Targeting Mitochondrial Fitness as a Strategy for Healthy Vascular Aging

Matthew J. Rossman[1*], Rachel A. Gioscia-Ryan[1*], Zachary S. Clayton[1], Michael P. Murphy[2,3] and Douglas R. Seals[1]

[1]Department of Integrative Physiology, University of Colorado Boulder, Boulder, CO, USA

[2]MRC-Mitochondrial Biology Unit, University of Cambridge, Cambridge, UK

[3]Department of Medicine, University of Cambridge, UK

*Rossman and Gioscia-Ryan share first authorship

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Address for Correspondence

Douglas R. Seals
Department of Integrative Physiology
University of Colorado Boulder
354 UCB
Boulder, CO 80309
Phone: 303-492-5305
Fax: 303-492-6778
Email: Douglas.Seals@colorado.edu

ABSTRACT

Cardiovascular diseases (CVD) are the leading cause of death worldwide and aging is the primary risk factor for CVD. The development of vascular dysfunction, including endothelial dysfunction and stiffening of the large elastic arteries (i.e., the aorta and carotid arteries), contribute importantly to the age-related increase in CVD risk. Vascular aging is driven in large part by oxidative stress, which reduces bioavailability of nitric oxide and promotes alterations in the extracellular matrix. A key upstream driver of vascular oxidative stress is age-associated mitochondrial dysfunction. This review will focus on vascular mitochondria, mitochondrial dysregulation and mitochondrial reactive oxygen species (ROS) production and discuss current evidence for prevention and treatment of vascular aging via lifestyle and pharmacological strategies that improve mitochondrial health. We will also identify promising areas and important considerations ("research gaps") for future investigation.

Keywords: endothelial dysfunction, arterial stiffness, reactive oxygen species, oxidative stress, mitophagy

Cardiovascular diseases (CVD) remain the largest contributor to morbidity and mortality in both developed and many developing nations [1, 2]. Aging is by far the strongest risk factor for CVD, with >90% of all deaths occurring in adults 50 years of age and older [1, 2]. Importantly, the changing demographics of aging characterized by a shift toward older populations [3] predicts a progressive, marked increase in prevalence of CVD in the absence of effective intervention [4].

A key mechanism by which aging increases CVD risk is the development of vascular dysfunction [5, 6]. A number of adverse changes to the vasculature occur with aging, but two major clinically relevant expressions are endothelial dysfunction, as assessed by reduced arterial dilation in response to endothelium-derived nitric oxide (NO), and stiffening of the large elastic arteries (i.e., the aorta and carotid arteries) [5, 6]. In combination, endothelial dysfunction and arterial stiffening contribute to a “vascular aging” phenotype that drives much of the adverse effects of age on CVD.

Vascular Endothelial Dysfunction. The vascular endothelium is a single-cell layer lining the lumen of blood vessels. Endothelial cells play a critical role regulating vasomotor tone, metabolism, immune function, thrombosis and many other processes via synthesis and release of a variety of vasoactive molecules [7]. A major vasodilatory and largely vasoprotective molecule released by endothelial cells is NO, which is produced in response to mechanical (i.e., blood flow) and chemical (e.g., acetylcholine [ACh]) stimuli by the enzyme nitric oxide synthase (eNOS); eNOS catalyzes the generation of NO from L-arginine and oxygen, with NO subsequently diffusing to vascular smooth muscle cells where it induces vascular smooth muscle relaxation and vasodilation [7]. Endothelial dysfunction occurs with aging and is characterized by a decline in endothelium-dependent dilation (EDD), largely as a

consequence of reductions in NO, although changes in concentrations of vasoactive factors such as prostaglandins, endothelin-1, norepinephrine, angiotensin II also contribute [7].

NO-mediated EDD can be determined in pre-clinical models by assessing changes in artery diameter in response to flow *in vivo* [8, 9] or changes in diameter of isolated artery segments *ex vivo* in response to mechanical or pharmacological stimuli, such as ACh [10]. In humans, the gold-standard non-invasive assessment of NO-mediated EDD is brachial artery flow-mediated dilation (FMD), in which the change in brachial artery diameter in response to increases in blood flow is determined [10, 11]. Brachial artery FMD primarily assesses macrovascular (conduit artery) function. Microvascular (resistance vessel) function can be determined by measuring changes in blood flow in response to intra-arterial infusions of ACh and is primarily assessed in the forearm [10, 11]. These experimental approaches all demonstrate reduced endothelial function with aging in pre-clinical models and humans [12-17]. Endothelial dysfunction is the major antecedent of atherosclerosis [5, 18] and both reduced brachial artery FMD and lower forearm blood flow responses to ACh are independent predictors of CV events and CVD in middle-aged and older adults free from clinical disease in large, community-based cohort studies [19-21].

Large Elastic Artery Stiffening. The aorta and carotid arteries expand and recoil as blood is ejected into the arterial system by the left ventricle during systole [22]. This action limits arterial pulsatile pressures by providing a dampening function and protects the downstream microvasculature from potentially damaging fluctuations in blood pressure and flow [23]. Moreover, the elastic recoil of the aorta aids in the propulsion of blood to the periphery and maintains perfusion of the heart during diastole [22]. With aging, aortic stiffening results in blood being ejected into a stiffer aorta, which augments central systolic blood

pressure because the ejected pressure wave travels at a higher velocity in stiffer arteries and is reflected by points of impedance such that the returning pressure wave reaches the heart at mid-to-late systole [22, 24]. In addition, the greater forward moving pressure wave amplitude (from systolic ejection, prior to the return of wave reflections) is a major contributor to the age-related increase in central systolic blood pressure after age 60, particularly in women, as a consequence of a plateau or decrease in reflected wave amplitude [25, 26]. The augmented systolic blood pressure, in turn, contributes to isolated systolic hypertension and results in a loss of diastolic pressure augmentation, such that aortic pulse pressure is widened [22, 24]. Aortic stiffening therefore increases left ventricular afterload during systole, promoting left ventricular hypertrophy and dysfunction, and compromises coronary perfusion during diastole because of the reduced augmentation of diastolic pressure [24, 27]. The loss of pulsatility-dampening effects of the aorta and the carotid artery also allows for transmission of high pulsatile pressures to the delicate small vessels in the microcirculation, which is particularly harmful for high-flow, low-resistance organs such as the brain and kidney, and a potential causative factor in target organ damage [23].

Structural changes to arteries, functional influences (i.e., factors influencing vascular smooth muscle tone) and the stiffness of vascular smooth muscle cells contribute to large elastic artery stiffening with aging [28, 29]. The primary structural changes mediating arterial stiffening occur in the extracellular matrix and include degradation/fragmentation of elastin (e.g., by matrix metalloproteinases), an increase in the deposition of collagen and formation of advanced glycation end products (AGEs), which cross-link collagen fibers, increasing their stiffness [5, 30, 31]. Increased vascular smooth muscle tone is a consequence of changes such as reductions in NO and increased sympathetic nervous system, endothelin-1, and renin-

angiotensin aldosterone system activity [32-34]. These factors also influence the intrinsic stiffness of the vascular smooth muscle cells, which adds to the stiffness of the arterial wall [29].

The mechanical stiffness of the large elastic arteries can be determined *ex vivo* in pre-clinical models by directly measuring properties such as compliance by creating stress-strain curves [35, 36]. *In vivo*, arterial stiffness can be assessed in pre-clinical settings and humans with pulse wave velocity (PWV), which is a measure of the (regional) speed of the pulse wave generated by the heart when blood is ejected into the arterial system [22]. Aortic PWV is the predominant measure in rodents and carotid-femoral PWV is the reference standard measure of aortic stiffness in humans [10, 22]. Carotid-femoral PWV increases with aging and is a strong, independent predictor of CVD risk in older adults [37, 38]. Moreover, consistent with aortic stiffness-associated end organ damage, growing evidence supports an association between elevated carotid-femoral PWV and other age-related clinical disorders such as cognitive decline, dementia, including Alzheimer's disease, and decreases in renal function/chronic kidney disease [39-43]. The local distensibility of the carotid artery can also be determined in humans by measuring carotid artery compliance (the change in artery diameter for a given change in arterial pressure) and determining the carotid distensibility coefficient (i.e., changes in artery diameter normalized to diastolic lumen diameter) and/or carotid beta-stiffness index, which is largely independent of blood pressure [10, 22]. Carotid artery compliance is associated with incident stroke, independent of aortic stiffness [44].

***Mechanisms of Vascular Dysfunction with Aging*.** The primary molecular mechanisms of vascular aging are oxidative stress and chronic, low grade inflammation [45, 46] (Figure 1). Excessive production of reactive oxygen species (ROS) in combination with

unchanged or decreased abundance/activity of antioxidant enzymes (e.g., superoxide dismutase, SOD) results in the development of oxidative stress in arteries with aging [24, 45]. Excess superoxide rapidly reacts with NO to form the secondary reactive species peroxynitrite ($ONOO^-$), decreasing the bioavailability of NO [24, 45], causing endothelial dysfunction. Peroxynitrite is also the primary molecule that reacts with and oxidizes tetrahydrobiopterin (BH_4), an essential co-factor for NO production by eNOS [47]. Loss of BH_4 leads to eNOS uncoupling, whereby eNOS produces more superoxide and less NO, exacerbating oxidative stress and decreasing bioavailable NO and endothelial cell function [47]. Excess ROS also can activate pro-inflammatory networks such as those regulated by the transcription factor nuclear factor kappa B (NFkB), which upregulates the production of pro-inflammatory cytokines that can impair vascular function and activate other ROS producing systems and enzymes, creating an adverse feed-forward (vicious) cycle of inflammation and oxidative stress [24, 45].

This overall state of oxidative stress and inflammation also contributes to arterial stiffening with aging by altering the structural properties of the arterial wall. Production of collagen by fibroblasts is stimulated by superoxide-related oxidative stress [30, 48, 49]. Matrix metalloproteinases are upregulated and elastin content is lower in aorta of SOD-deficient mice, consistent with the concept that elastin degradation is induced by oxidative stress [50]. Vascular oxidative stress also promotes transforming growth factor β signaling and this, in turn, stimulates inflammation, which further reinforces arterial stiffness via activation of the pro-oxidant enzyme, NADPH oxidase [48]. AGEs interact with the receptor for AGEs to activate NFkB-regulated pro-inflammatory pathways and oxidative stress, which ultimately perpetuates arterial stiffening and further increases production of AGEs [51].

Mitochondrial dysfunction is emerging as a key source of vascular oxidative stress and contributor to age-related vascular dysfunction. The remaining sections of this article will focus on mitochondrial dysfunction as a driver of vascular aging and review current evidence for prevention/treatment of age-associated vascular dysfunction via lifestyle and pharmacological strategies that improve mitochondrial health. We will also discuss current “research gaps” and future directions for the field.

VASCULAR MITOCHONDRIA, MITOCHONDRIAL DYSREGULATION AND ROS

Mitochondria are cytoplasmic organelles that are present in the majority of cell types in the human body, including vascular endothelial and smooth muscle cells. Mitochondria are often referred to as the “powerhouse” of the cell for their role in ATP production by oxidative phosphorylation, which occurs via a series of electron transfers through the respiratory chain in the mitochondrial inner membrane that is coupled to ATP synthesis by the FoF1-ATP synthase by the protonmotive force across the inner membrane. However, mitochondria are also vital for a number of additional cellular processes, including regulation of metabolism, calcium homeostasis, immune function, cell growth and stem cell function, and cell death pathways. Although mitochondrial density in vascular tissues is considerably lower than other tissues such as skeletal muscle, liver and heart [52, 53], increasing evidence indicates that these organelles are critical for maintenance of cellular and tissue homeostasis in the vasculature. This topic has been reviewed in detail elsewhere [54-61], but below we briefly summarize some of the key roles of mitochondria in the vasculature.

A first important distinction is to consider the vascular cell type in question, as the density and subcellular distribution of mitochondria vary between endothelial and vascular

smooth muscle cells, and indeed even among the same cell types in different vascular beds [54, 60]. In general, unlike in highly metabolically active tissues with greater ATP demand, the principal role of mitochondria in the vasculature appears to be cellular signaling rather than energy provision [54].

Cellular energy demand is quite low in endothelial cells, and ATP demand is met primarily via glycolysis. However, endothelial mitochondria are critical in the regulation of calcium homeostasis, apoptosis/necrosis, cellular response to stress, and immune and inflammatory pathways. An essential feature of these roles is the regulated production of signaling molecules including redox-active molecules (reactive oxygen, nitrogen, and other species; mtROS), mitochondrial DNA, mitochondria-derived peptides and damage-associated molecular pattern molecules (DAMPs), which exert effects intra- and extra-cellularly [62]. Importantly, there is crosstalk between mitochondrial and nuclear signaling pathways, whereby mitochondria-derived signaling is both influenced by and can influence nuclear events including gene expression [63].

Similarly, in vascular smooth muscle cells, mitochondria have an important role in cellular signaling. Mitochondria are involved in signaling pathways for regulation of vascular smooth muscle cell growth and proliferation (e.g., TGF-beta activity) [64], as well as maintenance of the dynamic balance among synthesis and breakdown of extracellular structural proteins, including collagen and elastin (e.g., matrix metalloproteinase enzyme activities) [65]. There is also emerging evidence demonstrating interplay between mtROS signaling and inflammatory pathways known to be important for regulating vascular smooth muscle cell function, including those involving NFkB and the NLRP3-inflammasome [66-69], further highlighting the crucial role of mtROS in vascular homeostasis.

Mitochondrial ROS. The signaling functions of vascular mitochondria are thought to be mediated in large part by the production of ROS at low, physiological levels. However, the dysregulation of this mtROS production also has the potential to lead to pathophysiological sequelae that disrupt other mitochondrial functions, cellular homeostasis, and ultimately vascular function.

The production of ROS by mitochondria can occur at several sites (Figure 2), including but not limited to the electron transport proteins, and this topic has been reviewed in detail elsewhere [54, 60, 70]. The most important sites for ROS production within mitochondria appear to be complexes I and III. These ROS are thought to be critical transducers of signaling mediated by mitochondria, leading to post-transcriptional modification of proteins and interactions with immune and inflammatory cellular pathways, although the mechanistic details are still uncertain. In the vasculature, the proximal mtROS species is superoxide, which is generated primarily at the electron transport chain in the mitochondrial inner membrane via interaction between oxygen and unpaired electrons, influenced by the proton motive force and the redox state of the coenzyme Q pool and integrity of intrinsic electron transport chain proteins [54, 58, 60, 70]. Superoxide is released into the matrix (complex I) or into both the matrix and intermembrane space (complex III); it can also undergo dismutation to hydrogen peroxide by the antioxidant enzyme manganese superoxide dismutase (MnSOD) [59, 60, 62, 70]. Hydrogen peroxide is also generated de novo on the surface of the mitochondrial outer membrane or in the intermembrane space mitochondria by $p66^{SHC}$, a growth factor adapter protein referred to as a sensor/marker and “master regulator” of mitochondrial redox signaling whose activity is indicative of the rate of mtROS production [71]. In addition, NADPH oxidase 4 (NOX4) is viewed as a primarily mitochondrial isoform of the NOX monoamine oxidase family

of enzymes that contributes to mitochondrial hydrogen peroxide generation [72], although more research is needed to confirm the mitochondrial specificity of NOX4.

Mitochondria as Source and Target of Oxidative Stress. Mitochondria are not only a key source of cellular ROS production but are also particularly vulnerable to potential damage caused by these molecules. The extensive lipid bilayer membranes, circular DNA lacking the protective histones of nuclear DNA, and numerous enzymes and proteins that characterize mitochondria all represent potential targets for ROS-induced damage, which, in turn, has adverse effects on mitochondrial function [73]. Although mitochondria have endogenous antioxidant defense mechanisms, including MnSOD, catalase, the glutathione/glutathione peroxidase systems and the thioredoxin/peroxiredoxin pathway [74], excessive levels of mtROS can overwhelm these defense systems, resulting in mitochondrial oxidative stress. As such, oxidative damage to mitochondria results in an abundance of less healthy mitochondria. Importantly, mitochondrial quality control mechanisms exist to degrade dysfunctional mitochondria/mitochondrial components by mitophagy (organelle-specific form of autophagy) and generate new mitochondria by mitochondrial biogenesis (e.g., by PGC-1α-regulated processes) [75]. In addition, a balance in the mitochondrial dynamics processes of fission and fusion is critical for maintaining mitochondrial health/function and regulating mtROS production, at least in part by effects on bioenergetic function and mitochondrial membrane potential (e.g., reducing hyperpolarization) [76]. Indeed, dysregulation of mitochondrial dynamics processes—characterized by excess fission relative to fusion—promotes endothelial inflammation in an NFkB-dependent manner [77] and is necessary for the cellular senescence-associated inflammatory phenotype induced by angiotensin II [78]. All of these mitochondrial stress response/defense and quality control pathways become impaired in settings of excess

mtROS such as aging, ultimately resulting in a pool of less healthy mitochondria, which may perpetuate mitochondrial dysfunction in part by further increasing mtROS [79].

In summary, due to the fundamental role of certain key mitochondrial processes as drivers of excess mtROS, they may be considered hallmarks of impaired mitochondrial health. Notable examples of this include: increased production of superoxide and other mtROS; mitochondrial DNA damage; decreased endogenous antioxidant defenses (e.g., MnSOD content/activity); dysregulated mitochondrial quality control (e.g., impaired mitophagy, decreased mitochondrial biogenesis and related PGC-1α signaling); impaired bioenergetic function with uncoupling of electron transport from ATP production; altered balance of mitochondrial dynamics resulting in excessive fission/insufficient fusion; and an overall reduction in the ability of mitochondria to adequately respond to stress (Figure 2). Therefore, interventions that target these processes and attenuate excessive mtROS have the potential to improve overall mitochondrial quality or “fitness,” with associated wide-ranging salutary effects on overall cellular function.

Stress Response as an Indicator of Mitochondrial Fitness*.* Loss of the ability to respond to stress is a common feature of the aging process [80] and many disease states. Mitochondria are vital for the ability of cells to maintain or restore homeostasis following exposure to stress—termed resistance and resilience, respectively [81]. Robust cellular stress resistance mediated by mitochondria is well established in cardiac tissues in the setting of cardioprotection against ischemia/reperfusion injury, for example [82]. Mitochondria are also vital for cellular adaptation following exposure to mild stressors through a process termed “mitohormesis”, as in the case of exercise training [83]. In contrast, dysregulation of mitochondrial health can lead to inadequate stress response, resulting in cellular damage or

death. Indeed, impairment of mitochondrial stress resistance may be an integrative hallmark of mitochondrial dysregulation (Figure 2). Known vascular stressors, including hypoxia, inflammation, hyperglycemia, hyperlipidemia, oxidized low-density lipoprotein, and cigarette smoke, stimulate ROS production in mitochondria, activating signaling pathways that allow mitochondria-mediated adaptation to stress or initiation of cell death events [59, 61]. Importantly, robust mitochondrial stress response appears to be a feature of healthy vascular function, whereas vascular disease is characterized by impaired mitochondrial stress response [60, 84, 85].

Functional Implications of Vascular Mitochondrial Dysfunction and Excessive mtROS. Although vascular mitochondrial production of ROS at physiological levels is critical for maintenance of cellular homeostasis, excessive levels of mtROS have detrimental effects on key aspects of vascular physiology.

Endothelial function. Excessive production of mitochondria-derived superoxide may contribute to vascular oxidative stress and reduce the bioavailability of NO, either directly via formation of peroxynitrite or indirectly by uncoupling of eNOS. These events are further propagated by peroxynitrite-mediated inhibition of an appropriate upregulation of the mitochondrial antioxidant enzyme MnSOD [86]. Decreased NO bioavailability leads to impairments in endothelial function (as described above) but may also contribute to further mitochondrial dysregulation. NO has a key regulatory role in PGC-1α signaling and mitochondrial biogenesis [87]. Moreover, NO acts as a tonic inhibitor of complex IV of the mitochondrial respiratory chain; as such, decreases in NO bioavailability may also augment mitochondrial superoxide production by the electron transport chain as this tonic inhibition is removed [60]. Mitochondria-derived hydrogen peroxide is a key signaling molecule in the

vasculature, as a compensatory vasodilatory mechanism for reduced NO bioavailability in the microvasculature and coronary arterioles in atherosclerotic heart disease [60]. However, as with superoxide, excessive levels of hydrogen peroxide production, either de novo or as a result of superoxide dismutation, can disrupt vascular homeostasis, including activation of NFkB with resultant prothrombotic and proinflammatory effects [60, 88].

Arterial stiffening. The majority of evidence suggests that oxidative stress is a critical upstream mechanism driving arterial stiffening with aging, although there is some indication for potential sex differences in the role of oxidative stress in this process [89, 90]. Regardless, there is growing evidence that mtROS are a key source of this oxidative stress [72, 91, 92]. Excessive mtROS in vascular smooth muscle cells may induce aberrant signaling in growth factor (e.g., transforming growth factor β1) and proteolytic enzyme (e.g., matrix metalloproteinase) pathways that leads to overproduction of collagen and accelerated elastin degradation [50, 65, 93]. Further, mtROS are now recognized as important activators of pro-inflammatory signaling [67, 68] that in vascular smooth muscle cells that is also implicated in mediating structural changes in arteries [88, 91, 94]. Finally, excessive levels of mtROS may also contribute to oxidative stress-driven formation of AGEs and subsequent cross-linking of collagen in the arterial wall [95].

Vascular disease is characterized by excessive mtROS production and altered mitochondrial health. Given the current understanding of mitochondrial biology and function specifically in vascular cells, the concept that excess mtROS and associated alterations in mitochondrial health may play an important causative role in vascular dysfunction and disease is compelling. In the following section, we outline current experimental evidence supporting a

link between mitochondrial dysregulation and vascular dysfunction across a range of experimental settings and disease states, with a focus on vascular aging.

The first line of evidence of an association between mitochondria and vascular dysfunction comes from cross-sectional studies in which chronic disease states characterized by vascular dysfunction are accompanied by elevated mtROS and/or markers of altered vascular mitochondrial health. Mitochondrial DNA damage is elevated in arteries from apolipoprotein E-null (to promote atherosclerosis) mice [96] as well as in plaques from human patients with atherosclerosis [97] and in circulating cells from patients with diabetes mellitus and atherosclerotic CVD [98]. Consistent with these observations, mitochondrial bioenergetics and mitophagy are impaired in naturally aged and aged atherosclerosis-susceptible mice [99], and excessive mtROS levels and disruption of mitochondrial dynamics are evident in endothelial cells from mice [100] and patients with diabetic vascular disease [101]. Moreover, rodent models of diabetic vascular disease demonstrate an impaired mitochondrial stress response to exercise [84, 85].

These associations between mitochondrial oxidative stress and vascular dysfunction are corroborated by experimental approaches involving pharmacological manipulation and genetic knockout approaches to alter mtROS *ex vivo* and *in vivo*. For example, chronic low doses of angiotensin-II in mice elevate mtROS and induce endothelial dysfunction, at least in part by hyperacetylation-mediated impairment of MnSOD secondary to inactivation of the mitochondrial NAD^+-dependent deacetylase sirtuin 3 [102, 103]. Heterozygous knockout of the key mitochondrial antioxidant MnSOD to experimentally increase mtROS results in endothelial dysfunction [104] and acceleration of arterial stiffening with age [50]. Knockouts of $p66^{SHC}$ [105] and NOX4 [72], which recapitulate settings of decreased mtROS, exhibit preserved

vascular function. Mice expressing a defective mitochondrial DNA polymerase with resulting excessive mitochondrial DNA damage exhibit accelerated aging, including development of vascular dysfunction [106]. In contrast, treating arteries *ex vivo* or supplementing rodents *in vivo* with mitochondria-targeted antioxidants to decrease mitochondrial oxidative damage ameliorates vascular endothelial dysfunction in spontaneously hypertensive rats and rats with angiotensin II-induced hypertension [107, 108]. Similarly, mitochondria-targeted antioxidant administration improves cutaneous microvascular function in patients with chronic kidney disease [109] and EDD of arterioles isolated from adipose tissue biopsies in patients with type 2 diabetes [110]. Taken together, the evidence from multiple experimental approaches, including genetically manipulated rodents, disease models and clinical populations indicates that mitochondrial oxidative stress may be a key upstream mechanism underlying vascular dysfunction.

Primary vascular aging is accompanied by elevated mtROS and reduced mitochondrial fitness*.* Accumulating evidence also indicates that mitochondrial oxidative stress and associated impairments in mitochondrial fitness underlie the vascular dysfunction accompanying primary aging in the absence of clinical disease. Arterial mtDNA quantity decreases with aging in mice and is associated with reduced mitochondrial respiration and arterial stiffening [106]. Excessive mtROS and activation of $p66^{SHC}$ in the face of reduced or unchanged abundance of MnSOD have been observed in vascular tissues from aged rodents (Figure 3) with corresponding impairments in vascular function [111, 112], decreased mitophagy, evidence of reduced mitochondrial quality control [112, 113], and greater susceptibility to acute mitochondrial stress [111, 113, 114]. In humans, expression of MnSOD is lower in vascular endothelial cells obtained by endovascular biopsies from older adults with

impaired FMD compared with a young adult reference group [115], and impaired mitochondrial bioenergetics, elevated mtROS and impairments in EDD have been observed in biopsied arterial segments from older vs. young adults [116, 117]. More direct evidence for excessive mtROS-mediated suppression of vascular function with primary aging comes from studies in which *acute* scavenging of mtROS *ex vivo* in arteries from old mice and older adult humans or *in vivo* (humans) with the mitochondria-targeted antioxidant MitoQ reverses age-related endothelial dysfunction [111, 117, 118].

Overall, there is strong observational and experimental evidence supporting a critical role for mitochondria in maintenance of vascular homeostasis. Vascular mitochondrial dysfunction, characterized by excessive production of mtROS and other markers of reduced mitochondrial health and fitness, is an important feature of vascular dysfunction and disease, including in the setting of primary aging. As such, interventions targeting excess mitochondrial oxidative stress and mitochondrial dysfunction hold strong promise for preserving vascular function with aging. In the following section, we discuss interventions for the prevention and treatment of age-associated vascular dysfunction and their effects on mitochondrial oxidative stress and mitochondrial fitness arteries.

Prevention and Treatment of Vascular Aging by Improving Mitochondrial Health. In this section we will discuss prevention and treatment strategies that may modulate mitochondrial fitness. Our focus will be on lifestyle-based approaches and compounds supported by translational evidence of efficacy in the context of vascular aging. We will discuss: 1) strategies with evidence of mitochondrial effects in the vasculature, starting with aerobic exercise and then emerging pharmacological approaches, including “nutraceuticals” (natural compounds),

which target many of the pathways and processes thought to mediate the beneficial effects of aerobic exercise; 2) approaches documented to improve vascular function with aging for which direct evidence of mitochondria-specific effects in the vasculature are currently lacking; and 3) promising therapies that have not yet been tested for treating vascular aging.

Aerobic Exercise

Regular aerobic exercise is advanced as a “first-line” healthy lifestyle strategy for reducing CVD risk with aging. It is likely that much of the beneficial effects of aerobic exercise on CVD risk – after accounting for its effects on traditional risk factors [119, 120] -- are mediated by the ability of exercise to counteract the adverse effects of aging on arteries [24]. Indeed, both cross-sectional comparisons of exercising and non-exercising adults and intervention studies of previously sedentary individuals support an overall protective effect of aerobic exercise for vascular aging [10, 17, 121, 122]; effects that are largely attributed to its suppression of oxidative stress and inflammation (see [24, 123]). We will next summarize the evidence for mitochondria-related mechanisms as mediators of the effects of aerobic exercise on vascular function with aging.

Mitochondrial mechanisms of aerobic exercise in the vasculature. Aerobic exercise has well-documented effects on mitochondrial health and homeostasis in non-vascular tissues. Evidence that similar beneficial mitochondrial adaptations may occur in the vasculature in the setting of prevention of age-related vascular dysfunction is derived from the observation that habitually exercising older men do not exhibit a decline in EDD with aging and this preservation of EDD is associated with expression of endothelial cell MnSOD similar to young adult controls [115]. Regular aerobic exercise also enhances resistance to potentially

harmful/adverse factors (i.e., stress resistance), many of which act on mitochondria [124]. For example, older exercising adults are protected, in part, against the decline in EDD in response to experimental ischemia-reperfusion injury [125], which can drive mtROS production [126]. Collectively, these data are consistent with beneficial effects of aerobic exercise on mitochondria in the vasculature observed in pre-clinical models of disease [84, 85] and young healthy animals [127].

The vascular mitochondrial effects of exercise in the setting of primary aging have been determined in lifelong and late-life intervention studies in mice with and without access to a running wheel, a translational approach to simulate voluntary aerobic exercise. Lifelong aerobic exercise initiated at three months of age prevented age-associated declines in EDD by increasing NO bioavailability and suppressing mtROS, as indicated by the absence of improvement in EDD isolated carotid arteries in response to incubation with a mitochondria-targeted antioxidant in exercise-trained but not sedentary animals [128]. Lifelong exercise also prevented the increase in aortic mitochondrial superoxide production with aging observed in sedentary mice [128].

As a late-life intervention, voluntary aerobic exercise restored NO-mediated EDD in old mice to young adult levels [114, 129], which was associated with a suppression of aortic mitochondrial superoxide production [114] (Figure 4). In addition, voluntary aerobic exercise reversed age-associated impairments in mitochondrial stress resistance. Incubation of arteries with the mitochondrial stressor rotenone, a complex I inhibitor which can induce mtROS, (further) impaired EDD in old control mice, which was completely prevented in old exercising mice [114]. Moreover, intra-luminal exposure of arteries to a simulated Western diet – high glucose, high palmitate – mtROS-mediated stress exacerbated endothelial dysfunction in

arteries from old mice; these effects were ameliorated in old exercised mice [114]. Consistent with an overall improvement in mitochondrial health, voluntary aerobic exercise normalized aortic protein markers of mitochondrial biogenesis, dynamics and stress resistance/metabolism and mitochondrial quality control processes [114] (Figure 4). Interestingly, data from genetically eNOS deficient young mice and mice treated with the eNOS inhibitor L-NAME while undergoing chronic exercise training suggest that NO plays a permissive role in vascular mitochondrial adaptations to exercise [130], in line with evidence for NO as a key regulator of PGC-1α. How NO-mitochondrial signaling is altered by aging, and whether an improvement in NO bioavailability is a requisite “upstream” factor for vascular adaptations to exercise with aging, remains to be determined.

Improvements in aortic mitochondrial health also are associated with the beneficial effects of aerobic exercise training on age-related arterial stiffening. In old rats, chronic exercise training via forced treadmill exercise reduced aortic stiffness (aortic PWV) to young adult levels, attenuated age-associated increases in mtROS and mitochondrial swelling (an indicator of mitochondrial permeability transition pore opening/calcium handling), and normalized mitochondrial bioenergetics/ATP production and mtDNA content to levels similar to young controls [131]. These changes were associated with lower collagen content and preserved elastin abundance in aortas from old exercise-trained rats, suggesting a link between improved mitochondrial health and the structural composition of the arterial wall [131].

Collectively, these data implicate improvements in vascular mitochondrial health as a primary mechanism underlying the favorable effects of aerobic exercise on endothelial function and arterial stiffening with aging. It remains to be determined whether novel “time-efficient” forms of exercise (e.g., high-intensity interval training, inspiratory muscle strength training) or

other aerobic exercise-inspired lifestyle strategies (e.g., heat therapy) [28, 132-134] transduce beneficial effects on vascular function by improving mitochondrial health, which has been suggested to be a primary mechanism of action of these therapies in other tissues [135, 136]. Additionally, the mitochondria-related mechanisms by which aerobic exercise improves vascular function are incompletely understood. As such, more research is needed to assess contributions of associated molecular and cellular processes, including autophagy, calcium handling and mito-nuclear communication (see “Research Gaps”).

Pharmacological Compounds Studied in the Context of Vascular Aging

Aerobic exercise activates numerous mitochondrial signaling pathways and processes to ultimately improve vascular function with aging. These individual pathways induced by aerobic exercise can be considered therapeutic targets, which can be activated (or suppressed) by specific pharmaceutical or nutraceutical compounds. In this section, we will discuss examples of this general approach with compounds supported by translational (pre-clinical to clinical) evidence of efficacy for healthy vascular aging.

Mitochondria-targeted antioxidants. Due to the centrality of mtROS- and mitochondrial oxidative damage-linked mediators of age-associated vascular dysfunction and the ability of aerobic exercise to counteract mtROS-related vascular aging, decreasing vascular mtROS is an attractive therapeutic option. The development of mitochondria-targeted antioxidants, such as MitoQ, offers an innovative therapeutic strategy for decreasing mtROS and associated oxidative damage to ultimately improve vascular function with aging. MitoQ is a compound consisting of a derivative of the naturally occurring antioxidant ubiquinol conjugated to a lipophilic cation, triphenylphosphonium (TPP) [137]. The lipophilic nature and positive

charge of the compound enables MitoQ to cross cell membranes and accumulate at the matrix face of the mitochondrial inner membrane, where it is well-positioned to decrease mitochondrial oxidative damage [137]. These features of the compound circumvent limitations of traditional exogenous antioxidants (e.g., vitamins C and E) such as inefficient cellular uptake and an inability to accumulate at the cellular sources of ROS, which likely contributed to their lack of efficacy in large-scale clinical trials [138]. Many traditional antioxidants also typically have very short half-lives, whereas MitoQ is a recyclable antioxidant: the active (reduced) form of MitoQ is regenerated via reaction with complex II in the electron transport chain, allowing for sustained antioxidant activity [137].

The primary antioxidant effects of MitoQ are attributed to its ability to act as a chain breaking antioxidant, blocking lipid peroxidation [137, 139, 140]. MitoQ may also affect (decrease) superoxide production by reverse electron transport at complex I of the electron transport chain, although this mechanism is less well established [141, 142]. Although some *ex vivo* data suggest MitoQ may be pro-oxidant [143], this is likely a consequence of the experiments being performed in water-based tissue culture medium, in which any quinol will "redox cycle" and generate ROS/hydrogen peroxide [139, 143]; the amount of MitoQ that is free in water *in vivo* is negligible, so redox cycling does not seem to occur in this biological setting [137, 139, 144]. The precise sites of mtROS and oxidative damage affected by MitoQ *in vivo* remain to be fully characterized, particularly in humans.

Initial evidence for the efficacy of MitoQ for reducing mitochondrial oxidative stress/damage and improving age-associated vascular dysfunction was determined with chronic administration of MitoQ to young and old mice for 4 weeks in the drinking water [111]. MitoQ supplementation completely reversed age-related endothelial dysfunction by restoring

NO bioavailability, with no effects in young adult mice [111] (Figure 5). The benefits of MitoQ were attributable to the antioxidant moiety of the compound, as supplementation with the mitochondria-targeting TPP moiety alone had no effects on EDD [111]. The primary mechanism responsible for the beneficial effects of MitoQ on endothelial function was a decrease in mtROS bioactivity, as evidenced by MitoQ treatment-associated abolition of tonic mtROS-associated suppression of EDD in old mice, i.e., in old mice treated with MitoQ there was no improvement in EDD with acute MitoQ incubation, as was observed in arteries from old control mice [111]. Consistent with an overall improvement in mitochondrial homeostasis in arteries, MitoQ supplementation normalized expression of p-p66^{SHC}, MnSOD, mitochondrial electron transport chain complex IV and PGC-1α in aortas from old mice to those of the young controls [111]. MitoQ also reversed the aging-induced decrease in mitochondrial resistance to stress, as shown by protection against rotenone-induced impairment in *ex vivo* EDD in old MitoQ-supplemented mice [111].

These effects were recently translated to older adult humans with impaired endothelial function at baseline in a small, randomized control (crossover design) pilot study [118]. Oral supplementation with MitoQ (20 mg/day) was well-tolerated without serious adverse effects over 6 weeks, consistent with other clinical trials with MitoQ up to 1 year in length [118, 145, 146]. MitoQ supplementation increased NO-mediated endothelial function, assessed by brachial artery flow mediated dilation, by 42% over placebo conditions [118] (Figure 5). As in old mice, the primary mechanism responsible for the improvement in endothelial function was decreased mtROS-associated suppression of endothelial function, as shown by an acute increase in FMD in response to a single supratherapeutic dose of MitoQ (160 mg) after placebo treatment, but not after chronic MitoQ supplementation [118] (Figure 6). MitoQ

supplementation also decreased levels of plasma oxidized low-density lipoprotein, a circulating marker of oxidative modification of lipids [118].

In mice, MitoQ supplementation completely ameliorated age-associated increases in aortic stiffness without affecting (the already normal) aortic stiffness in young adult mice [147] (Figure 7). Consistent with this finding, MitoQ supplementation decreased aortic stiffness in older humans exhibiting age-related increases in aortic stiffness assessed at baseline (i.e., subjects with carotid-femoral PWV values >2 standard deviations from the group mean of young subjects in the Framingham Heart Study [148]), but had no effect in subjects with normal (low) levels of aortic stiffness [118] (Figure 7). The aortic de-stiffening effects of MitoQ were associated with an attenuation of declines in aortic elastin content and function in old mice. It is unlikely that structural changes in the arterial wall would occur over 6 weeks in humans, suggesting mechanisms other than changes in elastin content contributed to the decrease in aortic stiffness in humans. Rather, reduced vascular smooth muscle tone and/or stiffness secondary to enhanced NO signaling, i.e., effects on “functional” determinants of arterial stiffening, presumably mediated these beneficial effects in older humans. As mitochondrial ROS appear to stimulate sympathetic vasoconstrictor nerve activity [149], it is possible that MitoQ may also have decreased aortic stiffness through reductions in alpha-adrenergic mediated vascular smooth muscle tone. Collectively, these observations suggest that MitoQ and potentially other strategies directly targeting mitochondrial oxidative stress may be viable options for treating vascular dysfunction with aging; however, the pilot study findings must first be confirmed in a larger and longer duration clinical trial.

In addition to its effects in primary aging, there is some evidence from animal studies of beneficial effects of supplementation with MitoQ in disease settings. MitoQ prevents the

development of endothelial dysfunction in spontaneously hypertensive stroke-prone rats [107, 150] and improves endothelial function and arterial stiffness in doxorubicin-treated mice, a model of chemotherapy-associated accelerated vascular aging. In humans, clinical trials are ongoing investigating the efficacy of MitoQ for improving vascular function in populations with clinical diseases/disorders of aging, including chronic kidney disease, heart failure, peripheral artery disease, mild cognitive impairment and chronic obstructive pulmonary disease (COPD), with some evidence of an improvement in endothelial function in patients with COPD following acute and chronic MitoQ supplementation [151].

The therapeutic potential of targeting mitochondrial oxidative damage to ameliorate vascular dysfunction is consistent with the findings of randomized clinical trials (see [152] for meta-analysis) in a variety of patient populations showing beneficial vascular effects of ubiquinol and ubiquinone, which, although not biochemically modified to target mitochondria, are thought to act, at least in part, on mitochondria. Indeed, improved mitochondrial fitness and reduced oxidative stress with ubiquinol/ubiquinone as a mechanism is supported by experimental evidence in some, albeit not all, clinical studies [153]. Moreover, in old mice, the mitochondria-targeted peptide SS-31 improved cerebrovascular function by improving NO bioavailability and reducing mtROS [154]. However, as discussed below, this compound likely acts on mitochondrial function via multiple mechanisms.

Activators of autophagy, mitophagy and mitochondrial quality control

Mitochondrial quality control – the aforementioned collection of molecular processes by which new, healthy mitochondria are produced (mitochondrial biogenesis) and damaged mitochondria are degraded by mitophagy -- becomes dysregulated with aging in the vasculature [99, 113, 155] resulting in an accumulation of dysfunctional mitochondria. Exercise

activates mitochondrial quality control processes (e.g., biogenesis, mitophagy), and the beneficial effects of exercise on vascular mitochondria may be partially attributable to improved quality control.

Several small molecules appear to improve mitochondrial quality control in the setting of vascular aging. For example, trehalose, a disaccharide found in mushrooms and honey, is known to enhance autophagy and mitophagy [113, 155, 156]. Four weeks of oral trehalose supplementation reverses age-associated declines in NO-mediated EDD by decreasing oxidative stress and reduces aortic stiffness in old mice [113, 155] (Figure 8). Although aspects of the improvements in vascular function can be attributed to a global (vs. organelle-specific) induction of autophagy [155], trehalose reversed age-related decreases in aortic Parkin and SIRT3 and increased abundance of BNIP3 (a Bcl-2 family protein that primarily localizes to the mitochondrial outer membrane) and PGC-1α, indicating specific activation of mitochondrial quality control pathways [113]. These changes were accompanied by normalization of the mitochondrial redox/stress sensor $p66^{SHC}$ in aortas from old trehalose-supplemented mice, indicating improved mitochondrial health with trehalose treatment [113] (Figure 8). Recent evidence suggests that trehalose supplementation also improves NO-mediated EDD in middle-aged and older adults, but the role of enhanced mitophagy and/or mitochondrial quality control in this improvement in endothelial function is unknown [157] (Figure 8). In models of accelerated vascular aging, trehalose supplementation also improved endothelial function and reduced stiffness of resistance vessels (mesenteric arteries) in spontaneously hypertensive adult rats [158] and improved mesenteric artery endothelial function in obese diabetic mice [159].

The natural polyamine spermidine is another autophagy/mitophagy-activating dietary compound that extends lifespan and healthspan in lower organisms [160, 161], and dietary intake of spermidine is inversely associated with all-cause mortality and CVD risk in humans [162]. Spermidine delays cardiac aging in rodents, which is attributed to activation of autophagy and mitophagy in cardiomyocytes [162]. In terms of vascular aging, spermidine restores age-related impairments in NO-mediated endothelial function and reverses age-associated aortic stiffening [163]. The improvements in vascular function in old mice are mediated by reductions in oxidative stress and cross linking of structural proteins, as shown by decreases in aortic abundance of AGEs [163]. Although the vascular effects of spermidine were shown to be dependent on the activation of autophagy, the contribution of mitophagy *per se* was not fully elucidated in this study [163]. More recently, spermidine supplementation in late middle-aged (18 month) hyperlipidemic mice was shown to reverse age-associated declines in aortic mitochondrial respiration and reduce inflammation and atherosclerosis by enhancing mitophagy [99]. Spermidine also restores NO signaling *ex vivo* in endothelial cells isolated from veins of patients with diabetes [164]. Although chronic spermidine supplementation is reported to be safe in humans and may improve cognitive function in older adults [165, 166], there currently are no clinical trials in older adults focused on vascular function.

***Modulators of cellular energy sensing pathways*.** Another mitochondria-focused therapeutic approach involves strategies to increase cellular levels of NAD^+, which are decreased with aging and increased by exercise (and caloric restriction, described below). The primary goal of increasing NAD^+ levels is activation of sirtuins, which, in turn, deacetylate histones and other proteins to alter gene expression and promote mitochondrial biogenesis,

antioxidant defenses and cellular stress resistance, while also decreasing carbon stress within mitochondria [167]. Of particular note, NAD^+ is also an activator of the mitochondrial sirtuin, SIRT3, which targets mitochondrial proteins such as MnSOD [102, 103]. Levels of NAD^+ can be increased by oral supplementation with NAD^+ precursors such as nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR), which are intermediates of an endogenous NAD^+ salvage pathway [168].

Supplementation with NMN increases aortic NAD^+ production and SIRT1 abundance, improves endothelial function by increasing NO bioavailability and reducing oxidative stress [169, 170] and reduces aortic stiffness in old mice [169]. Increases in aortic MnSOD were also observed with acute NMN incubation of arteries, supporting a benefit of NAD^+-boosting on mitochondrial antioxidant defenses [169]. The mitochondria-specific effects of NMN were also shown in the cerebrovasculature, where NMN improved cerebral blood flow by suppressing mtROS [170]. Moreover, in primary cerebrovascular endothelial cell cultures from old rats, NMN administration normalized NO production, which was accompanied by decreased mtROS production and improved mitochondrial membrane potential, ATP production and respiration [170]. All of these effects were abrogated by shRNA-mediated knockdown of SIRT1 and 2, indicating sirtuin activation is necessary for the beneficial mitochondrial effects of NMN on endothelial cells [170]. Interestingly, NMN supplementation did not appear to affect mitochondrial biogenesis, but did rescue age-related reductions in gene expression of subunits of the electron transport chain encoded by the mitochondrial genome, which may have explained improved electron transport chain function and decreased mtROS production [170, 171].

There are currently limited data in humans regarding the effects of NAD^+ precursors on vascular aging. Greater dietary intake of niacin, another NAD^+ precursor, is associated with higher endothelial function and lower oxidative stress in older adults [172]. A small pilot study in middle-aged and older adults showed that chronic supplementation with NR increased circulating NAD^+ levels and reduced aortic stiffness and blood pressure, particularly in subjects with elevated blood pressure [173]. No effects on endothelial function were observed in this pilot study, although a longer and larger clinical trial is currently underway to establish the cardiovascular effects of NR in older adults (NCT03821623). The effects of NMN supplementation in humans is currently unknown, but clinical trials are ongoing (NCT03151239). No studies have investigated vascular mitochondrial effects of NR or NMN supplementation in humans. However, a recent clinical trial in insulin-resistant obese men reported that NR did not affect skeletal muscle mitochondrial parameters, including mitochondrial respiration, content and morphology, although NR did not effectively increase NAD^+ metabolite concentration in skeletal muscle this study [174]. It remains to be determined if NR (or other NAD^+-boosting approaches) increases vascular NAD^+ in humans and if this impacts mitochondrial and/or vascular function with aging.

Prevention and Treatment of Vascular Aging: Strategies Currently Lacking Direct Evidence for Mitochondrial Effects. A number of lifestyle and pharmaceutical or nutraceutical approaches with clearly established effects on mitochondria in non-vascular tissues have been assessed for improving vascular function with aging. Presently, however, direct evidence supporting the vascular mitochondria-specific effects of these strategies as mediators of improvements in vascular function is lacking. These interventions will be reviewed

next, with a focus on observations pointing to the possible mitochondrial effects of these strategies.

Diet composition. There are clear, well-established benefits for reducing CVD risk of certain broad dietary patterns, which are typically high in fruits and vegetables, whole grains, low-fat dairy and associated with low to moderate consumption of lean meats and fish [175, 176]. The CVD risk-reducing effects of these dietary patterns may be attributed, in part, to improvements in vascular function. For example, the DASH and Mediterranean diets improve both endothelial function and arterial stiffness through mechanisms involving decreased oxidative stress and inflammation [177, 178]. Although many components (i.e., specific foods and/or bioactive ingredients) of these diets, such as dietary nitrates, co-enzyme Q10, omega-3 or other fatty acids, are thought to modulate mitochondrial function, it is currently uncertain if the beneficial effects of broad dietary patterns are dependent on these individual components and/or their effects on mitochondria. For example, the Mediterranean diet is rich in monounsaturated fatty acids, which modulate SIRT1 to activate PGC-1α signaling and increase mitochondrial biogenesis [179], but whether these effects contribute to improved vascular function with a Mediterranean diet is unknown. As such, more research is needed to determine the direct mitochondrial health-promoting effects in the vasculature of certain dietary patterns and/or specific components of these dietary patterns, several of which are discussed in more detail below.

In contrast to healthy dietary patterns, “sub-optimal” dietary patterns are associated with elevated CVD risk and accelerated vascular aging. In older adults with CVD risk factors, diets high in saturated fats further impair endothelial function [180, 181] and may promote arterial stiffening. Moreover, a Western diet (high in saturated fats and sugar, low in fiber) reduces

endothelial function and increases arterial stiffening with aging in mice [182-184]. These adverse effects of a Western diet on vascular function are mediated by increased superoxide-associated suppression of endothelial function [184], and the primary source of superoxide may be mitochondria [128]. Indeed, cell culture studies have demonstrated that glucose and palmitate – two key compounds which may be elevated in the circulation in the setting of Western diet consumption – induce mtROS production and dysfunction in endothelial cells [185, 186]. In agreement with these observations, aortic mtROS bioactivity is higher and EDD is lower across the lifespan in mice fed a Western diet vs. age-matched mice. EDD is completely restored with *ex vivo* incubation of arteries with a mitochondrial antioxidant, indicating the Western diet-associated endothelial dysfunction is mediated by excess mtROS [128]. Interestingly, the adverse effects of the Western diet on endothelial function and mtROS are prevented in mice given access to a running wheel, consistent with the mitochondrial stress resistance-enhancing effects of regular aerobic exercise discussed previously [128]. Whether aerobic exercise also protects against Western diet-induced arterial stiffening by reducing mtROS and/or other mitochondrial mechanisms is currently unknown (see Research Gaps).

Energy intake. Caloric restriction, characterized by a sustained 10-40% reduction in caloric intake without malnutrition, is the most well studied lifestyle strategy for extending maximal lifespan and healthspan in model organisms. Caloric restriction also promotes healthy vascular aging. In older adult humans, caloric restriction-based weight loss improves endothelial function [187] and reduces carotid artery and aortic stiffness [188]. Lifelong caloric restriction preserves EDD by maintaining NO bioavailability and prevents aortic stiffening [189, 190]. Moreover, later life caloric restriction restores NO bioavailability and endothelial function

in old mice [191] and rats [192] to levels observed in young adult controls. The primary mechanisms responsible for the effects of CR include a decrease in oxidative stress and inflammation, likely via modulation of cellular energy sensing pathways dysregulated with aging, including adenosine AMP-activated protein kinase (AMPK)-, sirtuin- and mammalian target of rapamycin (mTOR)-regulated cellular signaling cascades [193].

Mitochondria are integral to many of these energy sensing pathways, as effectors (e.g., activation of cell death pathways) and for coordination of cellular responses (e.g., via mito-nuclear communication). Accordingly, CR has been reported to have numerous effects on mitochondria in non-vascular tissues (primarily skeletal muscle) in rodents and humans, including decreased mtROS production, increased mitochondrial antioxidant defense, improved mitochondrial bioenergetic function and efficiency, increased mtDNA quantity and quality and enhanced resistance to apoptosis. CR may also increase mitochondrial biogenesis, although results are equivocal [194, 195]. These CR-induced improvements in mitochondrial function are thought to be accomplished by activation of mitochondrial quality control processes, such as autophagy and mitophagy, secondary to activation of AMPK and sirtuins and inhibition of mTOR in response to reduced nutrient availability. Specifically, low levels of glucose, amino acids and insulin downregulate mTOR and insulin-like growth factor 1 (IGF-1) signaling pathways, decreasing protein synthesis and stimulating autophagy in the cell. Low nutrient levels also increase the AMP to ATP ratio, which, in turn, stimulates AMPK-regulated energy producing (catabolic) processes, while inhibiting anabolic metabolism. Bioavailability of NAD^+ (relative to NADH) and acetyl CoA (relative to CoA) also increase during fasting/CR, activating sirtuins, which deacetylate transcription factors such as FOXOs and PGC-1α,

controlling the expression of genes regulating cellular stress resistance and mitochondrial biogenesis.

Despite the clear connections between CR and mitochondrial health, the direct effects of CR on mitochondria in the vasculature are not well established. Lifelong and short-term later life CR increase aortic abundance and/or activity of MnSOD [189-191], consistent with improved vascular mitochondrial antioxidant defenses. In addition, CR prevents the increase in mtROS bioactivity in cerebrovascular endothelial cells from old rats [196]. These changes in mitochondrial redox state are associated with evidence of increased SIRT1 and reduced mTOR signaling with lifelong CR, supporting CR-associated modulation of nutrient sensing pathways as upstream mechanisms [190]. Additional support for vascular mitochondria modulation by CR comes from work with alternative CR-mimicking pharmaceutical and/or nutraceutical compounds targeting energy sensing pathways [193] (discussed next). There is also growing evidence that at least some of the benefits of CR can be achieved by lifestyle-based CR-mimicking strategies, collectively referred to as intermittent fasting [193]. Whether intermittent fasting is effective for reversing vascular dysfunction in older adults and the potential (mitochondrial) mechanisms involved remain to be determined. Initial observations suggest that time-restricted feeding (a form of intermittent fasting) without weight loss does not improve vascular function in healthy late middle-aged and older men and women. Thus, the mitochondria-specific effects of CR on vascular function have not yet been fully elucidated and the efficacy of lifestyle-based CR-mimicking strategies for age-associated vascular dysfunction remains under investigation.

Pharmaceutical CR-mimetics. CR-mimicking strategies are typically pharmaceutical (synthetic) or nutraceutical (natural) modulators of the energy sensing pathways thought to be

responsible for the beneficial effects of CR. The NAD^+-boosting/sirtuin-activating strategies discussed previously is an example of this approach.

Resveratrol. The naturally occurring polyphenol resveratrol is another putative CR-mimicking approach, which is often attributed to the ability of the compound to activate sirtuins, although this compound is non-specific with several off-target effects. Indeed, chronic resveratrol administration in mice elicits many physiological and molecular changes characteristic of chronic CR in non-vascular tissues including improved insulin sensitivity and mitochondrial biogenesis [197]. Resveratrol also improved endothelial function in middle-aged and old rodents [198, 199], which was associated with increased expression of eNOS and decreased superoxide generation by NADPH oxidase [199]. Moreover, resveratrol prevented the “aging-like” increase in aortic stiffness with a high-fat/high-sugar diet in non-human primates [200]. Incubation with resveratrol of arterial segments from older adults with hypertension and dyslipidemia improved EDD via a mechanism involving activation of eNOS and decreased oxidative stress, accompanied by enhanced mitochondrial antioxidant defense (increased MnSOD expression) [201]. Acute and/or chronic resveratrol supplementation improved endothelial function in healthy estrogen-deficient postmenopausal women [202] and middle-aged and older overweight/obese adults [203-205]. More recently, in glucose-intolerant older adults, 6 weeks of resveratrol supplementation improved reactive hyperemia, which is partly influenced by endothelial function, while also favorably modulating skeletal muscle mitochondria-related gene transcripts associated with mitochondrial dysfunction and oxidative phosphorylation and increasing mitochondrial number [206]. These effects are consistent with the notion of enhanced mitochondrial biogenesis with CR/sirtuin activation and suggest the

vascular benefits of resveratrol might be related, at least in part, to enhanced mitochondrial function, although more research is needed.

AMPK and mTOR. Modulation of other energy sensing pathways via activation of AMPK or inhibition of mTOR have also been shown to affect vascular aging. Sustained activation of AMPK with AICAR, which can stimulate mitochondrial biogenesis in a PGC-1α-dependent manner [207], improves endothelial function by decreasing oxidative stress but without affecting NO bioavailability in old mice [208]. The anti-inflammatory drug salsalate also activates AMPK, which may be one of multiple mechanisms by which this compound improves endothelial function and reduces aortic stiffness in older mice and humans [209-212]. Inhibition of mTOR with rapamycin ameliorates age-related impairments in NO-mediated endothelial function by suppressing oxidative stress and reverses aortic stiffening, which is associated with reductions in collagen abundance [213]. More research is needed to translate these findings to humans and delineate the role of changes in mitochondrial function in response to pharmacological modulation of these energy sensing pathways in the aging vasculature.

Nitrates/nitrites/NO-boosting. Reversing declines in NO bioavailability holds great promise as a strategy for prevention and treatment of vascular dysfunction with aging. NO bioavailability can be enhanced by increasing NO production via upregulation of eNOS activity/expression and/or increasing flux of the nitrate-nitrite-NO (eNOS independent) pathway [214, 215]. Targeting the nitrate-nitrite-NO pathway is considered a more promising and effective approach because of eNOS dysfunction with aging and chronic disease, and the fact that supplementation with inorganic nitrites and nitrates improves endothelial function and arterial stiffness in both pre-clinical models and human subjects, including healthy older adults [36, 215-219]. Although the predominant mechanism for the beneficial effects of nitrates and

nitrites is the serial conversion of these precursor molecules to NO, nitrite and/or nitrate also suppress oxidative stress and inflammation independent of NO [215]. One mechanism by which nitrites and nitrates exert these effects may be via improved mitochondrial function [220]. Indeed, accumulating evidence suggests that restoration of NO signaling induces mitochondrial biogenesis and improves mitochondrial function [84, 85, 130]. In terms of vascular aging, oral sodium nitrite supplementation reverses age-related reductions in EDD in old mice by restoring NO bioavailability, which may be mediated by a suppression of mtROS and other favorable effects on mitochondrial health [221]. However, whether nitrite and/or nitrate improve vascular function by reducing mtROS and/or improving mitochondrial function in humans is currently unknown.

Polyphenols/flavonoids. As described above, mitochondrial dysfunction is a driver of age-associated increases in pro-inflammatory signaling, primarily via excessive production of mtROS, release of mtDNA (i.e., damage-associated molecular patterns) and as an inducer of cellular senescence [222]. Polyphenols/flavonoids are a class of compounds with potent anti-inflammatory properties that may represent a viable strategy for treating vascular dysfunction with aging by enhancing mitochondrial fitness. For example, curcumin, a naturally occurring phenol with anti-inflammatory (and antioxidant) properties found in the Indian spice turmeric, rescues age-related impairments in endothelial function by increasing NO bioavailability and decreasing oxidative stress and normalizing age-associated increases in aortic stiffness [223]. Curcumin supplementation also increases aortic MnSOD expression in old mice [223], suggesting improved mitochondrial antioxidant defenses and decreased mtROS, which are consistent with findings in liver and kidney tissue from curcumin treated diabetic obese mice [224, 225]. Curcumin was recently shown to improve endothelial function in older adults by

increasing NO bioavailability and lowering oxidative stress [226]; however, it is currently unknown if decreased mtROS contributed to the overall decrease in oxidative stress in the curcumin-treated group. Other agents with anti-inflammatory action have also been found to favorably modulate vascular dysfunction in humans with aging and/or age-associated chronic diseases, including TNFa antagonists [227, 228] and IL-1 [229] and NFkB [209-212] inhibitors, but additional research is needed to determine the mitochondrial effects of these and other such compounds.

Emerging Mitochondrial Therapies and Targets to Improve Vascular Function. This section of the review will highlight examples of promising therapies that have not yet been fully explored but hold promise for improving mitochondrial function to promote healthy vascular aging.

Mitochondrial cardiolipin. Cardiolipin is a key inner mitochondrial membrane phospholipid, which plays important roles in a variety of mitochondrial functions including calcium handling, import of proteins into the mitochondria, mitochondrial dynamics and assembly/stabilization of complexes of the electron transport chain [230]. Regarding the latter, cardiolipin is thought to enable a favorable organization of the electron transport chain complexes and the cristae for optimal electron transport chain function (e.g., supercomplex formation), which has been claimed to be associated with lower mtROS production. Cardiolipin is particularly vulnerable to damage due to lipid peroxidation being initiated by mtROS, which decreases function and content of cardiolipin and exacerbates mitochondrial dysfunction including increasing mtROS production. Cardiolipin content decreases with aging [231, 232] and in peripheral blood mononuclear cells is positively correlated with endothelial function in

diabetic and non-diabetic humans [110]. Szeto-Schiller tetra-peptides (e.g., SS-31) are an emerging therapy which have been proposed to act by concentrating at the mitochondrial inner membrane and stabilizing cardiolipin, potentially decreasing mtROS production. These peptides have documented efficacy for improving physiological function in a variety of pre-clinical settings [233]. Regarding vascular aging, SS-31 treatment improved cerebrovascular EDD by increasing NO availability in old mice [154]. SS-31 also normalized mtROS production and restored basal respiration in cerebrovascular endothelial cells from old rats to levels observed in cells from young animals (92). Early phase clinical trials in humans have shown mixed results on various physiological functions, but SS-31 or related compounds targeting cardiolipin have not yet been assessed for improving vascular function in older adults.

***Gut microbiome*.** Unfavorable age-related changes to the gut microbiome promote endothelial dysfunction and arterial stiffening, likely via production of adverse, gut microbiota-derived metabolites such as trimethylamine oxide [234]. Conversely, metabolites derived from microbiota metabolism may also have health-promoting effects, some of which occur via mitochondrial mechanisms. One such metabolite is ellagitannin-derived urolithin A, which activates mitophagy [235]. Urolithin A treatment preserved mitochondrial function with aging and extended lifespan of *C. elegans* and improved skeletal muscle function in rodents [235]. Moreover, acute and chronic administration of the compound appears to be safe and well-tolerated in sedentary older adults and modulates plasma acylcarnitine and skeletal muscle gene expression profiles consistent with effects on mitochondria [236]. More research is needed to determine the physiological effects of urolithin A and other gut microbiome-associated compounds for treating vascular dysfunction with aging.

***Metformin and other pharmaceuticals*.** Many existing pharmaceutical agents have "off-target" mitochondrial effects and show potential for being "repurposed" for treatment of vascular dysfunction. For example, metformin is a first line clinical therapy for lowering blood glucose levels in type 2 diabetes. Metformin reduces CVD risk at least in part through mechanisms independent of its effects on blood glucose, including potentially enhancing vascular function [237]. Indeed, metformin improves endothelial function and reduces arterial stiffness in a number of clinical populations. Although several mechanisms, including anti-inflammatory actions [238], may contribute to the beneficial effects of metformin on vascular function, metformin acts in part by interacting with complex I in mitochondria [239], so improved mitochondrial health/function appears to be important in its mode of action. For example, in diabetic mice, metformin improved endothelial function by modulating dynamin-related protein 1-associated mitochondrial fission and decreasing mtROS in an AMPK-dependent manner [240]. Other pharmaceuticals with efficacy for improving vascular function by mechanisms involving modulation of mitochondrial function include diabetes drugs such as the thiazolidinediones rosiglitazone and pioglitazone [241, 242], which stimulate peroxisome proliferator-activated receptor gamma, and antihypertensive agents including angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers [243].

Interactions Among Mitochondria-Targeted Therapies and Other Interventions. Although therapeutic strategies that decrease mitochondrial oxidative stress and augment mitochondrial health hold promise for enhancing vascular function with aging, it is important to consider how free-living conditions differ from carefully controlled experimental settings. Older adults are commonly prescribed multiple medications, and many, although not enough, engage in various

healthy lifestyle strategies that modulate vascular health. As discussed previously, physiologic levels of mtROS are critical transducers of the signaling events necessary for adaptations to stress, including responses to exercise training [83]. For example, hormetic mtROS levels (i.e., mitohormesis) are now recognized as important signals for exercise training-induced mitochondrial biogenesis, antioxidant enzyme upregulation, immune system responses, insulin responsiveness, growth factor signaling, angiogenesis, and vascular reactivity [83, 244-246]. As such, it is possible that dampening or completely eliminating mtROS via supplementation with mitochondria-targeted compounds might prevent some of these beneficial adaptations. It is therefore important to understand how mitochondria-targeted therapeutic strategies may interact with lifestyle and other pharmacologic and nutraceutical interventions.

There are mixed data regarding the interaction between general antioxidant compounds and exercise training, with some studies finding that supplementation with general antioxidants (e.g., such as vitamins C and E) and compounds with antioxidant effects (e.g., resveratrol) blunt exercise-induced physiological adaptations, and other studies finding no negative interaction between antioxidant supplementation and exercise [83, 247]. In contrast, some nutraceutical approaches such as dietary nitrate supplementation may enhance adaptations to exercise training [248]. Some of the differences among studies have been attributed to variations in dose, duration, and timing of antioxidant administration relative to exercise, supporting the importance of hormetic levels of mtROS for adaptation to exercise. There is limited evidence investigating the interaction between mitochondria-targeted antioxidants and exercise training, but a study by Shill et al. found that MitoQ supplementation in healthy young adults did not alter exercise-training adaptations at the whole-body, skeletal muscle, and circulating angiogenic cell levels [249]. Future work is needed to determine how vascular

adaptations to exercise in the setting of primary aging are influenced by concomitant administration of mitochondria-targeted compounds.

CONCLUSIONS, RESEARCH GAPS AND FUTURE DIRECTIONS

Aging is the major risk factor for CVD due importantly to the development of vascular dysfunction, in particular endothelial dysfunction and large elastic artery stiffening. In this review we have discussed the central role of mitochondrial dysregulation as a key pathophysiological substrate in mediating age-associated vascular dysfunction. We have also reviewed mitochondrial targets of established therapies and novel, emerging treatments directly targeting mitochondrial health/function for preventing and/or reversing age-related vascular dysfunction. There remain several important knowledge gaps in the field; the following represent some potential future, biomedically significant directions for research related to mitochondria and vascular aging (Figure 9).

1) Mitochondrial mechanisms of vascular aging: More research is needed to determine the influence of dysregulation of specific mitochondrial functions with aging on age-associated vascular dysfunction. Additional insight into the mitochondrial mechanisms underlying vascular aging would facilitate the development of new therapies. Notable mechanisms deserving further study include impaired mito-nuclear communication, calcium handling, mitochondrial membrane potential and mitochondrial dysfunction-associated cellular senescence.

2) *mtROS production and signaling*: The identity of the mitochondrial ROS and associated signaling pathways responsible for promoting vascular aging have not been fully elucidated. For example, it remains to be determined if and how superoxide is released into the mitochondrial matrix and/or intermembrane space exits the mitochondria (e.g., via anion

channels) to react with NO in the cell to reduce NO bioavailability. Alternatively, superoxide may not actually exit the mitochondria, but NO may diffuse into the mitochondria and react with superoxide there, leading to a decrease in NO in the rest of the cell. The relative contributions of other mitochondrial ROS (e.g., hydrogen peroxide) to vascular aging (vs. acting as signaling molecules) also remains to be fully characterized.

3) *Mitochondrial mechanisms of established therapies for vascular aging:* There are number of established therapies for healthy vascular aging with documented, beneficial effects on mitochondrial function in other tissues (e.g., skeletal muscle); whether these mitochondrial effects are also observed in the aging vasculature requires confirmation. Encouraging evidence exists for caloric restriction, select dietary patterns, novel modes of exercise training and exercise-inspired lifestyle and pharmacological approaches, but more research is needed to establish the safety and efficacy of these strategies.

4) *Translation of promising mitochondrial therapies:* Pre-clinical and early phase clinical (translational) results support the potential efficacy of a number of promising mitochondria-acting pharmaceutical and nutraceutical approaches (e.g., mitochondria-targeted antioxidants, NAD^+-boosting supplements, autophagy/mitophagy activating compounds). However, larger clinical trials (e.g., phase II and possibly multicenter trials) are needed to confirm these preliminary observations and fully translate these interventions to inform public health/clinical guidelines.

5) *Development of new mitochondria-targeted therapies:* New therapies targeting established “hallmarks” of mitochondrial dysfunction such as impaired stress

resistance/resilience should be developed; promising compounds in development should be assessed in the context of vascular aging (e.g., mitophagy activation with urolithin A).

6) *Extension to clinical populations:* Promising existing strategies for improving mitochondrial health and promoting healthy vascular aging should be translated to other clinical populations characterized by elevated CVD risk (e.g., chronic kidney disease, type II diabetes) or “accelerated” vascular aging (e.g., chemotherapy-treated cancer survivors).

7) *Role of sex differences:* Potential sex differences in responsiveness to both lifestyle and pharmacological strategies must be determined [202, 250-252] and the role of vascular mitochondria in mediating sex differences should be established. Appropriate pre-clinical approaches and experiments and/or large, properly powered clinical trials are needed to accomplish this goal.

8) *Interactions between different prevention and treatment strategies:* It remains to be determined whether there are interactions among mitochondria-targeted therapies and other health promoting medications, supplements, and lifestyle factors, and if such interactions are beneficial or detrimental. Pre-clinical models could provide valuable initial insights, but large clinical studies will ultimately be needed to more fully elucidate these complex, but clinically important, issues in order to promote the public health benefits of these strategies in the most evidence-based and informed manner.

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CONFLICT OF INTEREST

MPM consults for MitoQ Ltd.

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FIGURE LEGENDS

Figure 1. Mechanisms of age-associated vascular dysfunction and related clinical disorders. Aging is associated with mitochondrial dysfunction-induced increases in reactive oxygen species (ROS) and oxidative stress and increases in pro-inflammatory cytokine signaling and chronic low-grade inflammation. Together, these processes induce vascular dysfunction, featuring: (lower left) large elastic artery stiffening mediated by degradation of elastin fibers (blue), increased deposition of collagen (brown), and greater crosslinking of structural proteins by advanced glycation end-products (dashed connecting lines); and (right) vascular endothelial dysfunction characterized by reduced nitric oxide (NO) bioavailability and endothelium dependent dilation. These and other changes to arteries, in turn, increase the risk of developing cardiovascular diseases, chronic kidney disease, and Alzheimer's disease and related dementias.

Figure 2. Mechanisms of age-associated mitochondrial dysfunction. Aging is associated with dysregulated mitochondrial quality control featuring reduced mitochondrial biogenesis (upper left) and reduced mitophagy (upper right), increased mitochondrial fission (upper middle right), reduced mitochondrial fusion (lower middle right), reduced mitochondrial stress resistance (lower right), increased mitochondrial DNA damage (middle left of mitochondria image) and increased bioactivity of mitochondrial reactive oxygen species (e.g., superoxide and other reactive oxygen species [ROS], middle of mitochondria image) relative to antioxidant defenses (e.g., manganese superoxide dismutase [SOD], lower right of mitochondria).

Figure 3. Aging is associated with excess vascular mitochondrial superoxide production and lower mitochondrial superoxide dismutase. Vascular **(A)** mitochondrial superoxide production and **(B)** p66SHC are higher in old (Old Control) relative to young (Young Control) mice. The mitochondrial isoform of superoxide dismutase (SOD), manganese SOD (MnSOD), is lower in **(C)** aorta from old control compared with young control mice and **(D)** arterial endothelial cells from healthy older adult humans relative to young adult controls. Data are mean ± SEM. $^{*}P < 0.05$ YC vs. OC. Data from [111] and [115].

Figure 4. Voluntary aerobic exercise-associated improvement in vascular endothelium dependent dilation is associated with favorable changes in markers of mitochondrial biogenesis, bioenergetics, dynamics, superoxide bioactivity and stress resistance in arteries of old mice. Ten weeks of voluntary aerobic exercise in old mice: restores **(A)** vascular endothelial function and aortic abundance of markers of **(B)** mitochondrial biogenesis (PGC-1α) and **(C)** mitochondrial energy sensing (SIRT3), accompanied by lower abundance of **(D)** a marker of mitochondrial fission (Fis-1) and **(E)** mitochondrial superoxide bioactivity; and increases **(F)** mitochondrial stress resistance (rotenone-induced suppression of endothelial function). Data are mean ± SEM. $^{*}P < 0.05$ Young Control vs. Old Control. Data from [114].

Figure 5. Chronic oral MitoQ supplementation improves vascular endothelial function in both old mice and older adult humans. Oral MitoQ supplementation improves vascular endothelial function in **(A)** old mice and **(B)** healthy older adult humans. Data are mean ± SEM. $^{*}P < 0.05$ Young Control vs. Old Control; $^{\#}P < 0.05$ Old Control vs. Old MitoQ or Older Adult Placebo vs. Older Adult MitoQ. Data from [111] and [118].

Figure 6. Chronic oral MitoQ supplementation improves vascular endothelial function by decreasing mitochondrial oxidative stress-related suppression of endothelial function in both mice and humans. Oral MitoQ supplementation improves vascular endothelial function by reducing mitochondrial oxidative stress-related suppression of EDD as indicated by no further improvement in EDD following **(A)** *ex vivo* treatment of mouse carotid arteries with MitoQ and **(B)** a single supra-therapeutic oral dose of MitoQ in humans. Data are mean ± SEM. $^{*}P < 0.05$ Young Control (- acute MitoQ) vs. OC (- acute MitoQ) or Older Adult Placebo (- acute MitoQ) vs. Older Adult MitoQ (- acute MitoQ); $^{\#}P < 0.05$ Old Control (- acute MitoQ) vs. Old Control (+ acute MitoQ) or Older Adult Placebo (- acute MitoQ) vs. Older Adult Placebo (+ acute MitoQ) . Data from [111] and [118].

Figure 7. Chronic oral MitoQ supplementation reduces arterial stiffness in old mice and in older adult humans with elevated arterial stiffness. Data from our laboratory demonstrating that oral MitoQ supplementation reduces arterial stiffness in **(A)** old mice and **(B)** older healthy humans with normal age-related increases in aortic stiffness (PWV > 7.6 m/s; right-hand panels [148], red shading) but not in young mice or older adults without normal age-related aortic stiffening at baseline (green shading) (left-hand panels). Data are mean ± SEM. $^{*}P < 0.05$ Pre MitoQ Supplementation vs. Post MitoQ Supplementation. Data from [147] and [118].

Figure 8. Oral trehalose supplementation is associated with improvements in vascular endothelium dependent dilation, aortic superoxide bioactivity, arterial stiffness, human

microvascular endothelial function and markers of mitochondrial quality control in the vasculature of old mice. Four weeks of oral trehalose supplementation results in **(A)** improved vascular endothelium dependent dilation, **(B)** decreased aortic superoxide bioactivity, and **(C)** reduced age-related arterial stiffness in old mice, **(D)** improved microvascular function in humans, and changes in markers of mitophagy Parkin **(E)** and BNIP **(F)**, **(G)** mitochondrial bioenergetics (SIRT3), and **(H)** mitochondrial oxidative stress (phosphorylated p66SHC) in vascular tissue from mice indicative of enhanced mitochondrial fitness. Data are mean ± SEM. $^{*}P < 0.05$ Young Control vs. Old Control or Baseline vs. 12 weeks of trehalose supplementation. Data from [155], [113] and [157].

Figure 9. Research gaps and future directions. NAD, Nicotinamide Adenine Dinucleotide; NO, Nitric Oxide; RCTs, Randomized Controlled Trials.

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Figure 1.

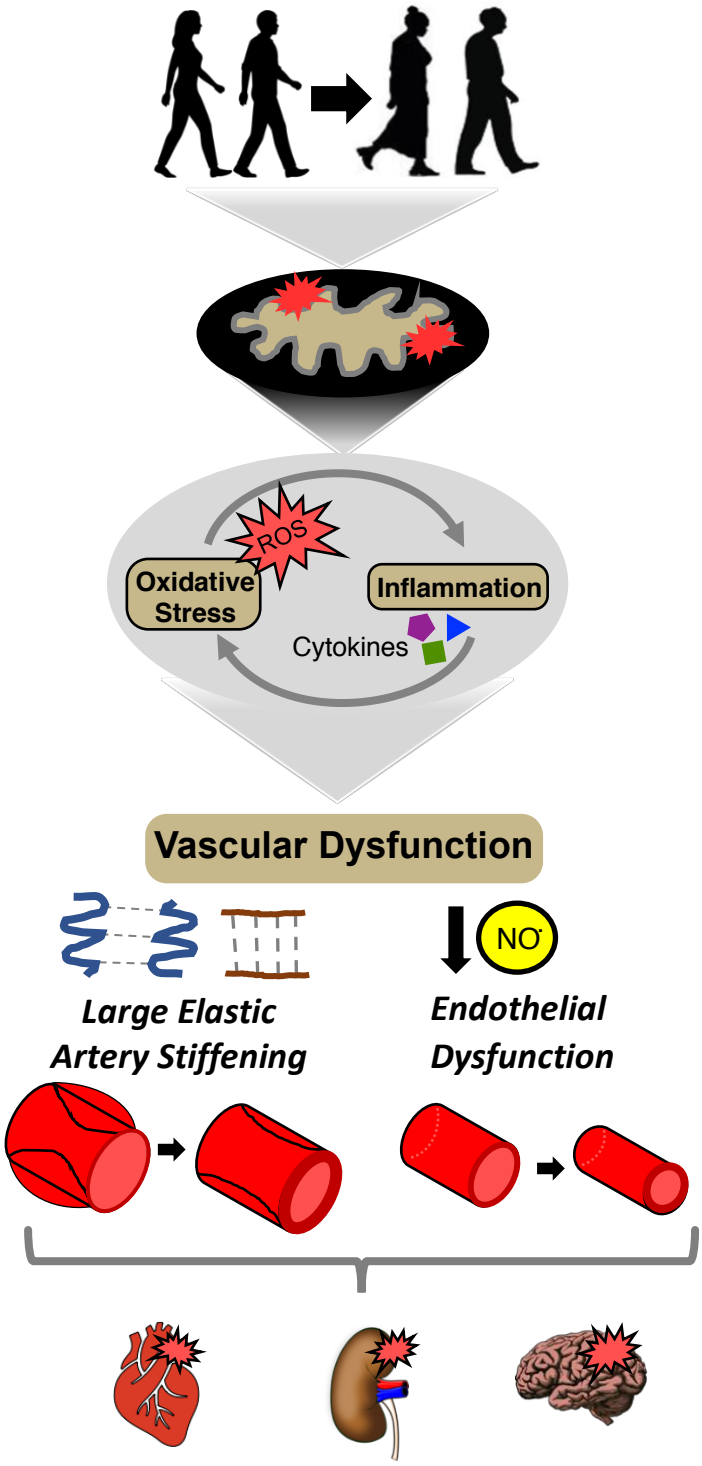

ROS
Oxidative Stress
Inflammation
Cytokines
Vascular Dysfunction
NO
Large Elastic Artery Stiffening
Endothelial Dysfunction

Figure 2.

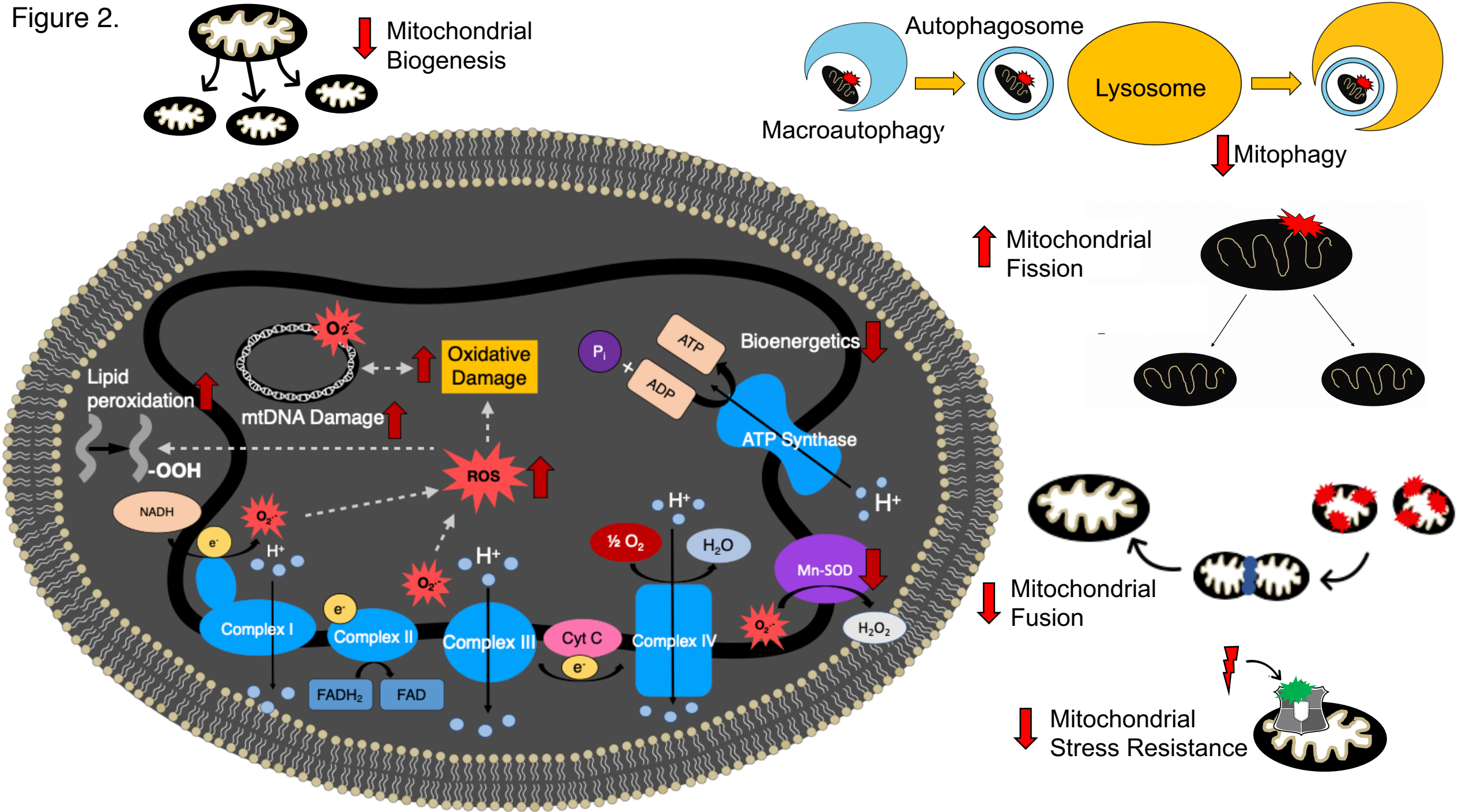

Mitochondrial Biogenesis
Autophagosome
Lysosome
Macroautophagy
Mitophagy
Mitochondrial Fission
Mitochondrial Fusion
Mitochondrial Stress Resistance
Lipid peroxidation
-OOH
mtDNA Damage
Oxidative Damage
ROS
NADH
Complex I
Complex II
Complex III
Cyt C
Complex IV
FADH2
FAD
Pi
ATP
ADP
Bioenergetics
ATP Synthase
½ O2
H2O
Mn-SOD
H2O2
H+
e-

Figure 3.

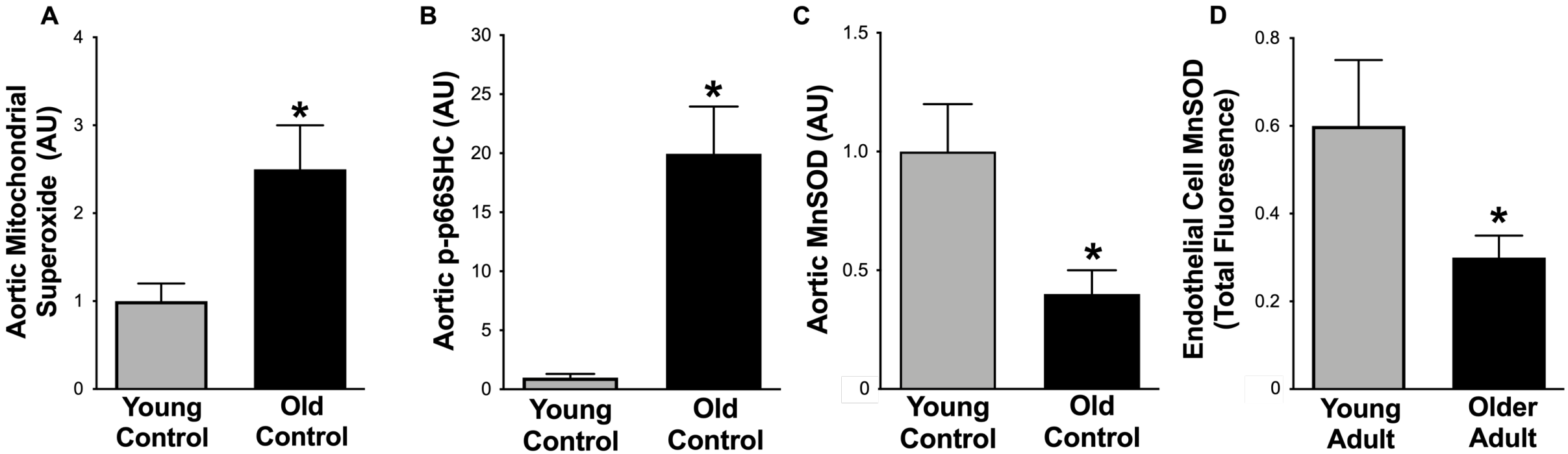

A
Aortic Mitochondrial Superoxide (AU)
4
3
2
1
0
*
Young Control
Old Control
B
Aortic p-p66SHC (AU)
30
25
20
15
10
5
0
*
Young Control
Old Control
C
Aortic MnSOD (AU)
1.5
1.0
0.5
0
*
Young Control
Old Control
D
Endothelial Cell MnSOD (Total Fluoresence)
0.8
0.6
0.4
0.2
0
*
Young Adult
Older Adult

Figure 4.

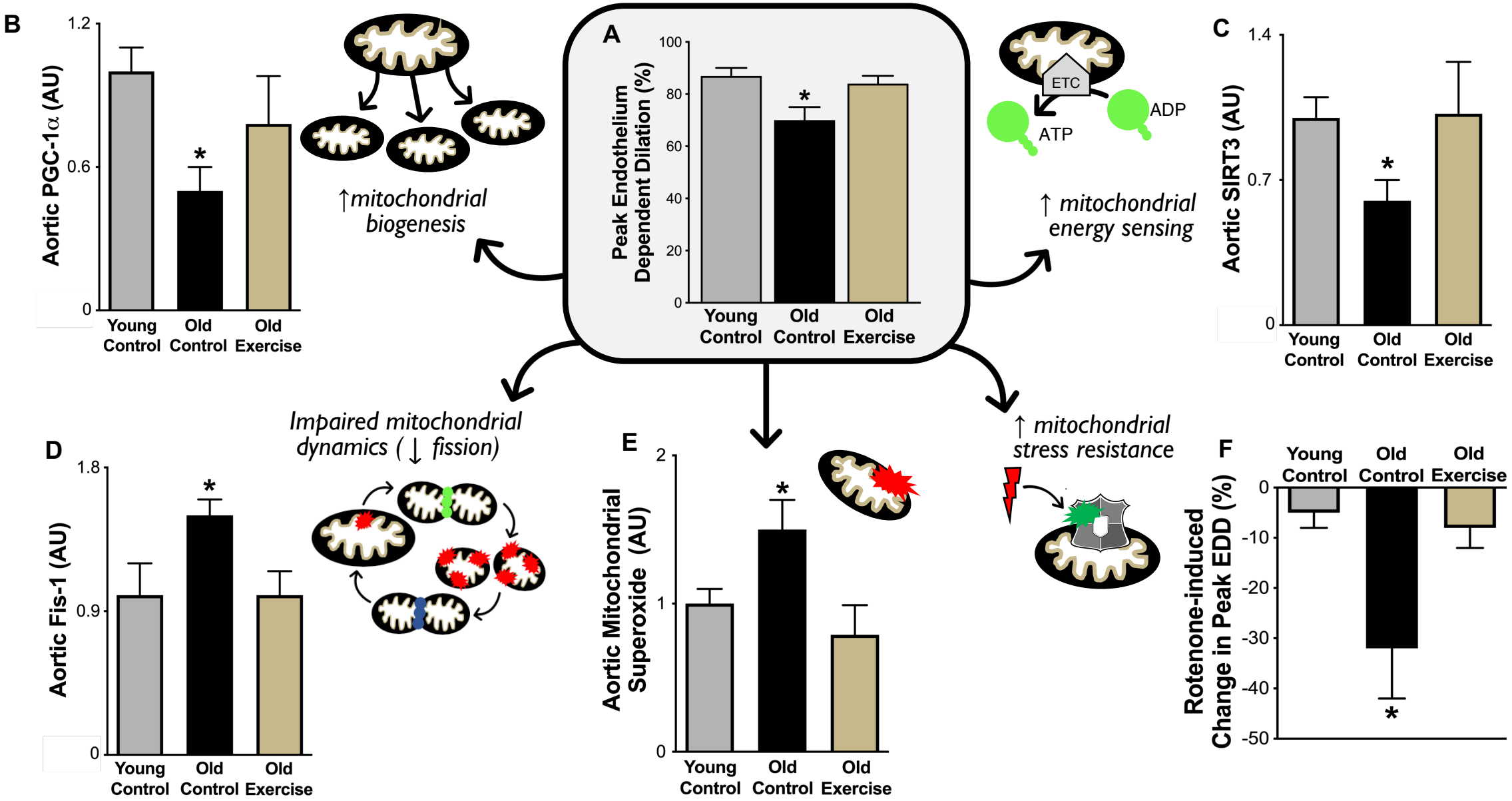
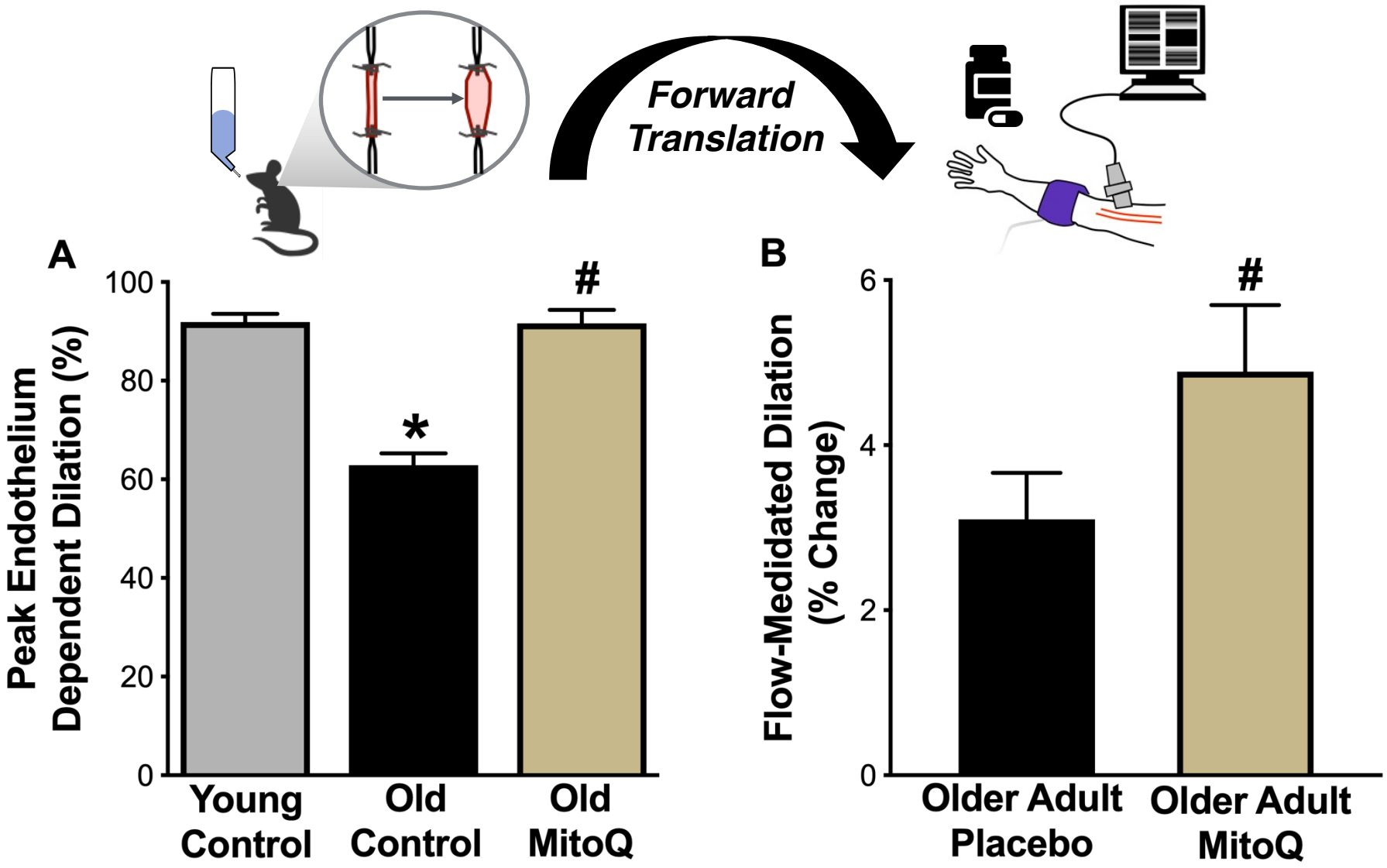

A
Peak Endothelium Dependent Dilation (%)
100
80
60
40
20
0
Young Control
Old Control
Old Exercise
B
Aortic PGC-1α (AU)
1.2
0.6
0
Young Control
Old Control
Old Exercise
↑mitochondrial biogenesis
ETC
ADP
ATP
↑ mitochondrial energy sensing
C
Aortic SIRT3 (AU)
1.4
0.7
0
Young Control
Old Control
Old Exercise
Impaired mitochondrial dynamics (↓ fission)
D
Aortic Fis-1 (AU)
1.8
0.9
0
Young Control
Old Control
Old Exercise
E
Aortic Mitochondrial Superoxide (AU)
2
1
0
Young Control
Old Control
Old Exercise
↑ mitochondrial stress resistance
F
Rotenone-induced Change in Peak EDD (%)
0
-10
-20
-30
-40
-50
Young Control
Old Control
Old Exercise

Figure 5.



Gioscia-Ryan et al., 2014. *J Physiol.*

Rossman et al., 2018. *Hypertension*

Figure 6.

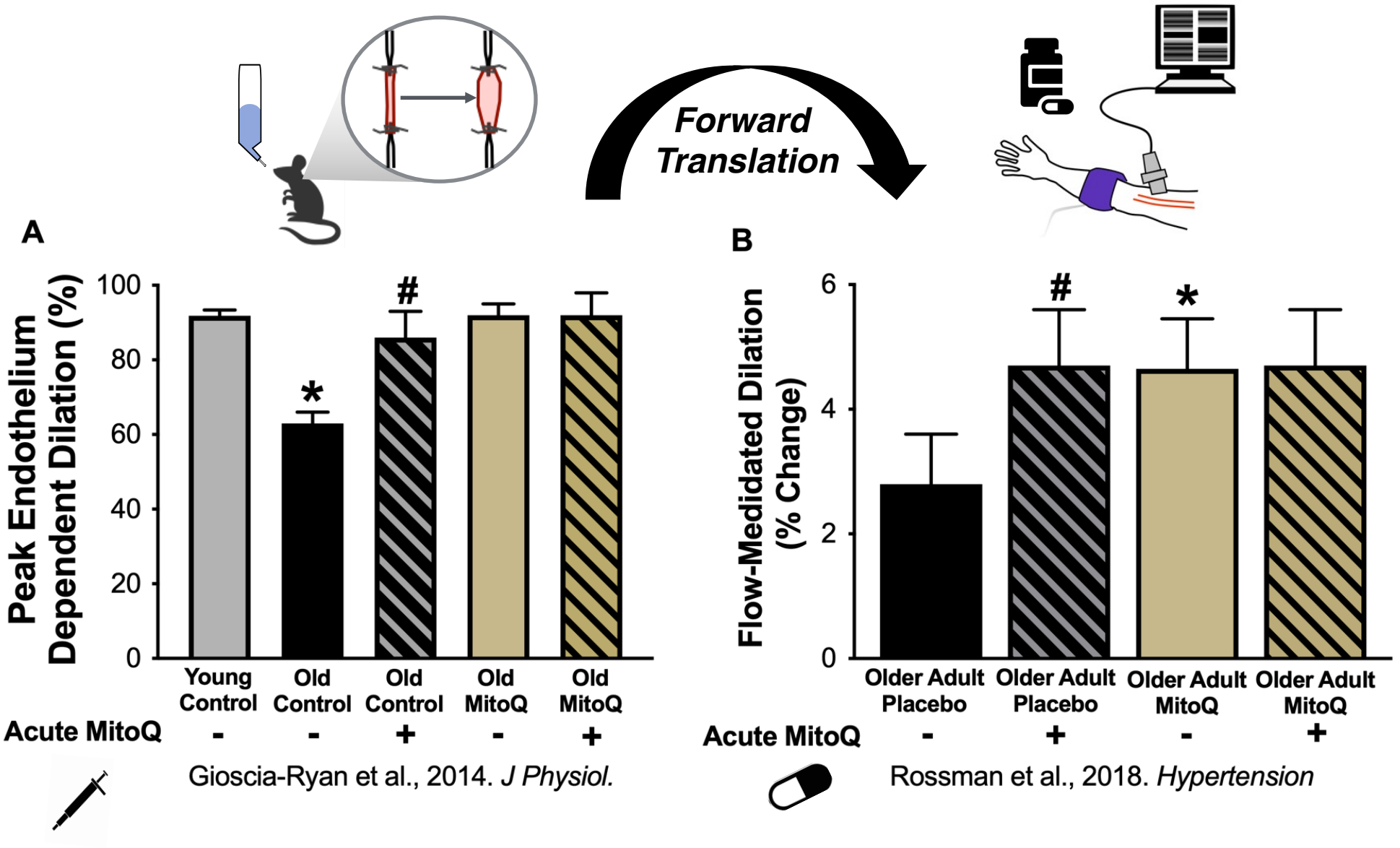
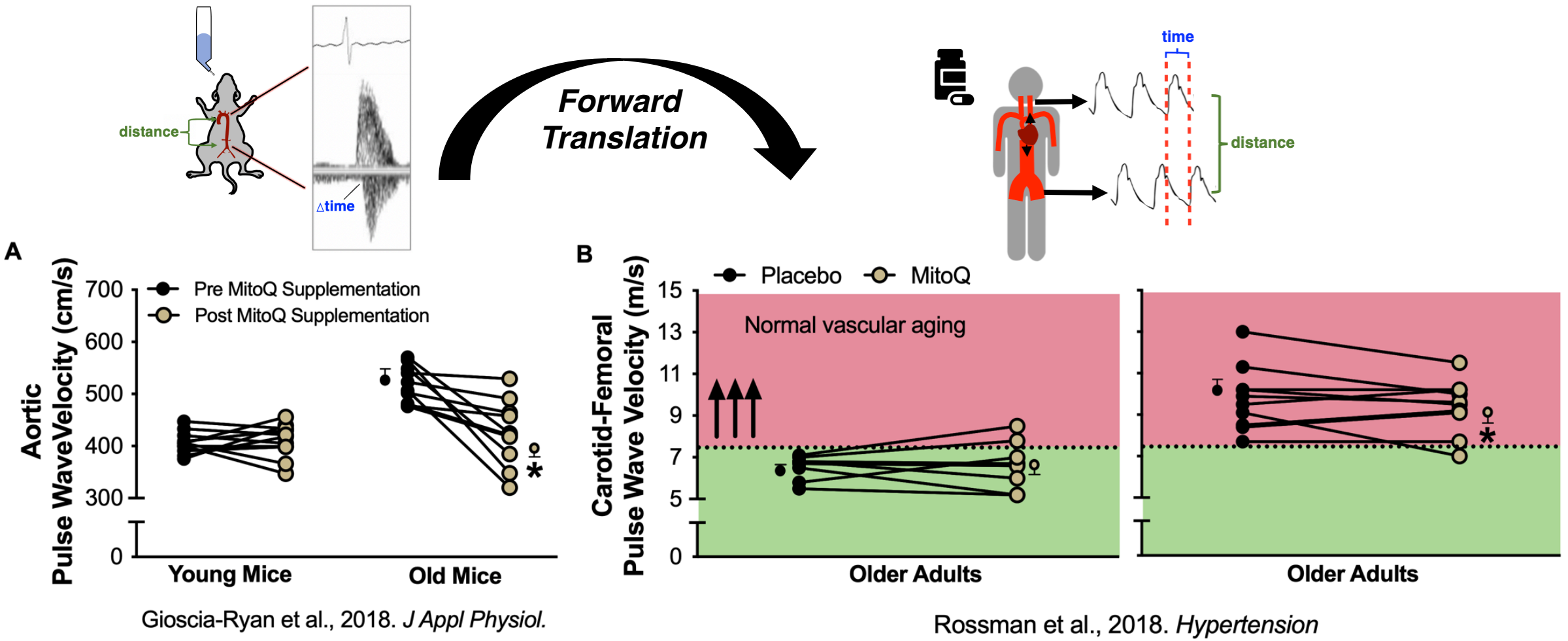

Forward Translation
A
Peak Endothelium Dependent Dilation (%)
100
80
60
40
20
0
*
#
Young Control
Old Control
Old Control
Old MitoQ
Old MitoQ
Acute MitoQ
-
-
+
-
+
Gioscia-Ryan et al., 2014. J Physiol.
B
Flow-Mediated Dilation (% Change)
6
4
2
0
#
*
Older Adult Placebo
Older Adult Placebo
Older Adult MitoQ
Older Adult MitoQ
Acute MitoQ
-
+
-
+
Rossman et al., 2018. Hypertension

Figure 7.



Gioscia-Ryan et al., 2018. *J Appl Physiol.*

Rossman et al., 2018. *Hypertension*

Figure 8.

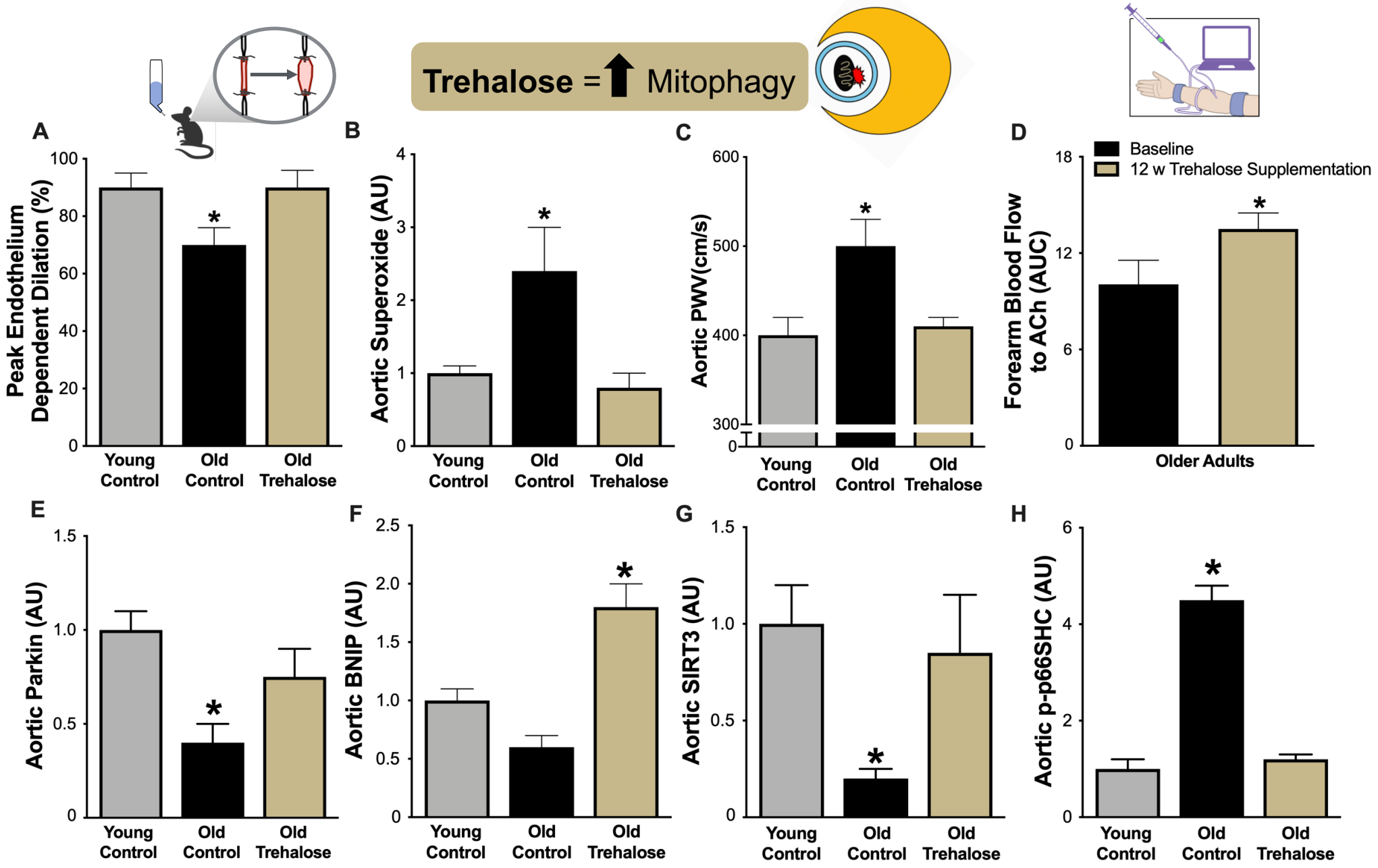
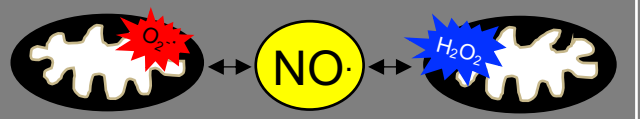
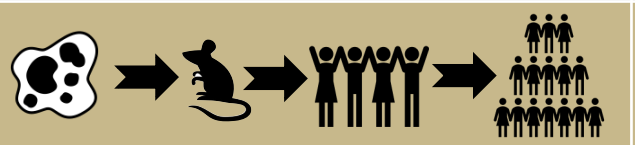
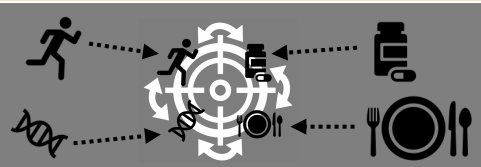

Trehalose = Mitophagy
A
Peak Endothelium Dependent Dilation (%)
100
80
60
40
20
0
Young Control
Old Control
Old Trehalose
B
Aortic Superoxide (AU)
4
3
2
1
0
C
Aortic PWV(cm/s)
600
500
400
300
0
D
Baseline
12 w Trehalose Supplementation
Forearm Blood Flow to ACh (AUC)
18
12
6
0
Older Adults
E
Aortic Parkin (AU)
1.5
1.0
0.5
0
F
Aortic BNIP (AU)
2.5
2.0
1.5
1.0
0.5
0
G
Aortic SIRT3 (AU)
1.5
1.0
0.5
0
H
Aortic p-p66SHC (AU)
6
4
2
0

Figure 9.

Issue	Research Gap	Future Direction
	The influence of several "non-ROS" mitochondrial cellular functions on vascular aging	Determine the roles of dysregulated mitochondrial-nuclear communication, calcium handling, mitochondrial membrane potential, and mitochondrial modulation of cellular senescence on vascular aging
	The specific mitochondrial ROS-related signaling pathways responsible for vascular aging	Determine the interactions between mitochondrial superoxide and NO, other mitochondrial derived ROS (e.g. H_2O_2) and vascular aging
	The role of improved mitochondrial function in mediating the beneficial effects of strategies that promote healthy vascular aging	Determine the role of increased mitochondrial fitness in the effects of caloric restriction, healthy dietary patterns, aerobic exercise and pharmaceutical/nutraceutical interventions on vascular aging
	Translation of promising mitochondrial targeted therapies for healthy vascular aging to humans	Clinical trials to determine the effects of novel mitochondrial peptides, mitophagy activators and other emerging mitochondrial-modulating lifestyle and pharmacological therapies on vascular aging
	Translation of strategies for improving mitochondrial function and promoting healthy vascular aging to patient populations with elevated CVD risk	Clinical trials to determine the effects of mitochondrial-targeted interventions on vascular function in adults with traditional CVD risk factors (e.g. type 2 diabetes) and patients at high risk of CVD (e.g. chemotherapy-treated cancer survivors; patients with chronic kidney disease)
	Potential sex differences in responsiveness to mitochondrial-targeted interventions for improving vascular aging	Preclinical studies and/or clinical trials to determine efficacy of mitochondrial health-enhancing strategies on vascular aging in females vs. males, including estrogen-deficient postmenopausal women
	Interactions (synergistic or detrimental) between lifestyle and pharmacological mitochondrial-targeted therapies for improving vascular aging	Preclinical studies and/or clinical trials to determine the interactive effects of specific mitochondrial fitness-promoting interventions for enhancing vascular aging (e.g. MitoQ + aerobic exercise)