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## Life-Long Caloric Restriction Reduces Oxidative Stress and Preserves Nitric Oxide Bioavailability and Function in Arteries of Old Mice

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### Abstract

Aging impairs arterial function through oxidative stress and diminished nitric oxide (NO) bioavailability. Life-long caloric restriction (CR) reduces oxidative stress, but its impact on arterial aging is incompletely understood. We tested the hypothesis that life-long CR attenuates key features of arterial aging. Blood pressure, pulse wave velocity (PWV) (arterial stiffness), carotid artery wall thickness and endothelium-dependent dilation (EDD) (endothelial function) were assessed in young (Y: 5–7 mo), old ad libitum (Old AL: 30–31 mo.) and life-long 40% CR old (30–31 mo.) B6D2F1 mice. Blood pressure was elevated with aging ( $P < 0.05$ ) and was blunted by CR ( $P < 0.05$  vs. Old AL). PWV was 27% greater in old vs. young AL fed mice ( $P < 0.05$ ), and CR prevented this increase ( $P < 0.05$  vs. Old AL). Carotid wall thickness was greater with age ( $P < 0.05$ ), and CR reduced this by 30%. CR effects were associated with amelioration of age-related changes in aortic collagen and elastin. Nitrotyrosine, a marker of cellular oxidative stress, and superoxide production was greater in old AL vs. young ( $P < 0.05$ ) and CR attenuated this increase. Carotid artery EDD was impaired with age ( $P < 0.05$ ); CR prevented this by enhancing NO and reducing superoxide-dependent suppression of EDD (Both  $P < 0.05$  vs. Old AL). This was associated with a smaller age-related increase in NADPH oxidase activity and p67 expression, with increases in superoxide dismutase (SOD), total SOD and catalase activities (All  $P < 0.05$  Old CR vs. Old AL). Lastly, CR normalized age-related changes in the critical nutrient sensing pathways SIRT-1 and mTOR ( $P < 0.05$  vs. Old AL). Our findings demonstrate that CR is an effective strategy for attenuation of arterial aging.

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#### Author Contributions

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#### Conflict of Interest / Disclosures

We have no disclosures or conflict of interest

## Keywords

Aging; Arteries; Oxidative Stress; Calorie Restriction

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## Introduction

Older age is a major risk factor for development of cardiovascular disease (CVD) (Lakatta 2003). Aging appears to exert its pathological influence primarily via adverse functional and structural effects on arteries, including the large elastic arteries (aorta and carotid arteries) (Lakatta 2003). In particular, aging is associated with elevated systolic blood pressure, large elastic artery stiffening, arterial wall hypertrophy and impaired endothelial function in the absence of clinical disease (Tanaka *et al.* 2000; Lakatta 2003; Seals *et al.* 2011), all of which are predictors of incident CV events and key features of a phenotype coined “arterial aging” (Lakatta 2003; Widlansky *et al.* 2003; Mitchell *et al.* 2010). The mechanisms of arterial aging are incompletely understood, but reduced nitric oxide (NO) bioavailability secondary to enhanced arterial oxidative stress is believed to play an important role (Taddei *et al.* 2001; Spier *et al.* 2004; Brandes *et al.* 2005; Seals *et al.* 2011).

Age-related arterial oxidative stress is associated with increased production of reactive oxygen species (ROS), including superoxide anion (Sun *et al.* 2004; Rippe *et al.* 2010). This is, in turn, at least partly mediated by increases in expression and activity of the oxidant enzyme NADPH oxidase (Hamilton *et al.* 2002; Donato *et al.* 2007a; Durrant *et al.* 2009), in the face of either lower or unchanged antioxidant enzymes (Donato *et al.* 2007a; Durrant *et al.* 2009; Trott *et al.* 2009). Reduced NO bioavailability with aging is caused primarily by superoxide reacting with NO to form peroxynitrite, leading to elevated nitration of tyrosine residues on arterial proteins (nitrotyrosine) (Donato *et al.* 2007a; Durrant *et al.* 2009; Lesniewski *et al.* 2009). Collectively, such oxidative stress-driven biochemical events lead to arterial dysfunction (Moncada *et al.* 1991; Soucy *et al.* 2006; Seals *et al.* 2011; Fleenor *et al.* 2012).

Life-long caloric restriction (CR), defined as a reduction in energy intake (typically 40% less than ad libitum) without malnutrition, is associated with enhanced physiological function in advanced age in several mammalian species (*Rattus norvegicus*, *Mus musculus*, and *Macaca mulatta*) (Weindruch & Sohal 1997; Masoro 2005). The beneficial physiological effects of calorie restriction are thought to be mediated through nutrient-sensing pathways such as SIRT-1 and mTOR (Fontana *et al.* 2010). Importantly, life-long caloric restriction is associated with dramatically attenuated development of CVD in non-human primates (Colman *et al.* 2009). We and others have shown that life-long caloric restriction provides cardioprotection during ischemia (Edwards *et al.* 2010) and improves endothelial vasodilatory function (Csiszar *et al.* 2009), respectively, in old rodents. However, despite these promising observations regarding potential benefits on cardiovascular aging, effects of life-long caloric restriction on the interactions between blood pressure, large artery function wall thickness, and nitric oxide bioavailability are unknown.

Utilizing our well-established mouse model of age-related arterial dysfunction (Donato *et al.* 2009; Durrant *et al.* 2009; Lesniewski *et al.* 2009; Fleenor *et al.* 2010; Lesniewski *et al.* 2011a), we hypothesized that life-long caloric restriction would prevent vascular aging and associated age-related increases in blood pressure. We further postulated that this protective effect would be associated with greater NO bioavailability and reduced superoxide-related oxidative stress. The latter, would, in turn, be associated with suppression of age-related increases in NADPH oxidase expression, along with enhanced arterial SOD. Finally, we

hypothesized that arterial expression and activity of two key nutrient-sensing pathways, SIRT-1 and mTOR, would be preserved with life-long caloric restriction.

## Results

### Animal Characteristics

Body mass and soleus muscle mass were not different with aging, but were lower in the old CR vs. AL mice ( $P < 0.05$ , Table 1). Heart mass was greater in the old AL vs. young AL and old CR mice ( $P < 0.05$ ), but heart mass normalized to total body mass actually was greatest in the old CR group ( $P < 0.05$ ). Soleus muscle mass was lower in the old CR mice vs. other two groups ( $P < 0.05$ ), but greater in the CR animals when normalized to body mass ( $P < 0.05$ ). Old AL animals had greater absolute and relative (to body mass) epididymal white adipose tissue mass vs. young AL mice ( $P < 0.05$ ). Old CR mice had lower absolute epididymal adipose tissue mass vs. the other two groups, and lower relative adipose mass vs. old AL controls (Table 1). Total plasma cholesterol was not affected by aging or life-long CR (Table 1). In contrast, plasma triglycerides were greater with age in the AL animals, but markedly lower in the old CR group (Table 1). Spontaneous cage activity was not different with aging in ad libitum fed mice ( $P > 0.05$ ), but was greater in old CR vs. young and old AL animals (both  $P < 0.05$ , Table 1). Soleus muscle citrate synthase activity was reduced with aging in the ad libitum fed animals ( $P < 0.05$ ), but was not different in old CR mice compared with the other 2 groups (both  $P > 0.05$ ) (Table 1).

### Arterial Blood Pressure and Large Elastic Artery Stiffness and Wall Thickness

Systolic and diastolic blood pressure increased by ~20 mmHg with age in AL mice ( $P < 0.05$ ), which was largely prevented by life-long CR (Figure 1 A & B). Heart rate did not differ significantly among groups (Young:  $699 \pm 10$ , Old AL:  $714 \pm 18$ , Old CR:  $690 \pm 21$  beats/min; all  $P > 0.05$ ). Aortic pulse wave velocity (PWV) was 27% higher in old compared with young AL mice ( $P < 0.05$ ), and this age-related increase was completely prevented by life-long CR (Figure 1 C). Carotid artery medial wall cross-sectional area increased markedly with age in AL mice ( $P < 0.05$ ), and this wall thickening was attenuated by ~50% with aging in mice undergoing life-long CR (Figure 1 D).

### Large Elastic Artery Structural Components

Aging resulted in a 167% increase in total aortic collagen content in AL mice ( $P < 0.05$ ), whereas this increase was entirely prevented in old CR animals (Figure 2 A). Results were similar for the medial (Young:  $18 \pm 5$ , Old AL:  $75 \pm 12$ , Old CR:  $25 \pm 7$ , % total positive area) and adventitial (Young:  $39 \pm 10$ , Old AL:  $73 \pm 8$ , Old CR:  $21 \pm 6$ , % total positive area) layers. Protein expression of precursor collagen I, a key modulator of total arterial collagen, was elevated by 190% in old AL compared with young AL controls (Figure 2 B), and this was completely prevented by life-long CR (Figure 2 B).

Total aortic elastin content declined modestly with aging in the AL mice, but was preserved in life-long CR mice (Figure 2 C). These differences were the same as those in the media alone as elastin is highly localized in the medial layer (see representative images Figure 2 C). Aging resulted in a 290% increase in aortic matrix metalloproteinase -9, a protease that breaks down elastin, and the majority of this increase was prevented by life-long CR (Figure 2 D).

### Large Elastic Artery Endothelial Function, NO Bioavailability and eNOS

Endothelium-dependent dilation (EDD) to ACh was impaired in isolated carotid arteries of old compared with young AL mice (maximal and dose response,  $P < 0.05$ ; Fig 3 A), whereas sensitivity to ACh ( $IC_{50}$ ) was not different ( $P > 0.05$ ). Life-long CR prevented the age-related

reduction in EDD for the entire dose response, although maximal dilation to ACh was slightly lower in the old CR vs. young AL mice ( $P<0.05$ ). Incubation with  $N^G$ -nitro-L-arginine methyl ester (L-NAME) reduced maximal EDD in the carotid arteries of all groups (All  $P<0.05$  vs. ACh alone; Figure 3 A) and abolished group differences (All  $P>0.05$ ; Figure 3 A). Life-long CR restored the age-related reduction in the NO component of EDD in old mice to that of young animals (Figure 3 B). Aortic eNOS protein expression was not different in young and old AL mice ( $P>0.05$ ; Figure 3 C), whereas life-long caloric restriction increased eNOS protein by 85% compared with AL mice ( $P<0.05$ , Figure 3 C). There were no differences in carotid artery dilation (All  $P>0.60$ ) or sensitivity (All  $P>0.4$ ) to the endothelium-independent dilator sodium nitroprusside (SNP) among the groups (maximal dilation to SNP, Young:  $98 \pm 2$ , Old AL:  $99 \pm 1$ , Old CR:  $99 \pm 1$  % dilation).

### Arterial Oxidative Stress and Superoxide Suppression of EDD and NO Bioavailability

Aortas from old AL mice had greater nitrotyrosine content, a cellular marker of oxidative stress, compared with young AL ( $P<0.05$ ), which was blunted by life-long CR (25kd band, Figure 3 D; 55kd band Supplemental Figure 1 C). Incubation with the superoxide scavenger, TEMPOL, restored carotid artery EDD ( $P<0.05$ ) in old AL mice, but had no effect in young AL or old CR mice (Figure 4 A). TEMPOL administration selectively increased EDD in carotid arteries from old AL mice in a NO-dependent manner ( $P<0.05$ ), whereas TEMPOL did not alter EDD or NO mediated EDD in young AL or old CR mice ( $P>0.05$ ; Figure 4 A; Supplemental Figure 1 A & B). Superoxide production was 10-fold higher in aortic segments from old compared with young AL mice ( $P<0.05$ ), whereas old CR animals had values similar to the young AL group (Figure 4 B;  $P>0.05$ ).

### Enzymatic Oxidant and Antioxidant Expression and Activity

Old AL mice demonstrated greater aortic NADPH oxidase activity and expression of the p67 subunit of NADPH oxidase compared with young AL animals (Figure 4 C and D,  $P<0.05$ ) and these age-associated changes were prevented by CR (Figure 4 C and D,  $P<0.05$ ).

Protein expression of CuZn, Mn and EC SOD isoforms, and catalase, as well as total SOD and catalase enzyme activities did not differ in the young and old AL mice ( $P>0.05$ , Figure 5). Life-long CR induced increases ( $P<0.05$ ) in CuZnSOD, MnSOD and ECSOD expression, and increased total SOD and catalase enzyme activities compared with the AL groups.

### Arterial SIRT-1 and mTOR

Aortic SIRT-1 protein was reduced with aging in AL mice ( $P<0.05$ ), but expression was completely preserved in life-long CR mice (Figure 6 A). The activity of the SIRT-1 enzyme, a protein deacetylase, was assessed by the amount of acetylated P53 (note that changes are opposite in direction to activity). Aortic expression of acetylated P53 was increased (SIRT-1 activity was decreased) with aging in AL mice ( $P<0.05$ ; Figure 6 B), and this was prevented by life-long CR.

Old AL mice tended to have higher total aortic mTOR protein expression ( $P=0.10$ ; Figure 6 C) and old CR mice had a similar age-related increase, reaching significance compared with young AL mice ( $P<0.05$ ). In contrast, phosphorylated mTOR (serine 2448) was increased by 93% with aging AL mice ( $P<0.05$ ; Figure 6 D) and this was prevented by life-long CR. The ratio of phosphorylated to total mTOR, used as a measure of mTOR activity, tended to be greater with aging in AL mice ( $P=0.06$ ; Figure 6 E), whereas life-long CR was associated with markedly lower aortic mTOR activation compared with old AL mice, and somewhat lower than young AL animals (Both  $P<0.05$ ).

## DISCUSSION

Our results demonstrate that life-long CR can prevent or significantly lessen multiple adverse features of arterial aging as well as age-related increases in arterial blood pressure. Our findings also provide broad insight into the mechanisms underlying the vasoprotective effects of life-long CR, which include preservation of major structural proteins (collagen and elastin), NO bioavailability, superoxide production, and expression and activity of the oxidant enzyme NADPH oxidase at young adult-like levels, as well as enhancement of key antioxidant enzymes. Finally, our data show that arterial aging is associated with potentially deleterious changes in expression and/or activity of the key cellular nutrient sensing pathways SIRT-1 and mTOR, effects that are ameliorated by life-long CR.

### Arterial Blood Pressure, Large Elastic Artery Stiffness, and Wall Thickening

Our results demonstrate for the first time in combination that life-long CR largely prevents age-related increases in systolic and diastolic arterial blood pressure, completely prevents aortic stiffening and significantly reduces large elastic artery wall hypertrophy. These findings are biomedically important in that these characteristics of cardiovascular aging change subclinically with aging in humans and are independent predictors of future CV events and clinical CVD (Lakatta & Levy 2003). Our findings are consistent with previous reports from both intervention and cross-sectional studies in humans that shorter-term energy-restriction and reduced body weight are associated with lower blood pressure and carotid wall thickness (Walford *et al.* 1992; Fontana *et al.* 2004). In contrast, the recent CR clinical trial (CALERIE) has not shown significant changes in blood pressure after 6 months of 25% CR in non-obese human subjects (Lefevre *et al.* 2009). Our results extend these prior observations by showing that life-long energy restriction can partially or totally prevent the increases in stiffening of large elastic arteries that occurs with adult aging.

We cannot discern the temporal sequence of these cardiovascular-protective effects of life-long CR in the present study. It is believed that large elastic artery stiffening contributes to age-associated increases in systolic blood pressure, which, in turn, stimulates compensatory wall thickening to normalize wall stress (Najjar *et al.* 2005). However, these interactions are complex and many other factors (structural, autonomic, hemodynamic, hormonal, etc.) are involved in determining arterial pressure and properties of large elastic arteries with aging. Such influences likely explain the variance in the relative changes observed among these outcomes in response to life-long CR in our study.

### Structural Proteins

Large elastic artery stiffening with aging is thought to be mediated primarily by changes in the major structural proteins involving increases in collagen and fragmentation of elastin (Fornieri *et al.* 1992; Nosaka *et al.* 2003; Wang *et al.* 2007; Fleenor *et al.* 2010). In the present investigation, the absence of aortic stiffening with life-long CR was associated with a lack of accumulation of collagen and maintenance of elastin in the aorta, as reported previously (Fornieri *et al.* 1999). Our findings further suggest that the inhibition of collagen accumulation by life-long CR may be mediated, at least in part, by suppression of collagen production, as indicated by the absence of increases in precursor collagen I in our old CR mice. Moreover, the preservation of aortic elastin with life-long CR in the present study was associated with inhibition of age-related increases in MMP-9, an elastin-degrading enzyme, suggesting the possible importance of CR-induced suppression of that arterial elastase. Thus, our findings suggest that CR likely prevents age-related increases in aortic stiffness by suppressing collagen production and elastin degradation.



## NO-Dependent Endothelial Function

Endothelial dysfunction develops with aging as indicated by impaired EDD in response to chemical (ACh) and mechanical (flow) stimulation as a result of reduced NO bioavailability (Celermajer *et al.* 1992; Taddei *et al.* 2001; Spier *et al.* 2004; Lesniewski *et al.* 2009). The cause and effect relation between reduced NO bioavailability and the major constitutive NO producing enzyme, eNOS, is less clear, as protein expression and activation of eNOS has been reported to be augmented, attenuated and similar eNOS in arteries from rodents and endothelial cells from humans (Cernadas *et al.* 1998; van der Loo *et al.* 2000; Sun *et al.* 2004; Donato *et al.* 2009; Durrant *et al.* 2009; Trott *et al.* 2009).

It has been reported that life-long CR is associated with preserved EDD to ACh in the rat aorta (Csiszar *et al.* 2009). Our results extend these findings by showing that the preservation of large artery endothelial function in old CR mice is mediated by maintained NO bioavailability, and that these events are associated with increases in the expression of the eNOS, consistent with the effects of other vasoprotective lifestyle interventions in aged animals such as exercise training (Spier *et al.* 2004; Durrant *et al.* 2009). Our observations regarding NO likely have important clinical implications given that NO bioavailability is a critical component of a healthy endothelial phenotype (Forstermann & Munzel 2006). Moreover, NO is anti-proliferative (Garg & Hassid 1989) and modulates large elastic artery stiffness (Fitch *et al.* 2001) and structural proteins (Myers & Tanner 1998), suggesting a possible role for this mechanism in several of the anti-vascular aging effects of life-long CR observed in the present study.

## Endothelial Function and Oxidative Stress

Endothelial dysfunction with aging is mediated by excessive superoxide-dependent oxidative stress (van der Loo *et al.* 2000; Taddei *et al.* 2001; Csiszar *et al.* 2002; Donato *et al.* 2007a). Consistent with this, acute administration of superoxide anion scavengers such as ascorbic acid or TEMPOL improve EDD and NO bioavailability with aging (Taddei *et al.* 2001; Sun *et al.* 2004; Durrant *et al.* 2009). A previous study in aortic rings demonstrated that TEMPOL restores EDD in old AL, but not life-long CR rats (Csiszar *et al.* 2009). Here we extend these findings by showing that life-long CR preserves NO-mediated large artery endothelial function with aging by suppressing superoxide-associated vascular oxidative stress. The latter is supported by several lines of evidence, including functional data using TEMPOL to scavenge superoxide and L-NAME to inhibit NO production, and biochemical analyses showing that aortic nitrotyrosine, a marker of cellular oxidative stress, and superoxide production, measured by the gold standard approach of electron paramagnetic resonance (EPR) spectroscopy, were markedly lower in old CR compared with AL animals, and similar to young AL mice.

Our results also provide novel insight into the cellular mechanisms that may contribute to life-long CR inhibition of oxidative stress with arterial aging. In the present study, CR increased protein expression and activity of SOD, a critical enzymatic antioxidant that protects NO as it diffuses from the endothelium to vascular smooth muscle cells (Jung *et al.* 2003). Our results are consistent with the effects of life-long CR on SOD expression in other tissue types such as skeletal muscle (Sreekumar *et al.* 2002). We also show here for the first time that life-long CR results in increased aortic activity of catalase, a key antioxidant enzyme that removes hydrogen peroxide, without an increase in protein expression, indicating a post-translational modification of the enzyme resulting in enhanced activity. This is similar to a previous report of increased catalase activity with CR non-vascular tissues (Mote *et al.* 1991). In addition to boosting antioxidant enzymes, our data indicate that life-long CR inhibits aortic expression of the major superoxide-producing oxidant enzyme in arteries, NADPH oxidase. Activation of this enzyme is broadly implicated in arterial aging

(Hamilton *et al.* 2002; Durrant *et al.* 2009). Together, our findings suggest that both enhanced antioxidant enzymes and inhibition of NADPH oxidase may be involved in the suppression of superoxide-related oxidation stress by life-long CR.

### Nutrient Sensing Pathways

The longevity and physiological benefits of life-long CR have been linked to alterations in nutrient sensing pathways, particularly SIRT-1 (Cohen *et al.* 2004; Dali-Youcef *et al.* 2007) and mTOR (mammalian target of rapamycin)(Fontana *et al.* 2010). These pathways are activated (SIRT-1) or inhibited (mTOR) during periods of reduced energy availability and such changes are associated with reduced oxidative stress in several tissues (Lindsley & Rutter 2004; Feng *et al.* 2005; Csiszar *et al.* 2009). The present data are the first to demonstrate in arteries that life-long CR prevents potentially adverse age-related changes in protein expression and/or the signaling state of these pathways. Specifically, life-long CR preserved aortic SIRT-1 expression and activity (measured via acetylated P53) with aging. Previously, we reported age-related reductions in SIRT-1 protein expression in mouse aorta and human endothelial cells (Donato *et al.* 2011), and others have documented the importance of SIRT-1 expression related to endothelial function in healthy arteries (Mattagajasingh *et al.* 2007; Tajbakhsh & Sokoya 2012). In an earlier study, we demonstrated that short-term CR could restore arterial SIRT-1 protein concentrations in old mice (Rippe *et al.* 2010). Preservation of aortic SIRT-1 mRNA expression was not observed with life-long CR in a rat model of aging (Csiszar *et al.* 2009), but the differences with our studies may be due to assessment of SIRT mRNA compared with protein and deacetylase activity, or the rodent species used.

In the present investigation, we also found that aortic mTOR signaling (phosphorylation at serine 2448) was increased with aging in AL mice, but maintained at young adult levels with life-long CR, similar to previous findings in non-vascular tissues (Wu *et al.* 2009). Together, our findings suggest that CR may preserve the function of important signaling pathways that regulate cellular energy status with aging in arteries, laying the foundation for future mechanistic studies on this topic.

### Short-term vs. Long-term CR

Results of a previous study from our laboratory suggest that many of the effects of life-long CR on vascular endothelial function, NO and oxidative stress also may be conferred by shorter-term CR (Rippe *et al.* 2010). In this earlier investigation, we reported that 8 weeks of ~30% CR with attendant acute weight loss restored NO-mediated EDD in carotid arteries of old B6D2F1 mice, and that this was associated with reductions in arterial oxidative stress and NADPH oxidase protein expression, as well as selective increases in antioxidant enzymes. Several other vascular features of arterial aging assessed in the present investigation, including arterial blood pressure, stiffness, wall hypertrophy and structural proteins were not assessed in this earlier study on short-term CR. Moreover, findings regarding the cardiovascular benefits of shorter-term energy intake restriction in humans have been inconsistent (Fontana *et al.* 2004; Fontana *et al.* 2007; Lefevre *et al.* 2009). As such, further investigation of the cardiovascular-protective effects of short- vs. long-term CR will be required to gain more definitive insight.

### Limitations

In the present study, we sought to gain initial insight into mechanisms by which life-long CR may preserve arterial function with aging. We focused on selective mechanisms relating to structural proteins, NO, oxidative stress and nutrient sensing pathways. Several other mechanisms not studied here may contribute to reduced oxidative stress with life-long CR in old mice including reductions in mitochondrial superoxide production, enhanced eNOS

coupling and/or increases in other antioxidant or stress resistance pathways. Moreover, our findings related to the important nutrient sensing pathways of SIRT-1 and mTOR and vascular protection with aging by life-long CR are associative. As such, pharmacological and/or genetic approaches will be required to provide direct cause and effect evidence among these events. Finally, it is possible that some of the beneficial effects of life-long CR on aging arteries may be due to, at least in part, to increases in spontaneous activity, which has been observed in other rodent models of life-long CR. This cannot be determined experimentally because increased activity is a fundamental part of the CR phenotype. Moreover, it is possible that such increases in spontaneous activity could have induced an “exercise training effect”. If so, this could, in turn, explain the preservation of vascular function with aging in CR animals because models of aerobic exercise training such as treadmill running (Donato *et al.* 2005; Donato *et al.* 2007b) and voluntary wheel running (Durrant *et al.* 2009) have previously been shown to reverse some expressions of vascular dysfunction in old rodents. However, such exercise training studies consistently showed an increase citrate synthase activity in skeletal muscle in the exercising animals, which is a well established marker of the exercise training effect. In the present study, CR was not associated with increases in citrate synthase. Thus, the preservation of arterial structure and function with aging in the CR animals cannot be attributed to an exercise training effect per se.

## Perspectives and Conclusions

With the changing demographics of aging leading to unprecedented numbers of older adults, it is imperative that we establish the efficacy of strategies to delay, slow and even prevent the development of arterial dysfunction and CVD with aging. There is accumulating evidence that energy restriction is an effective lifestyle intervention for preserving many aspects of physiological function with aging. In the present study, we extend insight into the physiological benefits of life-long CR by providing the first direct evidence that CR partly or completely prevents large elastic artery stiffening, wall hypertrophy, endothelial dysfunction, reductions in NO bioavailability and increases in arterial blood pressure with aging in mice. We also provide novel and comprehensive mechanistic insight into the role of suppression of oxidative stress in mediating these favorable effects of life-long CR in the prevention of arterial aging, and advance the possible involvement of two key nutrient sensing pathways, SIRT-1 and mTOR, in the vasoprotection conferred by long-term energy restriction.

## Methods

### Animals

Young (Y, 5–7 months) and old *ad libitum* (Old AL, 30–31 months) and old life-long calorically restricted (Old CR, 30–31 months) male B6D2F1 mice were obtained from the National Institute on Aging rodent colony. In NIH life-long CR mice, calorie restriction is initiated at 14 weeks of age at ~10% below ad libitum, increased to ~25% restriction at 15 weeks and to ~40% restriction at 16 weeks, and maintained throughout the life of the animal. All mice (n=43 University of Colorado; n=61 University of Utah) were housed for at least 2–3 months in an animal care facility at the University of Colorado at Boulder or the University of Utah on a 12:12 light:dark cycle at 24 ° C and fed the appropriate NIH-31(Y or Old AL) or NIH-31 fortified diet (Old CR). See supplemental material (Supplemental Figure 2) for full nutritional breakdown of these diets or access the NIA link ([http://www.nia.nih.gov/sites/default/files/nia\\_dietspdf\\_0.pdf](http://www.nia.nih.gov/sites/default/files/nia_dietspdf_0.pdf)). All animal procedures conformed to the *Guide to the Care and Use of Laboratory Animals* (version 8, revised 2011) and were approved by the University of Colorado and Utah Animal Care and Use Committee.



## Arterial Blood Pressure

Blood pressure was assessed non-invasively in the conscious state by determining the tail blood volume with a volume pressure recording (VPR) sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT) as previously described in detail (Feng *et al.* 2008; Rateri *et al.* 2011). This method has been validated versus arterial catheter blood pressure (Feng *et al.* 2008). Blood pressure and heart rate recordings were made in a quiet and warm (24 °C) environment. Mice were placed in restrainers on a heating unit and given 15–20 minutes to acclimate and reach a steady tail skin temperature of (30–35 °C). Each session consisted of 5–10 acclimatization measures and documentation of stable values, followed by 20 experimental measures. Measures with aberrant movement/behavior or inadequate tail volume/flow values were excluded and remaining values used to calculate mean values for each animal. This method is quite reproducible in our laboratories as the coefficient of variation for this method is 5% for systolic and 7% diastolic blood pressure (n=10) (Seals, unpublished).

## Arterial Stiffness

Several (2–3) weeks before euthanasia, aortic pulse wave velocity (PWV) was measured as described previously (Reddy *et al.* 2005; Fleenor *et al.* 2012). Briefly, mice were anesthetized under 2% isoflurane in a closed chamber anesthesia machine (V3000PK, Parkland Scientific, Coral Springs, FL) for ~1–3 min. Anesthesia was maintained using a nose-cone and mice were secured in a supine position on a heating board (~35 °C) to maintain body temperature. Velocities were measured with 20-MHz Doppler probes (Indus Instruments, Webster, TX) at the transverse aortic arch and ~4 cm distal at the abdominal aorta simultaneously and collected using WinDAQ Pro+ software (DataQ Instruments, Akron, OH). After velocities were collected, a precise measure of the distance between the probes was obtained using a scientific caliper and recorded. Absolute pulse arrival times were indicated by the sharp upstroke, or foot, of each velocity waveform analyzed with WinDAQ Waveform Browser (DataQ Instruments, Akron, OH). Aortic PWV is then calculated as the quotient of the separation distance and difference in absolute arrival times.

## Endothelial Function: Modulation by Superoxide and NO

Measurements of endothelial dependent dilation (EDD) and endothelial independent dilation (EID) in isolated carotid arteries studied *ex vivo* were performed using a method previously described in detail (Donato *et al.* 2009; Lesniewski *et al.* 2009; Donato *et al.* 2011). The carotid arteries were used as a large elastic artery for these measurements because we have previously established age-associated impairments in oxidative stress-associated NO-mediated endothelial dysfunction in this model (Durrant *et al.* 2009; Lesniewski *et al.* 2009; Fleenor *et al.* 2012). Moreover, use of the carotid arteries for this functional measurement allowed biochemical analyses, which require large tissue volumes, to be performed on the aortic samples, thus significantly reducing the number of animals required to complete the overall assessments of vascular function and structure.

Briefly, mice were euthanized by exsanguination via cardiac puncture while under isoflurane anesthesia. Both the right and left carotid arteries were excised and placed in myograph chambers (DMT Inc.) perfused by physiological saline solution (PSS) that contained 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl<sub>2</sub>, 1.17 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer and 1 g/100 ml BSA, pH 7.4 at 37 °C, cannulated onto glass micropipettes and secured with nylon (11-0) suture. Once cannulated, both carotid arteries were warmed to 37 °C and pressurized and allowed to equilibrate for ~1 hour. All arteries were submaximally precontracted with phenylephrine (2 μM) and increases in luminal diameter in response to increasing concentrations of the endothelium-dependent dilator, acetylcholine (ACh:1×10<sup>-9</sup>

to  $1 \times 10^{-4}$  M) and endothelium-independent dilator, sodium nitroprusside (SNP:  $1 \times 10^{-10}$  to  $1 \times 10^{-4}$  M) were determined. Responses to ACh were repeated in the presence of the NO synthase inhibitor, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 0.1mM, 30-minute incubation) to determine the contribution of NO. To determine superoxide (oxidative stress) suppression of EDD, measurements were repeated in the contra-lateral vessel following a 60-minute incubation in the presence of the superoxide scavenger, TEMPOL (1mM) (Zhang *et al.* 2003; Qamirani *et al.* 2005; Didion *et al.* 2006). NO bioavailability was determined from the maximal EDD in the absence or presence of L-NAME in each vessel according to the following formula: NO bioavailability (%) =  $\frac{\text{Max Dilation}_{\text{ACh+L-NAME}} - \text{Max Dilation}_{\text{ACh}}}{\text{Max Dilation}_{\text{ACh+L-NAME}}}$

### Arterial Wall Thickness and Histochemistry

Carotid arteries and thoracic aortas were saved in optimal cutting temperature (OCT) solution, frozen and sliced into 8-micron sections. Each mouse carotid artery or aorta had 3 to 4 sections per slide, which were averaged. Slides were batched to have equal numbers of each group and stained for wall thickness via Masson TriChrome (carotid), collagen via Picosirius red (aorta) and elastin via Verhoff's Van Gieson (aorta), as previously described (Fleenor *et al.* 2010; Raub *et al.* 2010). All area (total, medial layer and adventitial layers) and color threshold quantification was performed with ImageJ software (NIH, Bethesda, MD, USA). For measures of medial cross-sectional area (wall thickness) trichrome stained carotid arteries, the lumen border and the outer medial border were traced in ImageJ and internal areas measured. These areas were used to calculate medial cross sectional area and were calculated as the outer media border area minus the lumen area. Green channel images from a RGB stack were used for collagen (Aikawa *et al.* 1998; Fleenor *et al.* 2010) and the 8-bit grey scale was used for elastin analyses (Raub *et al.* 2010). The same color threshold was applied to all collagen and elastin samples. Content was calculated as the percentage of the selected area (arterial wall) with positive color.

### Arterial Protein Expression and Enzyme Activities

To obtain sufficient tissue to perform measurements of protein expression and enzyme activity, the thoracic aorta was excised, cleared of surrounding tissues while maintained in ice-cold (4 ° C) PSS (similar composition as above, but at pH of 7.4 at 4°C), and then frozen in liquid nitrogen. Whole artery lysates were prepared as previously described (Donato *et al.* 2011; Lesniewski *et al.* 2011a). Briefly, 15 µg of protein with 2 mol/L dithiothreitol was loaded into polyacrylamide gels, separated by electrophoresis and transferred onto a nitrocellulose membrane. The membrane was treated with 5% nonfat dry milk in TBS with 0.05% Tween (TBST) overnight at 4°C. Membranes were then washed with TBST and incubated overnight at 4°C in primary antibody. Extracellular SOD (ECSOD)(1:500, Sigma-Aldrich, St. Louis, MO) endothelial NO synthase (eNOS) (1:1000, BD Biosciences, San Jose, CA), catalase (1:250, Abcam, Cambridge MA), collagen I precursor (1:1000, Millipore, Billerica, MA), copper zinc (CuZn) SOD (1:2000, Stressgen, Ann Arbor, MI), manganese (Mn) SOD (1:2000, Stressgen, Ann Arbor, MI), mTOR and phosphorylated mTOR serine 2448 (1:1000, Cell Signaling, Danvers, MA), actelyated p53 (1:500, Cell Signaling, Danvers, MA), p67phox-NADPH oxidase (p67, 1:1000, BD Biosciences, San Jose, CA), SIRT-1 (1:1000, Abcam, Cambridge, MA), and nitrotyrosine (1:1000, Abcam, Cambridge, MA), expression were measured by standard western blotting techniques using an HRP-conjugated secondary antibody (Jackson Immunological, West Grove, PA) and Supersignal ECL (Pierce, Rockford, IL). Bands were visualized using a digital acquisition system (ChemiDoc-It, UVP, Upland, CA) and quantified using ImageJ software (NIH, Bethesda, MD). To account for differences in protein loading, expression is presented normalized to GAPDH (1:1000, Cell Signaling, Danvers, MA) expression. Data are then normalized to the mean of the young control group.

NADPH oxidase activity was determined in aortic lysates (10 µg protein) using the Amplex Red Xanthine/Xanthine Oxidase Assay kit (Invitrogen, Carlsbad, CA), according to manufacturer instructions with NADPH (200 µmol/L/reaction) as the reaction substrate, as previously described (Durrant *et al.* 2009).

Total SOD and catalase activity were determined in aortic lysates (1 µg protein) using SOD and Catalase Activity Assay kits (Cayman Chemical, Ann Arbor, MI) according to manufacturer instructions as previously described (Durrant *et al.* 2009). MMP-9 was measured in aortic lysates (10 µg protein) by ELISA (Thermo / Fisher Scientific Waltham, MA) as previously described (Lesniewski *et al.* 2011b).

### Arterial Superoxide Production

Production of superoxide was measured by EPR spectrometry using the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Alexis Biochemicals). CMH was prepared in ice-cold deoxygenated Krebs-HEPES buffer (mmol L<sup>-1</sup>: NaCl, 99.01, KCl 4.69, CaCl<sub>2</sub> 2.50, MgSO<sub>4</sub> 1.20, K<sub>2</sub>HPO<sub>4</sub> 1.03, NaHCO<sub>3</sub> 25.0, glucose 11.10, Na-HEPES 20.00; pH 7.4) containing 0.1 mmol L<sup>-1</sup> diethylenetriamine-penta-acetic acid, 5 µmol L<sup>-1</sup> sodium diethyldithiocarbamate and pretreated with Chelex (Sigma) to minimize auto-oxidation of the spin probe. Three-millimeter aortic rings were washed once in PSS and again in modified Krebs-HEPES buffer. Rings were then incubated for 60 min at 37°C in 200 µL Krebs-HEPES buffer containing 0.5 mmol L<sup>-1</sup> CMH and analyzed immediately on an EMX Plus EPR spectrometer (Bruker, Rheinstetten, Germany). Instrument settings were: microwave frequency 9.83 Ghz, centerfield 3480 G, sweep 80 G, modulation amplitude 3.3 G, microwave power 40 mW, microwave attenuation 7, and receiver gain 30. A total of six sweeps were conducted lasting 8.7 seconds per sweep. The running average of the six sweeps was collected with the double integration (area under and over the baseline) of the triplet used to display the magnitude of the signal. The magnitude of this signal directly relates to the amount of superoxide that has been trapped by the CMH.

### Cage Activity

Mice were individually housed in clear Plexiglass cages and studied for a period of 72 hours using the Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH). Animals were acclimatized to the chambers for 5 days prior to data collection and maintained at approximately 24 °C with a 12-hour light/dark cycle. Food and water were freely available for ad libitum animals and CR animals were fed at the same time (9 A.M. MST as was standard in the control cages). Mouse movements in the X, Y, and Z planes were monitored by laser beam interruption. The sum of the movement in these planes was summed over each 72-hour period then averaged divided by 3 for average activity per 24 hours.

### Citrate Synthase Activity

Soleus citrate synthase activity was assessed as described previously (Tweedie *et al.* 2011). Briefly, frozen soleus samples were lysed in homogenization buffer (1 mM EDTA and 50 mM Triethanolamine in dH<sub>2</sub>O) with a handheld homogenizer. Soleus lysate protein concentration was assessed by Pierce BCA Assay and aliquots of lysate were brought up to standardized protein concentrations with homogenization buffer. Citrate synthase activity was determined in triplicate in a 96 well plate. The reaction mix consisted of 10 µL standardized protein lysate, 7 µL Oxaloacetic acid (2 mM) and 200 µL of reaction buffer (Tris Buffer, dH<sub>2</sub>O, 200 µM Acetyl CoA, 200 µM DTNB, and 10% Triton X). Plates were read at 412 Absorbance over 5 minutes to determine citrate synthase activity and expressed as mM/ug protein lysate /min.

## Statistics

For animal and vessel characteristics and protein expression / activity, group differences were determined by one-way analysis of variance (ANOVA). For all dose responses, group differences were determined by repeated measures ANOVA. An LSD post hoc test for equal variances was used for preplanned comparisons where appropriate. Data are presented as mean  $\pm$  SEM. Significance was set at  $P < 0.05$ .

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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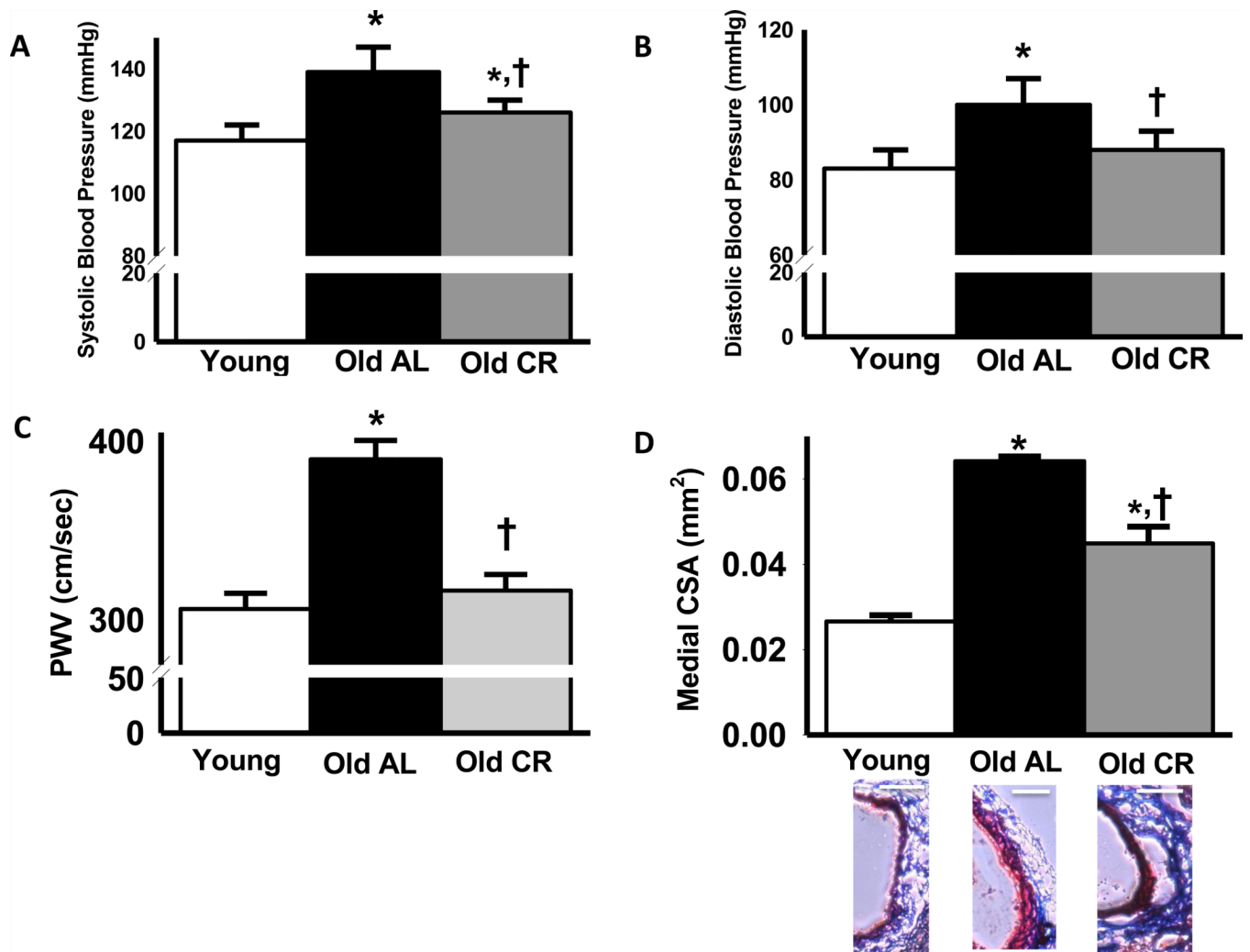
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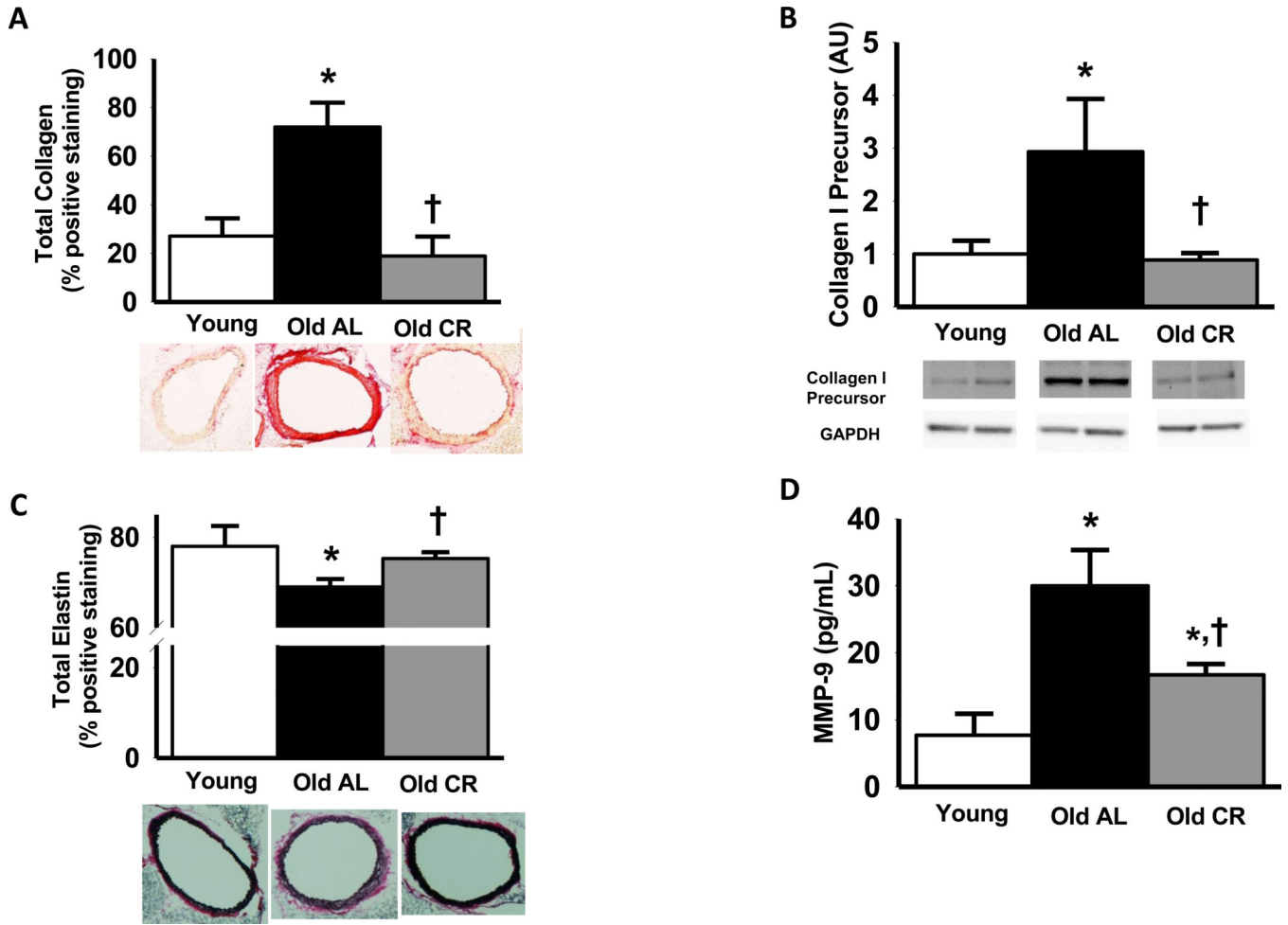
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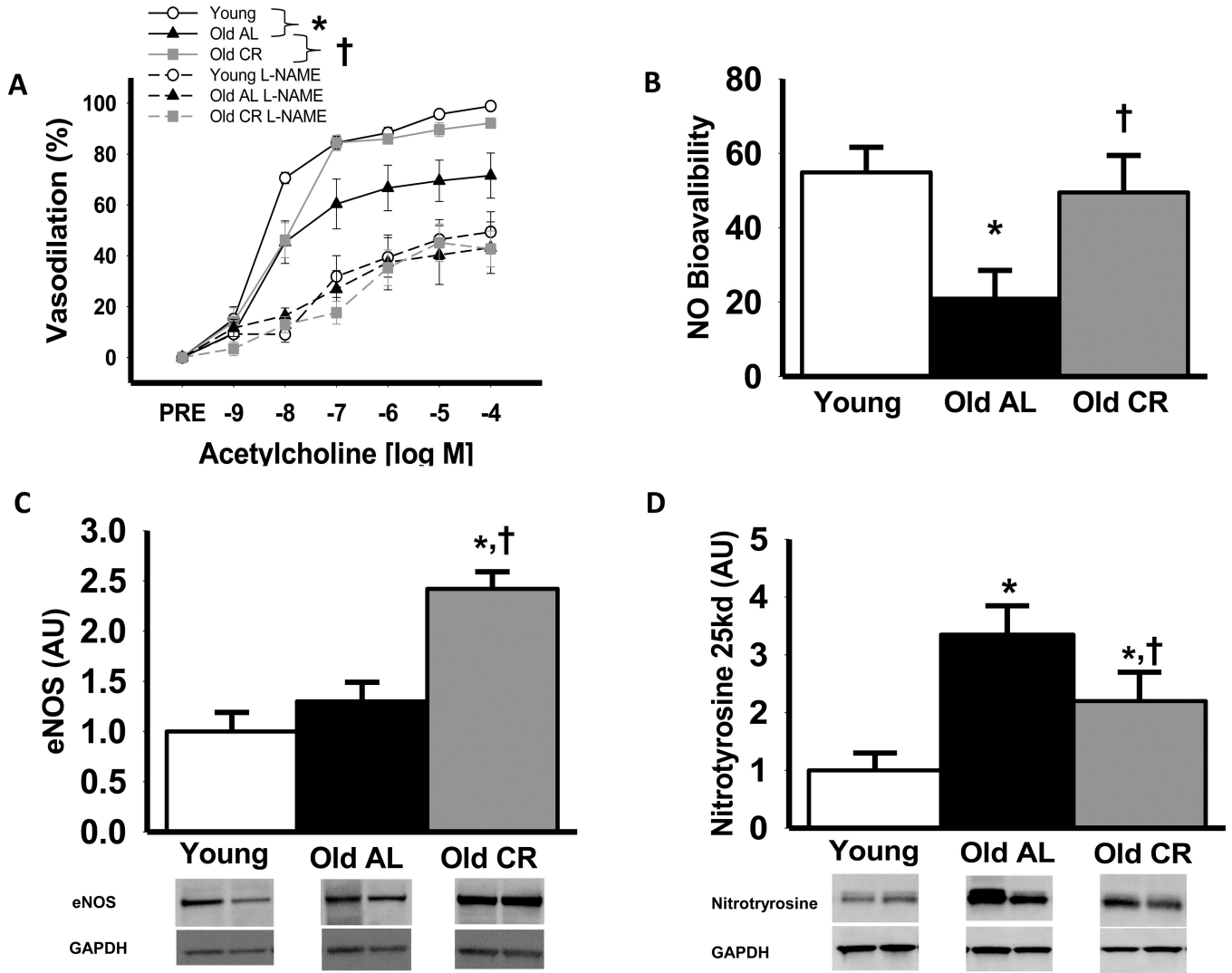
**Figure 1.**

Systolic (A) and diastolic (B) blood pressure (A&B:  $n = 9-11$  per group), aortic pulse wave velocity (PWV) (C) and carotid medial artery cross sectional area (CSA; D) in young, old ad libitum (AL) and old life-long caloric restricted (CR) B6D2F1 mice (C&D:  $n = 7-8$  per group). Representative images of trichrome stained carotid artery wall thickness are provided below the summary graph. White Scale bars are equal to 100 microns. Values are means  $\pm$  s.e.m. \* $P < 0.05$  vs. Young. †  $P < 0.05$  vs. Old AL

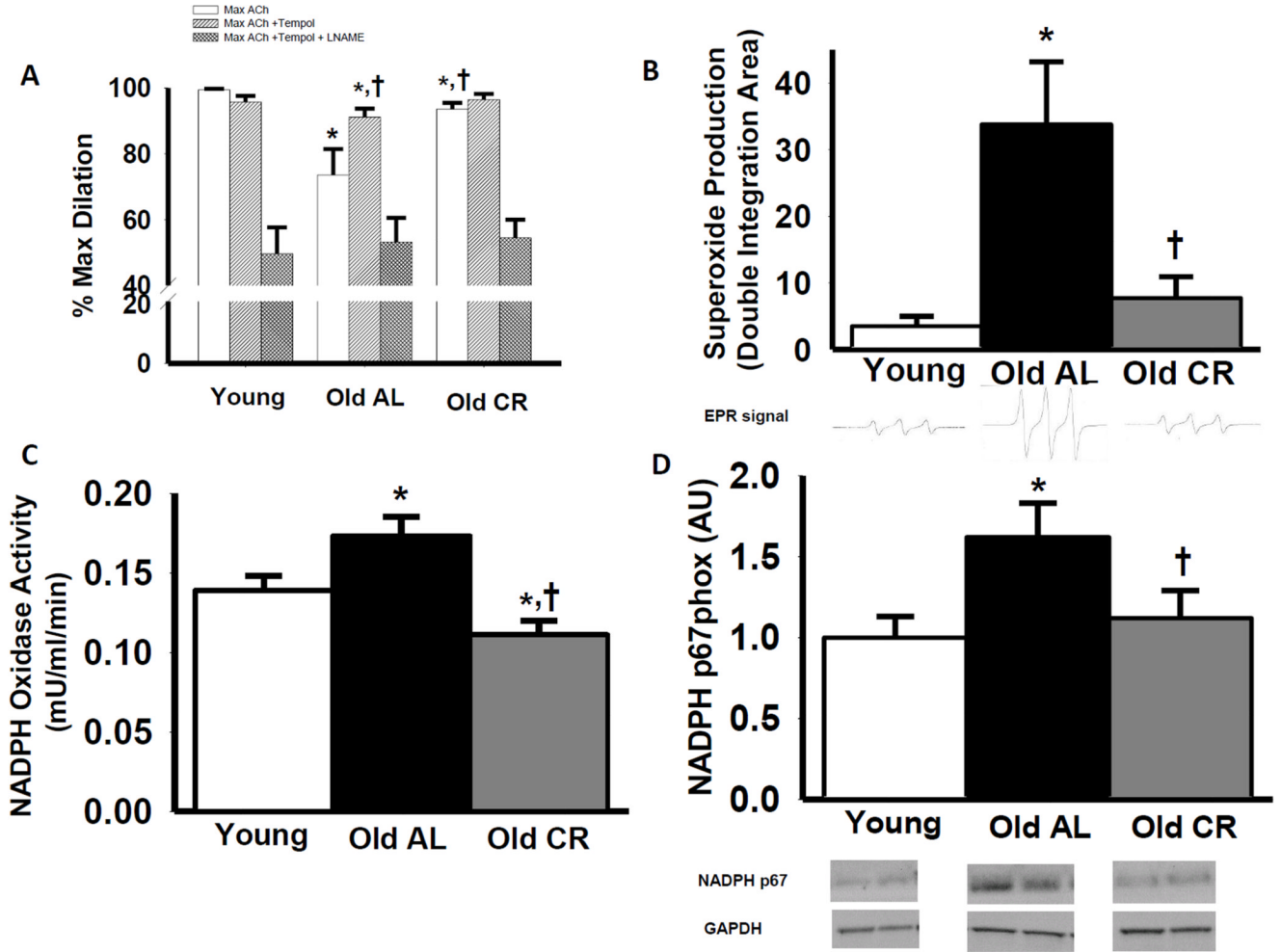


**Figure 2.** Total thoracic aortic collagen (A), precursor collagen I (B), elastin (C) and matrix metalloproteinase 9 (MMP-9; D) content, in young, old ad libitum (AL) and old life-long caloric restricted (CR) B6D2F1 mice ( $n = 6-10$  per group). Representative images for collagen and elastin content in thoracic aorta are provided below the summary graphs. Collagen I is expressed relative to GAPDH to account for differences in protein loading and presented normalized to the Young mean. Representative blots shown below the summary graph. Values are means  $\pm$  s.e.m. \* $P < 0.05$  vs. Young. †  $P < 0.05$  vs. Old AL

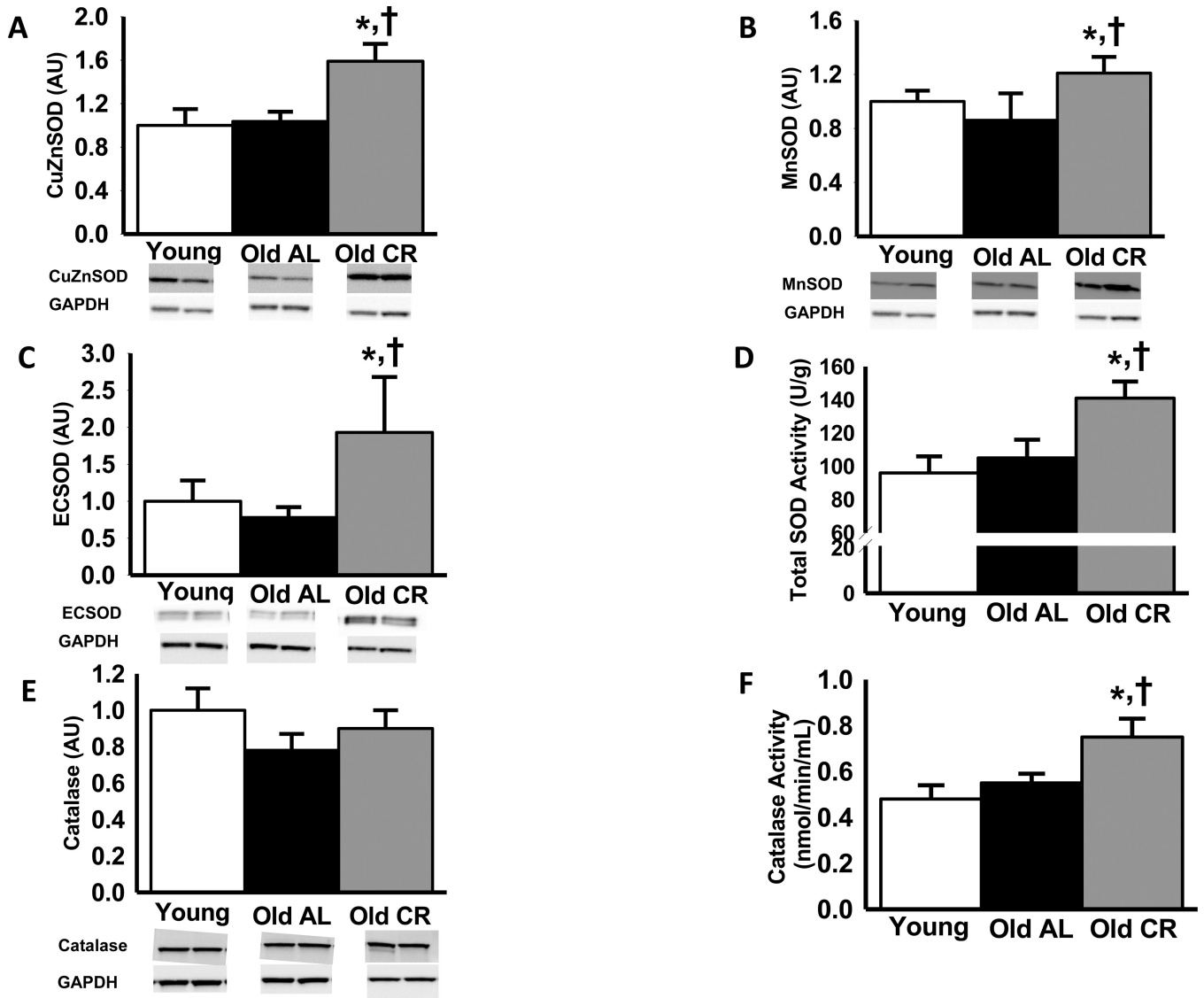




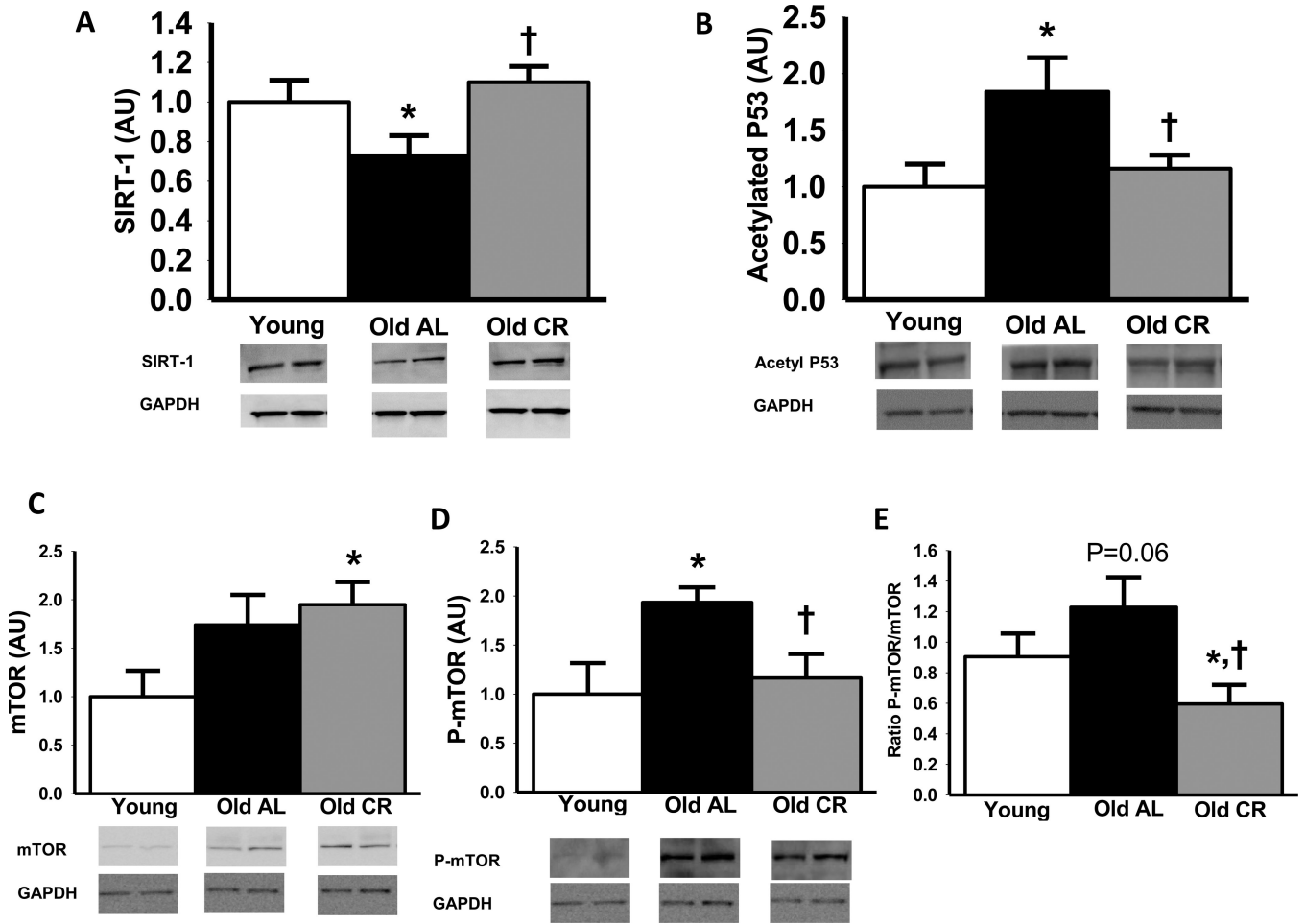
**Figure 3.** Vasodilation of carotid arteries to acetylcholine (ACh) alone or after pre-treatment with the NOS inhibitor, L-NAME (A), nitric oxide (NO) component of ACh-mediated vasodilation (NO bioavailability) in carotid arteries; NO bioavailability (%) = Maximum DilatationACh + L-NAME - Maximum DilatationACh (B) (A&B:  $n = 8-12$  per group), aortic eNOS content (C) and nitrotyrosine abundance, (D) an oxidative stress marker, in young, old ad libitum (AL) and old life-long caloric restricted (CR) B6D2F1 mice; (C&D:  $n = 6-9$  per group) eNOS and nitrotyrosine are expressed relative to GAPDH to account for differences in protein loading and presented normalized to the Young mean. Representative blots shown below the summary graph (C&D). Values are means  $\pm$  s.e.m. \* $P < 0.05$  vs. Young. †  $P < 0.05$  vs. Old AL



**Figure 4.** Maximal vasodilation of carotid arteries to acetylcholine (ACh) alone or to ACh after pre-treatment with TEMPOL in the absence or presence of the NOS inhibitor, L-NAME (A), superoxide production (assessed by electron paramagnetic resonance spectroscopy (B), aortic NADPH oxidase activity (C) and aortic NADPH oxidase p67 (NADPH p67 phox) subunit (D) protein expression in in young, old ad libitum (AL) and old life-long caloric restricted (CR) B6D2F1 mice ( $n = 6-17$  per group). NADPH p67 is expressed relative to GAPDH to account for differences in protein loading and presented normalized to the Young mean. Representative electron paramagnetic resonance of CMH shown below summary graph (B) Representative blots shown below the summary graph (C&D). Values are means  $\pm$  s.e.m. \*  $P < 0.05$  vs. Young ACh alone, †  $P < 0.05$  vs. Old ACh alone



**Figure 5.** Aortic protein expression of the copper zinc (CuZn) (A), manganese (Mn) (B) and extra cellular (EC) (C) isoforms of superoxide dismutase (SOD), total aortic SOD activity (D), aortic catalase protein expression (E) and catalase activity (F) from young and old ad libitum (AL) and old life-long caloric restricted (CR) B6D2F1 mice ( $n = 7-10$  per group). Protein expression is expressed relative to GAPDH to account for differences in protein loading and shown normalized to the Young mean. Representative blots shown below the summary graph. Values are means  $\pm$  s.e.m. \* $P < 0.05$  vs. Young. †  $P < 0.05$  vs. Old AL



**Figure 6.** Aortic sirtuin (SIRT)-1 (A), acetylated P53 (B), total mammalian target of rapamycin (mTOR; C), Ser2448- phosphorylated mTOR (D) and ratio of total to phosphorylated mTOR (E) protein expression from young and old ad libitum (AL) and old life-long caloric restricted (CR) B6D2F1 mice ( $n = 6-11$  per group). Protein expression is expressed relative to GAPDH to account for differences in protein loading and shown normalized to the Young mean. Representative blots shown below the summary graph. Values are means  $\pm$  s.e.m. \* $P < 0.05$  vs. Young. †  $P < 0.05$  vs. Old AL

**Table 1**

## Animal Characteristics

	YOUNG	OLD AL	OLD CR
Age, mo	6.1 ± 0.5	30.2 ± 0.4 *	30.6 ± 0.7 *
Food intake, g/day	4.8 ± 0.1	4.1 ± 0.1 *	3.2 ± 0.0 * <sup>†</sup>
Body mass, g	42.6 ± 1.2	40.5 ± 1.3	27.5 ± 0.06 * <sup>†</sup>
Heart, mg	223 ± 7	258 ± 7 *	214 ± 13 <sup>†</sup>
Heart:BW, g/g x 100	0.55 ± 0.02	0.67 ± 0.03 *	0.80 ± 0.06 * <sup>†</sup>
Soleus, mg	11 ± 1	13 ± 2	10 ± 1 <sup>†</sup>
Soleus:BW, g/g x 100	0.05 ± 0.00	0.06 ± 0.00	0.07 ± 0.00 *
WAT, mg	684 ± 37	972 ± 108 *	346 ± 33 * <sup>†</sup>
WAT:BW, g/g x 100	1.58 ± 0.08	2.26 ± 0.21 *	1.27 ± 0.13 <sup>†</sup>
Total cholesterol, mg/dl	70 ± 11	72 ± 13	70 ± 10
Triglycerides, mg/dl	67 ± 12	130 ± 20 *	15 ± 4 * <sup>†</sup>
Total daily activity, beam breaks / day	866 ± 79	915 ± 92	1215 ± 83 * <sup>†</sup>
Soleus citrate synthase activity, mMol * µg protein <sup>-1</sup> * min <sup>-1</sup>	4472 ± 665	2960 ± 417 *	3338 ± 772

Data are mean±SE. AL, ad libitum; CR, caloric restricted; BW, mass to body weight ratio; WAT, epididymal white adipose tissue.

\* P<0.05 vs. YOUNG;

<sup>†</sup> P<0.05 vs. OLD AL.