TELOMERE DYSFUNCTION: ROLE IN ARTERIAL AGING AND HYPERTENSION

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

The University of Utah

December 2013

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The role of telomere dysfunction, defined as telomere shortening that leads to telomere uncapping, in arterial aging and hypertension is currently unexplored. Understanding the role that telomere dysfunction plays in arterial aging could lead to novel therapies and interventions designed to blunt, stop, or even reverse the arterial dysfunction that precedes cardiovascular diseases. Likewise, understanding the role that arterial telomere dysfunction plays in the pathogenesis of hypertension could ultimately produce therapies and interventions that treat or reverse high arterial blood pressure. To gain insight into the role of telomere dysfunction in arterial aging and hypertension, we first determined the association between telomere dysfunction, cellular senescence, and inflammation in aging and hypertension in human arteries. Next, we determined if arterial telomere uncapping, independent of telomere shortening, leads to cellular senescence and ensuing inflammation and oxidative stress, and subsequent arterial endothelial dysfunction, increased arterial blood pressure, and pulse wave velocity in a mouse model of telomere uncapping. Our findings demonstrate that arterial telomere uncapping occurs with advancing age and is associated with hypertension independent of telomere length. Furthermore, our results reveal that telomere uncapping leads to arterial dysfunction that is comparable

to that seen in arterial aging and hypertension. We believe these studies lay the clinical and mechanistic foundation for future studies aimed at establishing the prognostic value of telomere uncapping as a biomarker for CVDs.

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ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation for the guidance and friendship of my primary mentor, Anthony J Donato, and my co-mentor, Lisa A Lesniewski. Completion of this project was only possible because of their unwavering support of my ideas and goals. All experiments were performed in the Translational Vascular Physiology Laboratory at the University of Utah and Veteran's Affairs Medical Center-Salt Lake City, Geriatric Research Education and Clinical Center. Garrett Morgan and Anthony J Donato contributed to all aspects of the projects, including the conception and design of the experiments, collection, analysis, and interpretation of data, and drafting and revision of manuscripts. Stephen J Ives, Lisa A Lesniewski, Russell S Richardson, Eros Denchi, Ashley Walker, Grant Henson, Richard M Cawthon, Robert HI Andtbacka, and R Dirk Noyes contributed to the collection and analysis of data as well as the revision of manuscripts. Additionally, Russell S Richardson, Richard M Cawthon, and Eros Denchi contributed reagents, analytical tools, and specimens that were essential to completing this project.

CHAPTER 1

INTRODUCTION

Cardiovascular Disease, Arterial Aging, and Hypertension

Role of Aging in Cardiovascular Disease

Cardiovascular diseases (CVD), largely defined as stroke and heart diseases, such as coronary artery disease, heart failure, and cardiac arrest, are the predominant killers of Americans, accounting for ~752,000 deaths per year, according to current statistics from the Centers for Disease Control[1]. CVDs are considered diseases of aging, as they are the leading causes of death for Americans 65 years of age or older, causing ~35% of all deaths in this age group[1]. Additionally, the prevalence of CVDs among Americans increases progressively with advancing age from ~5.5% in 25-44 year olds to ~41% in people 65 years of age or older, which illustrates the chronic nature of these diseases[1]. Thus, CVDs can be considered true diseases of aging.

This trend is also true for hypertension, which is defined as an arterial blood pressure greater than 139/89 mmHg[2]. Hypertension is a major risk factor for heart disease and stroke and is thought to be responsible for 62% of stroke-related deaths and 49% of deaths from heart disease worldwide, according to the World Health Organization[2]. Indeed, the medical community often considers hypertension a distinct

form of CVD and the most pervasive age-related CVD[2]. Similar to other CVDs, hypertension is also a chronic disease that increases in prevalence with age from ~11% in 20-44 years olds to ~75% in people 75 years of age or older[1, 2].

Role of Age-Related Arterial Dysfunction in CVD

Heart disease, stroke, and hypertension are all diseases currently recognized to be caused in part by arterial dysfunction[3, 4]. Age-related alterations to arteries are thought to lead to a dysfunctional phenotype that precedes CVDs[3, 5, 6]. Importantly, the dysfunctional phenotype that develops in arteries with advancing age can occur in the absence of overt CVD and conventional CVD risk factors[7]. This supports the idea that these changes are a primary effect of normal aging that may lead to the development of heart disease and stroke.

Large landmark studies, like the Baltimore Longitudinal Study on Aging (BLSA) and the Framingham Heart Study, have demonstrated that the age-associated phenotype of arteries involves the development of a dysfunctional arterial endothelium, loss of elasticity in the arterial wall, and intimal media thickening[5, 8-10]. This phenotype is common to humans, nonhuman primates, as well as rodents[11-13]. These changes contribute to an increase in arterial tone, which may underlie age-related increases in arterial blood pressure[14]. These changes also lead to arterial stiffening, which is manifested by age-associated increases in arterial pulse wave velocity (PWV)[3, 10]. Increased PWV reflects the increased velocity of blood traveling through an artery, which indicates a stiffer artery caused by greater tone or loss of

elasticity due to structural remodeling of the artery[15]. Large epidemiological studies, including the BLSA, have shown that PWV increases with advancing age in humans and is an independent predictor of future cardiovascular events[10, 16, 17].

High arterial blood pressure, or hypertension, is itself a powerful risk factor for age-related heart disease and stroke. The seventh report of the Joint Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure states that for individuals between the ages from 40 to 89, there is nearly a doubling in mortality from heart disease and stroke for every 20 mmHg increase in systolic blood pressure (SBP) and 10 mmHg increase in diastolic blood pressure (DBP)[2]. Interestingly, DBP is a better predictor of CVD risk before age 50, while SBP is a more powerful CVD risk factor thereafter[2, 14]. DBP may increase until the fifth decade of life and level off over the next decade, or even slightly decrease later in life as a consequence of age-related remodeling of the arterial wall[2, 3, 14]. The BLSA and other large studies have found that age-related widening of pulse pressure (SBP-DBP), which is another indicator of arterial stiffening, is a better predictor of CVD risk than SBP or DBP alone[5, 18, 19].

Arterial aging represents an important area of study related to CVDs, as understanding how these changes occur in arteries could lead to novel therapies and interventions designed to blunt, stop, or even reverse the arterial dysfunction that precedes CVDs. As hypertension is the most prevalent age-related CVDs in the US and one of the most powerful predictors of death from heart disease and stroke[2], understanding the mechanisms that underlie age-related changes in arteries that lead to hypertension could ultimately produce therapies and interventions to treat or

reverse high blood pressure. Importantly, the cellular and molecular biology underlying these age-associated changes in arteries that lead to hypertension and more overt CVDs are not completely understood.

Mechanisms of Arterial Aging and Hypertension

Role of Endothelial Dysfunction in Arterial Aging and Hypertension

Arterial endothelial dysfunction, or a reduced endothelium-dependent vasodilatory response, is one of the most clinically important age-related changes to arteries that precedes hypertension and other CVDs[9]. This form of endothelial dysfunction occurs with advancing age in humans, rodents, and nonhuman primates alike[9, 20-22]. Endothelial dysfunction in conduit arteries and arterioles is thought to lead to increases in arterial blood pressure and PWV, and subsequently more overt CVDs like heart disease and stroke[23-29]. Endothelial dysfunction is also considered a major therapeutic target for reducing CVD morbidity and mortality[4]. Importantly, this can occur in the absence of CVD and major CVD risk factors, which indicates that endothelial dysfunction is a primary effect of normal aging[7, 9].

Several large prospective studies, including the Framingham Offspring Cohort, have shown that arterial endothelial dysfunction occurs with advancing age and predicts future CVD, CVD prognosis, and cardiovascular events. Celermajer *et al.* (1994)[9] showed that endothelial dysfunction begins to occur around the sixth decade of life, and Kitta (2009)[28], Perticone (2001)[25], and Modena (2002)[26] *et al.* showed that impairments in endothelial function predicted heart disease and stroke onset after

follow-ups ranging from 2-5 years. Endothelial dysfunction is also associated with hypertension[23, 29]. Results from the Framingham Offspring Cohort, which is a large prospective epidemiological study initiated in the early 1970s, showed that the severity of hypertension is predicted by the degree of endothelial dysfunction[29]. Furthermore, Rossi *et al.* (2004) found that postmenopausal women with normal arterial blood pressure that exhibit endothelial dysfunction have a six-fold greater risk of developing hypertension than those without endothelial impairments[23].

Basic Biology of the Arterial Endothelium

The entire mammalian circulatory system is lined by a single layer of vascular endothelial cells that make up the tunica intima of blood vessels[30]. The vascular endothelium in arteries performs many vital functions that vary from one segment of the arterial tree to another as well as one organ system to another[30, 31]. These include the maintenance of blood in a fluid state; exchange of fluid and molecules between the blood and surrounding tissues; participation and facilitation of the immune response; and, finally, the control of arterial blood pressure in response to changes in blood flow by regulating arterial tone in conduit arteries and arterioles[30, 31]. Endothelial cells can regulate the membrane potential of vascular smooth muscle cells via gap junctions, but also release vasodilator and vasoconstrictor substances that act to control tone[30-32]. Studies in animals have demonstrated that removal of the endothelium in arteries abolishes flow and acetylcholine-mediated vasodilatory

responses[32, 33]. These early studies established that these vasodilatory responses represent endothelium-dependent dilation (EDD).

One of the most important vasodilatory substances released by the endothelium is nitric oxide (NO), which is produced by endothelial nitric oxide synthase (eNOS) in response to increased blood flow[30-32, 34]. The increased shear forces of blood acting on the arterial wall following an increase in blood flow is sensed as shear stress by receptors linked to calcium channels on the surface of endothelial cells[30, 34]. Calcium influx from these receptors ultimately leads to eNOS activation and NO release onto vascular smooth muscle cells[30, 34]. eNOS activation also occurs in response to acetylcholine (ACh), serotonin, bradykinin, or thrombin binding to muscarinic receptors, which are ligand-gated calcium channels, on endothelial cells[34]. Indeed, the first study of endothelial regulation of arterial tone in humans, conducted by Ludmer *et al.* (1986), involved the infusion of ACh into coronary arteries and assessing the subsequent vasodilatory responses[35]. Both mechanisms cause calcium influx, which activates calmodulin and calmodulin-dependent kinases, ultimately leading to eNOS activation[34]. Other endothelium-derived factors like prostacyclin can also contribute to regulation of tone by causing EDD[31, 36].

eNOS converts oxygen and arginine to citrulline and NO in a reaction that depends on several essential cofactors, including tetrahydrobiopterin, flavin adenine dinucleotide, flavin mononucleotide, and nicotinamide adenine dinucleotide phosphate (NADPH)[34, 37, 38]. NO relaxes vascular smooth muscle cells by activating guanylate cyclase to generate cGMP, which activates protein kinase G[34, 37]. This leads

to the opening of calcium-gated potassium channels and hyperpolarization of vascular smooth muscle cell membranes[34, 37], thus exerting a tonic relaxing effect to maintain arterial blood pressure in the face of increasing flow or chemical stimulation[30]. *In vivo* inhibition of NO by N^G--nitro-L-arginine methyl ester (LNAME) or N^G-monomethyl-L-arginine (LNMMA) raises blood pressure[31, 39] and markedly attenuates flow and ACh-induced vasodilatory responses in humans and rodents [22, 40, 41].

Mechanisms of Age-Related Arterial Endothelial Dysfunction

Arterial endothelial dysfunction is often defined as the failure of the endothelium to release NO and cause local vasodilation in response to acetylcholine[7, 42]. This metric of endothelial dysfunction, assessed in many different arterial beds and by different techniques, occurs with advancing age [9, 32, 35, 43-45] and is predictive of morbidity and mortality in heart disease, stroke, and hypertension[23-26, 28, 29].Whether endothelial dysfunction is indeed a cause of hypertension, or if hypertension causes endothelial dysfunction, is a question still under debate. Mechanistic studies suggest that endothelial dysfunction leads to hypertension, as *in vivo* eNOS inhibition in humans and rodents leads to hypertensive blood pressures[31, 39]. However, a few studies in young adults and children seem to suggest that high arterial blood pressure early in life increases the risk of endothelial dysfunction later in life[46, 47]. Generally speaking, an increase in arterial tone from impairments in endothelial function will lead to increased blood pressure and, if impairments are chronic in nature, could lead to the development of hypertension over time. Likewise, endothelial dysfunction-

mediated increases in tone could potentially lead to increased arterial PWV with advancing age due to an artery developing a less responsive, or stiffer, phenotype.

It is often difficult to determine the source of arterial endothelial dysfunction with advancing age. Endothelial dysfunction could be the result of reductions in eNOS expression, eNOS uncoupling due to deficiencies in cofactors like tetrahydrobiopterin (BH_4), reduced L-arginine levels, endothelial cell apoptosis, poor diffusion of NO to vascular smooth muscle, poor responsiveness of vascular smooth muscle to NO, or increased reaction of NO with reactive oxygen species[7, 31, 48]. Arterial NO production in response to ACh has been shown to decrease with aging in humans and rodents[49, 50]. The cause of reduced NO production is unclear, as arterial eNOS expression and activation have been shown to increase, decrease, and remain unchanged with advancing age[51-54]. Likewise, arterial BH_4 levels have been shown to decrease or remain unchanged with aging[55, 56]; however, human and rodent data suggest that BH_4 bioactivity is reduced with aging, as supplementation partially restores age-related endothelial dysfunction[56-58]. L-arginine levels in arteries appear to be stable with advancing age, as L-arginine administration does not improve EDD in older human and rodents[55, 59]. Arterial endothelial cells from older nonhuman primates have shown increased apoptosis and reduced endothelial cell density associated with endothelial dysfunction[11].

In experimental settings, researchers use NO donors, like sodium nitroprusside, to assess the responsiveness of vascular smooth muscle cells to NO to determine whether endothelium-independent arterial dysfunction underlies attenuated ACh

responses[33, 60]. Using this technique, several lines of evidence suggest that responsiveness of arterial smooth muscle to NO is not impaired with advancing age in humans and rodents[22, 44, 49]. Antioxidants and ROS scavengers like 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) are used in experimental settings to alleviate the consumption of NO by reactive oxygen species (ROS) and determine to what extent these reactions underlie reduced ACh responses with advancing age[22]. NO consumption by ROS may be a particularly important mechanism underlying age-related endothelial dysfunction, as studies utilizing this approach have demonstrated that acute administration of antioxidants and ROS scavengers improves arterial EDD in older humans and rodents[22, 49, 61].

Role of Arterial Remodeling in Arterial Aging and Hypertension

Age-related arterial remodeling in mammals is largely considered to be increased intimal media thickness and arterial fibrosis, or the breakdown of elastin and deposition of collagen[12, 13, 22, 62, 63]. Arterial remodeling is an important age-related mechanism that underlies the arterial stiffening that leads to increases in arterial blood pressure and PWV, and subsequently more overt CVDs like heart disease and stroke[10, 16, 17, 64]. Several large epidemiological studies, including the BLSA, have shown that intimal media thickness increases with advancing age and is a good predictor of future cardiovascular events[65-67]. Indeed, intimal media thickness performs as well or better than many conventional CVD risk factors[67]. Arterial fibrosis has also been shown to increase with advancing age, and is thought to be the primary factor driving age-

related increases in arterial stiffness[12, 22, 64, 68]. Therefore, blood pressure changes in older adults may be largely due to intimal media thickening and fibrosis-mediated arterial stiffening.

Basic Structural Biology of Arteries

Mammalian arteries are composed of a tunica intima that consists of a layer of endothelial cells and the tunica media consisting of the vascular smooth muscle and extracellular matrix proteins like elastin and collagen[31, 69]. The tunica intima lines the lumen of arteries, while the tunica media forms the tubular structure[69]. The size of arteries, the lumen diameter, and the thickness and composition of the tunica media varies widely between different arteries and arterial beds[69]. Likewise, the degree to which arterial tone is controlled by the endothelium varies across arterial beds[31]. In general terms, the thicker the tunica media and greater the collagen to elastin ratio, the stiffer the artery[3, 68].

Mechanisms of Age-Related Arterial Remodeling

Arterial remodeling arises when structural changes occur to the tunica intima and tunica media of arteries with advancing age. Intimal media thickening, as the term implies, is a thickening of both the tunica intima and tunica media layers[12, 63]. Intimal media thickening occurs primarily as a result of the pathologic hyperplasia of vascular smooth muscle[12, 70]. Important mechanistic studies like one conducted in aged rats by Li *et al.* (1999) suggest that this process is the consequence of chronic cytokine and

chemokine activation of vascular smooth muscle cells[12]. Several large epidemiological studies have shown that carotid intimal media thickness increases 2-3 fold between the second and ninth decade of life[8, 67]. Intimal media thickening occurs with advancing age in humans, nonhuman primates, and rodents alike[11, 12, 63]. Importantly, this occurs in the absence of CVD and major CVD risk factors, which indicates that intimal media thickening is a primary effect of normal aging[63]. As mentioned, intimal media thickening is a potent predictor of CVDs, with increasing degrees of thickness more powerfully predicting onset of CVDs [67].

Arterial fibrosis, or the breakdown of elastin and deposition of collagen, is another important form of arterial remodeling that occurs with advancing age[12, 22, 68]. Elastin breakdown involves the enzymatic degradation of elastin as well as elastin calcification and fractures in arteries[12, 68]. This age-related increase in enzymatic degradation of elastin is linked to chronic inflammation in arteries[12]. Collagen deposition in arteries is also thought to occur as the result of inflammatory processes driven by increased transforming growth factor beta expression[12].

Role of Chronic Arterial Oxidative Stress in Arterial Aging and Hypertension

Chronic oxidative stress is the intracellular disturbance of the pro-oxidant antioxidant balance that leads to cellular damage, and is currently one of the most aggressively studied age- and hypertension-associated stressors in arteries. Markers of oxidative stress have been shown to be increased with advancing age in arterial cells

from humans and rodents[22, 51, 53, 71-73]. Recent evidence also shows that ROS levels and markers of oxidative stress are inversely related with arterial EDD in humans[74, 75] and linked to the development of hypertension[76-78]. Additionally, Eskurza *et al.* (2004) and Taddei *et al.* (2001) demonstrated that acute administration of antioxidants improves EDD in older humans[49, 61].

Arterial Sources of Oxidative Stress

ROS are highly reactive molecules that are generated from the partial reduction of molecular oxygen[31]. ROS that are commonly studied in mammalian arteries include superoxide, hydroxyl ion, and hydrogen peroxide[22, 31, 79, 80]. Arterial ROS can come from arterial cells[22, 71] as well as immune cells that are recruited to the arterial wall in response to injury or infection[31, 69, 81]. NADPH oxidase complexes (NOXs) are important endogenous mechanisms by which superoxide is produced in endothelial cells[51, 73, 82, 83] as well as immune cells[81]. NOXs are membrane-bound enzyme complexes that generate superoxide by transferring electrons from NADPH to molecular oxygen[82, 83]. NOXs 1, 2 and 3 are isoforms of the catalytic subunits of NADPH oxidase complexes responsible for the electron transfer to oxygen[82, 83]. NOXs are found in high abundance in phagocytes like macrophages and neutrophils, and NOX-generated superoxide is particularly important for the innate immune response to infection[82, 83]. Superoxide generated by NOXs in endothelial cells serves as an important second messenger that regulates endothelial cell growth and proliferation, endothelial cell barrier function, vasodilation, and vascular remodeling[31, 82, 83].

Arterial ROS can also be generated by electron leakage from the electron transport chain in mitochondria of arterial cells[31, 79]. Mitochondria are organelles that produce adenosine triphosphate (ATP) via oxidative phosphorylation, during which electrons are passed through the complexes of the electron transport chain to generate a proton gradient between the inner and outer mitochondrial membranes that drives ATP synthesis by ATP synthase[31]. Electrons that leak from complexes I and III can react with and reduce molecular oxygen in mitochondria to produce superoxide, which can leak from mitochondria into the cell or be converted to hydrogen peroxide by mitochondria-specific superoxide dismutases[31]. Under normal conditions, up to 4% of the oxygen in mitochondria is released into the cell as superoxide or hydrogen peroxide[31]. Uncoupling proteins create pores in the inner mitochondrial membrane to facilitate transfer of protons from outer to inner membrane, as well as hydroxyl ions from the inner to outer membrane, for the purposes of thermoregulation[84]. The accumulation of hydroxyl ions in the outer membrane can also lead to increased levels of ROS in the cytoplasm[84].

Oxidative phosphorylation in mitochondria is the major source of hydrogen peroxide in arterial cells, but this particular ROS is also the dismutated product of superoxide produced by superoxide dismutases (SODs)[31]. SODs convert superoxide and hydrogen into molecular oxygen and hydrogen peroxide[31]. Hydrogen peroxide is less reactive than superoxide, but is easily converted to the powerful oxidant hydroxyl ion via the Fenton reaction[85]. SODs, however, are considered important antioxidant enzymes in arterial cells[31, 69]. SOD1 is soluble and resides in the cytoplasm and

intermembrane space in mitochondria[86]. It is one of two SODs that require copper and zinc for normal function that are often referred to as Cu-ZnSODs[86]. SOD2 resides in the mitochondrial matrix, requires manganese for normal function, and is often referred to as MnSOD[86]. Finally, SOD3 is an extracellular SOD anchored to extracellular matrix that also depends on copper and zinc for normal function[86]. Along with catalases and certain peroxidases, SODs are a vital defense against oxidative stress in arterial cells[31, 69].

Role of Oxidative Stress in Arterial Dysfunction

NO is a major target of ROS. ROS reactions with NO result in reductions in NO bioavailability, which leads to arterial endothelial dysfunction[48, 49, 52]. Superoxide reactions with NO also lead to production of peroxynitrite, which can in turn oxidize BH_4 leading to eNOS uncoupling[48, 52, 87, 88]. Uncoupled eNOS can produce superoxide, thus leading to a vicious cycle of oxidative stress and endothelial dysfunction[48, 89].

Evidence of a Role for Chronic Oxidative Stress in Arterial Aging and Hypertension

Many lines of evidence have shown that ROS levels increase with advancing age in arteries, and the balance of pro-oxidant and antioxidant enzymes shifts arteries towards a pro-oxidative phenotype. In addition to evidence that antioxidant treatment improves arterial EDD in humans[49, 61], overexpression of antioxidants in endothelial cells results in increased endogenous NO bioavailability and improved endothelial

function in rodents[90]. Superoxide and peroxynitrite levels have been shown to be increased with advancing age in arteries[22, 52, 53, 72, 78], coinciding with decreased NO levels[52]. NOXs and the transcription factor complex, nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), which upregulates NOXs, are also increased with advancing age in human arterial endothelial cells[73, 91], while SOD expression has been shown to be either reduced or unchanged in human and rodent arterial cells[51, 53, 73, 92]. Increased arterial NOX expression and NOX generated superoxide[76-78], as well as arterial SOD dysregulation[93], have all been linked to the pathogenesis of hypertension. Similarly, ROS generated by dysfunctional mitochondria in arteries also increases with advancing age[79], and has been linked to the development of hypertension[78, 94]. Interestingly, *in vitro* studies have shown increased superoxide levels and reduced NO levels in senescent, or aged, human vascular endothelial cells, which could be linked to increased NOX activity or uncoupled mitochondria[95-97].

Role of Chronic Arterial Inflammation in Arterial Aging and Hypertension

Chronic arterial inflammation is thought to play an important role in arterial aging and hypertension. Chronic ''low-grade'' arterial inflammation is the gradual accumulation of inflammatory cytokines and chemokines in arterial tissue with advancing age that is distinct from the inflammatory processes involved in the pathogenesis of overt CVDs like atherosclerosis and thrombosis[7]. It has been reported that inhibition of NFKB signaling improves arterial EDD in older humans and rodents[98,

[99], while inhibition of tumor necrosis factor alpha (TNFA) improves EDD in old rodents[100]. The accumulation of inflammatory mediators, including NFKB, interleukin 6 (IL6), TNFA, and monocyte chemotactic protein 1 (MCP1), occurs with advancing age in human arterial tissue[73, 91, 101, 102]. Increased arterial levels of interleukin 1 (IL1), IL6, TNFA, and MCP1 have also been reported in rodents[103, 104]. Chronic low-grade levels of interleukin 8 (IL8) in plasma are linked to arterial dysfunction[105]. Inflammatory mediators like NFKB, IL6, TNFA, and MCP1 have also all been linked to the development of hypertension[106-108]. Additionally, adhesion molecules, like P-selectin, L-selectin, vascular cell adhesion molecule 1 (VCAM1), and intercellular adhesion molecule 1 (ICAM1), have well-established roles in the atherosclerotic process[109, 110].

Arterial Sources of Inflammation

Similar to arterial ROS, inflammation in mammalian arteries is produced by either arterial cells[12, 73, 91] or immune cells recruited to the arterial wall[69, 111-113]. Immune cells can be recruited to the arterial wall in response to injury or infection. Certain inflammatory mediators released by arterial cells recruit immune cells to the sight of injury or infection, while other inflammatory mediators produced by both immune and arterial cells serve to promote immune cell migration in the arterial wall and retain them there[28, 104-106]. Furthermore, inflammatory mediators are produced to coordinate the proliferation, survival, and apoptosis of immune and arterial cells as needed to resolve injury or infection[28, 104-106]. Cell cycle arrest may also be important source of inflammation in arteries. Cells that are in a state of permanent cell cycle

arrest, or cellular senescence, release inflammatory mediators to reinforce the cell cycle arrest in an autocrine fashion and activate immune cell surveillance of the senescent cells[114]. Senescent human arterial cells have been shown *in vitro* to release IL1, IL6, IL8, TNFA, and MCP1[115, 116].

Role of Inflammation in Arterial Dysfunction

IL1 and TNFA are thought to be particularly important cytokines in arterial endothelial dysfunction because they can activate NFKB, which in turn can lead to upregulation of NOX1[73, 117]. NFKB also promotes transcription of a wide array of pro-inflammatory genes, including IL1, IL6, IL8, TNFA, and MCP1, which leads to a positive feedback loop[118-123]. Furthermore, NFKB is also activated by ROS, which can create a vicious cycle of ROS production[124]. These cytokines and chemokines perform many different functions that could potentially play a role in endothelial dysfunction. IL1 is a powerful cytokine that promotes adhesion of immune cells to the endothelium and transmigration through the endothelium[125]. It also acts as a local inducer of other cytokines and chemokines in arteries[125]. IL6 stimulates growth and differentiation of immune cells[126, 127], which could lead to NOX-mediated oxidative stress[81]. MCP1 is important for monocyte recruitment from bone marrow to the arterial wall[128], which could also lead to immune cell-induced ROS production[81]. P-selectin and L-selectin allow rolling of immune cells along endothelial cells, until VCAM1 and eventually ICAM1 slow immune cell rolling and allow transmigration across the endothelium to

occur[109, 110]. Once in the subendothelial layer, immune cells can promote atherogenesis as well as oxidative stress[81, 113].

Inflammation is also thought to play a role in arterial intimal media thickening and arterial fibrosis. Several cytokines and chemokines, such as IL8 and TNFA, can stimulate vascular smooth muscle cells to proliferate and migrate, which may be an important process underlying intimal media thickening[12, 129, 130]. TNFA stimulation of vascular smooth muscle cells can also lead to the expression and release of activated zinc-dependent endopeptidase type-2 metalloproteinase (MMP2)[12]. MMP2, like other MMPs, promotes migration of cells by breaking down extracellular matrix proteins to allow for movement through a tissue[12, 68]. This process can promote migration of vascular smooth muscle cells as well as breakdown of elastin in arteries, which can lead to fibrosis and stiffening of the artery[12, 68]. Collagen deposition in arteries is also thought to occur as a result of inflammation, mediated by increased transforming growth factor beta expression[12, 131]. Transforming growth factor beta is a potent profibrotic cytokine that stimulates type I collagen secretion from fibroblasts through a mitogen-activated protein kinase-dependent signaling pathway[131].

While these inflammation-mediated mechanisms of arterial dysfunction are interesting and merit assessment of their role in arterial aging and hypertension, we are not exploring these pathways in this project. For this project, our focus will be on inflammatory processes linked to oxidative stress and their major roles in arterial aging and hypertension.

Evidence of a Role for Chronic Inflammation in Arterial Aging and Hypertension

There is ample evidence of a role of chronic inflammation in arterial aging and hypertension. While many other inflammatory mediators are found in arteries, some important cytokines and chemokines linked with arterial aging include IL1, IL6, IL8, TNFA, and MCP1[73, 91, 101, 103, 104]. There is evidence of age-related immune cell infiltration into arterial tissue in rodents[132]. Additionally, NFKB, IL6, TNFA, and MCP1 have all been shown to play a role in the development of hypertension[106-108]. Because senescent human arterial cells, considered *in vitro* to be aged cells, develop a pro-inflammatory/pro-oxidative phenotype[95, 115, 116, 133], they may be an important source of chronic arterial inflammation and oxidative stress in aging and hypertension.

Potential Role of Cellular Senescence in Arterial Aging and Hypertension

Cellular senescence, or permanent cell cycle arrest, is considered cellular aging. Senescence occurs *in vitro* after a certain number of cell cycles and in response to excessive intracellular and extracellular stressors[114, 134]. The number of cell cycles to reach senescence *in vitro* varies across cell types[134]. Senescence occurs primarily in the G0/G1 phase of the cell cycle and is an important tumor suppressive mechanism that prevents passing damaged DNA to daughter cells or potential neoplastic transformation of damaged cells[114, 134]. Since being first described by Leonard Hayflick

as an *in vitro* phenomenon in human fibroblasts, the potential role of senescence in *in vivo* aging and disease has been difficult to assess and somewhat controversial[135].

However, markers of cellular senescence have been linked to aging and chronic disease. Recent studies have shown that senescent cells accumulate in tissues over the lifespan of humans and rodents[136, 137]. Likewise, the accumulation of senescent cells has been reported in diseased tissues, such as atherosclerotic plaques[138] and abdominal aortic aneurysms[139]. Baker *et al.* (2011) showed that clearance of senescent cells reversed aged and diseased phenotypes in a mouse model of accelerated aging[140]. This important study strongly suggested that there were phenotypic properties of senescent cells that were problematic to tissues, and potentially contribute to aging and chronic disease.

Mechanisms of Cellular Senescence

There are several causes of cellular senescence in mammalian cells, including excessive mitogenic signals[141], increases in extracellular or intracellular stressors like oxidative stress[142], chromatin disruptions[143], and DNA damage[144]. Double strand DNA breaks (DSBs) initiate a DNA damage response that results in temporary cell cycle arrest to allow DNA repair pathways time to repair breaks prior to a cell entering S phase during replication[144]. If DSBs are persistent or extensive, then permanent cell cycle arrest or even apoptosis will ensue[144]. Telomere dysfunction occurs over time, or over the course of continuing cell cycles, and is another mechanism thought to lead to cellular senescence[145, 146]. First described by Harley *et al.*, senescence triggered by

replication-dependent telomere dysfunction is often referred to as replicative senescence[145]. Importantly, telomere dysfunction triggers senescence by activating the same DNA damage response pathways as DSBs[147, 148]. Senescence is induced by the two major tumor suppressor pathways, known as the P53 and P16/pRB pathways[149]. Both of these pathways involve upregulation of cyclin-dependent kinase inhibitor proteins, like cyclin-dependent kinase inhibitors 1A (P21) and cyclin-dependent kinase inhibitors 2A (P16), in response to a variety of cellular stressors[149].

The P53 pathway depends on activation of the transcription factor, tumor suppressor protein P53 (P53), by a number of different signaling cascades[149]. One of the most important of these is the DSB-initiated DNA damage response pathway[144, 150]. Once activated, P53 promotes the transcription of P21, which induces cell cycle arrest by binding and inhibiting cell cycle-promoting cyclin-dependent kinases 2 and 4 (CDKs 2 and 4)[151]. P21 binds to CDK 2 and 4 by displacing cyclins E and D, respectively, which inhibits their activity and thus arrests the cell cycle[151]. P53 also promotes the transcription of bcl-2 associated X protein, which induces apoptosis in damaged cells[152, 153]. Thus, P53 is one of the most important mediators of the general stress response in cells. Abnormal P53 function results in damaged cells escaping senescence or apoptosis, which increases the risk of tumorigenesis. Indeed, many cancers in humans are linked to loss-of-function mutations in the P53 gene[154, 155], whereas gain-of-function mutations in the P53 gene lead to tumor regression in mice[156]. P53 can also be activated in response to excessive mitogenic signals, which represents another tumor suppressive function. This occurs through activity of the ARF variant of cyclin-

dependent kinase inhibitor 2A (P14ARF in humans and p19ARF in rodents), which stabilizes P53 concentrations in the cell by inhibiting double minute 2 protein (MDM2)-mediated ubiquitylation and subsequent protease degradation[157].

The P16/pRB pathway involves P16-mediated inhibition of CDK 4, which prevents activation of retinoblastoma-like protein 1 (pRB)[151]. This in turn prevents pRB from activating the cell cycle-promoting transcription factor E2F4[158]. The P16/pRB pathway is also activated by a variety of stressors[149]. Many of the same stressors activate both pathways, such as telomere dysfunction, and there appears to be crosstalk between the pathways, as P21 can inhibit the same CDKs that activate pRB[149, 151]. The preference toward one pathway versus another appears to be cell type-specific[149, 159], with variation across species[160]. For example, telomere dysfunction can lead to activation of the P53 or P16/pRB pathway in human cells, but will only trigger the P53 pathway in rodent cells[160]. The general consensus seems to be that senescence via the P53 pathway is activated primarily by DNA damage and telomere dysfunction, while the P16/pRB pathway is linked primarily to mitogenic stress, chromatin disruptions, or general cellular stress[149, 159, 161].

The Senescence-Associated Secretory Phenotype

In vitro senescent cells are characterized by a pro-inflammatory pro-oxidative senescence-associated phenotype, or SASP[114]. The release of inflammatory mediators likely reinforces cell cycle arrest in an autocrine fashion and activates immune cell surveillance of senescent cells[114]. The SASP occurs within a few days of senescence

induction in cells and appears to be irreversible due to stable chromatin modifications around clusters of SASP genes[158, 162, 163]. The SASP profile in various cell types, including vascular endothelial cells, from humans and rodents has been characterized by *in vitro* studies using comprehensive arrays of inflammatory cytokines and chemokines[114, 162, 164-167]. Interestingly, besides the release of some core SASP factors, senescence induced by different mechanisms in different cell types and species results in very different SASPs[114]. P16/pRB-induced senescence has not been shown to lead to a SASP in human cells[114, 168].

The SASP is largely divided into three categories including soluble signaling factors, secreted proteases, and soluble nonproteins[114]. Soluble signaling factors, including interleukins, chemokines, and growth factors, are some of the SASP elements that are most relevant to arterial aging and hypertension. IL6 is the most ubiquitous core SASP cytokine, which is released during P53/P21-induced senescence *in vitro* in a variety of human cell types[169-171], including arterial cells[172]. Likewise, the release of IL1, IL8, TNFA, and MCP1 has been linked to P53/P21-induced senescence in human arterial cells *in vitro*[115, 172]. Secreted proteases, such as MMP2, are also released by senescent human cells[173, 174], and could play a role in arterial aging and hypertension by mediating arterial fibrosis. Finally, secreted soluble nonproteins may contribute directly to arterial endothelial dysfunction. P53/P21-induced senescence leads to increased ROS production, which also appears to be required for maintenance of cell cycle arrest as inhibition of ROS with antioxidants rescues P53/P21-induced senescence[175]. Human arterial endothelial cells that had undergone replicative

senescence *in vitro* exhibited elevated levels of hydrogen peroxide and superoxide as well as reductions in NO[95, 176]. This SASP profile is possibly due to mitochondrial uncoupling or alterations in eNOS[95, 177]. Several lines of evidence support a major role for NFKB in the induction and maintenance of the P53/P21-induced SASP in human and rodent cells[96, 178, 179]. Indeed, inhibition of NFKB allowed cells to escape P53/P21-induced senescence and reduced oxidative stress in rodents[96, 179]. This alludes to the importance of the SASP in reinforcing P53/P21-induced senescence and the importance of NFKB in promoting the SASP.

Evidence of a Role for P53/P21-Induced Senescence in Arterial Aging and Hypertension

P53/P21-induced senescence, and associated SASP, has never been assessed in normal human arterial aging or hypertension. However, recent studies have linked increased arterial expression of senescence effector proteins like P21 and P53 to atherosclerosis[138] and chronic obstructive pulmonary disease[116] in humans. Most relevantly, Marchand *et al.* (2011) showed greater *P21* mRNA expression in arteries from nine older coronary artery bypass graft patients compared with eleven middle aged patients[137]. Furthermore, age-related telomere dysfunction has been reported in human arteries taken from cadavers[180-182]. As telomere dysfunction occurs over time, P53/P21-induced senescence triggered by telomere dysfunction could be an important source of chronic inflammation and oxidative stress in arterial aging.

Potential Role of Telomere Dysfunction in Arterial Aging and Hypertension

Basic Telomere Biology

Telomeres are the natural ends of chromosomes in most eukaryotes[183]. First discovered by Elizabeth Blackburn in 1978, telomeric DNA consists of noncoding repeats of the hexamer TTAGGG[184, 185]. Telomeres are approximately 10-15kb in length in humans[146,186] and 20-50 kb in rodents[187], and they end in 50-500bp 3' overhang in all mammalian cells[188, 189]. Telomere length is inherited and varies greatly across different species, tissues, and even between individual cells within a tissue[146,186].

The fundamental purpose of mammalian telomeres is to prevent chromosome ends from resembling DSBs. Recognition of chromosome ends as DSBs could result in a DNA damage response at telomeres and subsequent P53/P21-induced senescence[147, 148]. Telomeres prevent this by forming a secondary structure known as the t-loop, which is said to "cap" telomeres[190]. The t-loop structure also prevents the DSB repair pathway, known as nonhomologous end-joining (NHEJ), from being initiated at chromosome ends[191]. NHEJ activity at chromosomes could result in end-fusions of adjacent chromosome ends, which would cause aneuploidy, or an abnormal number of chromosomes, and substantially increase the risk of neoplastic transformation of cells[191, 192].

Another purpose of telomeres is to prevent degradation of genomic DNA via the end-replication problem[145, 146] or postreplication processing[189]. The end-replication problem is the incomplete replication of DNA during each synthesis phase due to

inherent inefficiencies in the DNA replication machinery[145, 146, 193]. Telomeres effectively provide a source of ‘‘junk’’ DNA that can be lost each cycle without compromising important coding regions of the genome. Postreplication processing by nucleases could also cause degradation of coding regions of the genome without the presence of telomeres[189]. This form of DNA degradation is thought to be origin of the 3’ overhang at telomeres[189]. Postreplication processing by nucleases is prevented by the t-loop structure of telomeres[190].

First described by Titia de Lange in human and rodent cells in 1999, the t-loop is formed when the very distal 3’ end of telomeres folds back along the telomere and invades telomeric DNA upstream of the chromosome end and base pairs with the local 5’ sequence, thus forming a loop[190]. The displaced G-rich 3’ telomeric DNA forms a d-loop inside the t-loop[194]. The size of the t-loop varies between telomeres within cells as well as between species[190]. Furthermore, the size of the t-loop does not seem important for it to perform its capping function. The t-loop is formed and maintained by several shelterin proteins, all of which were discovered by Titia de Lange[195]. The shelterin proteins consist of three telomeric DNA binding proteins, known as telomeric repeat binding protein 2 (TRF2), telomeric repeat binding protein 1 (TRF1), protection of telomeres 1 (POT1), and three structural support proteins, TRF1-interacting nuclear factor 2 (TIN2), TPP1, and repressor/activator protein 1 (RAP1)[195]. The functions of different shelterin proteins inform their relative importance for the maintenance of telomere capping.

TRF2 binds double stranded telomeric DNA, and is arguably the most important shelterin protein for maintaining telomere capping. TRF2 has been shown to independently form and maintain a t-loop structure with telomeric DNA *in vitro*[196]. TRF2 deletion results in a rapid DNA damage response at telomeres and subsequent P53/P21-induced senescence[148, 197], as well as activation of NHEJ at telomeres[191, 198]. Thus, deletion or knockdown of TRF2 is the preferred method for uncapping telomeres, independent of changes in telomere length. TRF2 has also been shown to localize to uncapped telomeres *in vitro* to prevent NHEJ between adjacent uncapped telomeres[199, 200].

TRF1 also binds double stranded telomeric DNA and assists in the actual looping of the t-loop[201, 202]. POT1 stabilizes the D-loop by binding to the single stranded telomeric DNA present in the D-loop to protect it from nucleolytic attack[203-205]. TIN2 binds to TRF2, TRF1, and TPP1 to provide a bridge between the double stranded and single stranded DNA binding shelterin components[206-210]. TPP1 binds to POT1[211], connecting it to TIN2 and recruiting it to nucleus[209, 212, 213]. Finally, RAP1 interacts with TRF2 and helps stabilize the shelterin complex[214, 215].

Mechanisms of Telomere Dysfunction

Telomere Shortening

Telomeres shorten over time in most mammalian somatic tissues, except in germ cells and some stem cell compartments[181, 216-218]. Telomere shortening in various tissues has been linked to a variety of chronic disease in humans[180, 219-222]. Telomere

shortening in most human tissues occurs at a rate of 25-100bp per year depending on the tissue[181, 218]. Telomeres that shorten beyond some critical length are thought to become uncapped and subsequently lead to P53/P21-induced senescence[146-148]. *In vitro* studies have clearly demonstrated that telomere shortening corresponds closely with replicative senescence[145, 146]. Telomere shortening occurs as a result of the end-replication problem[145, 193], as well as replication-independent mechanisms like nucleolytic attack[189] or genotoxic stress from UV radiation or ROS[216, 223]. Indeed, the guanine triplet of telomeric DNA appears to be uniquely susceptible to break-inducing oxidative damage relative to nontelomeric DNA[223].

Telomere length homeostasis is maintained over time by telomerase in some tissues[224]. Telomerase is a ribonucleoprotein complex first discovered by Elizabeth Blackburn and Carol Greider in 1985[225]. Telomerase consists of a catalytic subunit, known as telomerase reverse transcriptase (TERT), and the telomerase RNA component (TERC)[224]. During telomere elongation, TERT uses TERC as a template from which to extend telomeric repeats from the chromosome end[224]. In mammals, telomerase expression is detectable almost exclusively in germ and stem cells compartments as well as some highly proliferative somatic tissues[226-232]. Telomerase activity in human white blood cells is relatively high and then drops off after the second decade of life[229, 231]. Thus, alterations in telomerase activity are not thought to play a significant role in aging or chronic disease. However, increased telomerase activity in white blood cells has been linked to CVD risk[233] and plaque severity in atherosclerotic arteries[234]. Age-related reductions in telomerase activity have also

been observed in human microglial cells, which suggests that telomerase may play a role in Alzheimer's disease and dementias[235]. Currently, the potential role of telomerase in aging and chronic disease is unclear.

Telomere Uncapping

As mentioned, breakdown of the t-loop structure, or telomere uncapping, can result in a DNA damage response at telomeres and P53/P21-induced senescence in mammalian cells[147, 148, 236]. *In vitro* studies have demonstrated that telomere uncapping occurs with successive cell cycles and may be the mechanism underlying replicative senescence, as it coincides closely with P53/P21-induced senescence[134, 135,199, 200]. Telomere uncapping *in vitro* presumably occurs as a result of telomere shortening beyond a critical length[146-148]. *In vivo*, telomere uncapping could potentially occur as a result of genotoxic damage to the t-loop, dysfunctional shelterin proteins, or telomere shortening. Any compromise in the t-loop that exposes chromosome ends as DSBs will initiate an ataxia telangiectasia mutated protein (ATM)-dependent DNA damage response, which ultimately results in P53 activation[236-238]. ATM is a kinase that rapidly localizes to DSBs, or uncapped telomeres[236-238], and activates checkpoint kinases 1 and 2, which transduces the signal by phosphorylating P53[134, 135]. Phosphorylation of P53 blocks its binding by MDM2, thus preventing ubiquitylation and subsequent protease degradation of P53[239]. Stable intracellular concentrations of active P53 lead to P21 upregulation and ultimately cell cycle arrest[147, 148,239]. As mentioned previously, telomere uncapping can lead to P16/pRB-induced senescence

in some human cells, but not in rodent cells[160]. Interestingly, *in vitro* studies have reported that as few as five uncapped telomeres are sufficient to trigger P53/P21-induced senescence in human cells[200].

Well-described early events at both DSBs and uncapped telomeres include P53 binding protein 1 localization, which is thought to prevent 5' resection of broken DNA, and phosphorylation of histone H2A.X at serine 139 (p-H2A.X (ser139))[147, 148]. p-H2A.X (ser139) occurs on chromatin on either side of a DSB within minutes of the break occurrence or telomere uncapping and is thought to mark DSBs for recognition by additional DNA damage response factors[240]. ATM is thought to be the kinase responsible for p-H2A.X (ser139)[240].

Mouse Models of Telomere Dysfunction

Mice have relatively long telomeres, high telomerase activity, and less age-related telomere shortening than humans[241, 242]. This limits the utility of aged wild type mice as a model for human telomere dysfunction. Developing useful transgenic models can be challenging, as deletion of many shelterin and telomerase-related genes is embryonically lethal in mice. For example, null *TRF2*, *TRF1*, *dyskerin*, which is a telomerase stabilizing protein, and *TIN2* mutations are all embryonically lethal[236, 243-245]. Nonetheless, several transgenic mouse models of telomere shortening and telomere uncapping have been developed that mimic telomere dysfunction observed with advancing age and chronic disease in humans[226, 232, 246-252].

Models of Telomere Shortening

The most commonly studied mouse models of telomere shortening are models of telomerase deficiency. Several strains of mice deficient for the *TERT* gene have been created that lack the catalytic subunit of telomerase[246-248]. Another commonly studied model of telomerase deficiency involves deletion of the *TERC* gene to remove the RNA template from the telomerase complex[226, 232, 249-252]. Both models develop similar phenotypes, such as shortened telomeres, diminished tissue renewal in bone marrow, germ cells, and intestines, and impaired wound repair[226, 232, 246-252]. To shorten the relatively large telomeres of mice enough to induce telomere uncapping, these models require breeding animals for multiple generations[226, 232, 246-252]. Importantly, functional telomerase may not be essential for embryonic development in these mice, but phenotypes do occur at earlier time points in later generations of animals due to the inheritance of progressively shorter telomeres[226, 232, 248, 250]. Thus, these mice may have limited utility as true models of age-related telomere shortening in humans.

The most successful model of telomere shortening within a single generation has been the *POT1B* null mice[205, 253, 254]. Mouse shelterin has two *POT1* variants, *POT1A* and *POT1B*, while human shelterin has a single *POT1* variant[253]. *POT1A* deletion is embryonically lethal in mice, but *POT1B* null mice are viable, fertile, and exhibit marked telomere shortening, presumably due to nucleolytic degradation of the single stranded telomeric DNA in the d-loop[205, 253, 254]. However, this progressive telomere shortening does not appear to lead to complete telomere uncapping in many tissues[205, 253-255], which is possibly due to the large telomere length reserve in mice.

Additionally, the damage to telomeric DNA following *POT1B* deletion consists of single strand DNA breaks, which will initiate different DNA damage response, cell cycle arrest, and DNA repair pathways than DSBs[255].

Models of Telomere Uncapping

Since the same genotoxic stressors that can cause telomeres to shorten may also separately induce P53/P21-induced senescence[142, 223], it is often considered necessary to uncap telomeres independent of telomere shortening to truly assess the role of telomere dysfunction in P53/P21-induced senescence and its downstream effects. To date, only one mouse model of true telomere uncapping independent of telomere shortening has been developed. A conditional *TRF2* deletion mouse was developed in Titia de Lange's laboratory, in 2005[256]. Using a Cre-Lox inducible gene deletion system, floxed versions of exon 1 and 2 in the *TRF2* gene can be deleted upon activation of Cre-recombinase[236, 257]. Whole body *TRF2* deletion in these animals results in rapid widespread telomere uncapping as well as P53 and P21 upregulation[257]. Whole body *TRF2* deletion in mice is normally fatal.

Evidence of a Role for Telomere Dysfunction in Arterial Aging and Hypertension

Arterial telomere shortening has been shown to occur with advancing age and has been associated with the development of some CVDs. Chang *et al.* (1995), Aviv *et al.* (2001), and Okuda *et al.* (2000) all showed age-related telomere shortening in

human arterial tissue taken from cadavers (n=27-51)[180-182], while Ogami *et al.* (2004) and Okuda reported that telomere length was inversely correlated with atherosclerotic plaque severity[180, 219]. Telomere shortening has also been associated with P21/P53-induced senescence in atherosclerotic arteries[138]. The role of arterial telomere shortening in the pathogenesis of hypertension has not been assessed, but telomere shortening in white blood cells has been associated with hypertension. Bhupatiraju *et al.* (2012) and Yang *et al.* (2009) both demonstrated that hypertensive adults had shorter white blood cell telomeres than nonhypertensive adults[258, 259].

Importantly, age-related or chronic disease-associated telomere uncapping has not been described in any noncultured human tissue. Indeed, neither arterial telomere uncapping nor its link to P21/P53-induced senescence have been assessed in arterial cell culture models, animal models, or human tissues. Likewise, the association between arterial telomere shortening and P21/P53-induced senescence with advancing age has not been assessed. Finally, the roles of arterial telomere shortening, telomere uncapping, and P21/P53-induced senescence in the etiology of hypertension have not been assessed.

General Objectives and Approach

Project Novelty and Significance

The role of telomere dysfunction in arterial aging and hypertension is currently unexplored. Understanding the role that telomere dysfunction plays in arterial aging could lead to novel therapies and interventions designed to blunt, stop, or even reverse the arterial dysfunction that precedes CVDs. Likewise, understanding the role

that arterial telomere dysfunction plays in the pathogenesis of hypertension could ultimately produce therapies and interventions that treat or reverse high arterial blood pressure.

Research Questions

Our overall research questions for this project are; Does arterial telomere dysfunction, defined as telomere shortening that leads to uncapping, play a role in arterial aging and development of hypertension? Does telomere dysfunction lead to P53/P21-induced senescence in arteries, ensuing SASP, and subsequent arterial endothelial dysfunction, and increased arterial blood pressure and PWV?

Working Hypothesis

Our working hypothesis for this project is that arterial telomere dysfunction contributes to arterial aging and hypertension by leading to P53/P21-induced senescence and ensuing SASP, and ultimately arterial endothelial dysfunction, and increased arterial blood pressure and PWV.

General Approach

Our general approach for this project will be to first determine the association between telomere dysfunction, P53/P21-induced senescence, and SASP in aging and hypertension in human arteries. Next, we will determine if arterial telomere uncapping, independent of telomere shortening, leads to P53/P21-induced senescence

and ensuing SASP, and subsequent arterial endothelial dysfunction, increased arterial blood pressure, and PWV in a mouse model of telomere uncapping.

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CHAPTER 2

AGE-RELATED TELOMERE UNCAPPING IS ASSOCIATED WITH CELLULAR SENESCENCE AND INFLAMMATION INDEPENDENT OF TELOMERE SHORTENING IN HUMAN ARTERIES

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Morgan, R. G. *et al.* Age-related telomere uncapping is associated with cellular senescence and inflammation independent of telomere shortening in human arteries. *Am J Physiol Heart Circ Physiol*, doi:10.1152/ajpheart.00197.2013 (2013).

Abstract

Arterial telomere dysfunction may contribute to chronic arterial inflammation by inducing cellular senescence and subsequent senescence-associated inflammation. Though telomere shortening has been associated with arterial aging in humans, age-related telomere uncapping has not been described in noncultured human tissues and may have substantial prognostic value. In skeletal muscle feed arteries from 104 younger, middle-aged, and older adults, we assessed the potential role of age-related telomere uncapping in arterial inflammation. Telomere uncapping, measured by p-histone γ-H2A.X, ser139 localized to telomeres (chromatin immunoprecipitation; ChIP) and telomeric repeat binding factor 2 bound to telomeres (ChIP) was greater in arteries from older adults compared with those from younger adults. There was greater tumor suppressor protein p53 (P53)/cyclin-dependent kinase inhibitor 1A (P21)-induced senescence, measured by P53 bound to *P21* gene promoter (ChIP), and greater expression of *P21,* interleukin 8, and monocyte chemotactic protein 1 mRNA (RT-PCR) in arteries from older adults compared with younger adults. Telomere uncapping was a highly influential covariate for the age-group difference in P53/P21-induced senescence. Despite progressive age-related telomere shortening in human arteries, mean telomere length was not associated with telomere uncapping or P53/P21-induced senescence. Collectively, these findings demonstrate that advancing age is associated with greater telomere uncapping in arteries, which is linked to P53/P21-induced senescence independent of telomere shortening.

Introduction

Arterial telomere dysfunction may contribute to age-related arterial inflammation by inducing cellular senescence and subsequent senescence-associated inflammation. Telomeres are terminal sequences of TTAGGG repeats that make up the natural ends of chromosomes[1, 2]. Telomeres and telomere binding proteins, like telomeric repeat binding factor 2 (TRF2), form specialized structures that protect chromosome ends from being recognized as dsDNA breaks and initiating a dsDNA break response[3-5], which could induce cellular senescence through tumor suppressor protein p53 (P53)-mediated upregulation of cyclin-dependent kinase inhibitor 1A (P21)[5, 6]. Several *in vitro* studies in various human cell types have shown that replication-dependent breakdown of telomere structure, referred to as uncapping, can lead to P53 activation and P53/P21-induced senescence[5, 6]. Following *in vitro* telomere uncapping in human cells, phosphorylation of histone γ-H2A.X at serine 139 (p-H2A.X (ser139)) occurs at telomeric chromatin to aid in initiation of the dsDNA break response[5, 6] and TRF2 binds to telomeres to prevent chromosome end-fusions[7, 8]. Additional *in vitro* studies have described P53-mediated senescence-associated inflammation in human cells, characterized by the accumulation of cytokines and chemokines like interleukin 6 (IL6), interleukin 8 (IL8), and monocyte chemotactic protein 1 (MCP1)[9-13]. Consequently, senescence-associated inflammation has been implicated in aging and chronic disease[14, 15].

Many cardiovascular diseases (CVDs) are recognized as diseases of the arteries[16, 17] preceded by chronic low-grade arterial inflammation[18, 19]. Telomere uncapping with

advancing age might lead to P53/P21-induced senescence and subsequent inflammation in arteries. Though age-related telomere uncapping has not been assessed in noncultured human tissues, telomere shortening has been shown to occur over time in most human somatic tissues[1, 20, 21], including arteries[22-24]. Telomere shortening could contribute to arterial telomere uncapping with advancing age along with other factors like genotoxic stress or dysregulation of telomere binding proteins[20, 25]. Age-related dysregulation of the reverse transcriptase, telomerase (hTERT), could also contribute to arterial telomere uncapping by mediating telomere shortening[26]. Elucidating the potential role that telomere uncapping plays in chronic arterial inflammation may lead to the identification of novel biomarkers that are predictive of CVDs, as well as lifestyle and pharmacological interventions that blunt or even reverse age-related arterial inflammation.

Thus, an important unexplored hypothesis is that telomere uncapping occurs with advancing age in arteries and is associated with P53/P21-induced senescence and inflammation. To test this hypothesis, we measured p-H2A.X (ser139) localized to telomeres, TRF2 bound to telomeres, P53 bound to *P21* gene promoter, *P21*, *IL6, IL8,* and *MCP1* mRNA expression, mean telomere length, and active hTERT with advancing age in arteries from a large generalizable sample of human subjects.

Materials and Methods

Human Artery Biopsy Collection and General Sample Processing

Arterial biopsies were excised from patients undergoing a prophylactic melanoma-associated sentinel lymph node biopsy to rule out melanoma metastasis, at the Huntsman Cancer Hospital, University of Utah. A heterogeneous sample (n) of 104 subjects (62 males and 42 females; aged 21-93 yrs) consented to donate arterial biopsies for the study. A comprehensive outline of biometric, physiological, and medical characteristics for all subjects enrolled in the study was collected (Table 2.1). Subjects were grouped according to age at time of biopsy into younger adult ($\leq$ 40 yrs of age), middle-aged adult (41-60 yrs of age), and older adult ($\geq$ 61 yrs of age) age-groups. Although medical histories and prescription medication use were noted, there were no exclusions based on this information. Subject blood pressures were measured and recorded during physician consultations according to standard clinical blood pressure measurement guidelines[27]. Subjects with high lactate dehydrogenase (LDH) blood values were excluded from the study, as blood LDH levels are considered a strong indicator of melanoma metastasis when outside the normal range[28, 29]. Thus, all subjects were within our institutionally specified normal blood LDH range of 313-618 U/L. No subjects included in this study had previously received chemotherapy, as this criterion was a contraindication for surgery. The Institutional Review Boards of the University of Utah and the Salt Lake City Veteran's Affairs Medical Center approved all protocols, and written informed consent was obtained from all subjects prior to biopsy collection.

The arterial biopsies consisted of skeletal muscle feed arteries excised from the inguinal (e.g., hip adductors or quadriceps femoris) or axillary regions (e.g., serratus anterior or latissimus dorsi) and were free of melanoma cells[30]. Arterial biopsies were identified as skeletal muscle feed arteries by entry into muscle bed, gross anatomy, coloration, and pulsatile bleed pattern[30]. There were no differences in our outcomes between arteries from inguinal and axillary regions (all $P \geq 0.14$), and no interactions were found between biopsy source and age-group in any outcomes (all $P \geq 0.13$). Arterial biopsies were cleaned of adipose and connective tissue, and washed to remove residual blood cells. The average size of each artery was 2 mm in length, ~0.5 mm in luminal diameter, and approximately 10-20 mg in mass. Cleaned arteries were then snap frozen in liquid nitrogen and stored at -80˚C prior to performing the following outcomes. All samples were assayed in triplicate, and replicate means were used for analysis.

Telomere Uncapping

ChIP was used to determine the amount of p-H2A.X (ser139) (Santa Cruz Biotechnology, Inc.) localized to telomeres and TRF2 (Abcam) bound to telomeres. ChIPs were performed as described by Dahl and Collas (2008)[31] and analyzed via qPCR for telomere content as described by Cawthon (2009)[32]. Final values were expressed as the ratio of background corrected starting quantity (SQ) of telomeric DNA enriched by ChIP to telomeric DNA SQ in INPUT fraction. INPUTs represented 50% of telomeric DNA

present in corresponding ChIP and were used to control for tissue concentration in samples [ex: (p-H2A.X (ser139) SQ - background SQ)/INPUT SQ = final value].

P53/P21-induced Senescence

ChIPs were performed to assess P53 bound to *P21* gene promoter (EMD Millipore Corporation) as described above[31], using a sequence-independent qPCR assay with FastStart SYBR Green Master (Roche Diagnostics Corporation, Roche Applied Science). Additionally, *P21* mRNA expression was determined by qRT-PCR using the Quantitect Reverse Transcription kit (Qiagen, Inc.) and FastStart SYBR Green Master (Roche Diagnostics Corporation, Roche Applied Science) according to the manufacturer's protocols. Final mRNA SQs were generated by standard curve and expressed as a ratio of target mRNA SQ to *18s* rRNA SQ (18s rRNA QuantiTect Primer Assay: Qiagen, Inc.). 18s rRNA was used as a housekeeping gene transcript to control for tissue concentration in samples (ex: *P21* mRNA SQ/*18s* SQ = final value). *P21* mRNA primers: fwd-*gacctgtcactgtcttgta*, rev-*cctcttggagaagatcagccg***.**

Senescence-Associated Inflammation

IL6, *IL8*, and *MCP1* mRNA expression was determined by qRT-PCR as described above. *IL6* mRNA primers: fwd-*tacccccaggagaagattcc*, rev-*gccatctttggaaggttcag. IL8* mRNA primers: fwd-*aggtgcagttttgccaagga*, rev-*tttctgtgttggcgcagtgt.MCP1* mRNA primers: fwd-*tcgctcagccagatgcaatcaatg***,** rev-*tggaatcctgaacccacttctgct.*

Mean Telomere Length

A sequence-independent multiplex qPCR technique using a SYBR Green master mix with 0.625U AmpliTaq Gold 360 DNA polymerase (Life Technologies Corporation) was utilized to determine mean telomere length as described by Cawthon (2009)[32]. Telomeric DNA (T) SQs and *albumin* SQs*,* used as single copy gene (S) to control tissue concentration in samples, were generated by standard curve and mean telomere length was expressed as the T/S ratio. Mean telomere lengths, telomere ranges, and telomere shortening rates were generated by converting T/S ratios to bp of DNA using the formula: bp = 3330(T/S) + 3730, derived by Cawthon (2009)[32].

Active hTERT

ChIPs were performed to assess hTERT (Abcam) bound to telomeres as described above[31]. This technique allows for a sensitive and quantitative assessment of hTERT bound to telomeres versus simple protein expression or enzyme activity assays, which we believe gives us the most accurate representation of active hTERT *in vivo*.

Data Analysis

Primary outcomes included p-H2A.X (ser139) localized to telomeres, TRF2 bound to telomeres, P53 bound to *P21* gene promoter, mean telomere length, and active hTERT. Secondary outcomes included *P21*, *IL6, IL8,* and *MCP1 mRNA* expression. ANOVA tests were performed with least significance difference (LSD) post hoc tests to assess all age-group and tertile differences in all primary outcomes. Independent samples t-tests

were performed to assess age-group differences in all secondary outcomes. The Pearson correlation coefficient (r) was used to assess correlations between each outcome and between all outcomes and subject characteristics. To assess age-group differences in subject characteristics, ANOVA tests with LSD post hoc tests or Chi-squared tests were performed. Analysis of covariance tests with LSD post hoc tests were performed in all outcomes that correlated with continuous variable subject characteristics to determine the influence of covariates on the age-group differences in our outcomes. All covariates were tested for homogeneity across age-groups in all outcomes and covariate effect size was assessed using partial eta squared (η_p^2). Factorial ANOVA tests were performed to assess interactions between the effects of age-group and age-related disease or prescription medication use status in all outcomes. Independent samples t-tests or Chi-squared tests were performed to assess age-group differences in subgroup analyses of subject characteristics and all outcomes. Data are presented as mean±SEM normalized to younger adult age-group, mean±SEM, or %(n). Significance was set at $P < 0.05$.

Results

Arterial Telomere Uncapping

Telomere uncapping was greater with advancing age in human arteries. p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres were approximately two-fold greater in arteries from older adults compared with younger adults (all $P \leq 0.03$; Figure 2.1). p-H2A.X (ser139) localized to telomeres displayed a strong positive correlation with TRF2 bound to telomeres ($r = 0.67$, $P < 0.001$).

Arterial P53/P21-Induced Senescence and Senescence-Associated Inflammation

Consistent with greater telomere uncapping, P53/P21-induced senescence and senescence-associated inflammation were greater with advancing age in human arteries. There was almost three-fold more P53 bound to *P21* gene promoter in arteries from older adults compared with younger adults ($P = 0.03$; Figure 2.1). Accordingly, there was almost two-fold greater expression of *P21* mRNA in arteries from older adults compared with younger adults ($P = 0.02$; Table 2.2). There was also nearly two-fold higher *IL6* ($P = 0.09$), nearly eight-fold greater *IL8* ($P = 0.01$), and two-fold greater *MCP1* mRNA ($P = 0.03$) expression in arteries from older adults compared with younger adults (Table 2.2).

Influence of Telomere Uncapping on P53/P21-induced Senescence

p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres demonstrated a positive correlation with P53 bound to *P21* gene promoter ($r = 0.48$ and $r = 0.60$, respectively, all $P < 0.001$). Likewise, there was over three-fold greater P53 bound to *P21* gene promoter among subjects in the highest tertiles of p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres compared with those in the median and lowest tertiles (all $P \leq 0.04$; Figure 2.2). Analysis of covariance results indicated that p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres had large effects on the P53 bound to *P21* gene promoter age-group difference ($\eta_p^2 = 0.20$ and 0.33, respectively), accounting for 53% of the total age-related variance in this P53/P21-

induced senescence marker. Controlling for the influence of p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres, the adjusted age-group difference in P53 bound to *P21* gene promoter was no longer significant (older adults compared with younger adults; Δ $P = 0.03$ to $P = 0.13$ and 0.492, respectively).

Arterial Telomere Shortening

Progressive telomere shortening occurred with advancing age in human arteries, with mean telomere lengths of 13.4±0.5 kb in younger adults, 11.5±0.2 kb in middle-aged adults and 10.5±0.3 kb in older adults (all $P < 0.001$ compared with younger adults; $P = 0.03$ for older adults compared with middle-aged adults; Figure 2.3). There was a 61 bp/yr rate of shortening, estimated by the linear equation: $y = 61.2x + 14{,}900.7$ ($R^2 = 0.22$, $r = -0.47$, $P < 0.001$; Figure 2.3). Mean telomere length was not correlated with p-H2A.X (ser139) localized to telomeres ($r = -0.09$, $P = 0.20$), TRF2 bound to telomeres ($r = -0.15$, $P = 0.09$), or P53 bound to *P21* gene promoter ($r = -0.16$, $P = 0.08$). Additionally, there were no differences in these markers of telomere uncapping and P53/P21-induced senescence between subjects in the shortest (8.2-10.8 kb), median (10.9-12.5 kb), and longest (12.6-17.2 kb) tertiles of mean telomere length (all $P \geq 0.20$; Figure 2.4). There was no difference in active hTERT between age-groups in human arteries (all $P \geq 0.82$; Figure 2.3).

Influence of CVD Risk Factors and Prescription Medication Use

We observed greater systolic blood pressure (SBP), body mass index (BMI), hypertension incidence, and prescription blood pressure medication use with advancing age in our subjects (Table 2.1). P53 bound to *P21* gene promoter was positively correlated with SBP (r = 0.20, *P* = 0.04), and analysis of covariance results indicated that SBP was an influential covariate for the age-group difference in this P53/P21-induced senescence marker (older adults compared with younger adults; Δ *P* = 0.03 to 0.12, η_p^2 = 0.02). BMI was negatively correlated with mean telomere length (r = -0.20, *P* = 0.03), but was not an influential covariate for the age-group differences in this primary outcome (all *P* remained $\leq$ 0.02, η_p^2 < 0.01). Likewise, DBP was positively correlated with *MCP1* mRNA expression (r = 0.29, *P* = 0.01), but was not an influential covariate for the age-group difference in this marker of senescence-associated inflammation (*P* remained = 0.05, η_p^2 = 0.09). No interactions between the effects of age-group and age-related disease or prescription medication use status were found using a factorial ANOVA to compare means for all outcomes (all *P* $\geq$ 0.06).

To further assess the influence of prescription medication use on the age-group differences we observed , we identified and removed all subjects using potentially confounding prescription medications and repeated analyses for all outcomes in this subgroup of unmedicated subjects (unmedicated subject characteristics; Table 2.3). Arteries from unmedicated older adults had greater p-H2A.X (ser139) localized to telomeres, TRF2 bound to telomeres, P53 bound to *P21* gene promoter, and *P21*, *IL8*, and *MCP1* mRNA expression compared with younger adults (all *P* < 0.05). Arteries from

unmedicated older adults also had shorter mean telomere length compared with younger adults ($P < 0.01$), and there was no age-group difference in active hTERT ($P = 0.44$). SBP was positively correlated with P53 bound to the *P21* gene promoter in unmedicated subjects ($r = 0.35$, $P = 0.03$), and remained an influential covariate for the age-group difference in this marker of P53/P21 senescence (older adults compared with younger adults; $\Delta P = 0.04$ to $P = 0.34$, $\eta_p^2 = 0.05$).

Discussion

The key novel findings of the current study are as follows: Telomere uncapping was greater in arteries from older adults compared with those from younger adults. There was greater P53/P21-induced senescence in arteries from older adults compared with younger adults. Telomere uncapping was a highly influential covariate for the age-group difference in P53/P21-induced senescence. Despite progressive age-related telomere shortening in human arteries, mean telomere length was not associated with telomere uncapping or P53/P21-induced senescence. There was also no difference in active hTERT with advancing age in human arteries. Collectively, these findings demonstrate that advancing age is associated with greater telomere uncapping in arteries, which is linked to P53/P21-induced senescence independent of telomere shortening.

Arterial Telomere Uncapping

Age-related telomere uncapping could lead to P53/P21-induced senescence and ensuing inflammation in arteries. *In vitro* studies in human cells have shown that replication-dependent telomere uncapping can lead to a dsDNA break response at telomeres[3-5] and ultimately P53/P21-induced senescence[5, 6]. While greater telomere uncapping has been reported in $CD19^+$ B cells from radiation-resistant chronic lymphocytic leukemia patients compared with radiation-sensitive patients[33], to our knowledge, no studies have measured telomere uncapping with advancing age in noncultured human tissues. We reported greater telomere uncapping, as determined by p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres, in arteries from older adults compared with those from younger adults. These results establish that greater telomere uncapping is associated with advancing age in arteries, which represents a plausible precursor to P53/P21-induced senescence and senescence-associated inflammation.

In support of evidence that TRF2 binds to uncapped telomeres to prevent chromosome end-fusions [7, 8], TRF2 has also been shown to localize to nontelomeric dsDNA break sites and participate in the dsDNA break response in human cells[34]. However, TRF2 has other well-established functions that are essential for normal telomere structure and stability[3, 4]. Thus, greater TRF2 bound to telomeres in arteries with advancing age might also be a compensatory response to telomere shortening to maintain telomere structure and stability. Nonetheless, we found a strong positive

correlation between p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres, which reinforces *in vitro* evidence that TRF2 binds to uncapped telomeres [7, 8].

Arterial P53/P21-Induced Senescence and Senescence-Associated Inflammation

Age-related P53/P21-induced senescence and ensuing inflammation may contribute to the chronic inflammation in arteries that precedes many CVDs. In human cells, activated P53 binds to the *P21* gene promoter region, resulting in P21 upregulation and subsequent inhibition of cell cycle progression[5, 6]. Several *in vitro* studies in human cells have also shown that P53/P21-induced senescence results in increased expression of IL6, IL8, and MCP1[9-13]. Importantly, there is evidence that arterial P53/P21-induced senescence may play a role in carotid artery disease[35], chronic obstructive pulmonary disease[13], as well as popliteal artery aneurysm[36]. Marchand *et al.*(2011) reported greater *P21* mRNA expression in arteries from older coronary artery bypass graft patients compared with middle-aged patients[37]; however, it was unknown whether age-related P53/P21-induced senescence occurs in arteries from subjects that are more generalizable. We observed greater P53 bound to *P21* gene promoter and greater *P21* mRNA expression in normal arteries from older adults compared with those taken from younger adults. Likewise, we found higher *IL8* and *MCP1* mRNA expression in arteries from older adults compared with those from younger adults. These findings demonstrate that P53/P21-induced senescence and senescence-associated inflammation occur with advancing age in human arteries.

Influence of Telomere Uncapping on P53/P21-Induced Senescence

If age-related telomere uncapping results in P53/P21-induced senescence in arteries, there should be a close connection between these outcomes. Both p-H2A.X (ser139) localized to telomeres and TRF2 bound to telomeres demonstrated positive correlations with P53 bound to *P21* gene promoter, and were highly influential covariates for the age-group difference in this P53/P21-induced senescence marker. Combined, these two markers of telomere uncapping accounted for more than half of the variance in the P53 bound to *P21* gene promoter age-group difference. These results suggest that age-related telomere uncapping is a biologically relevant link to P53/P21-induced senescence in arteries.

Arterial Telomere Shortening

Age-related telomere shortening is a potential mechanism by which telomere uncapping, P53/P21-induced senescence, and subsequent inflammation occur in arteries. Telomere shortening with advancing age has been documented in most human somatic tissues[1, 20-22], and numerous *in vitro* studies in human cells have linked telomere shortening and senescence[1, 20, 38]. Arterial telomere shortening has been associated with both advancing age and atherosclerotic plaque development in studies with modestly sized subject samples (n = 27-51), using tissue taken from cadavers[22-24]. We showed progressive telomere shortening with advancing age in a large sample of human arteries obtained from living donors, which extends previous findings. We also found that mean telomere length was not associated with telomere uncapping or P53/P21-induced

senescence. Arterial telomere uncapping caused by the accumulation of genotoxic insults with advancing age might explain these lack of associations[20, 25]. These observations cast doubt on the biological relevance of telomere shortening as a mechanism underlying age-related telomere uncapping, P53/P21-induced senescence, and senescence-associated inflammation in arteries.

Telomere length homeostasis is maintained by hTERT activity[26], primarily in germ cells, white blood cells (WBCs), and adult stem cell compartments[39-41]. While a link between age and hTERT activity has not previously been tested in human arteries, a few studies have shown age-related declines in hTERT activity in human WBCs[40, 42]. Interestingly, Liu *et al.*(2005) found greater hTERT expression and activity in atherosclerotic arteries compared with nonatherosclerotic controls[43], and a recent study by Kroenke *et al.*(2011) has associated increased WBC hTERT activity with greater CVD risk [44]. We reported no difference in active hTERT with advancing age in human arteries. Thus, hTERT activity may be insufficient to prevent age-related telomere shortening in human arteries. However, a growing body of work suggests that hTERT may have important nontelomeric activities related to mitochondrial function and oxidative stress in human cells[45-47]. As mitochondria-derived reactive oxygen species are well-established mediators of chronic disease[48, 49], this new area of inquiry may reveal a role for arterial hTERT activity in CVDs related to mitochondrial dysregulation.

Influence of CVD Risk Factors and Prescription Medication Use

We noted higher levels of CVD risk factors like SBP and BMI, as well as greater incidence of hypertension and prescription blood pressure medication use, with advancing age in our subjects. These trends are similar to those observed in other human studies assessing arterial aging in large generalizable subject samples[16, 50, 51], but could have influence on the age-group differences reported in our outcomes. With the exception of SBP, no subject characteristics influenced any outcomes, nor were there interactions between the effects of age-group and age-related disease or prescription medication use status in any outcomes. Controlling for the influence of SBP did affect the age-group difference in P53 bound to *P21* gene promoter, which indicates a potentially interesting link between these measures. These analyses account for the impact of CVD risk factors and prescription medication use on age-related telomere uncapping and P53/P21-induced senescence.

To completely rule out any effect of prescription medication use on the age-group differences we observed, we performed analyses for all outcomes with only unmedicated subjects. The influence of age on telomere uncapping, P53/P21-induced senescence, senescence-associated inflammation, mean telomere length, and active hTERT in human arteries was not affected by prescription medication use. These results provide support for the age-group differences found in the larger more generalizable subject sample.

Conclusions

The goal of this study was to assess the potential role of telomere uncapping in chronic arterial inflammation. Our findings reveal that telomere uncapping occurs with advancing age in arteries, which is linked to P53/P21-induced senescence independent of telomere shortening. These studies lay the clinical foundation for future studies aimed at establishing the causal role of telomere uncapping in age-related arterial inflammation and subsequent CVD.

Acknowledgements

All experiments were performed in the Translational Vascular Physiology Laboratory at the University of Utah and Veteran's Affairs Medical Center-Salt Lake City, Geriatric Research Education and Clinical Center. Garrett Morgan and Anthony J Donato contributed to all aspects of the study, including the conception and design of the experiments, collection, analysis, and interpretation of data, and drafting and revision of the manuscript. Stephen J Ives, Lisa A Lesniewski, Richard M Cawthon, Robert HI Andtbacka, R Dirk Noyes, and Russell S Richardson contributed to the collection and analysis of data as well as revision of the manuscript. Additionally, Richard M Cawthon contributed reagents and analytical tools that were essential to completing this study.

Source of Funding

Awards from the National Institutes of Health: NIA-AG040297, NIA-AG029337, NIA-AG033196, NIA-AG033755 NHLBI-HL09183, The Department of Veterans Affairs

Merit Grant-E6910R, and a University of Utah Center on Aging Grant financially supported this work.

Conflict of Interest

The authors have no conflict of interest or disclosures to report.

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Table 2.1. Subject characteristics

Characteristic	Younger Adults (n=25)	Middle-aged Adults (n=43)	Older Adults (n=36)	*P*
Age (years)[a]	31.2±1.3	52.6±0.7	70.9±1.4	< 0.001*
Sex (M/F)[b]	13/12	26/17	23/13	0.641
BMI (kg/m^2)[a]	23.8±0.9	29.6±0.9	28.9±0.8	< 0.001*
SBP (mmHg)[a]	127.9±2.8	132.7±2.6	146.5±3.4	< 0.001*
DBP (mmHg)[a]	75.0±2.5	80.0±1.7	77.0±2.1	0.240
Medical history				
Hypertension[b]	0.0%(0)	32.6%(14)	55.6%(20)	< 0.001*
CAD[b]	0.0%(0)	4.7%(2)	11.1%(4)	0.172
PVD[b]	0.0%(0)	0.0%(0)	2.8%(1)	0.385
Prescription medications				
Calcium channel blockers[b]	0.0%(0)	9.3%(4)	19.4%(7)	0.049*
Beta blockers[b]	0.0%(0)	9.3%(4)	11.1%(4)	0.243
ACE inhibitors[b]	0.0%(0)	16.3%(7)	22.2%(8)	0.047*
Angiotensin blockers[b]	0.0%(0)	2.3%(1)	13.9%(5)	0.033*
Diuretics[b]	4.0%(1)	14.0%(6)	19.4%(7)	0.219

Data presented are [a]mean±SEM, [b]n, and [b]%(n) across age-groups. Terms: BMI-body mass index, SBP-systolic blood pressure, DBP-diastolic blood pressure, CAD-coronary artery disease, PVD-peripheral vascular disease, ACE-angiotensin-converting-enzyme.

Table 2.2. Arterial P53/P21-induced senescence and senescence-associated inflammation

Target mRNA	Fold Δ in expression (Older/Younger)	*P*
P53/P21-induced senescence		
P21	1.9±0.3	0.02*
Senescence-associated inflammation		
IL6	1.7±0.3	0.09
IL8	7.5±2.1	0.01*
MCP1	2.1±0.3	0.03*

Data presented are fold change in mean±SEM target mRNA expression in older adults compared with younger adults. Terms: P53-tumor suppressor protein p53, P21-cyclin-dependent kinase inhibitor 1A, IL6-interleukin 6, IL8- interleukin 8, MCP1-monocyte chemotactic protein 1.

Table 2.3. Unmedicated subject characteristics

Characteristic	Younger Adults (n=25)	Older Adults (n=15)	*P*
Age (years)[a]	31.2±1.3	68.2±1.8	< 0.001*
Sex (M/F)[b]	13/12	12/3	0.077
BMI (kg/m^2)[a]	23.8±0.9	28.0±1.2	0.004*
SBP (mmHg)[a]	127.9±2.8	142.5±4.7	0.004*
DBP (mmHg)[a]	75.0±2.5	79.5±3.4	0.143

Data presented are [a]mean±SEM and [b]n across age-groups. Terms: BMI-body mass index, SBP-systolic blood pressure, DBP-diastolic blood pressure.

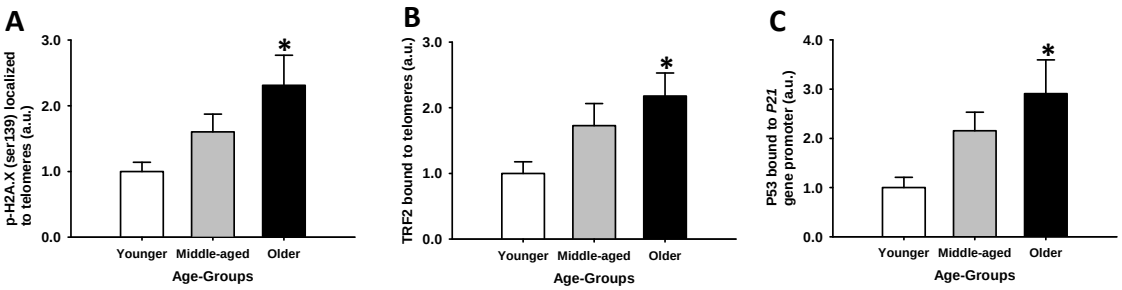

Figure 2.1. Arterial telomere uncapping and P53/P21-induced senescence. **A)** p-H2A.X (ser139) localized to telomeres, **B)** TRF2 bound to telomeres, and **C)** P53 bound to *P21* gene promoter across age-groups (all $*P \leq 0.03$). Terms: p-H2A.X (ser139)-p-histone γ-H2A.X (ser139), TRF2-telomeric repeat binding factor 2, P53-tumor suppressor protein p53, P21-cyclin-dependent kinase inhibitor 1A.

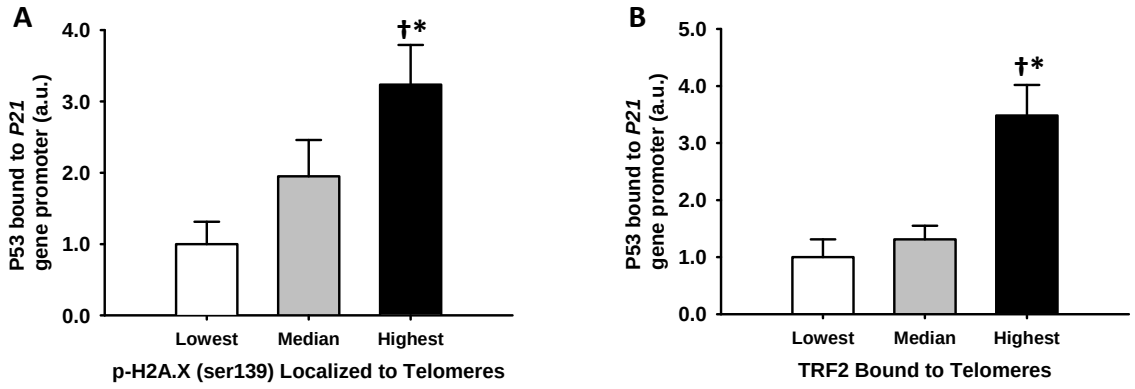

Figure 2.2. Influence of telomere uncapping on P53/P21-induced senescence. P53 bound to *P21* gene promoter across tertiles of telomere uncapping markers, including **A)** p-H2A.X (ser139) localized to telomeres and **B)** TRF2 bound to telomeres (all $^{*}P < 0.001$ compared with lowest tertile; all $^{\dagger}P \leq 0.04$ compared with median tertile). Terms: p-H2A.X (ser139)-p-histone γ-H2A.X (ser139), TRF2-telomeric repeat binding factor 2, P53-tumor suppressor protein p53, P21-cyclin-dependent kinase inhibitor 1A.

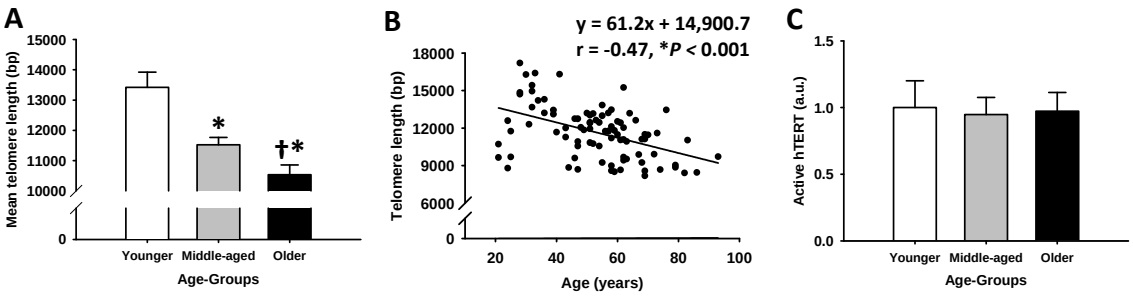

Figure 2.3. Arterial telomere shortening. **A)** Mean telomere length across age-groups (all *$P < 0.001$ compared with younger adults; $^{\dagger}P = 0.03$ for older adults compared with middle-aged adults), **B)** correlation between mean telomere length and age**,** and **C)** active hTERT across age-groups. Terms: hTERT-telomerase reverse transcriptase.

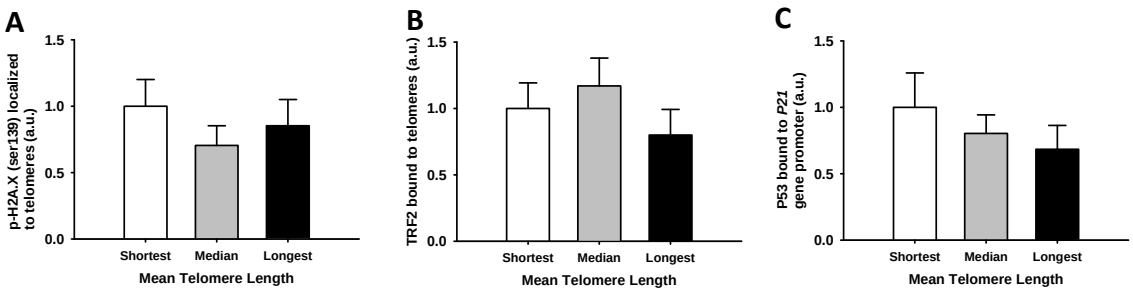

Figure 2.4. Influence of mean telomere length on telomere uncapping and P53/P21-induced senescence. **A)** p-H2A.X (ser139) localized to telomeres, **B)** TRF2 bound to telomeres, and **C)** P53 bound to *P21* gene promoter across tertiles of mean telomere length. Terms: p-H2A.X (ser139)-p-histone γ-H2A.X (ser139), TRF2-telomeric repeat binding factor 2, P53-tumor suppressor protein p53, P21-cyclin-dependent kinase inhibitor 1A.

CHAPTER 3

ROLE OF ARTERIAL TELOMERE DYSFUNCTION IN HYPERTENSION: RELATIVE CONTRIBUTIONS OF TELOMERE SHORTENING AND TELOMERE UNCAPPING

Abstract

Telomere shortening in arteries could lead to telomere uncapping and cellular senescence, which in turn could promote the development of hypertension. To assess the novel role of arterial telomere dysfunction in hypertension, we compared mean telomere length (qPCR), telomere uncapping (serine 139 phosphorylated histone γ-H2A.X (γ-H2) localized to telomeres: ChIP), and tumor suppressor protein p53 (P53)/cyclin-dependent kinase inhibitor 1A (P21)-induced senescence (P53 bound to *P21* gene promoter: ChIP) in arteries from 55 age-matched hypertensive and nonhypertensive subjects. Arterial mean telomere length was not different in hypertensive subjects compared with nonhypertensive subjects ($P = 0.29$). Arterial telomere uncapping and P53/P21-induced senescence were two-fold greater in hypertensive subjects compared with nonhypertensive subjects ($P = 0.04$ and $P = 0.02$, respectively). Arterial mean telomere length was not associated with telomere uncapping or P53/P21-induced senescence ($r = -0.02$, $P = 0.44$ and $r = 0.01$, $P = 0.50$, respectively), but telomere uncapping was a highly influential covariate for the hypertension group difference in P53/P21-induced senescence ($r = 0.62$, $P < 0.001$, $\eta_p^2 = 0.35$). Finally, telomere uncapping was a significant predictor of hypertension status ($P = 0.03$), while mean telomere length was not ($P = 0.68$). Collectively, these findings demonstrate that arterial telomere uncapping and P53/P21-induced senescence are linked to hypertension independent of mean telomere length, and telomere uncapping influences hypertension status more than mean telomere length.

Introduction

Arterial telomere dysfunction may contribute to the pathogenesis of hypertension by inducing cellular senescence. Telomeres are terminal sequences of TTAGGG repeats that make up the natural ends of chromosomes[1, 2]. Telomeres form specialized structures that protect chromosome ends from being recognized as dsDNA breaks and initiating a dsDNA break response[3-5], which can induce cellular senescence through tumor suppressor protein p53 (P53)-dependent expression of cyclin-dependent kinase inhibitor 1A (P21)[5, 6]. *In vitro* studies in various human cell types have shown that breakdown of telomere structure, referred to as telomere uncapping, leads to P53 activation and P53/P21-induced senescence[5, 6]. Replication and genotoxic stress-mediated telomere shortening beyond a critical telomere length may lead to uncapping in human cells[5-8]. Following *in vitro* telomere uncapping in human cells, phosphorylation of histone γ-H2A.X at serine 139 (γ-H2) occurs at telomeric chromatin to aid in the dsDNA break response and initiation of P53/P21-induced senescence[5, 6]. Importantly, cellular senescence has been implicated in the etiology of chronic diseases[9, 10], including cardiovascular diseases (CVDs)[11, 12].

While the role of arterial telomere dysfunction in hypertension has not been assessed, telomere shortening in white blood cells (WBCs) has been linked to hypertension [13, 14], and telomere shortening in arteries has been associated with abdominal aortic aneurysm[15] and atherosclerotic plaque development [16, 17]. Telomere shortening in arteries could lead to telomere uncapping and P53/P21-induced senescence, which in turn could promote the development of hypertension.

Interestingly, some studies have suggested that arterial telomere uncapping is more closely linked to P53/P21-induced senescence than telomere shortening in human cells[18, 19], including arterial tissues[20]. Insight into the relative contributions of arterial telomere shortening and telomere uncapping to the etiology of hypertension will lead to a more complete understanding of the potential role of telomere dysfunction in hypertension. This in turn could lead to novel therapeutic strategies that target telomere dysfunction to delay onset, attenuate severity, or even reverse hypertension.

Therefore, an important unexplored hypothesis is that arterial telomere dysfunction and P53/P21-induced senescence are associated with hypertension. To test this hypothesis, we compared mean telomere length, telomere uncapping (γ-H2 localized to telomeres), and P53/P21-induced senescence (P53 bound to *P21* gene promoter) in arteries from an age-matched sample of hypertensive and nonhypertensive subjects. Next, we used logistic regression to compare the influence of arterial mean telomere length and telomere uncapping on hypertension status in these subjects.

Materials and Methods

Arterial Biopsy Collection and General Sample Processing

Arterial biopsies were excised from patients undergoing a prophylactic procedure for a melanoma-associated sentinel lymph node biopsy at the Huntsman Cancer Hospital, University of Utah. A heterogeneous sample (n) of 55 age-matched subjects (34 males and 21 females) consented to donate arterial biopsies for the study.

A comprehensive outline of biometric, physiological, and medical characteristics for all subjects enrolled in the study was collected (Table 3.1). Subjects were classified as hypertensive or nonhypertensive according to prior hypertension diagnosis reported in medical history. Both medical histories and prescription medication use were noted, and subjects with prior diagnosis of CVD other than hypertension or metastatic melanoma were excluded. Subject blood pressures were measured and recorded during physician consultations according to standard clinical blood pressure measurement guidelines[21]. Subjects with high lactate dehydrogenase (LDH) blood values were excluded from the study, as blood LDH levels are considered a strong indicator of melanoma metastasis when outside the normal range[22, 23]. Thus, all subjects were within our institutionally specified normal range of 313-618 U/L, and LDH levels were not different between groups (all $P = 0.32$) nor correlated with any outcomes (all $P \geq 0.08$). No subjects included in this study had received chemotherapy, as this criterion was a contraindication for surgery. The Institutional Review Boards of the University of Utah and the Salt Lake City Veteran's Affairs Medical Center approved all protocols, and written informed consent was obtained from all subjects prior to biopsy collection.

The arterial biopsies consisted of skeletal muscle feed arteries excised from the inguinal (e.g., hip adductors or quadriceps femoris) or axillary regions (e.g., serratus anterior or latissimus dorsi) and were free of melanoma cells[24]. Arterial biopsies were identified as skeletal muscle feed arteries by entry into muscle bed, gross anatomy, coloration, and pulsatile bleed pattern[24]. There were no differences in our outcomes between arteries from inguinal and axillary regions (all $P \geq 0.10$), and no interactions

between the effects of biopsy source and group were found in any outcomes (all $P \geq 0.16$). Arterial biopsies were cleaned of adipose and connective tissue, and washed to remove residual blood cells. The average size of each artery was 2 mm in length, ~0.5 mm in luminal diameter, and approximately 10-20 mg in mass. Cleaned arteries were then snap frozen in liquid nitrogen and stored at -80˚C prior to performing the following outcomes. All samples were assayed in triplicate, and replicate means were used for analysis.

Mean Telomere Length

A sequence-independent multiplex qPCR technique using a SYBR Green master mix with 0.625U AmpliTaq Gold 360 DNA polymerase (Life Technologies Corporation) was utilized to determine mean telomere length as described by Cawthon[25]. Telomeric DNA (T) SQs and *albumin* SQs*,* used as single copy gene (S) to control tissue concentration in samples, were generated by standard curve and mean telomere length was expressed as the T/S ratio. Mean telomere lengths and telomere ranges were generated by converting T/S ratios to bp of DNA using the formula: bp = 3330(T/S) + 3730, derived by Cawthon[25].

Telomere Uncapping

ChIP was used to determine the amount of γ-H2 (Santa Cruz Biotechnology, Inc.) localized to telomeres. ChIPs were performed as previously described[20], and analyzed via qPCR for telomere content as described by Cawthon[25]. Final values were expressed

as the ratio of background corrected starting quantity (SQ) of telomeric DNA enriched by ChIP to telomeric DNA SQ in INPUT fraction. INPUTs represented 50% of telomeric DNA present in corresponding ChIP and were used to control for tissue concentration in samples [ex: (γ-H2) SQ - background SQ)/INPUT SQ = final value].

P53/P21-Induced Senescence

ChIPs were performed to assess P53 bound to *P21* gene promoter (EMD Millipore Corporation) as previously described[20], using a sequence-independent qPCR assay with FastStart SYBR Green Master (Roche Diagnostics Corporation, Roche Applied Science).

Data Analysis

Outcome measures were defined as mean telomere length, γ-H2 localized to telomeres, P53 bound to *P21* gene promoter, and hypertension status. Independent samples t-tests were performed to assess group differences in all outcomes. ANOVA tests were performed with least significance difference (LSD) post hoc tests to assess tertile differences in all outcomes. The Pearson correlation coefficient (r) was used to assess correlations between each outcome and between outcomes and subject characteristics. To assess group differences in subject characteristics, independent samples t-tests or Chi-squared tests were performed. Analysis of covariance tests with LSD post hoc tests were performed with all continuous variable subject characteristics with correlated outcomes to determine the influence of covariates on any group

differences in outcomes. All covariates were tested for homogeneity across groups within outcomes ($P = 0.67$) and covariate effect size was assessed using partial eta squared (η_p^2).

Logistic Regression

Forward and backward likelihood ratio (LR) logistic regression analyses were conducted to assess the influence of mean telomere length, γ-H2 localized to telomeres, and body mass index (BMI) on hypertension status (test statistic: Δ in -2 log likelihood). Logistic regression model goodness-of-fit was determined by comparing the significant predictors of hypertension status identified by forward and backward LR logistic regression analyses (good model of data: same predictors). Additionally, the Hosmer-Lemeshow (good model of data: significance value ≥ 0.05) and Nagelkerke's R^2 (pseudo R^2) statistics were used to assess logistic regression model goodness-of-fit. Significance was set at $P < 0.05$.

Results

Subject Characteristics

Approximately 83% of hypertensive subjects used one or more prescription medications to control blood pressure (n = 24; Table 3.1). By definition, no nonhypertensive subjects used prescription blood pressure medications. BMI was greater in hypertensive subjects compared with nonhypertensive subjects ($P = 0.01$;

Table 3.1), while sex, systolic blood pressure (SBP), diastolic blood pressure and pulse pressure were not different between groups (all $P \geq 0.09$; Table 3.1).

Arterial Telomere Dysfunction, P53/P21-Induced Senescence, and Hypertension

Arterial mean telomere length was not different between hypertensive and nonhypertensive subjects ($P = 0.29$; Figure 3.1). Arterial γ-H2 localized to telomeres was nearly two-fold greater in hypertensive subjects compared with nonhypertensive subjects ($P = 0.04$; Figure 3.1). Correspondingly, arterial P53 bound to *P21* gene promoter was over two-fold greater in hypertensive subjects compared with nonhypertensive subjects ($P = 0.02$; Figure 3.1). No subject characteristics were correlated with γ-H2 localized to telomeres or P53 bound to *P21* gene promoter (both $P \geq 0.09$).

Influence of Telomere Dysfunction on P53/P21-Induced Senescence

Mean telomere length was not correlated with γ-H2 localized to telomeres or P53 bound to *P21* gene promoter (r = -0.02, $P = 0.44$ and r = 0.01, $P = 0.50$, respectively). Additionally, there were no differences in P53 bound to *P21* gene promoter between subjects in the shortest (7.8-9.9 kb), median (10.6-12.4 kb), and longest (12.5-17.2 kb) tertiles of mean telomere length (all $P \geq 0.08$; Figure 3.2). γ-H2 localized to telomeres demonstrated a strong positive correlation with P53 bound to *P21* gene promoter (r =

0.62, $P < 0.001$). Likewise, there was almost six-fold greater P53 bound to *P21* gene promoter among subjects in the highest tertile of γ-H2 localized to telomeres compared with those from the lowest tertile ($P < 0.01$; Figure 3.2). Analysis of covariance results indicated that γ-H2 localized to telomeres had a large effect on the P53 bound to *P21* gene promoter group difference ($\eta_p^2 = 0.35$), accounting for 35% of the total hypertension-related variance in this P53/P21-induced senescence marker. Controlling for the influence of γ-H2 localized to telomeres, the adjusted group difference in P53 bound to *P21* gene promoter was no longer significant (Δ $P = 0.04$ to 0.24).

Influence of Telomere Dysfunction and BMI on Hypertension Status

Logistic regression results indicated that γ-H2 localized to telomeres and BMI were both significant predictors of hypertension status ($P = 0.03$ and 0.01, respectively; Table 3.2), while mean telomere length was not ($P = 0.68$; Table 3.2). Together, γ-H2 localized to telomeres and BMI explained 25% of the variance in hypertension status (pseudo $R^2 = 0.25$; Table 3.2) and correctly predicted hypertension status in over 67% of hypertensive subjects (Table 3.3). Backward and forward LR logistic regression analyses produced the same results (Tables 3.2 and 3.3), and generated identical Hosmer-Lemeshow test results (both $P = 0.78$; Table 3.2).

Discussion

The key novel findings of the current study are as follows: Arterial mean telomere length was not different in hypertensive subjects compared with nonhypertensive subjects. Arterial telomere uncapping and P53/P21-induced senescence were greater in hypertensive subjects compared with nonhypertensive subjects. Arterial mean telomere length was not associated with telomere uncapping or P53/P21-induced senescence, but telomere uncapping was a highly influential covariate for the group difference in P53/P21-induced senescence. Finally, telomere uncapping was a significant predictor of hypertension status, while mean telomere length was not. Collectively, these findings demonstrate that arterial telomere uncapping and P53/P21-induced senescence are linked to hypertension independent of mean telomere length, and telomere uncapping influences hypertension status more than mean telomere length.

Arterial Mean Telomere Length and Hypertension

Arterial telomere shortening could play a role in hypertension by leading to telomere uncapping and P53/P21-induced senescence. Here we showed no difference in mean telomere length in arteries from hypertensive subjects compared with those from nonhypertensive subjects. Furthermore, mean telomere length was not correlated with telomere uncapping or P53/P21-induced senescence. Telomere uncapping caused by reactive oxygen species[7, 8] or mechanical stress[26] in hypertension could explain the lack of associations between mean telomere length and telomere uncapping or P53/P21

senescence. While its role in hypertension has not previously been assessed, arterial telomere shortening has been associated with chronic obstructive pulmonary disease (COPD)[12], abdominal aortic aneurysm[15], and atherosclerosis[16, 17]. These data demonstrate that arterial mean telomere length is not associated with hypertension, telomere uncapping, or P53/P21-induced senescence, which suggests that arterial telomere shortening likely does not contribute to the pathogenesis of hypertension.

Telomere shortening in WBCs has been correlated with hypertension in medicated subjects[13, 14], increased pulse pressure in men[27], and pulmonary hypertension severity[12]. Arterial mean telomere length should be more relevant to the etiology of hypertension than that of WBCs, but the accessibility of blood makes WBCs a preferred source of tissue for researchers interested in the role of telomere dysfunction in CVD. Tissue specific differences in mean telomere length and rates of telomere shortening may account for the difference in our findings from those in studies with WBCs. Therefore, these results cast doubt on the biological relevance of telomere shortening in WBCs to hypertension.

Arterial Telomere Uncapping and Hypertension

Arterial telomere uncapping could play a role in hypertension by leading to P53/P21-induced senescence. We showed that telomere uncapping and P53/P21 senescence were greater in arteries from hypertensive subjects compared with those from nonhypertensive subjects, and these differences were independent of mean telomere length. We also reported that telomere uncapping demonstrated a strong

positive correlation with P53/P21 senescence. Interestingly, telomere uncapping was a highly influential covariate for the group difference in P53/P21 senescence, accounting for about 35% of the variance in this outcome. Prior to these findings, the role of telomere uncapping in hypertension was entirely unknown. The only previous study to measure arterial telomere uncapping reported greater γ-H2 localized to telomeres with advancing age, which was positively correlated with P53/P21 senescence independent of telomere shortening[20]. Arterial P53/P21-induced senescence has been linked to atherosclerosis[11] and COPD[12], but its association with hypertension was also previously unexplored. These results demonstrate that arterial telomere uncapping and P53/P21-induced senescence are associated with hypertension and establish the link between telomere uncapping and P53/P21-induced senescence. Thus, arterial telomere uncapping may contribute to the etiology of hypertension by leading to P53/P21-induced senescence.

Influence of Telomere Dysfunction on Hypertension Status

If arterial telomere dysfunction indeed plays a role in hypertension, then mean telomere length or telomere uncapping should influence hypertension status. Utilizing logistic regression analyses, we showed that telomere uncapping and BMI were significant predictors of hypertension status, while mean telomere length was not. Both telomere uncapping and BMI correctly predicted hypertension status in over two-thirds of hypertensive subjects and explained one-quarter of the variance in hypertension status. BMI was included in the logistic regression models because it was higher in

hypertensive subjects compared with nonhypertensive subjects and a well-established CVD risk factor. Thus, we felt it was necessary to account for the influence of BMI on hypertension status in our logistic regression model. These findings demonstrate that telomere uncapping influences hypertension status more than mean telomere length, which suggests that in arteries, telomere uncapping may contribute more to the development of hypertension than mean telomere length.

Influence of Subject Characteristics on Outcomes

Subject characteristics that include conventional CVD risk factors or prior CVD diagnoses could influence our hypertension-associated outcomes and hypertension status. To control for these potential confounds, we matched our subjects for age and excluded subjects with a prior diagnosis of CVDs other than hypertension. Nonetheless, our hypertensive subjects had higher BMI than nonhypertensive subjects. BMI was included in the logistic regression models to account for this group difference, as described above. Importantly, no subject characteristics were correlated with telomere uncapping or P53/P21-induced senescence. Thus, group differences in these outcomes were independent of subject characteristics.

Most hypertensive subjects used one or more prescription medications to control their blood pressure, and none of our nonhypertensive subjects took prescription blood pressure medications. While our hypertensive subjects' mean SBP was clinically hypertensive, SBP was not different between hypertensive and nonhypertensive subjects. This was not unexpected, as most of our hypertensive

subjects used blood pressure medications, and the mean age of both hypertensive and nonhypertensive subjects was nearly sixty-four years of age. To avoid misidentifying hypertensive subjects as nonhypertensive, we based our group classification on prior diagnosis of hypertension rather than SBP measured during physician consultations. Thus, the lack of group difference in SBP did not affect our outcomes. Collectively, these experimental controls and analyses account for the influence of CVD risk factors and CVDs on our hypertension-associated outcomes and hypertension status.

Conclusions

The goal of this study was to elucidate the potential role of arterial telomere dysfunction in hypertension. Our findings demonstrate that arterial telomere uncapping and P53/P21-induced senescence were linked to hypertension independent of mean telomere length. We also reported that telomere uncapping had greater influence on hypertension status than mean telomere length. These results establish the framework for more mechanistic studies aimed at determining the causal role of arterial telomere dysfunction in hypertension. Furthermore, these results provide insight into the relative contributions of arterial telomere shortening and telomere uncapping to the etiology of hypertension and a more complete understanding of the role of telomere dysfunction in CVD.

Acknowledgements

All experiments were performed in the Translational Vascular Physiology Laboratory at the University of Utah and Veteran's Affairs Medical Center-Salt Lake City, Geriatric Research Education and Clinical Center. Garrett Morgan and Anthony J Donato contributed to all aspects of the study, including the conception and design of the experiments, collection, analysis, and interpretation of data, and drafting and revision of the manuscript. Stephen J Ives, Lisa A Lesniewski, Richard M Cawthon, Robert HI Andtbacka, R Dirk Noyes, Ashley E Walker, and Russell S Richardson contributed to the collection and analysis of data as well as revision of the manuscript. Additionally, Richard M Cawthon contributed reagents and analytical tools that were essential to completing this study.

Funding Sources

Awards from the NIH: National Institute of Aging-AG040297, National Institute of Aging-AG029337, National Institute of Aging-AG033196, National Institute of Aging-AG033755, NIH Heart, Lung, and Blood Institute-HL09183, The Department of Veterans Affairs Merit Grant-E6910R, and a University of Utah Center on Aging Grant financially supported this work.

Conflict of Interest

The authors have no conflict of interest or disclosures to report.

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Table 3.1. Age-matched nonhypertensive and hypertensive subject characteristics

Characteristic	Nonhypertensive (n=26)	Hypertensive (n=29)	*P*
Age (years) [a]	63.7±1.5	63.8±2.4	0.48
Sex (M/F) [b]	16/10	18/11	0.48
BMI (kg/m^2) [a]	27.2±0.9	30.1±1.1	0.01*
SBP (mmHg) [a]	135.8±3.8	140.8±3.5	0.17
DBP (mmHg) [a]	78.4±2.4	78.5±2.4	0.48
PP (mmHg) [a]	57.5±2.5	62.3±2.5	0.09
Prescription medications			
Calcium channel blockers [b]	0.0%(0)	24.1%(7)	N/A
Beta blockers [b]	0.0%(0)	17.2%(5)	N/A
ACE inhibitors [b]	0.0%(0)	37.9%(11)	N/A
Angiotensin blockers [b]	0.0%(0)	17.2%(5)	N/A
Diuretics [b]	0.0%(0)	34.5%(10)	N/A

Data presented are [a]mean±SEM, [b]%(n) and [b]n across groups. Terms: BMI; body mass index**,** SBP; systolic blood pressure, DBP; diastolic blood pressure, PP; pulse pressure, ACE; angiotensin-converting enzyme.

Table 3.2. Logistic regression results

Backward LR		
Step 1	*Hosmer-Lemeshow (P = 0.47)*	*Pseudo R^2 = 0.26*
Predictor	Δ in -2 log likelihood	*P*
γ-H2	4.77	0.03*
mTL	0.18	0.68
BMI	6.49	0.01*
Step 2	*Hosmer-Lemeshow (P = 0.78)*	*Pseudo R^2 = 0.25*
Predictor	Δ in -2 log likelihood	*P*
γ-H2	4.76	0.03*
BMI	6.83	0.01*
Forward LR		
Step 1	*Hosmer-Lemeshow (P = 0.51)*	*Pseudo R^2 = 0.14*
Predictor	Δ in -2 log likelihood	*P*
BMI	5.48	0.02*
Step 2	*Hosmer-Lemeshow (P = 0.78)*	*Pseudo R^2 = 0.25*
Predictor	Δ in -2 log likelihood	*P*
BMI	6.83	0.01*
γ-H2	4.76	0.03*

Terms: γ-H2; p-histone γ-H2A.X (ser139), mTL; mean telomere length, BMI; body mass index.

Table 3.3. Logistic regression hypertension group classification table

Backward LR			
Observed	*Predicted (n)*		*%Correct*
Step 1	Nonhypertensive	Hypertensive	
Nonhypertensive	13	10	56.5
Hypertensive	10	16	61.5
Overall			59.2
Step 2	Nonhypertensive	Hypertensive	
Nonhypertensive	17	6	73.9
Hypertensive	10	16	61.5
Overall			67.3
Forward LR			
Observed	*Predicted (n)*		*%Correct*
Step 1	Nonhypertensive	Hypertensive	
Nonhypertensive	15	8	65.2
Hypertensive	10	16	61.5
Overall			63.3
Step 2	Nonhypertensive	Hypertensive	
Nonhypertensive	17	6	73.9
Hypertensive	10	16	61.5
Overall			67.3

Data presented are n predicted in groups and %(n) predicted correctly.

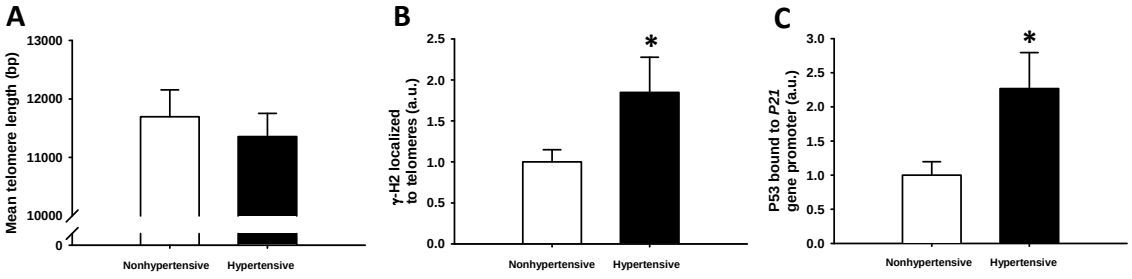

Figure 3.1. Arterial telomere dysfunction, P53/P21-induced senescence and hypertension. **A)** Mean telomere length, **B)** γ-H2 localized to telomeres and **C)** P53 bound to *P21* gene promoter across groups (both *$P \leq 0.04$). Data presented are mean±SEM normalized to control group. Terms: γ-H2; p-histone γ-H2A.X (ser139), P53; tumor suppressor protein p53, *P21*; cyclin-dependent kinase inhibitor 1A.

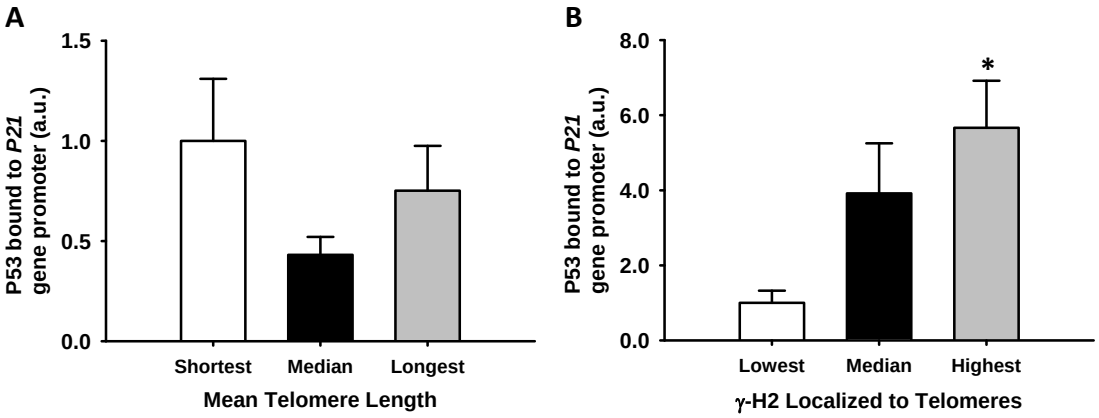

Figure 3.2. Influence of telomere dysfunction on P53/P21-induced senescence. **A)** P53 bound to *P21* gene promoter across tertiles of mean telomere length and **B)** P53 bound to *P21* gene promoter across tertiles of γ-H2 localized to telomeres (*$P < 0.01$ compared with lowest tertile). Data presented are mean±SEM normalized to control group. Terms: γ-H2; p-histone γ-H2A.X (ser139), P53; tumor suppressor protein p53, *P21*; cyclin-dependent kinase inhibitor 1A.

CHAPTER 4

ARTERIAL TELOMERE UNCAPPING: ROLE IN ARTERIAL DYSFUNCTION

Abstract

Telomere uncapping may lead to arterial aging and hypertension by inducing cellular senescence and subsequent senescence-associated secretory phenoptype (SASP). To test this unexplored hypothesis, we measured arterial telomere uncapping, mean telomere length, markers of tumor suppressor protein p53 (p53)/cyclin-dependent kinase inhibitor 1A (p21)-induced senescence, markers of inflammation and superoxide levels, as well as arterial endothelium-dependent dilation to acetylcholine (ACh), in the presence and absence of the superoxide scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) and endothelial nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (LNAME), in a novel Cre-lox inducible mouse model of telomere uncapping. We also measured arterial blood pressure and aortic pulse wave velocity (PWV). Telomeric repeat binding factor 2 (*Trf2)* gene deletion led to telomere uncapping ($P < 0.05$) and subsequent p53/p21-induced senescence ($P < 0.05$) in arteries. Arterial telomere uncapping also led to a SASP that was characterized by greater expression of inflammatory mediators and greater levels of superoxide (All $P < 0.05$). Telomere uncapping in arteries led to arterial endothelial dysfunction (ACh response; $P < 0.001$) that was mediated by superoxide (ACh+TEMPOL response vs. ACh alone; $P < 0.001$), but did not lead to a difference in endothelium-independent dilation ($P = 0.53$). The oxidative stress-mediated endothelial dysfunction was likely due to nitric oxide consumption by superoxide (ACh+TEMPOL+LNAME response vs. ACh+LNAME; $P > 0.16$). Telomere uncapping led to increased systolic, diastolic, and mean arterial blood pressure (all $P < 0.05$), but did not change aortic PWV (all $P > 0.05$). The greater arterial

superoxide levels were not due to differences in NADPH oxidase or superoxide dismutase expression (all $P \geq 0.14$). Collectively, our findings provide proof of concept that arterial telomere uncapping leads to arterial dysfunction that is comparable to that seen in arterial aging and hypertension.

Introduction

Telomere uncapping may lead to arterial aging and hypertension by inducing cellular senescence and subsequent senescence-associated secretory phenoptype (SASP). Telomeres are terminal sequences of TTAGGG repeats that make up the natural ends of chromosomes[1, 2]. Telomeres and telomere binding proteins, like telomeric repeat binding factor 2 (Trf2), form specialized structures that protect chromosome ends from being recognized as dsDNA breaks and initiating a dsDNA break response[3-5], which could induce cellular senescence through tumor suppressor protein p53 (p53)-mediated upregulation of cyclin-dependent kinase inhibitor 1A (p21)[5, 6]. Whereas, expression of senescence-inducing factors that activate p53 in response to excessive mitogenic signals, like the ARF variant of cyclin-dependent kinase inhibitor 2A (P14ARF in humans and p19ARF in rodents), are not involved in telomere uncapping triggered senescence[7].

Several *in vitro* studies in various mammalian cell types have shown that replication-dependent breakdown of telomere structure, referred to as uncapping, can lead to p53 activation and p53/p21-induced senescence[5, 6]. Likewise, *in vitro* and *in vivo* Trf2 knockdown both result in telomere uncapping and p53/p21-induced senescence[6, 8,

[9]. *In vivo*, age-related telomere uncapping is thought to occur as a result of genotoxic damage to the t-loop, dysfunctional shelterin proteins, or telomere shortening. Following *in vitro* telomere uncapping in mammalian cells, phosphorylation of histone γ-H2A.X at serine 139 (γ-H2) occurs at telomeric chromatin to aid in initiation of the dsDNA break response[5, 6].

In vitro studies have also described a SASP linked to p53/p21-induced senescence in a variety of mammalian cells, including vascular cells. This SASP is characterized by the accumulation of cytokines and chemokines like interleukin 1 (Il1), tumor necrosis factor alpha (Tnfa), and monocyte chemotactic protein 1 (Mcp1)[10-15], as well as increased superoxide and reduced nitric oxide (NO) levels[16, 17]. The SASP is thought to reinforce p53/p21-induced senescence in an autocrine/paracrine fashion, as well as mediate immune cell surveillance of senescence cells[18].

Many cardiovascular diseases (CVDs), including hypertension, are recognized as diseases of age-related arterial dysfunction[19-21] that result from chronic oxidative stress[22] and the accumulation of inflammatory mediators like Il1, Tnfa, and Mcp1 in arteries[23, 24]. Chronic oxidative stress can lead to arterial endothelial dysfunction by reducing NO bioavailability[25, 26], which in turn leads to increased arterial tone and potentially increased arterial pulse wave velocity (PWV) and blood pressure[27, 28]. The accumulation of reactive oxygen species, like superoxide, in arteries is thought to be due in part to inflammation-mediated upregulation of pro-oxidant enzymes like NADPH oxidases (Noxs 1-3)[22-24], and downregulation of anti-oxidant enzymes like superoxide dismutases (Sods 1-3)[29, 30].

Several studies have shown age-related telomere shortening in human arteries[31-33]. Morgan *et al.* (2013) reported greater γ-H2 localized to telomeres with advancing age in human arteries, which was positively correlated with p53/p21-induced senescence independent of telomere shortening[34]. Age-related telomere uncapping and p53/p21-induced senescence were also associated with greater expression of *MCP1* mRNA in arteries from older adults compared with younger adults[34]. Likewise, Morgan *et al.* (unpublished data) found that arterial telomere uncapping and p53/p21-induced senescence were associated with hypertension in age-matched adults and telomere uncapping was a significant predictor of hypertension status, independent of telomere length. Importantly, the effect of telomere uncapping *per se* on arterial dysfunction has not been assessed.

Telomere uncapping that causes p53/p21-induced senescence and subsequent inflammation and oxidative stress in arteries might lead to arterial aging and hypertension. Elucidating the role that telomere uncapping plays in arterial dysfunction may lead to the identification of novel biomarkers that are predictive of CVDs, as well as lifestyle and pharmacological interventions that blunt or even reverse arterial aging and hypertension. Thus, an important unexplored hypothesis is that arterial telomere uncapping results in p53/p21-induced senescence and subsequent SASP, which in turn leads to arterial endothelial dysfunction, increased arterial blood pressure, and aortic PWV. To test this hypothesis, we measured arterial telomere uncapping (γ-H2 localized to telomeres), mean telomere length, p53/p21-induced senescence and ensuing SASP,

as well as arterial endothelial function, blood pressure, and aortic PWV in a novel Cre-lox inducible mouse model of telomere uncapping.

Materials and Methods

Ethical Approval

All animal procedures conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23, revised 1996) and were approved by the University of Utah and Veteran's Affairs Medical Center-Salt Lake City (VAMC-SLC) Animal Care and Use Committees.

Animals

Young (4-6 months) rosa26-Cre $Trf2^{+/+}$ mice and rosa26-Cre $Trf2^{-/-}$ mice of mixed sexes were generated from breeding colonies at the University of Utah. $Trf2^{F/F}$ mice[35] were crossed with whole body expressing rosa26-Cre transgenic mice[36] to obtain compound rosa26-Cre $Trf2^{F/F}$ mice. Mice were maintained in a mixed 129/Sv C57/Bl6 genetic background. For the activation of Cre, 16- to 24-wk-old mice were injected intraperitoneally with tamoxifen (1 mg) daily for a total of 4-8 injections, based on body weight (BW) of each animal (200mg-tamoxifen/kg-BW)[36]. BW and gross phenotypes were assessed daily during tamoxifen treatment to monitor effects of Cre activation, and all mice were studied within 7-10 days of initiating tamoxifen treatment. Mice were euthanized by exsanguination via cardiac puncture while under isoflurane anesthesia. All mice (n = 53) were housed for at least 4-6 months in an animal care facility at the

University of Utah on a 12:12 light/dark cycle at 24 °C and fed ad libitum prior to study. All animal procedures conformed to the Guide to the Care and Use of Laboratory Animals (version 8, revised 2011) and were approved by the University of Utah Animal Care and Use Committee.

Genotyping and *Trf2* Deletion

DNA isolated from ear punches and aortas was used to detect $Trf2^{+/+}$,$Trf2^{F/F}$, $Trf2^{-/-}$ and rosa26-Cre loci by standard PCR and DNA gel as described by Denchi *et al.* (2006) and Jacks *et al.* (2007)[9, 36]. Cre activation resulted in loxP-directed deletion of exons 1 and 2 from the *Trf2* gene. Aortic *Trf2* mRNA expression was determined by qRT-PCR using the Quantitect Reverse Transcription kit (Qiagen, Inc.) and FastStart SYBR Green Master (Roche Diagnostics Corporation, Roche Applied Science) according to the manufacturer's protocols. Final mRNA SQs were generated by standard curve and expressed as a ratio of target mRNA SQ to *18s* rRNA SQ (18s rRNA QuantiTect Primer Assay: Qiagen, Inc.). *18s* rRNA was used as a housekeeping gene transcript to control for tissue concentration in samples (ex: *Trf2* mRNA SQ/*18s* SQ = final value).

Telomere Uncapping

ChIP was used to determine the amount of γ-H2 (Santa Cruz Biotechnology, Inc.) localized to telomeres in aortic tissue. ChIPs were performed as described by Dahl and Collas (2008)[37] and analyzed via qPCR for telomere content as described by Cawthon (2009)[38]. Final values were expressed as the ratio of background corrected starting quantity (SQ) of telomeric DNA enriched by ChIP to telomeric DNA SQ in INPUT fraction.

INPUTs represented 50% of telomeric DNA present in corresponding ChIP and were used to control for tissue concentration in samples [ex: (γ-H2 SQ - background SQ)/INPUT SQ = final value].

Mean Telomere Length

A sequence-independent multiplex qPCR technique using a SYBR Green master mix with 0.625U AmpliTaq Gold 360 DNA polymerase (Life Technologies Corporation) was utilized to determine mean telomere length in aortic tissue as described by Cawthon (2009)[38]. Telomeric DNA (T) SQs and *albumin* SQs, used as single copy gene (S) to control tissue concentration in samples, were generated by standard curve and mean telomere length was expressed as the T/S ratio. Mean telomere lengths were generated by converting T/S ratios to bp of DNA using the formula: bp = 3330(T/S) + 3730, derived by Cawthon (2009)[38].

p53/p21-Induced Senescence

p21 and *p19ARF* mRNA expression in aortic tissue was determined as described above. *p21* mRNA primers: fwd- cgagaacggtggaactttgac, rev- ccagggctcaggtagacctt and *p19ARF* mRNA primers: fwd- cgcaggttcttggtcactgt, rev- tgttcacgaaagccagagcg.

Senescence-Associated Secretory Phenotype

Il1, Tnfa, and *Mcp1,* mRNA expression in aortic tissue was determined by qRT-PCR as described above. *Il1* mRNA primers: fwd- cgaagactacagttctgccatt, rev-

atcttttggggtccgtcaact. *Tnfa* mRNA primers: fwd- ctgaacttcggggtgatcgg, rev-ggcttgtcactcgaattttgaga. *Mcp1* mRNA primers: fwd- gacgtttcagaggttctcagag**,** rev-gcattagcttcagatttacgggt.

Arterial Superoxide Production

Production of superoxide was measured by EPR spectrometry using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Alexis Biochemicals, Farmingdale, NY, USA). CMH was prepared in ice-cold deoxygenated Krebs-HEPES buffer (mM: NaCl, 99.01, KCl 4.69, $CaCl_2$ 2.50, $MgSO_4$ 1.20, K_2HPO_4 1.03, $NaHCO_3$ 25.0, glucose 11.10, Na-HEPES 20.00; pH 7.4) containing 0.1 mM diethylenetriaminepenta-acetic acid, 5 uM sodium diethyldithiocarbamate, and pretreated with Chelex (Sigma) 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 uL Krebs-HEPES buffer containing 0.5 mM CMH and analyzed immediately on an EMX Plus EPR spectrometer (Bruker, Rheinstetten, Germany). Instrument settings were 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.

Endothelial Function: Modulation by Superoxide and NO

Measurements of endothelial-dependent dilation (EDD) and endothelial-independent dilation (EID) in isolated carotid arteries studied *ex vivo* were performed using a method previously described in detail[39-41]. The carotid arteries were used as a large elastic artery for these measurements because we have previously established age-associated impairments in oxidative stress associated NO-mediated endothelial dysfunction in a mouse model[29, 39, 42]. Moreover, use of the carotid arteries for this functional measurement allowed biochemical analyses, which require large tissue volumes, to be performed on the aortic samples, thus significantly reducing the number of animals required to complete the overall assessments of vascular function and structure.

Briefly, mice were euthanized as described above. Both the right and left carotid arteries were excised and placed in myograph chambers (DMT Inc.) perfused by physiological saline solution (PSS) that contained 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1 g/100 mL BSA, pH 7.4 at 37 °C, cannulated onto glass micropipettes and secured with nylon (11–0) suture. Once cannulated, both carotid arteries were warmed to 37 °C and pressurized and allowed to equilibrate for approximately 1 h. All arteries were submaximally preconstricted with phenylephrine (2 uM) and increases in luminal diameter in response to increasing concentrations of the endothelium-dependent dilator, acetylcholine (ACh: 1 X 10^{-9} to 1 X 10^{-4} M), and endothelium-independent dilator, sodium nitroprusside (SNP: 1 1 X 10^{-10} to 1 X 10^{-4} M)

were determined. Responses to ACh were repeated in the presence of the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (LNAME, 0.1 mM, 30-min incubation) to determine the contribution of NO. To determine superoxide (oxidative stress) suppression of EDD, measurements were repeated in the contralateral vessel following a 60-min incubation in the presence of the superoxide scavenger, TEMPOL (1 mM)[43-45]. Sensitivity to ACh was calculated by EC50.

Arterial Blood Pressure

Several (2–3) days before and immediately after completing tamoxifen treatment, arterial blood pressure was assessed noninvasively in the conscious state by determining the tail blood volume with a volume pressure recording (VPR) sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT) as previously described in detail[46, 47]. This method has been validated vs. arterial catheter blood pressure[46]. Blood pressure and heart rate recordings were made in a quiet and warm (24 °C) environment. Mice were placed in restrainers on a heating unit and given 15–20 min to acclimate and reach a steady tail skin temperature of (30–35 °C). Each session consisted of 5–10 acclimatization measures and documentation of stable values, followed by 20 experimental measures. Measures with aberrant movement/behavior or inadequate tail volume/flow values were excluded and remaining values used to calculate mean values for each animal. This method is quite reproducible in our laboratories as the coefficient of variation for this method is 5% for systolic and 7% diastolic blood pressure (n = 10) (unpublished data).

Aortic Pulse Wave Velocity

Several (2–3) days before and immediately after completing tamoxifen treatment, aortic pulse wave velocity (PWV) was measured as described previously[42, 48]. Briefly, mice were anesthetized under 2% isoflurane in a closed chamber anesthesia machine (V3000PK, Parkland Scientific, Coral Springs, FL, USA) for approximately 1–3 min. Anesthesia was maintained using a nose-cone, and mice were secured in a supine position on a heating board (approximately 35 °C) to maintain body temperature. Velocities were measured with 20-MHz Doppler probes (Indus Instruments, Webster, TX, USA) at the transverse aortic arch and approximately 4 cm distal at the abdominal aorta simultaneously and collected using WinDAQ Pro + software (DataQ Instruments, Akron, OH, USA). After velocities were collected, a precise measure of the distance between the probes was obtained using a scientific caliper and recorded. Absolute pulse arrival times were indicated by the sharp upstroke, or foot, of each velocity waveform analyzed with WinDAQ Waveform Browser (DataQ Instruments). Aortic PWV is then calculated as the quotient of the separation distance and difference in absolute arrival times.

Arterial Sources of Superoxide

Nox1, Nox2, Nox3, Sod1, Sod2, and *Sod3* mRNA expression in aortic tissue was determined by qRT-PCR as described above. *Nox1* mRNA primers: fwd-ggttggggctgaacatttttc, rev-tcgacacacaggaatcaggat. *Nox2* mRNA primers: fwd-atgagttccacaccttccttc**,** rev- ggcttgagacaacctggtatta. *Nox3* mRNA primers: fwd-

tggcagtaaacgcctatctgt, rev- cggaacccagaataactcgtgta. *Sod1* mRNA primers: fwd-aaccagttgtgttgtcaggac, rev- ccaccatgtttcttagagtgagg. *Sod2* mRNA primers: fwd-cagacctgccttacgactatgg, rev- ctcggtggcgttgagattgtt. *Sod3* mRNA primers: fwd-ccttcttgttctacggcttgc**,** rev- tcgcctatcttctcaaccagg.

Data Analysis

For animal and vessel characteristics and molecular biological assays, group differences were determined by independent and paired samples t-tests. For all dose responses, group differences were determined by repeated-measures ANOVA. Data are presented as mean±SEM. Significance was set at $P < 0.05$.

Results

Animal Characteristics

Overall body mass as well as heart, liver mass, skeletal muscle, and epididymal white adipose tissue (WAT) mass was lower in rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$ mice ($P < 0.05$, Table 4.1). When normalized for body mass, only WAT was lower in rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$ mice ($P < 0.05$, Table 4.1).

Trf2 Deletion in Mice Leads to Telomere Uncapping and P53/p21-Induced in Arteries, Independent of Telomere Length

Tamoxifen treatment-induced Cre activation resulted in deletion of exons 1 and 2 from the *Trf2* gene in aortic tissue. *Trf2* deletion resulted in over a 5-fold knockdown of *Trf2* mRNA expression ($P < 0.05$; Figure 4.1B), and subsequent 1.5–fold greater telomere uncapping in aortas from rosa26-Cre $Trf2^{-/-}$ mice compared with aortas from rosa26-Cre $Trf2^{+/+}$ mice ($P < 0.05$; Figure 4.1C). The greater telomere uncapping in rosa26-Cre $Trf2^{-/-}$ aortas compared with rosa26-Cre $Trf2^{+/+}$ aortas occurred without a change in mean telomere length ($P = 0.48$; Figure 4.1D).

Telomere Uncapping in Mice Leads to p53/p21-Induced Senescence and SASP in Arteries

There was nearly 5-fold greater *p21* mRNA expression in aortas from rosa26-Cre $Trf2^{-/-}$ mice compared with aortas from rosa26-Cre $Trf2^{+/+}$ mice ($P < 0.05$; Figure 4.2A), accompanied by no difference in *p19ARF* mRNA expression ($P > 0.33$; Figure 4.2B). Accordingly, there was 1.5-fold and nearly 3-fold greater expression of *Il1, Mcp1* mRNA, respectively ($P < 0.05$; Figure 4.3A, B). There was 2-fold higher *Tnfa* mRNA expression in aortas from rosa26-Cre $Trf2^{-/-}$ mice compared with those from rosa26-Cre $Trf2^{+/+}$ mice, but this difference was not significant (all $P = 0.08$; Figure 4.3C). Additionally, there was almost 3–fold greater superoxide production ($P < 0.002$; Figure 4.3D) in aortas from rosa26-Cre $Trf2^{-/-}$ mice compared with aortas from rosa26-Cre $Trf2^{+/+}$ mice.

Telomere Uncapping in Mice Leads to Oxidative Stress-Mediated Arterial Endothelial Dysfunction

EDD to ACh was impaired ($P < 0.001$; Figure 4.4A) in isolated carotid arteries of rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$ mice (maximal dilation to ACh, rosa26-Cre $Trf2^{+/+}$: 92.2±1.5 vs. rosa26-Cre $Trf2^{-/-}$: 63.5±3.1 % dilation, $P < 0.001$). There was no difference in sensitivity to ACh (EC50) in the carotid arteries of rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$ mice ($P = 0.23$). Incubation with LNAME did not reduce EDD to ACh in the carotid arteries of rosa26-Cre $Trf2^{-/-}$ mice ($P = 0.19$ vs. ACh alone; Figure 4.4A), and abolished the group difference observed with ACh alone ($P = 0.11$; Figure 4.4A). There was no difference in carotid artery dilation ($P = 0.53$; Figure 4.4B) to SNP among the groups (maximal dilation to SNP, rosa26-Cre $Trf2^{+/+}$: 91.1±12.5 vs. rosa26-Cre $Trf2^{-/-}$: 86.8±3.1 % dilation).

Incubation with the superoxide scavenger, TEMPOL, restored carotid artery EDD to ACh in rosa26-Cre $Trf2^{-/-}$ mice ($P < 0.001$ vs. ACh alone; Figure 4.5A), had no effect in rosa26-Cre $Trf2^{+/+}$ mice ($P = 0.70$, vs. ACh alone; Figure 4.5A), and abolished the group difference observed with ACh alone ($P = 0.31$; Figure 4.5A). TEMPOL administration selectively increased EDD in carotid arteries from rosa26-Cre $Trf2^{-/-}$ mice in a NO-dependent manner ($P > 0.16$ vs. ACh+LNAME alone; Figure 4.5B), whereas TEMPOL did not alter NO-mediated EDD to ACh in rosa26-Cre $Trf2^{+/+}$ mice ($P > 0.82$ vs. ACh+LNAME alone; Figure 4.5B).

Telomere Uncapping in Mice Leads to Hemodynamic Changes *In vivo*

Systolic, diastolic, and mean arterial blood pressure increased by approximately 12 mmHg, 19 mmHg, and 15 mmHg, respectively, following *Trf2* deletion in rosa26-Cre $Trf2^{-/-}$ mice ($P < 0.05$; Figure 4.6A-C). Systolic, diastolic, and mean arterial pressure blood pressure did not change following Cre activation in rosa26-Cre $Trf2^{+/+}$ mice ($P \geq 0.39$; Figure 4.6A-C). Systolic blood pressure was approximately 13 mmHg higher in rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$mice ($P < 0.05$; Figure 4.6A). Heart rate decreased following Cre activation in rosa26-Cre $Trf2^{+/+}$mice (Pre-rosa26-Cre $Trf2^{+/+}$: 663.2±36.1 vs. Post-rosa26-Cre $Trf2^{+/+}$: 513.7±23.4 beats/min; $P < 0.05$). Heart rate also decreased following *Trf2* deletion in rosa26-Cre $Trf2^{-/-}$ mice (Pre-rosa26-Cre $Trf2^{-/-}$: 575.6±30.4 vs. Post-rosa26-Cre $Trf2^{-/-}$: 506.2±31.0 beats/min; $P < 0.05$). There was no change in aortic PWV following *Trf2* deletion in rosa26-Cre $Trf2^{-/-}$ mice or Cre activation in rosa26-Cre $Trf2^{+/+}$ mice (all $P > 0.05$; Figure 4.7). PW-generated heart rate decreased following Cre activation in rosa26-Cre $Trf2^{+/+}$mice ($P < 0.05$), but did not change following *Trf2* deletion in rosa26-Cre $Trf2^{-/-}$ mice ($P = 0.29$).

Telomere Uncapping in Mice Does Not Lead to Increased NOX or Decreased SOD Expression in Arteries

There were no differences in expression of *Nox1, Nox2,* or *Nox3 mRNA* in aortas from rosa26-Cre $Trf2^{-/-}$ mice compared with aortas from rosa26-Cre $Trf2^{+/+}$ mice (all $P \geq 0.14$). Likewise, there were no differences in *Sod1, Sod2,* or *Sod3* mRNA expression in

aortas from rosa26-Cre Trf2$^{-/-}$ mice compared with aortas from rosa26-Cre Trf2$^{+/+}$ mice (all $P \geq 0.15$).

Discussion

The key novel findings are as follows. *Trf2* deletion leads to telomere uncapping and subsequent p53/p21-induced senescence in arteries. Arterial telomere uncapping also leads to a SASP that is characterized by greater expression of inflammatory mediators and greater levels of superoxide. Telomere uncapping in arteries leads to arterial endothelial dysfunction that is mediated by superoxide, but does not lead to a difference in endothelium-independent dilation. The oxidative stress-mediated endothelial dysfunction is likely due to NO consumption by superoxide. Telomere uncapping leads to increased systolic, diastolic, and mean arterial blood pressure, but does not change aortic PWV. The greater arterial superoxide levels are not due to increased *Nox* or decreased in *Sod* expression. Collectively, our findings provide proof of concept that arterial telomere uncapping leads to arterial dysfunction that is comparable to that seen in arterial aging and hypertension.

Telomere Uncapping and p53/p21-Induced Senescence in Arteries

Arterial telomere uncapping should lead to p53/p21-induced senescence in arterial cells. Here we showed that arterial telomere uncapping results in greater *p21* mRNA expression in arteries from rosa26-Cre Trf2$^{-/-}$ mice compared with rosa26-Cre Trf2$^{+/+}$ mice. Furthermore, there is no difference in *p19ARF* mRNA expression or mean

telomere length in arteries from rosa26-Cre Trf2$^{-/-}$ mice compared with rosa26-Cre Trf2$^{+/+}$ mice. p19ARF is upregulated and activates p53 in response to strong mitogenic signals and other nontelomere uncapping related cellular stressors[7]. Likewise, telomere shortening can result from genotoxic stressors that could induce p53/p21-induced senescence independently of telomere uncapping[49]. These findings suggest that the p53/p21-induced senescence we reported is independent of p53 activation from other stress response pathways, which indicates that telomere uncapping *per se* is likely the cause of arterial p53/p21-induced senescence.

The first evidence for age-related telomere uncapping in noncultured human tissues showed greater telomere uncapping in arteries from older adults compared with younger adults, independent of age-related telomere shortening[34]. Telomere uncapping was a highly influential covariate for p53/p21-induced senescence, while mean telomere length was not correlated with p53/p21-induced senescence[34]. These results cast doubt on the biological relevance of telomere shortening as a mechanism underlying age-related telomere uncapping and p53/p21-induced senescence in arteries. Morgan *et al.* (unpublished data) also demonstrated that arterial telomere uncapping and p53/p21-induced senescence were greater in age-matched hypertensive compared with nonhypertensive adults, independent of telomere length. Likewise, telomere uncapping was a highly influential covariate for p53/p21-induced senescence while mean telomere length was not correlated with p53/p21-induced senescence, which suggests that telomere length does not play a role in the etiology of hypertension. The present results provide proof of concept that telomere uncapping *per se* can lead to p53/p21-induced

senescence in arteries, which corresponds closely with cross-sectional evidence for the role of telomere uncapping in arterial aging and hypertension.

Telomere Uncapping and SASP in Arteries

Arterial telomere uncapping should lead to a SASP in arterial tissue. Here we showed that telomere uncapping results in higher expression of inflammatory mediators, like Il1, MPC1, and Tnfa, and greater superoxide levels in arteries from rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$ mice. The role of chronic arterial inflammation and oxidative stress in arterial aging and hypertension is well documented in humans and rodents[22-24, 50, 51]. These results demonstrate that arterial telomere uncapping leads to a pro-inflammatory/pro-oxidative SASP in arteries, which provides a potential mechanistic link between telomere uncapping and arterial dysfunction.

Telomere Uncapping and Arterial Endothelial Dysfunction

An arterial telomere uncapping-mediated SASP may lead to arterial endothelial dysfunction through NO consumption by ROS, like superoxide. Here we showed that telomere uncapping leads to impaired ACh-mediated EDD in arteries from rosa26-Cre $Trf2^{-/-}$ mice compared with rosa26-Cre $Trf2^{+/+}$ mice, whereas, there is no difference in endothelium-independent dilation between groups, assessed by SNP dose response. NO inhibition by LNAME abolishes the group difference in ACh-mediated arterial EDD. Treatment with the superoxide scavenger, TEMPOL, restores the impaired EDD to ACh in

arteries from rosa26-Cre Trf2$^{-/-}$ mice and abolishes the group difference, but does not alter EDD to ACh following NO inhibition by LNAME. Thus, the arterial endothelial dysfunction in rosa26-Cre Trf2$^{-/-}$ mice is likely due to NO consumption by superoxide.

Interestingly, while TEMPOL abolishes the group difference, arterial EDD to ACh does not appear to be restored completely in rosa26-Cre Trf2$^{-/-}$ mice. The remaining impairment in EDD in rosa26-Cre Trf2$^{-/-}$ mice may be due other SASP related factors, like additional ROS, or a general decrease in arterial cell viability. Age- and hypertension-associated arterial endothelial dysfunction[27, 52] and the effects of chronic inflammation and oxidative stress on endothelial function[22, 26, 53] are well documented in humans and rodents. Likewise, there is cross-sectional evidence for a role of telomere uncapping in arterial aging and hypertension[34]. These findings demonstrate that the arterial telomere uncapping leads to oxidative stress-mediated endothelial dysfunction.

Telomere Uncapping and *In vivo* Hemodynamic Changes

Arterial telomere uncapping-mediated arterial endothelial dysfunction may lead to increased arterial tone, which could result in increased arterial blood pressure and PWV. Here we showed that telomere uncapping leads to increased systolic, diastolic, and mean arterial blood pressure in rosa26-Cre Trf2$^{-/-}$ mice. However, telomere uncapping does not lead to increased aortic PWV in rosa26-Cre Trf2$^{-/-}$ mice. While the increased blood pressure we observed could be due to telomere uncapping-mediated dysfunction in nonarterial tissues, such as kidneys[54], arterial telomere uncapping induced increases in blood pressure support cross-sectional evidence for a link between

arterial telomere uncapping and the etiology of hypertension (Morgan *et al.*, unpublished data). In addition to well-characterized age-related increases in arterial blood pressure and PWV in humans and rodents[22, 52, 55], the effect of arterial endothelial dysfunction on arterial tone-mediated increases in arterial blood pressure have been suggested by both large epidemiological studies in humans and mechanistic studies in animals[27, 28]. Therefore, we believe these results provide proof of concept that telomere uncapping leads to increased blood pressure, potentially via increased tone due to endothelial dysfunction. We found no change in aortic PWV following telomere uncapping, which suggests that telomere uncapping-mediated endothelial dysfunction does not lead to increases in arterial tone sufficient to affect arterial stiffness.

Telomere Uncapping, NOX, and SOD Expression in Arteries

The age-related accumulation of reactive oxygen species (ROS), like superoxide, in arteries is typically associated with increased expression of *Noxs* and decreased expression of *Sods*[22, 29, 30]. However, the greater superoxide levels in arteries from rosa26-Cre Trf2$^{-/-}$ mice compared with rosa26-Cre Trf2$^{+/+}$ mice is not due to differences in expression of *Nox1, Nox2, Nox3, Sod1, Sod2*, or *Sod3* mRNA. These results suggest that telomere uncapping-mediated increases in superoxide levels are likely not the result of the canonical Nox or Sod-dependent pathways previously linked to oxidative stress in arteries.

Conclusions

The goal of this study was to establish that telomere uncapping results in arterial dysfunction. Our findings demonstrate that arterial telomere uncapping leads to p53/p21-induced senescence and a subsequent SASP in arteries that is characterized by greater expression of inflammatory mediators and increased levels of superoxide. Telomere uncapping also leads to arterial endothelial dysfunction that is mediated by superoxide, and increased systolic, diastolic, and mean arterial blood pressure. We believe these studies provide the mechanistic foundation for future studies aimed at establishing the prognostic value of telomere uncapping as a biomarker for CVDs.

Acknowledgements

All experiments were performed in the Translational Vascular Physiology Laboratory at the University of Utah and Veteran's Affairs Medical Center-Salt Lake City, Geriatric Research Education and Clinical Center. Garrett Morgan and Anthony J Donato contributed to all aspects of the study, including the conception and design of the experiments, collection, analysis, and interpretation of data, and drafting and revision of the manuscript. Lisa A Lesniewski, Eros Denchi, Ashley Walker, Grant Henson, Richard M Cawthon, Colton Bramwell, and Cutter Bach contributed to the collection and analysis of data as well as revision of the manuscript. Additionally, Richard M Cawthon and Eros Denchi contributed reagents, analytical tools, and specimens that were essential to completing this study.

Source of Funding

Awards from the National Institutes of Health: NIA-AG040297, NIA-AG029337, NIA-AG033196, NIA-AG033755 NHLBI-HL09183, and a University of Utah Center on Aging Grant financially supported this work.

Conflict of Interest

The authors have no conflict of interest or disclosures to report.

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Table 4.1. Animal characteristics

Characteristic	**rosa26-Cre Trf2$^{+/+}$** (n=16)	**rosa26-Cre Trf2$^{-/-}$** (n=23)
Age, months	5.8±0.2	4.2±0.1
Body mass, g	26.8±0.7	21.2±0.9*
Heart, mg	145±9.0	120±5.6*
Heart:BW, g g^{-1} x 100	0.49±0.04	0.56±0.02
Liver, g	1.75±0.3	1.10±7.1*
Liver:BW, g g^{-1} x 100	6.6±1.1	5.1±0.1
Quadricep, mg	191±28	149±11
Quadricep:BW, g g^{-1} x 100	0.62±0.11	0.70±.06
WAT, mg	282±39	136±23*
WAT:BW, g g^{-1} x 100	0.94±0.1	0.52±0.1*

Data are mean ± SEM.BW, mass to body weight ratio; WAT, epididymal white adipose tissue. *P < 0.05 vs. rosa26-Cre Trf2$^{+/+}$ mice.

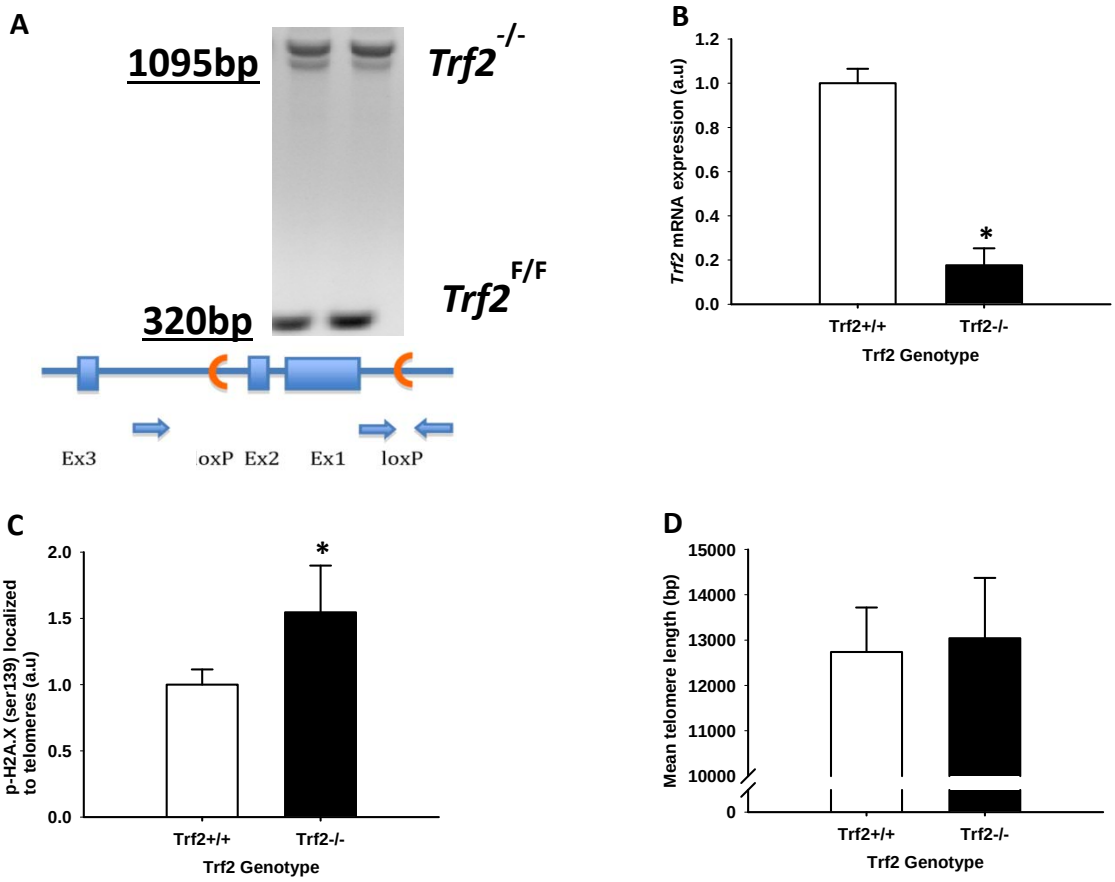

Figure 4.1. *Trf2* deletion in mice leads to telomere uncapping and p53/p21-induced in arteries, independent of telomere length. Representative DNA gel and diagram demonstrating aortic loxP directed deletion of exons 1 and 2 from the telomeric repeat binding factor 2 (*Trf2*) gene (A), aortic telomere uncapping, assessed by ChIP for γ-H2 localized to telomeres (B), aortic mRNA expression of telomeric repeat binding factor 2 (*Trf2*) (C), and mean telomere length (D) from rosa26-Cre $Trf2^{+/+}$ and rosa26-Cre $Trf2^{-/-}$ mice (B-D: n = 6–11 per group). mRNA is expressed relative to 18s to account for differences in total RNA and presented normalized to the rosa26-Cre $Trf2^{+/+}$ mean. ChIP data are expressed relative input DNA to account for differences in cell number and presented normalized to the rosa26-Cre $Trf2^{+/+}$ mean. Values are means ± SEM. *$P < 0.05$ vs. rosa26-Cre $Trf2^{+/+}$ mice. Terms: γ-H2; p-histone γ-H2A.X (ser139).

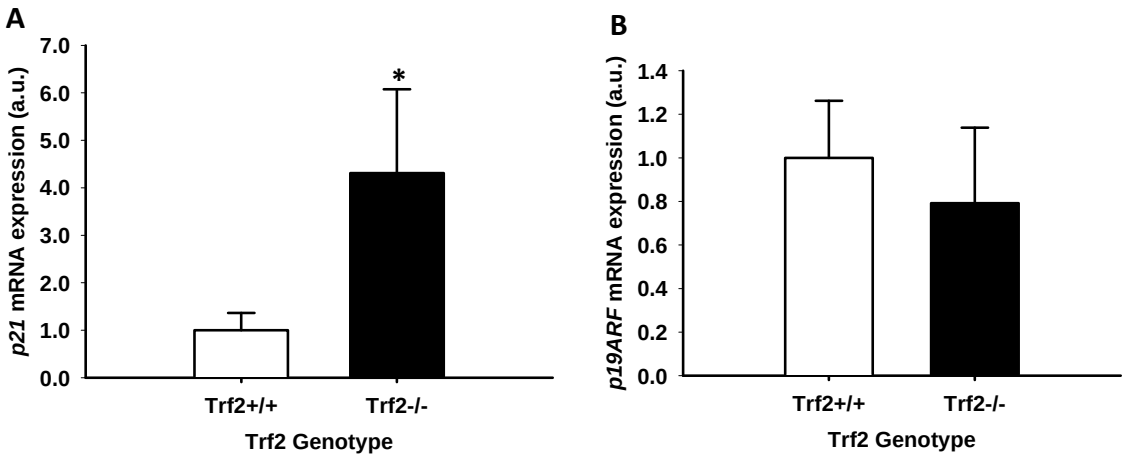

Figure 4.2. Telomere uncapping in mice leads to p53/p21-induced senescence in arteries. Aortic mRNA expression of the cyclin-dependent kinase inhibitor 1A (*p21*) (A) and cyclin-dependent kinase inhibitor 2A, ARF variant (*p19ARF*) (B) from rosa26-Cre $Trf2^{+/+}$ and rosa26-Cre $Trf2^{-/-}$ mice (A,B: n = 6–8 per group). mRNA is expressed relative to 18s to account for differences in total RNA and presented normalized to the rosa26-Cre $Trf2^{+/+}$ mean. Values are means $\pm$ SEM. *$P < 0.05$ vs. rosa26-Cre $Trf2^{+/+}$ mice.

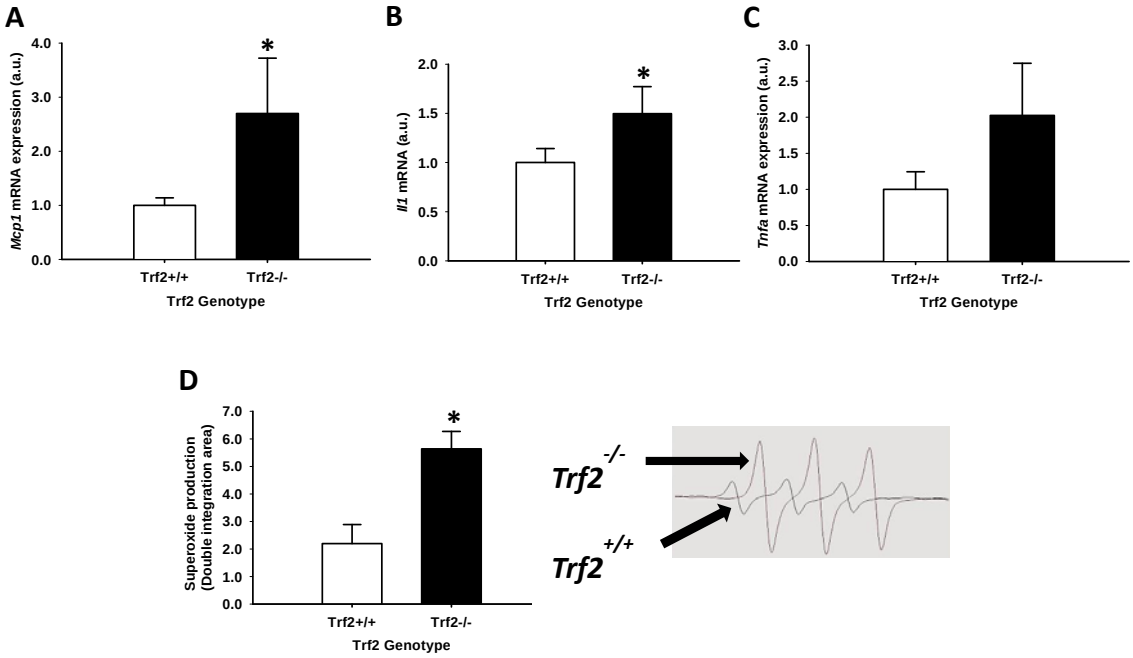

Figure 4.3. Telomere uncapping in mice leads to SASP in arteries. Aortic mRNA expression of interleukin 1 (*Il1*) (A), tumor necrosis factor alpha (*Tnfa*) (B), monocyte chemotactic protein 1 (*Mcp1*) (C), and aortic superoxide production (assessed by electron paramagnetic resonance spectroscopy) (D) from rosa26-Cre Trf2$^{+/+}$ and rosa26-Cre Trf2$^{-/-}$ mice (A-D: n = 6–8 per group). mRNA is expressed relative to 18s to account for differences in total RNA and presented normalized to the rosa26-Cre Trf2$^{+/+}$ mean. Representative electron paramagnetic resonance of CMH shown adjacent to summary graph (B). Values are means ± SEM. *P < 0.05 vs. rosa26-Cre Trf2$^{+/+}$ mice.

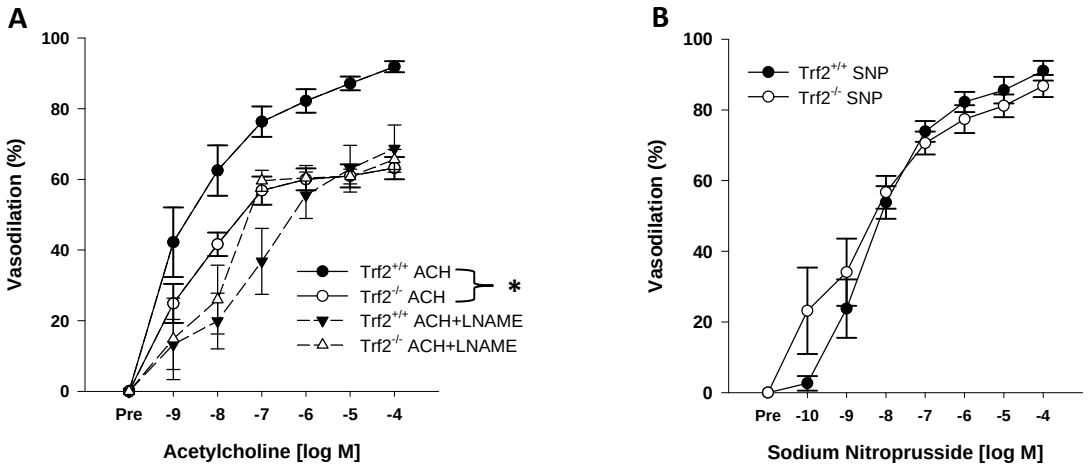

Figure 4.4. Telomere uncapping in mice leads to arterial endothelial dysfunction. Vasodilation of carotid arteries to acetylcholine (ACh) alone or after pretreatment with the NOS inhibitor, L-NAME (A), and vasodilation of carotid arteries to sodium nitroprusside (SNP) (B) (A,B: n = 7–10 per group). Values are means ± SEM. **P* < 0.05 vs. rosa26-Cre $Trf2^{+/+}$ mice.

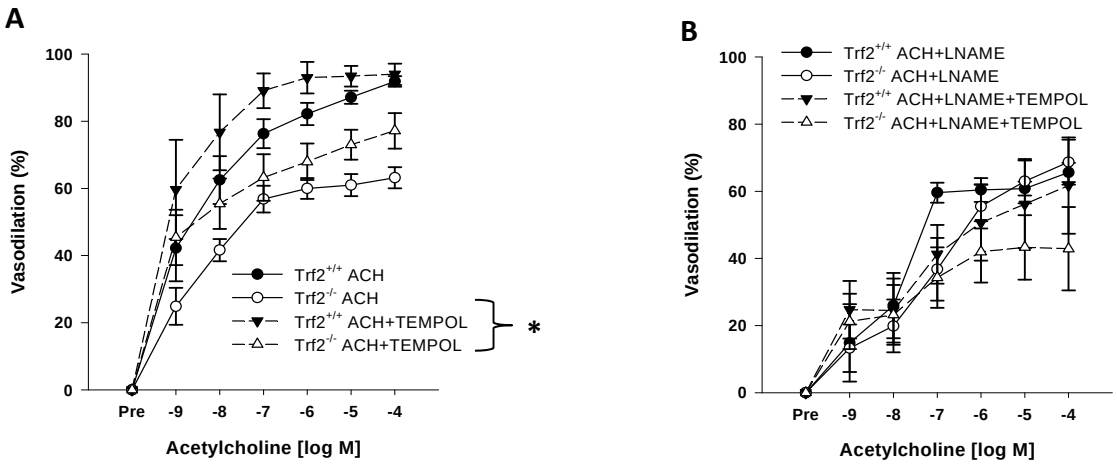

Figure 4.5. Telomere uncapping in mice leads to oxidative stress- mediated arterial endothelial dysfunction. Vasodilation of carotid arteries to acetylcholine (ACh) alone or after pretreatment with the TEMPOL (A), and vasodilation of carotid arteries to acetylcholine (ACh) after pretreatment with the NOS inhibitor, L-NAME, in the absence or presence of TEMPOL (B) (A,B: n = 6–10 per group). Values are means ± SEM. *P < 0.05 vs. ACh alone.

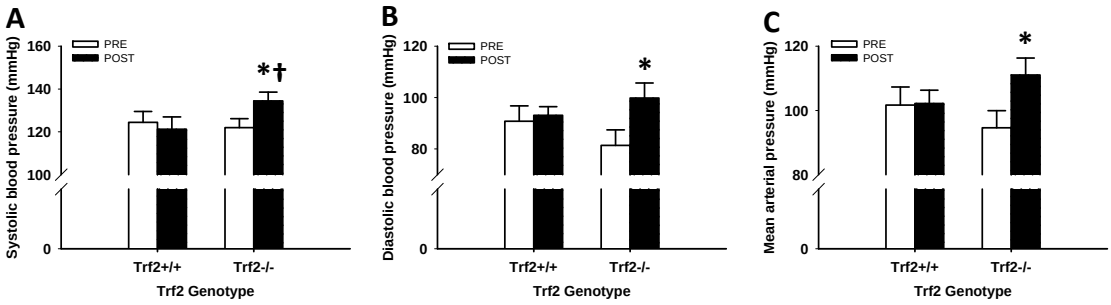

Figure 4.6. Telomere uncapping in mice leads to increased arterial blood pressure. Systolic (A), diastolic (B), and mean (C) arterial blood pressure (A-C: n = 6–8 per group). Values are means ± SEM. **P* < 0.05 following *Trf2* deletion in rosa26-Cre $Trf2^{-/--}$ mice. †*P* < 0.05 vs. rosa26-Cre $Trf2^{+/+}$ mice.

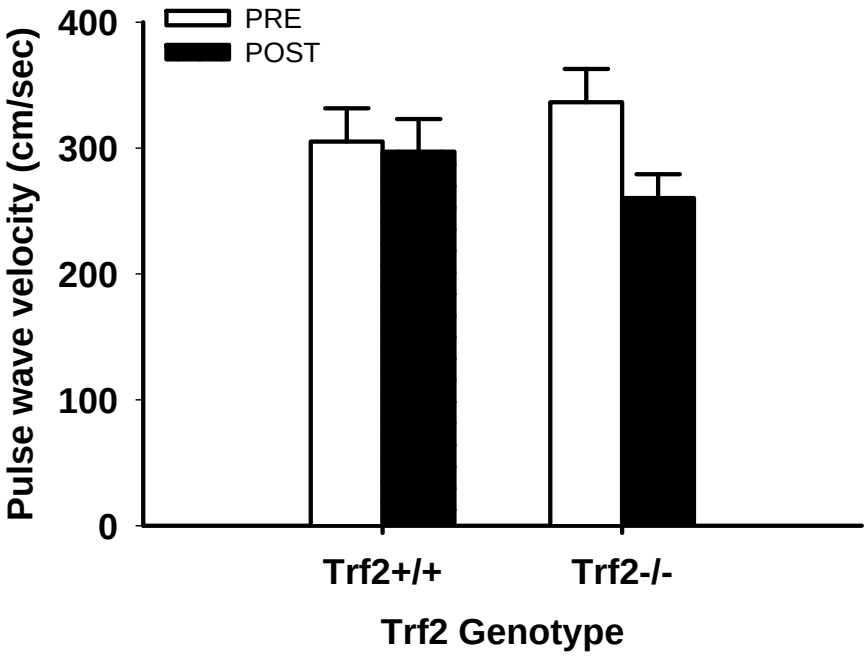

Figure 4.7. Telomere uncapping in mice does not lead to changes in aortic pulse wave velocity. Aortic pulse wave velocity (PWV; n = 6–8 per group). Values are means ± SEM. all $P > 0.05$ vs. rosa26-Cre $Trf2^{+/+}$ mice.

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

General Objectives and Main Findings

The general objectives of this project were to determine the association between telomere dysfunction, P53/P21-induced senescence, and ensuing SASP in aging and hypertension in human arteries. Additionally, we wanted to determine the casual relationship between arterial telomere uncapping and arterial dysfunction.

The primary objectives of our first study were to assess the role of telomere dysfunction and P53/P21-induced senescence in chronic arterial inflammation. Our findings reveal that telomere uncapping occurs with advancing age in human arteries, which is linked to P53/P21-induced senescence independent of telomere shortening. These age-related differences were associated with greater inflammation in arteries from older compared with younger adults. The primary objectives of our second study were to assess the roles of telomere dysfunction and P53/P21-induced senescence in the etiology of hypertension in humans. Similar to the results of our first study, we found that arterial telomere uncapping and P53/P21-induced senescence were linked to hypertension independent of mean telomere length in humans. We also reported that

telomere uncapping had greater influence on hypertension status than mean telomere length.

The primary objectives of our third and final study were to determine whether arterial telomere uncapping results in P53/P21-induced senescence and subsequent SASP, which in turn leads to arterial endothelial dysfunction, increased arterial blood pressure, and aortic pulse wave velocity in a novel Cre-lox inducible mouse model of telomere uncapping. Our findings provide proof of concept that arterial telomere uncapping leads to P53/P21-induced senescence and a subsequent SASP in arteries that is characterized by greater expression of inflammatory mediators and increased levels of superoxide. The greater arterial superoxide levels are not due to increases in NOX or decreases in SOD expression. Arterial telomere uncapping also leads to arterial endothelial dysfunction that is mediated by superoxide. The oxidative stress-mediated arterial endothelial dysfunction is likely due to NO consumption by superoxide. Finally, telomere uncapping leads to increased systolic, diastolic, and mean arterial blood pressure, but without alterations in aortic PWV.

Alternative Explanations and General Limitations

Chapter 2

To our knowledge, no other studies have measured telomere uncapping with advancing age in noncultured human tissues prior to our first study. Furthermore, it was unknown whether age-related P53/P21-induced senescence occurs in arteries from subjects that are more generalizable than CVD patients. The results of our first study

extend previous findings that telomere shortening is associated with both advancing age and atherosclerotic plaque development in arterial tissue taken from cadavers[1-4]. Likewise, our findings support those from a study conducted with arteries from coronary artery bypass graft patients that demonstrated age-related P53/P21-induced senescence[5]. Interestingly, we found that mean telomere length was not correlated with telomere uncapping or P53/P21-induced senescence. Telomere uncapping caused by the accumulation of genotoxic insults with advancing age might explain these lack of associations[6, 7]. We believe these observations cast doubt on the biological relevance of telomere shortening as a mechanism underlying age-related telomere uncapping, P53/P21-induced senescence and senescence-associated inflammation in arteries.

While the findings of our first study demonstrate that telomere uncapping accounts for most of the age-group difference in P53 bound to *P21* gene promoter, this difference cannot be completely explained by telomere uncapping with advancing age. Other stressors, such as the accumulation of DNA breaks[8], excessive mitogenic signals[9], or oxidative stress[10], may lead to P53/P21-induced senescence with advancing age in arteries. Likewise, a P53/P21-induced senescence-mediated SASP is undoubtedly not the only source of arterial inflammation with advancing age. Pro-inflammatory mediators may also be expressed and released in arteries in response to injury or infection[11].

Chapter 3

Prior to our second study, the roles of telomere uncapping and P53/P21-induced senescence in hypertension were entirely unknown. Our findings support the previously reported association between P53/P21-induced senescence and CVD, as P53/P21-induced senescence has been linked to carotid artery disease[12], chronic obstructive pulmonary disease[13], and popliteal artery aneurysms[14]. Similar to the findings of our first study, the results of our second study demonstrate that arterial mean telomere length is not associated with telomere uncapping, P53/P21-induced senescence, or hypertension, which suggests that arterial telomere shortening likely does not contribute to the pathogenesis of hypertension. These findings generally do not support the reported link between arterial telomere shortening and CVD. While its role in hypertension has not previously been assessed, arterial telomere shortening has been associated with chronic obstructive pulmonary disease[13], abdominal aortic aneurysm[15], and atherosclerosis[2, 16]. Interestingly, telomere shortening in white blood cells has been correlated with hypertension in medicated subjects[17, 18]. Tissue specific differences in mean telomere length and rates of telomere shortening may account for the difference in these findings from those in studies with white blood cells. As arterial mean telomere length should be more biologically relevant to the etiology of hypertension than that of white blood cells, these results cast doubt on the importance of telomere shortening in hypertension.

Likewise, the findings of our second study demonstrate that telomere uncapping accounts for much of the group difference in P53 bound to *P21* gene promoter;

however, this difference cannot be completely explained by telomere uncapping in hypertension. Other hypertension-associated cellular stressors, such as oxidative stress, may lead to P53/P21-induced senescence[10, 19]. Furthermore, hypertension is a complex disease with many factors that could potentially contribute to its pathogenesis[20]. Thus, telomere uncapping is certainly not the only or even primary factor influencing the development and onset of hypertension.

Chapter 4

Our third study is the first to establish that arterial telomere uncapping leads to P53/P21-induced senescence, ensuing SASP, and subsequent arterial dysfunction. The results from this study support the findings of our first and second studies. Interestingly, the greater superoxide levels we observed in arteries following telomere uncapping is not due to increases in expression of NOXs or decreases in expression of SODs. Thus, the arterial telomere uncapping triggered SASP may not be the result of the canonical NOX and SOD-dependent pathways previously linked to inflammation and oxidative stress in arteries[21-23]. Mitochondrial uncoupling may be a potential source of superoxide production linked to P53/P21-induced senescence in arteries, as *in vitro* studies in replicatively senescent human vascular cells reported increased superoxide levels and mitochondrial dysfunction[24].

We showed that arterial telomere uncapping leads to impaired ACh-mediated endothelial dilation, and superoxide scavenging by TEMPOL abolished the group difference in an NO-dependent manner. Thus, a portion of the observed arterial

endothelial dysfunction is likely due to NO consumption by superoxide. Interestingly, while TEMPOL abolished the group differences in ACh-mediated endothelial dilation, these responses did not appear to be restored fully in rosa26-Cre TRF2$^{-/-}$ mice. The remaining impairment in endothelial dysfunction may be due to other SASP related factors, such as additional ROS, or just a general decrease in arterial cell viability. We also showed that telomere uncapping leads to increased arterial blood pressure, which corresponds well the associations between arterial telomere uncapping, P53/P21-induced senescence, and hypertension we reported in our second study. However, the increased blood pressure we observed could be due to telomere uncapping-mediated dysfunction in nonarterial tissues, such as kidneys[25]. We found no change in aortic PWV following telomere uncapping, which suggests that telomere uncapping-mediated endothelial dysfunction does not lead to increases in arterial tone sufficient to affect arterial stiffness.

We acknowledge several general limitations to these studies. The descriptive cross-sectional study design utilized in the first and second studies precludes any inferences of causality regarding the role of telomere dysfunction in chronic arterial inflammation or hypertension. Similarly, a prospective study design could show age-related increases in telomere uncapping and P53/P21-induced senescence more conclusively, as well as demonstrate how well telomere dysfunction predicts the development and onset of hypertension in previously nonhypertensive subjects. Finally, our third study did not utilize pharmacological or genetic interventions to mitigate to the effects of telomere uncapping on arterial dysfunction. While establishing the

efficacy of an intervention would be interesting, it is unclear how relevant or impactful such results would be for human populations. It is not possible to recap telomeres following deletion of *TRF2*, nor is it reasonable to knockdown the P53/P21-induced senescence pathway due to the inevitable risk of neoplastic cell transformation[26]. Clearance of senescent cells using pharmacological treatments represents a potential strategy for ameliorating the worst effects of P53/P21-induced senescence, but this approach is purely hypothetical at this time. Thus, of all the pathways thought to be involved in telomere uncapping-mediated arterial dysfunction, currently the only actionable targets for intervention include the production of inflammatory mediators and ROS. As anti-inflammatory and antioxidant treatments and therapies have well-established efficacy in mitigating age- and hypertension-associated arterial dysfunction[27-29], we did not see a particularly compelling reason to utilize such interventions in this study.

General Implications and Future Directions

The overall goals for this project were to determine if arterial telomere dysfunction, defined as telomere shortening that leads to uncapping, plays a role in arterial aging and the development of hypertension. Additionally, we wanted to establish that telomere dysfunction leads to P53/P21-induced senescence in arteries, ensuing SASP, and subsequent arterial dysfunction. Our findings demonstrate that arterial telomere uncapping occurs with advancing age and is associated with hypertension independent of telomere length. Furthermore, our results reveal that

telomere uncapping leads to arterial dysfunction that is comparable to that seen in arterial aging and hypertension. Thus, we believe these studies lay the clinical and mechanistic foundation for future studies aimed at establishing the prognostic value of telomere uncapping as a biomarker for CVDs.

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