide synthase inhibition by L-NAME (Fig. 2A). Dietary rapamycin treatment improved EDD ($P < 0.01$) and NO bioavailability in old mice compared to untreated old mice and restored these measurements to those of young controls (Fig. 2B). Rapamycin treatment was without effect on EDD or NO bioavailability in young mice (Fig. 2C). There were no differences in endothelium-independent dilation to sodium nitroprusside (SNP) with aging or rapamycin treatment in either young or old mice (Fig. 2D). There were no differences in total aortic eNOS protein expression with aging or rapamycin treatment in old mice (Fig. 2E). Still, aging tended to decrease ($P = 0.1$) and rapamycin tended to increase ($P = 0.1$ vs. old) phosphorylation of eNOS at the activating site, ser1177, in old mice (Fig. 2E).

Maximal dilation to ACh was reduced with aging ($P < 0.05$) (Fig. 3A). Both dietary rapamycin and in vitro treatment with the superoxide dismutase mimetic, TEMPO, restored maximal dilation in old mice (both $P < 0.05$) (Fig. 3A). TEMPO treatment of carotid arteries from young untreated and rapamycin-treated mice was without effect on maximal dilation (Fig. 3A). In contrast to untreated old mice, in vitro treatment of carotid arteries with TEMPO did not improve maximal dilation in arteries from old rapamycin-treated mice (Fig. 3A). These findings indicate that increased oxidative stress underlies the observed reductions in EDD with aging and suggest that rapamycin improves EDD by reducing oxidative stress.

L-NAME treatment of isolated arteries removed all group and treatment differences (Fig. 3A), indicating that the impairments with aging and improvements in dilation after rapamycin or TEMPO in old mice result from alterations in NO bioavailability downstream of increased oxidative stress. Superoxide production, measured by electron paramagnetic resonance (EPR) (Fig. 3B), and nitrotyrosine abundance (Fig. 3C), markers of oxidative stress, were increased in the aorta with aging ($P < 0.05$), and this was reversed after dietary rapamycin treatment ($P < 0.05$). The protein expression of the oxidant enzyme NOX4 tended to be increased with aging ($P = 0.10$) and reduced after rapamycin ($P = 0.07$) (Fig. 3D). Likewise, gene expression for another NADPH oxidase isoform, NOX2, was increased with advancing age ($1.0 \pm 0.4$ vs. $12.1 \pm 8.1$ AU, $P < 0.05$) and reduced after rapamycin treatment in aortas from old ($0.5 \pm 0.2$ AU, $P < 0.05$ vs. old), but not young ($1.7 \pm 0.4$ AU) mice. Although not different with aging, the expression of the antioxidant extracellular superoxide dismutase (ecSOD) was increased in the aortas of old mice after dietary rapamycin compared to both young and old untreated mice ($P < 0.01$) (Fig. 3E).

**Dietary rapamycin reduces large elastic artery stiffness in old mice and selectively modifies arterial wall composition**

Large elastic artery stiffness, assessed by in vivo arterial pulse-wave velocity (PWV), was increased with aging ($P < 0.001$) and was improved ($P < 0.05$ vs. old), but not restored to young values ($P < 0.01$ vs. young), in old mice after rapamycin treatment (Fig. 4A). Rapamycin treatment was without effect on PWV in young mice (Fig. 4A). The intima-media area of the aorta was increased in old mice ($P < 0.05$), but rapamycin treatment had no effect on intima-media area in young or old mice (Fig. 4B).

To determine whether the structural changes underlie changes in large artery stiffness with aging and rapamycin, aortic collagen (assessed by picrorhiosin red stain), elastin (assessed by Verhoff’s Van Geison stain, advanced glycation end products (AGEs), assessed by immunohistochemistry), and calcification (assessed by Von Kossa staining) were assessed on histological aortic sections. With aging in the aorta, there was increased collagen ($P < 0.01$) (Fig. 4C), decreased elastin ($P < 0.01$) (Fig. 4D), and increased abundance of AGEs ($P < 0.01$) (Fig. 4E). There was no evidence of calcification of the aorta with aging (data not shown).

In old mice, dietary rapamycin reduced aortic collagen ($P < 0.05$) (Fig. 4C), but was without effect on elastin content (Fig. 4D). In contrast, in young rapamycin-treated mice, collagen was higher and elastin was lower in the aorta compared to untreated young mice (Fig. 4C,D), despite there being no differences in PWV. AGEs were reduced ($P < 0.05$) in aortas of old, but not young mice after dietary rapamycin treatment (Fig. 4E).

**mTOR inhibition increases AMPK activation and cell cycle regulatory proteins and decreases a marker of senescence in aorta of old mice**

Phosphorylation, but not total protein expression, of AMPK was lower in aorta of old compared to young mice ($P < 0.05$, Fig. 5). Dietary rapamycin treatment in old mice did not impact total AMPK protein expression, but increased phosphorylation of AMPK ($P < 0.01$)
**Discussion**

The novel findings of the present study are that (a) compared with young mice, mTOR activation was increased in the large arteries of old mice and was associated with vascular oxidative stress, impaired endothelial function, increased large elastic artery stiffness, decreased AMPK activation, and increased senescence marker p19, but no change in the expression of cell cycle-related proteins, PTEN or p27kip, and (b) dietary rapamycin treatment of old mice reversed the arterial aging phenotype and was associated with decreased oxidative stress, increased arterial AMPK activation, increased expression of PTEN and p27kip, and decreased senescence marker p19. We also provide evidence that although rapamycin can lead to reductions in glucose tolerance in young although rapamycin can lead to reductions in glucose tolerance in young mice, mTOR activation was increased in the large arteries of old mice and that mTOR inhibition is associated with reduced expression of both of these NOX isoforms in old mice. This is similar to changes reported for isoforms of NADPH oxidase after CR (Rippe et al., 2010). We
Rapamycin and AMPK activation

AMPK activation, in response to low cellular energy status, leads to a reduction in mTOR signaling via inhibition of S6K (Gwinn et al., 2008). Likewise, inhibition of mTOR after rapamycin treatment of MCF-7 breast cancer cells leads to increased AMPK activation, indicating a reciprocal relation between these energy-sensing pathways (Zakikhani et al., 2010). In addition to effects on metabolism, AMPK activation increases eNOS activation in cultured endothelial cells, suggestive of a

Rapamycin and beneficial effects of CR

Here, we find that dietary rapamycin improves, but does not completely normalize, aortic stiffness in old mice. This reduction in arterial stiffness was associated with a rapamycin-induced decrease in collagen and AGEs in the aorta in the absence of changes in intima-media thickness or aortic elastin content. The findings of the present study are consistent with a ‘CR mimetic’ effect of rapamycin on age-associated large elastic artery stiffening, and an incomplete phenocopy may be due to the shorter length of treatment compared to lifelong CR. Furthermore, the reduction in AGES observed in the present study suggests that the beneficial effects of rapamycin on large elastic artery stiffness may be mediated, at least in part, by a reduction in the cross-linking of collagens by AGES, an effect that may be related to the improved glucose metabolism after rapamycin treatment in old mice (Beisswenger et al., 1993).
vasoprotective effect (Murakami et al., 2006). Our laboratory has previously demonstrated that pharmacological activation of AMPK, via in vivo administration of AICAR, reverses superoxide-mediated suppression of endothelium-dependent dilation in the carotid arteries of old mice, although this effect did not result from an increase in NO bioavailability, but rather from increased reliance on another endothelial vasodilator, endothelium-derived hyperpolarizing factor (EDHF) (Lesniewski et al., 2012). Here, we demonstrate the cross talk between mTOR inhibition and AMPK activation in arteries, with rapamycin treatment of old mice leading to increased arterial AMPK activation. However, unlike direct pharmacological activation of AMPK by AICAR, increased vasodilation in old mice after rapamycin resulted from increased NO bioavailability. Still, the beneficial effects of both rapamycin and AICAR on arterial endothelial vasodilation appear to be predominately mediated through a reduction in the suppression of vasodilation by oxidative stress. This effect to reduce oxidative stress and improve vasodilation via either AICAR or rapamycin occurs regardless of the endogenous vasodilator (i.e. NO or EDHF) being impacted.

Rapamycin, cell fate, and senescence

Advancing age is associated with cellular senescence (Herbig et al., 2006). Here, we demonstrate that although aging did not impact the expression of the tumor suppressor, PTEN, or the cell cycle inhibitor, p27kip, their expressions were increased after rapamycin treatment. PTEN is a negative regulator of protein kinase B (AKt) signaling with antiproliferative effects and a reduction in PTEN-mediated inhibition of AKt in old mice may underlie the reduction in p27kip (Sun et al., 2014). p27kip is a member of the Cip/kip family of cyclin-dependent kinase inhibitors that act to block progression through the G1/S transition of the cell cycle (Pestell et al., 1999). In addition, we found that rapamycin reversed the age-associated increase in p19, a cyclin-dependent kinase inhibitor that is associated with cellular senescence. Taken together, these results indicate that rapamycin treatment in old mice may reduce growth signaling in arteries and perhaps favor the clearing of dysfunctional senescent cells/tissue, which could in part explain the improvements in arterial vasodilation and stiffness we observed.

Differential effects of rapamycin on glucose/insulin metabolism in young and old

Although not a primary outcome of our study, our results are consistent with those of previous investigations (Lamming et al., 2012; Fang et al., 2013), indicating that short-term rapamycin treatment in young mice leads to glucose and insulin intolerance. In contrast, long(er) term
Fig. 5 Expression and activation of adenosine monophosphate-activated protein kinase (AMPK) and cell cycle-related proteins in aortas of young and old untreated and old rapamycin-treated mice. (A) Total (open bars) and the ratio of phosphorylated (P-) to total (hashed bars) AMPK in aortas excised from young and old untreated and rapamycin-treated mice \((N = 5–6/group)\). Expression of (B) phosphatase and tensin homolog, PTEN, and (C) the cyclin-dependent kinase inhibitor, p27kip, in aortas excised from young and old untreated and rapamycin-treated mice \((N = 5–6/group)\). Expression was normalized to GAPDH and blot images are provided in panel (D). * denotes difference from young, and †denotes difference from old. Differences were assessed by one-way ANOVA with LSD post hoc. Data are means ± SEM, \(P \leq 0.05\).

**Rapamycin/mTOR inhibition: a potential CR mimetic?**

Treatment with rapamycin, from 20 weeks up to 2 years, improved metabolic function and reduced adiposity in mice (Harrison et al., 2009; Anisimov et al., 2011; Fang et al., 2013). Here, we find that short-term rapamycin treatment of old mice improves glucose tolerance. In young mice, rapamycin treatment increased collagen and decreased elastin in the aortas, and although such differences should act to increase large elastic artery stiffness, these structural changes were not sufficient to change aortic PWV. When applied to our young mice, the findings of Fang et al. (2013) suggest that the deleterious effects of short-term rapamycin treatment on glucose-insulin metabolism would resolve if treatment were sustained. However, it is not known if a longer period of treatment in young mice might also lead to potentially deleterious changes in arterial structure or function. Although earlier reports demonstrating lifespan extension and beneficial metabolic effects of long-term rapamycin treatment (initiated in middle age) (Harrison et al., 2009; Miller et al., 2014) argue against a deleterious effect of prolonged treatment, no information is available on other biomarkers, including vascular function.

**Summary and future directions**

Age-associated vascular dysfunction is an important contributing factor to increased CVD risk in older adults. Our results indicate that increased mTOR activation may underlie vascular oxidative stress, endothelial dysfunction, increases in large elastic artery stiffness, and structural adaptations in the vessel wall with advancing age. Dietary rapamycin treatment reversed these deleterious arterial phenotypes and increased the expression of proteins involved in the control of the cell cycle. Our findings suggest that treatment with rapamycin or other ‘rapalogs’ holds promise for the treatment of arterial aging and, therefore, the potential prevention of age-associated CVD. Future studies should explore the role of the individual mTOR complexes in age-associated vascular dysfunction, as well as the impact of rapamycin on autophagy and cellular senescence.

**Experimental procedures**

**Ethical approval**

All animal procedures conformed to the Guide to the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 2010) and were approved by the University of Colorado at Boulder, University of Utah and VAMC-SLC Animal Care and Use Committees.
Animals

Young male B6D2F1 mice were obtained from Charles River Inc, and old male mice were purchased from the aging colonies maintained at Charles River Inc. for the National Institute on Aging. All mice were housed in standard mouse cages in an animal care facility at the VAMC-SLC or at the University of Colorado at Boulder on a 12-h:12-h light:dark cycle. Mice were fed either a custom control (Purina 5LG6/122 PPM Eudragit 3/8 Pellet, Test Diet) or rapamycin supplemented diet (14 mg kg⁻¹ diet in Purina 56L6/122 PPM Eudragit 3/8 Pellet, Test Diet) for 6 weeks, as described by Harrison et al. (2009). Assuming a daily food intake of approximately 5 g day⁻¹ and an approximate 30-g mouse, this yields a dose of 2.24 mg rapamycin kg⁻¹ body weight day⁻¹. Food and water were supplied ad libitum. Glucose tolerance was assessed by intraperitoneal glucose tolerance test (GTt) as previously described (Lesniewski et al., 2007; Donato et al., 2012). Briefly, in the morning after a 2-h fast, blood glucose was collected from a tail nick (5 μl) and assessed with a Precision Xceed Pro Glucose Analyzer. Glucose was then administered (2 g glucose kg⁻¹ body weight), and blood glucose was measured in whole blood at times 15, 30, 45, 60, and 90 min after injection. Area under the curve for glucose (AUCglc) during the GTt was calculated. Prior to tissue harvest, mice were euthanized via exsanguinations by cardiac puncture while under isoflurane anesthesia (Donato et al., 2009; Durrant et al., 2009). Whole blood from untreated and rapamycin-treated old mice collected at sacrifice was used for total white blood cell and differential assessment. To do so, a standard veterinary differential was performed with 100 WBC counted and types identified thereby giving a percent for each type of WBC.

Ex vivo arterial endothelium and vascular smooth muscle assessment

To assess endothelial function, carotid arteries were excised, cleared of surrounding tissue and cannulated in the stage of a pressure myograph (DMT Inc, Atlanta, GA, USA). Arteries were preconstricted with 2 μmol phenylephrine, and endothelium-dependent dilation and the NO contribution to dilation were measured in response to the cumulative addition of acetylcholine (1 × 10⁻⁹ to 1 × 10⁻⁴ mol L⁻¹) in the absence or presence of the nitric oxide synthase inhibitor, L-NAME (0.1 mmol L⁻¹, 30 min), as described previously (Durrant et al., 2009). To assess superoxide-mediated suppression of endothelial function, acetylcholine dose responses in the absence and presence of L-NAME were performed in the contralateral carotid artery after incubation with the superoxide dismutase mimetic, TEMPOL (1 mmol/L, 1 h). Endothelium-independent dilation was assessed in response to sodium nitroprusside (1 × 10⁻⁹ to 1 × 10⁻⁴ mol L⁻¹) (Durrant et al., 2009). Vessel diameters were measured by MyoView software (DMT, Inc., Atlanta, GA, USA). All data−response data are presented as percent of possible dilation after preconstriction to phenylephrine. Arteries failing to achieve ≥20% preconstriction were excluded. Sensitivity was defined as the concentration of ACh or SNP that elicited 50% of the maximal response (IC50). Sensitivities (IC50) were calculated using BioDataFit 1.02. A regression was used to fit a sigmoidal model to individual dose responses yielding a dose for the half maximal response in log M units.

Superoxide production

Production of superoxide was measured by electron paramagnetic resonance (EPR) spectrometry using the spin probe 1-hydroxy-3-methoxy carbonyl-2,2,5,5-tetramethylpyrroline (CMH, Alexis Biochemicals). Stock solutions of CMH were prepared in ice-cold deoxygenated Krebs–HEPES buffer (mmol L⁻¹: NaCl, 99.01, KCl 4.69, CaCl₂ 2.50, MgSO₄ 1.20, K₂HPO₄ 1.03, Na₂ICO₃ 25.0, glucose 11.10, and Na₂HEPES 20.00; pH 7.4) containing 0.1 mmol L⁻¹ diethylenetriaminepenta- acetic acid and 5 μmol L⁻¹ sodium diethylthioclormate and pretreated with Chelex (Sigma, Saint Louis, MO, USA) to minimize auto-oxidation of the spin probe. Three-millimeter aortic rings were washed once in PSS and again in modified Krebs–HEPES buffer. Rings were then incubated for 60 min at 37 °C in 200 μL Krebs–HEPES buffer containing 0.5 mmol L⁻¹ CMH and analyzed immediately on an MS300 X-band EPR spectrometer (Magnettech, Berlin, Germany). Instrument settings were as follows: microwave frequency 9.83 Ghz, centerfield 3480 G, sweep 80 G, modulation amplitude 3.3 G, microwave power 40 mW, microwave attenuation 7, and receiver gain 30. A total of six sweeps were conducted lasting 8.7 s per sweep. The running average of the six sweeps was collected with the double integration (area under and over the baseline) of the triplet used to display the magnitude of the signal. The magnitude of this signal directly relates to the amount of superoxide that has been trapped by the CMH.

Pulse-wave velocity

To assess large artery stiffening, aortic PWV was measured as described previously (Henson et al., 2014). Briefly, mice were anesthetized under 2% isoflurane in a closed chamber anesthesia machine (V3000 PK, Parkland Scientific, Coral Springs, FL) for ~1–3 min. Anesthesia was maintained via nose-cone and mice were secured in a supine position on a heating board (~35 °C) to maintain body temperature. Velocities were measured with 4-mm piezoelectric crystal, 20-MHz Doppler probes (Indus Instruments, Webster, TX, USA) at the transverse aortic arch, and ~4-cm distal at the abdominal aorta and collected using WinDAQ Pro+ software (DataQ Instruments, Akron, OH, USA). Absolute pulse arrival times were indicated by the sharp upstroke, or foot, of each velocity waveform analyzed with WinDAQ Waveform Browser (DataQ Instruments). Aortic pulse-wave velocity was then calculated as the quotient of the separation distance, assessed to the nearest half millimeter by engineering caliper (typically ~4 cm) and difference in absolute arrival times.

Western blots

Because both carotid arteries were used for functional measures and to provide adequate tissue for measurement of proteins, the thoracic aorta was excised and used for protein expression assays. Histological samples were saved as described below and the remaining aorta was cleared of perivascular adipose tissue while maintained in 4 °C PSS. Cleared aorta was then frozen in liquid nitrogen. Whole artery lysates were prepared as previously described (Durrant et al., 2009). Protein expression was assessed by standard Western blot procedures using primary antibodies against ribosomal S6K (56K, 1:1000; 32 kDa; Cell Signaling, Danvers, MA, USA), phosphorylated S6K (p-S6K, 1:1000; 32 kDa; Cell Signaling), phosphoAKT (Thr172 p-AMPK, 1:1000; 62 kDa; Cell Signal- ing), nitrotyrosine (NT, 1:100; 25/55/160 kDa; Abcam, Cambridge, MA, USA), phosphorylated eNOS (ser1177 p-eNOS, 1:1000; 140 kDa; BD Transduction, San Jose, CA, SUA), ser1177 phosphorylated eNOS (ser1177 p-eNOS, 1:1000; 140 kDa; Cell Signaling), nitrotyrosine (NT, 1:100; 25/55/160 kDa), Abcam, Cambridge, MA, USA), NOX4 (1:650; 67 kDa; Abcam), extracellular superoxide dismutase (ecSOD, 1:500; 31/35 kDa; Sigma), AMPK (1:500; 62 kDa; Cell Signaling), phosphorylated AMPK (Thr172 p-AMPK, 1:1000; 62 kDa; Cell Signaling), PTEN (1:1000; 54 kDa; Cell Signaling), p27kip (1:1000; 27 kDa; Cell Signaling) and GAPDH (1:1000; 37 kDa; Cell Signaling).

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appropriate HRP-conjugated secondary antibodies (Jackson Immunological, West Grove, PA, USA), and Supersignal ECL (Pierce, Rockford, IL, USA). Bands were visualized using a digital acquisition system (ChemiDoc-It, UVP, Upland, CA, USA or Bio-Rad ChemiDoc XRS+ with ImageLab Software) and quantified using ImageJ 1.42 software (NIH, Bethesda, MD, USA). To account for differences in protein loading, expression is normalized to GAPDH expression. Representative images are provided. The ratio of phosphorylated to total protein is calculated from the bands for a given sample lysate.

Quantitative PCR

mRNA expression for p19 and Nox2 was measured in lysed aortas by qRT–PCR using the QuantiTect Reverse Transcription kit (Qiagen, Inc., Valencia, CA, USA) and FastStart SYBR Green Master (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer’s protocols. Fold change in mRNA expression was calculated as the fold difference in expression of target mRNA to 18s rRNA for each animal (ΔCt = Ct(target) – Ct(18s)). 18s rRNA QuantiTect Primer Assay (Qiagen, Inc.). p19 mRNA primers: fwd-CCGAGGTCCCTTGTCACGTG and rev-TGTTCACGAAAGCGAGG; Nox2 mRNA primers: fwd-CCAGGAAACAGCTAATT and rev-CTAGCCGCTGTATTGGATCTT.

Histology

Two-millimeter rings of thoracic aorta with perivascular tissue intact were removed from the thoracic aorta directly distal to the greater curvature of the aortic arch and embedded in optimal cutting temperature (OCT) medium. Rings were sectioned (7 μm) and mounted on glass slides for histological analysis. Intima-media area was measured on Masson’s trichrome (HT15, Sigma) stained sections of aorta using ImageJ. Collagen was quantified by picrosirius red stain as described previously (Donato et al., 2013; Henson et al., 2014), and green channel images from a RGB stack were utilized for densitometric quantification with ImageJ (NIH, Bethesda, MA, USA). Elastin was quantified by Verhoff’s Van Geison stain as described previously (Donato et al., 2013; Henson et al., 2014), and 8-bit gray-scale images were utilized for densitometric quantification with ImageJ (NIH, Bethesda, MA, USA). Elastin was quantified by Verhoff’s Van Geison stain as described previously (Donato et al., 2013; Henson et al., 2014), and 8-bit gray-scale images were utilized for densitometric quantification with ImageJ (NIH, Bethesda, MA, USA). Elastin was quantified by Verhoff’s Van Geison stain as described previously (Donato et al., 2013; Henson et al., 2014), and 8-bit gray-scale images were utilized for densitometric quantification with ImageJ (NIH, Bethesda, MA, USA). Elastin was quantified by Verhoff’s Van Geison stain as described previously (Donato et al., 2013; Henson et al., 2014), and 8-bit gray-scale images were utilized for densitometric quantification with ImageJ (NIH, Bethesda, MA, USA).

Statistics

Repeated-measures analysis of variance (ANOVA) was performed to assess differences in Ach and SNP dose responses as well as for GTT curves. One-way ANOVAs were performed for all other analyses. Least-squares differences post hoc tests were performed where appropriate. Data are presented as mean ± SEM. Significance was set at P < 0.05.

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Conflict of interest

The authors have no conflict of interest to disclose.

Author contributions

LA Lesniewski, AJ Donato, and DR Seals contributed to all aspects of the study, including the conception and design, data collection and analysis, and manuscript preparation. TJ LaRocca, AE Walker, BR Lawson, ML Zigler, MW Blimline, GC Bosshardt, DW Trott, and GD Henson contributed to the collection and analysis of the data and revision of the manuscript.

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