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Aging Compounds Western Diet-Associated Large Artery Endothelial Dysfunction in Mice: Prevention by Voluntary Aerobic Exercise

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Abstract

We tested the hypothesis that aging will exacerbate the negative vascular consequences of exposure to a common physiological stressor, i.e., consumption of a “western” (high fat/high sucrose) diet (WD) by inducing superoxide-associated reductions in nitric oxide (NO) bioavailability, and that this would be prevented by voluntary aerobic exercise. Incremental stiffness and endothelium-dependent dilation (EDD) were measured in the carotid arteries of young (5.4 ± 0.3 mo, $N=20$) and old (30.4 ± 0.2 mo, $N=19$) male B6D2F1 mice fed normal chow (NC: 17% fat, 0% sucrose) or a western diet (40% fat, 19% sucrose) diet and housed in either standard cages or cages equipped with running wheels for 10–14 weeks. Incremental stiffness was higher in old NC ($P<0.05$) and both young ($P<0.01$) and old ($P<0.01$) WD fed mice compared with young NC mice, but WD did not further increase stiffness in the old mice. In cage control mice, EDD was 17% lower in both NC fed old mice and young WD fed mice ($P<0.05$). Consumption of WD by old mice led to a further 20% reduction in EDD ($P<0.05$). Incremental stiffness was 28% lower and EDD was 38% greater in old WD fed mice with access to running wheels vs. old WD fed control mice ($P<0.05$) and not different from young NC fed controls. Wheel running also tended to improve EDD (+9%, $P=0.11$), but not incremental stiffness in young WD fed mice. Ex vivo treatment with the superoxide scavenger TEMPOL and NO inhibitor L-NAME abolished these respective effects of age, WD and voluntary running on EDD. Ingestion of a WD induces similar degrees of endothelial dysfunction in old and young adult B6D2F1 mice, and these effects are mediated by a superoxide-dependent impairment of NO bioavailability. However, the combination of old age and WD, a common occurrence in our aging society, results in a marked, additive reduction in endothelial function. Importantly, regular voluntary aerobic exercise reduces arterial stiffness and protects against the adverse influence of WD on endothelial function in old animals by preventing superoxide suppression of NO. These findings may have

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important implications for arterial aging and the prevention of age-associated cardiovascular diseases.

Keywords

vasodilation; aging; western diet; obesity; exercise

1. Introduction

Cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality in modern societies, and older age is the primary risk factor for CVD (Lakatta and Levy 2003; Redberg and others 2009). The mechanisms by which aging increases risk of CVD are incompletely understood, but the development of vascular endothelial dysfunction is believed to be a major contributor (Lakatta and Levy 2003; Seals and others 2011). Vascular endothelial dysfunction with aging, as indicated by impaired endothelium-dependent dilation (EDD), is mediated by reductions in the endothelium-derived dilator nitric oxide (NO) (Seals and others 2011; Taddei and others 2001) and results from excessive vascular superoxide production (Seals and others 2011; Taddei and others 2001). Thus, lifestyle and other factors that influence age-associated impairments EDD by modulating superoxide-dependent NO bioavailability may have important implications for the prevention/treatment of age-related CVD.

A common lifestyle factor that may interact with older age to impair arterial function is consumption of a high fat/high sugar or “western” diet (WD). In young adults or animals, a high fat diet often, although not always, reduces EDD (Donato and others 2012; Erdei and others 2006; Keogh and others 2005; Woodman and others 2005), and there is evidence that WD-associated reductions in EDD result from reduced NO bioavailability and oxidative stress (Erdei and others 2006; Turk and others 2005). Likewise, It is unknown, however, if aging exacerbates the effects of WD on large artery endothelial function (i.e., reduces “resistance” to this pathological influence) and, if so, if this is mediated by superoxide-associated reductions in NO bioavailability.

It is also of clinical interest to determine if “healthy” lifestyle behaviors can counteract the combined effects of WD and aging on vascular function. In this context, we have reported that regular daily wheel running improves vascular function with aging in mice (Durrant and others 2009; Lesniewski and others 2011). It is not known, however, if voluntary aerobic exercise can protect against the combined negative influence of aging and WD on endothelial function, nor if such an effect would be attributable to reduced superoxide suppression of NO.

Here, we tested the hypothesis that aging exacerbates the deleterious consequences of a WD on large artery EDD and that this is mediated by superoxide-mediated reductions in NO. To do so, we used a well-established model of arterial aging (Durrant and others 2009; Lesniewski and others 2009; Lesniewski and others 2011) to assess carotid artery EDD *ex vivo* in the presence or absence of pharmacological inhibition of NO production (L-NAME) and scavenging of superoxide (TEMPOL) in young (~6 mo) and old (~30 mo) adult mice fed either a standard chow or WD. We also sought to gain insight into the potential protective influence of voluntary aerobic exercise on WD-induced endothelial dysfunction in old mice, and the possible role of reduced superoxide suppression of NO in mediating this effect. Finally, we used the opportunity afforded by the *ex vivo* assessments of vascular function to gain initial insight into the interactive effects of aging, WD and exercise on carotid artery stiffness. To address these aims, we studied groups of WD-fed young and old

mice allowed access to voluntary running wheels, and compared their responses to those of the (non-exercising) groups.

2. Materials and Methods

2.1 Ethical Approval

All animal procedures conformed to the Guide to the Care and Use of Laboratory Animals (revised 2011) and were approved by the University of Colorado at Boulder Animal Care and Use Committee. Mice were housed in an animal care facility at the University of Colorado at Boulder on a 12:12 light:dark cycle. Euthanasia for tissue collections was performed by exsanguination via cardiac puncture under 2–5% isoflurane (inhaled) anesthesia.

2.2 Animals

Old male B6D2F1 mice were obtained from the National Institute on Aging rodent colony maintained at Charles River Inc. and young mice were obtained from the commercial colony maintained at Charles River Inc. Mice were allowed to acclimate to housing at the University of Colorado at least one month prior to being placed on special diet, in running wheel cages or used for study. Young (5.4 ± 0.3 mo) and old (30.4 ± 0.2 mo) male B6D2F1 mice were fed ad libitum either a normal chow diet (NC: 8640 Harlan Teklad 22/5 Standard Rodent Chow; protein: 29%, carbohydrate: 55%, fat: 17% by kcal containing 0% sucrose by weight) or a WD supplemented with vegetable shortening and beef tallow (WD: Harlan Teklad custom diet TD.96132, adjusted fat diet; protein: 19%, carbohydrate: 41%, fat: 40% by kcal, containing 19% sucrose by weight) as used previously (Lesniewski and others 2007). The composition of fats in the WD was 41% saturated, 17% trans, 35% monounsaturated (cis) and 7% polyunsaturated (cis). The animals were fed ad libitum and housed in standard mouse cages or in cages fitted with running wheels for 10–14 weeks prior to euthanasia. Running distance was monitored daily. Food intake was monitored weekly and kcal per day was calculated based on the caloric density of each diet (NC: 3.0 kcal/gram; WD: 4.5 kcal/gram). The vivarium temperature was kept at 74–76° F. Normal chow fed cage control mice were housed 3–5 per cage. WD fed mice and mice in running wheel cages were singly housed in order to allow for daily monitoring of running distance and weekly assessment of food intake for individual mice as described previously (Donato and others 2012; Durrant and others 2009; Lesniewski and others 2011). All mice were given nesting squares as enrichment. Procedures including animal monitoring, tissue collection and wheel running were described in detail previously (Durrant and others 2009; Lesniewski and others 2009; Lesniewski and others 2011).

2.3 Carotid Artery Endothelial Function and Incremental Stiffness

Both carotid arteries were excised, placed in myograph chambers (DMT, Inc., Atlanta, GA) containing EDTA-buffered physiological salt solution (PSS), and cannulated onto glass micropipettes with nylon (11-0) suture. Arteries were warmed to 37° C, pressurized to 50 mmHg intraluminal pressure (Durrant and others 2009; Lesniewski and others 2009) and allowed to equilibrate for 60 minutes (Durrant and others 2009; Lesniewski and others 2009; Lesniewski and others 2011). One of the excised arteries was used to assess EDD to acetylcholine and the other was used to examine the role of superoxide in reducing EDD by pre-treatment with superoxide dismutase (SOD) mimetic, 4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL). To do so, following the initial 60-min equilibration one of the arteries was sub-maximally pre-constricted with phenylephrine ($2 \mu\text{mol/L}$) (Donato and others 2012; Lesniewski and others 2009; Lesniewski and others 2011) and those failing to achieve 15% constriction were discarded. Because TEMPOL has been demonstrated to reduce pre-constriction to phenylephrine, artery viability was

determined prior to the addition of TEMPOL by verifying that the artery achieves at least 15% pre-constriction to phenylephrine in the absence of TEMPOL (Durrant and others 2009). Increases in luminal diameter were measured in response to the cumulative addition of the endothelium-dependent dilator, acetylcholine (1×10^{-9} to 1×10^{-4} mol/L). To determine the contribution of NO to dilation, responses to acetylcholine were repeated in the presence of NG-nitro-L-arginine methyl ester (L-NAME, 0.1 mmol/L, 60 min). To determine if aging and/or high fat-high sucrose diet impacts the ability of the smooth muscle to dilate, changes in lumen diameter was assessed in response to the cumulative addition of the endothelium-independent dilator, sodium nitroprusside (SNP, 1×10^{-10} to 1×10^{-4} mol/L). Last, the arteries were incubated in calcium-free PSS for at least 1 hr to abolish any myogenic vasoconstriction and maximal dose of sodium nitroprusside was added to the bath to ensure maximal dilation of the artery. Lumen diameter was measured under these conditions and used in the calculations of percent pre-constriction and percent vasodilation as described below. To determine the contribution of superoxide to dilation, dose responses to acetylcholine in the presence and absence of L-NAME were repeated in the contra-lateral vessel after a 60-min incubation with TEMPOL (1 mmol/L) (Durrant and others 2009) and percent pre-constriction, dose responses (repeated measures ANOVA), maximal dilation and sensitivity to acetylcholine and sodium nitroprusside were compared with the untreated artery.

After all functional measures were complete, the non-TEMPOL treated carotid artery was incubated in Ca^{2+} -free PSS for 1 hour. Lumen diameter and medial wall thickness were measured at 50 mmHg and then in response to increases in intraluminal pressure (5 and 20 – 200 mmHg, in 20 mmHg increments). Lumen diameters during the passive pressure – diameter relations were recorded and the incremental stiffness was determined using the calculated circumferential stress and circumferential stretch as previously described (Fleenor and others 2010; Lesniewski and others 2009; Muller-Delp and others 2002). A calculated wall thicknesses for each pressure step was used in the determination of circumferential stress and was calculated based on the assumption of a constant wall volume and the measured wall thickness at 50 mmHg (Fleenor and others 2010; Lesniewski and others 2009; Muller-Delp and others 2002).

Vessel segments were imaged and diameters measured by MyoView software (DMT, Inc., Atlanta, GA). All dose response data are presented on a percent possible vasodilation basis as described previously (Durrant and others 2009; Lesniewski and others 2009; Lesniewski and others 2011). The maximal diameter of the artery used in the following calculations was determined as described above at 50 mmHg prior to assessing passive pressure-diameter relations. As previously described (Lesniewski and others 2009), functional artery data are presented on a percent basis. Pre-constriction was calculated as percentage of maximal diameter according to the following formula:

$$\text{Pre-constriction (\%)} = [(D_m - D_p) / D_m] * 100$$

Where D_m is the maximal lumen diameter measured in calcium free PSS, D_p is the steady state lumen diameter after the addition of a sub-maximal 2 μM dose of phenylephrine.

Vasodilator responses were recorded as actual diameters and expressed as a percentage of maximal possible vasodilator response according to the following formula:

$$\text{Relaxation (\%)} = [(D_s - D_p) / (D_m - D_p)] * 100$$

where D_m is maximal inner diameter at 50 mm Hg measured in calcium free PSS, D_s is the steady-state inner diameter recorded after the addition of either acetylcholine or sodium nitroprusside, and D_p is the steady-state inner diameter following pre-constriction before the first addition of drug.

In addition to assessing group and treatment differences across the entire dose responses, the maximal vasodilation and the sensitivities (IC_{50}) were also assessed and used in the analyses to describe the dose response relations. Maximal vasodilation was defined as the largest percent dilation achieved during the dose responses. Sensitivity was determined by fitting a four parameter sigmoidal curve to the percent dilation data using BioDataFit 1.02 (<http://www.changbioscience.com/stat/ec50.html>).

2.4 Statistics

For animal and vessel characteristics, maximal vasodilation and sensitivity to acetylcholine, group differences were determined by one-way analysis of variance (ANOVA). Least squares difference post hoc tests were used where appropriate. Repeated measures ANOVAs with least squares differences post hoc testing were employed to determine differences among dose response curves. Data are presented as mean $\pm$ S.E.M. Significance was set at $P<0.05$.

3. Results

3.1 Effects of Aging and WD

3.1.1 Animal and Carotid Artery Characteristics—Because mice reduced the total grams of food consumed per day when fed WD, food intake in kcal did not differ among groups. Body mass did not differ in young and old mice on a NC diet but was ~10% greater in young ($P=0.1$) and ~17% greater in old ($P<0.01$) WD mice. Heart mass was greater in the old NC group (absolute) and in both NC and WD fed old groups (heart:body mass ratio) ($P<0.01$), whereas gastrocnemius muscle mass was lower in the old groups ($P<0.01$). Liver mass was did not differ among the groups. Epididymal white adipose mass was greater in old WD fed mice (absolute mass) and in the WD fed groups (relative to total body mass) ($P=0.05$ to <0.01). Animal characteristics data is shown in Table 1. Maximal diameter of the carotid artery was greater in old NC and WD fed compared to young NC fed mice (Table 2). Incremental stiffness, determined from the passive pressure-diameter relation, was higher in old NC ($P<0.05$) and both young ($P<0.01$) and old ($P<0.01$) WD fed mice compared with young NC mice. WD did not further increase stiffness in the old mice, perhaps due to a ceiling effect (Table 2, $N=6-8$ /group).

3.1.2 Pre-constriction to Phenylephrine—There were no differences in pre-constriction in response to phenylephrine with either aging or WD (Table 2). Compared with pre-constriction in the same vessel in the absence of the NOS inhibitor, L-NAME, incubation with L-NAME significantly increased phenylephrine-induced pre-constriction in young NC and WD fed mice (both $P<0.01$), but not in the NC or WD fed old mice (Table 2). Treatment with the superoxide dismutase mimetic, TEMPOL, reduced phenylephrine-induced pre-constriction of the contralateral carotid artery in all groups except young NC (Table 2, $P<0.05$). Lastly, in the TEMPOL treated arteries, L-NAME increased pre-constriction in arteries from both young and old WD fed mice (Table 2, $P<0.05$). These data suggest that NO contributes to vascular tone in young, but not old mice, and further suggests that increased superoxide suppresses basal NO bioavailability after WD.

3.1.3 Effects of Aging and WD on EDD—In NC fed mice, the dose response to acetylcholine, assessed by repeated measures ANOVA, was reduced with aging (Fig 1 A,

$P<0.01$). WD impaired the acetylcholine-mediated dose response in young mice and further impaired the response in old mice (Fig 1 A, both $P<0.05$). Maximal vasodilation was determined for each individual dose response and the group mean is presented. The differences in maximal vasodilation (Fig 1C, open bars) mirrored those observed in the dose responses, i.e., aging ($P<0.01$) in NC fed mice and WD in both young ($P<0.05$) and old ($P<0.05$) mice resulted in a reduction in maximal vasodilation. Sensitivity (IC_{50}) to acetylcholine did not differ with either aging or WD in young or old mice (Table 2).

3.1.4 Nitric Oxide Bioavailability—Dose responses to acetylcholine were reduced after L-NAME incubation in all groups (Fig 1B, all $P<0.01$), indicating that NO was contributing to acetylcholine-mediated dilation under all conditions. Most importantly, all age and diet-related differences observed in the dose responses (Fig 1A) and maximal vasodilation (Fig 1C, open bars) to acetylcholine were abolished after incubation with L-NAME (Fig 1B and C, hashed bars). Sensitivity to acetylcholine was reduced after L-NAME in all groups except the WD fed old mice (Table 2). Collectively, these results indicate that impairments in the dose responses and maximal vasodilation to acetylcholine with aging and in response to WD are mediated by reductions in NO bioavailability.

3.1.5 Superoxide Impairs Vasodilation and Nitric Oxide Bioavailability—In young mice, TEMPOL treatment eliminated the WD-associated reduction in the dose response (Fig 2A) and maximal vasodilation (Fig 2E) to acetylcholine that was observed in the untreated contralateral artery (Fig 2A, $P=0.20$). In both NC and WD fed old mice, the dose responses (Fig 2B) and maximal vasodilation (Fig 2F) to acetylcholine were higher in the TEMPOL treated artery compared with the untreated contralateral artery (all $P<0.05$). Furthermore, in the TEMPOL treated arteries, L-NAME reduced the dose response (Fig 2 C&D, all $P<0.05$) and the maximal vasodilation (Fig 2 E&F, all $P<0.05$) in response to acetylcholine in all groups, such that no differences between diet or treatment groups remained after L-NAME. After pretreatment with TEMPOL, L-NAME reduced sensitivity to acetylcholine in the old NC fed mice (Table 2, $P<0.01$). These findings indicated that there is an increase in the superoxide mediated suppression of vasodilation with aging and after WD in both young and old mice, and that the age- and WD-associated endothelial dysfunction results from a superoxide-mediated suppression of NO bioavailability.

3.2 Effects of Voluntary Wheel Running in WD Fed Mice

3.2.1 Animal and Carotid Artery Characteristics—When allowed access to running wheels in their home cages, WD fed old mice ran 4.1 ± 1.7 km/day compared with 10.4 ± 0.6 km/day in young WD fed mice ($P<0.05$). Voluntary running was associated with lower body mass ($P<0.01$) despite a 13% increase in food intake ($P=0.05$) in young mice compared to cage control age- and diet-matched mice. Voluntary running also decreased body mass in old WD mice ($P<0.01$) although food intake did not differ ($P=0.52$) compared to age- and diet-matched cage control mice. Epididymal adipose tissue mass was lower in young and old (both $P<0.01$) WD-voluntary running: compared with non-exercising WD fed age-matched mice. Animal characteristics for WD-voluntary running mice are provided in Table 1. Maximal diameter of the carotid artery was not different between after voluntary running in WD fed young or old mice compared to age- and diet-matched cage control mice (Table 1). Incremental stiffness was lower after voluntary running in old WD ($P<0.05$), but not in young WD fed mice (Table 2, $N=5-6$ /group) compared with age-matched WD fed cage control mice.

3.2.2 Pre-constriction to Phenylephrine—There was no difference in pre-constriction between the TEMPOL treated and untreated carotid arteries from young WD-voluntary running mice ($P>0.05$), nor was there an effect of L-NAME on pre-constriction in the

TEMPOL treated arteries from this group (Table 2). However, pre-constriction to phenylephrine was lower in TEMPOL treated carotid arteries from old voluntary running WD fed mice compared to arteries without TEMPOL pretreatment ($P<0.05$) and combined L-NAME increased pre-constriction in the TEMPOL treated old WD fed voluntary running mice (Table 2, both $P<0.05$). These data suggest that voluntary running is increasing the contribution of NO to vascular tone in the WD fed old mice.

3.2.3 Effects of Voluntary Running on Endothelium Dependent Dilation in WD fed mice—In young WD fed mice, voluntary running produced a modest (9%) increase in NO-associated EDD such that neither the dose response (Fig 3A) nor the maximal vasodilation (Fig 3B & D) to acetylcholine were significantly different from young NC fed controls in the absence or presence of L-NAME. There was no effect of voluntary wheel running on sensitivity (Table 2) to acetylcholine (Fig 3 A&B) in the absence or presence of L-NAME in young WD fed mice (Table 3, both $P=0.1$). In WD fed old mice, voluntary running increased NO-associated EDD such that the dose response to acetylcholine was increased to levels not different from young NC fed mice (Fig 3A) and the maximal vasodilation in response to acetylcholine was increased by ~40% (Fig 3B & E) without altering sensitivity (Table 2) to acetylcholine. These results demonstrate that voluntary running restores EDD and NO bioavailability in both young and old WD fed mice, eliminating both the effects of aging and WD.

3.2.4 Voluntary Running Reduces the Superoxide-Mediated Suppression of Endothelium Dependent Dilation with Aging and After WD—There were no differences in the dose response, maximal vasodilation or sensitivity (Table 2) to acetylcholine between TEMPOL treated and untreated arteries after voluntary running in young or old WD fed mice (Fig 3C, D and E). These data suggest the exercise-associated improvements in endothelium-dependent dilation in WD fed mice resulted from an alleviation of the superoxide-mediated suppression of vasodilator function.

3.3 Endothelium Independent Dilation

Endothelium independent dilation and sensitivity to sodium nitroprusside did not differ with aging, WD, voluntary wheel running or TEMPOL treatment (data not shown). This lack of effect in the sodium nitroprusside responses indicates that the aforementioned differences in vasodilation to acetylcholine observed between the age, diet and exercise groups were the consequences of altered NO production/release by the endothelium and not the result of altered sensitivity of the vascular smooth muscle to NO.

4. Discussion

The main novel findings of the present study are as follows. First, ingestion of a WD containing sucrose and a similar fat content to the average consumption by adults in modern societies induces large artery endothelial dysfunction in young mice and compounds age-associated endothelial dysfunction in old mice. Second, this adverse influence of WD in old animals is mediated by increased superoxide-dependent reductions in NO-bioavailability compared with aging or WD alone. Finally, voluntary aerobic exercise prevents WD-induced large artery endothelial dysfunction in old mice by preventing the associated increase in superoxide suppression of NO-mediated dilation, and also reduces arterial stiffness in old WD-fed animals.

4.1 WD, Aging and Endothelial Dysfunction

In the present study, we found that WD and aging each impair large artery endothelial function by ~20% in mice. The combination of age and WD represents a “double insult” that

may be common in modern societies in which ever increasing numbers of older adults are consuming WDs. Given that endothelial dysfunction is considered a key antecedent to the development of CVD, our results suggest that chronic exposure to WD in older adults may significantly worsen the overall risk factor burden associated with primary vascular endothelial aging.

Our results indicate that WD impairs large artery function in young mice to approximately the same degree (% decrease) as in old mice. Thus, there was no obvious age-related increase in the vulnerability of large arteries to WD-induced dysfunction. Nevertheless, the combination of WD and old age exerts an additive negative effect on endothelial function. A similar deleterious influence on endothelial dysfunction has been shown in aortic rings of adult (9 mo) rats in response to prolonged (7 mo) exposure to high fat/high sucrose diet (Roberts and others 2005). The present findings also are consistent with recent cross sectional observations from our laboratory that the presence of adverse (risk) factors such as preclinical elevations in plasma low-density lipoprotein cholesterol and fasting blood glucose exert an additive influence on endothelial function in older adults (DeVan and others 2013; Walker and others 2009).

Previous studies indicate that endothelial dysfunction with aging is mediated by reductions in endothelium-dependent NO bioavailability as a consequence of excessive superoxide bioactivity (Donato and others 2007; Durrant and others 2009; Lesniewski and others 2009). Moreover, there is evidence that WD may induce endothelial dysfunction via similar mechanisms in young animals (Erdei and others 2006; Turk and others 2005). In the present study, we used the NO inhibitor L-NAME in conjunction with the superoxide-scavenging compound TEMPOL to investigate the mechanisms by which old age and WD exert their independent and interactive effects on vascular endothelial dysfunction. It is possible that as a SOD mimetic, TEMPOL could increase the availability of other vasoactive reactive oxygen species by converting superoxide to hydrogen peroxide, thus possibly confounding the interpretation of the results. However, we do not believe that increased hydrogen peroxide plays such a role in the TEMPOL-mediated improvements in function observed here. In addition to its role as a SOD mimetic, TEMPOL also acts to both increase catalase activity, which aids in the removal of hydrogen peroxide and decreases the production of hydroxyl radicals (Knight 1998; Krishna and others 1996; Schnackenberg 2002). Using TEMPOL in the vessel bath, we found that the worsening of EDD in old mice by exposure to a WD was mediated by an exaggeration of the superoxide-dependent reduction in NO bioavailability. Together, these results suggest that ingestion of a WD further reduces NO bioavailability in old animals, and that this is caused by further stimulation of superoxide bioactivity resulting in even greater endothelial dysfunction. Our findings also indicate that these events are endothelium-specific and not due to differences in vascular smooth muscle sensitivity to NO because dilation in response to an NO donor (SNP) was not affected by aging or WD.

Another potential contributor to the vasodilator dysfunction observed with aging and WD may be an increase in stiffness of the large arteries, which may limit their capacity for dilation. To address this possibility, we measured the passive pressure-diameter relation and arterial wall thickness to calculate incremental stiffness of the carotid artery, a commonly used assessment of the passive mechanical properties of the artery (Lesniewski and others 2009; Muller-Delp and others 2002; Padilla and others 2011). We found that incremental stiffness was elevated with aging and with WD in young mice, but that no further increase in stiffness was observed in old mice with WD. The latter may be due to a “ceiling effect” in the old mice, which already demonstrated increased stiffness under baseline (normal chow) conditions. This dissociation of the effects of WD on endothelial function and arterial

stiffness in old mice, suggests that changes in intrinsic stiffness did not play an important mechanistic role in mediating endothelial dysfunction in the old mice in response to WD.

4.2 Effects of Voluntary Aerobic Exercise

We employed voluntary wheel running, an established model of voluntary aerobic exercise in humans, to determine if regular aerobic exercise prevents or lessens the combined effects of aging and WD on endothelial function (Durrant and others 2009; Lesniewski and others 2011). Using this model we previously showed that voluntary running restored EDD in old mice ingesting a normal (low fat) chow diet (Durrant and others 2009; Lesniewski and others 2011). (Durrant and others 2009; Lesniewski and others 2011). Here we extend these findings by demonstrating that voluntary running largely prevents the negative impact of WD on endothelial function in old animals, while also improving carotid artery stiffness in old animals consuming a WD. Consistent with our previous observation of lower daily running distance in old compared with young mice fed NC diet (Durrant and others 2009), this vascular protective effect was observed despite the fact that the old WD fed mice ran only ~40% as much as the young mice (4.1 ± 1.7 vs. 10.4 km/day). Thus, even a modest (compared with young mice) voluntary aerobic exercise stimulus was sufficient to prevent the combined adverse effects of aging and consuming a WD on endothelial function and increases in carotid artery stiffness. Our observations here also are in agreement with recent studies in humans showing that regular aerobic exercise exerts a protective influence on endothelial function against the combined effects of older age and the presence of preclinical elevations in risk factors for CVD (DeVan and others 2013; Walker and others 2009).

We also provide insight into the mechanisms by which voluntary aerobic exercise exerts its endothelial-preserving effects in old animals in response to the adverse influence of a WD. Specifically, the *ex vivo* vessel responses to L-NAME and TEMPOL indicate that voluntary running preserved endothelial dysfunction in the old animals exposed to WD by preventing superoxide-dependent reductions in NO bioavailability. We previously reported a similar effect of voluntary running in old mice fed a NC diet (Durrant and others 2009). In that study, the reduced oxidative stress appeared to be mediated by a reduction in the expression and activity of the oxidant enzyme, NADPH oxidase, and also was associated with an increase in the antioxidant defenses, including increases in the expression of extracellular superoxide dismutase (SOD) and the activities of manganese, copper zinc and extracellular SOD. It is likely that these mechanisms also were involved in mediating the improvements in endothelial function observed in the old WD fed mice after voluntary wheel running.

The signal(s) inducing improvements in endothelial function with voluntary aerobic exercise are incompletely understood. It is well established, however, that aerobic exercise performed with large muscle groups improves endothelial function beyond the circulation of the active skeletal muscles (Padilla and others 2011), as seen here with the carotid artery. These effects are likely mediated in part by systemic increases in shear stress during each exercise bout, although non-shear stress-dependent mechanisms such as changes in (presently unknown) circulating humoral factor(s) released by skeletal muscle, fat or other tissues during exercise also may contribute (Padilla and others 2011). Indeed, in rodents, bulk blood flow to the brain increases significantly during both maximal and submaximal running exercise resulting in an increase in blood flow to areas of the brain associated with locomotor activities, vision, equilibrium and cardiorespiratory control (Delp and others 2001). This increase in total brain blood flow is conducted through the carotid arteries and, thus, may constitute a chronic increase in the shear stimulus that contributes to the observed improvements in vasodilator function and the reductions in arterial stiffness in old WD fed mice. The present findings show that daily moderate voluntary aerobic exercise has powerful vasoprotective effects against the combined adverse influences of WD and old age.

Here, voluntary running did not completely restore EDD in young WD fed mice. This may be the result of the lesser dysfunction in this group compared with the combination of WD and aging in the old mice. That is, the greater dysfunction of old mice represents a lower baseline from which to show improvements by an intervention. A recent report found that voluntary wheel running was sufficient to protect coronary resistance arteries against the effects of similar type of WD in young C57/BL6 mice (Park and others 2012). However, the impairment in coronary artery EDD induced by WD consumption was much greater (50%) compared with the 17% reduction in carotid artery EDD observed in the young mice in the present study. It also is possible that mouse strain- and artery type/location-related differences explain the different interactions between WD and exercise in the two studies. Indeed, previous reports reveal substantial variability in the endothelial responses in different vascular beds to high-fat feeding or exercise in young animals and humans (Laughlin and others 2001; Newcomer and others 2005; Woodman and others 2005). It remains to be determined why the beneficial effects of voluntary running were either diminished (endothelial) function or absent (incremental stiffness) in young mice fed WD.

4.3 Experimental Issues and Limitations

The old mice consuming a WD had a greater body mass and epididymal fat mass than both young and old NC fed animals. It is possible that these differences in body or fat mass contributed to the effects of WD on endothelial function in the old mice. However, WD produced the same degree of endothelial dysfunction in both the young and old animals, despite a lack of effect on body mass and a much smaller increase in body fat in young compared with old animals. Thus, there was a clear dissociation between WD feeding-associated changes in body mass, epididymal fat mass and endothelial function in the present study.

5. Conclusions

The results of the present study show that aging and WD have an additive effect in producing endothelial dysfunction in mice via superoxide-associated suppression of NO. Importantly, regular voluntary aerobic exercise largely prevents the combined adverse influence of aging and WD, while also reducing arterial stiffness in old WD-fed animals. Given the growing population of older adults, the high prevalence of consumption of a WD and the role of vascular dysfunction on cardiovascular risk, the present results have important implications for arterial aging and the prevention of age-associated CVD.

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Figure 1. Endothelium dependent dilation in young and old, normal chow and western diet fed mice

Dose responses to acetylcholine (A) in carotid arteries from young (Y) normal chow (NC) (N=9), old (O) NC (N=8), Y western diet (WD) (N=9) and O WD (N=8) fed mice. Dose responses to acetylcholine in the presence (B) of the nitric oxide synthase (NOS) inhibitor, L-NAME in Y and O, NC and WD fed mice. Maximal vasodilation (C) to acetylcholine (ACh) in carotid arteries from Y and O, NC and WD fed mice in the absence or presence of the nitric oxide synthase inhibitor, L-NAME. * Denotes group difference from Y NC, † denotes group differences from O NC, ‡ denotes group differences from Y WD, § Denotes difference with L-NAME from ACh alone. Differences in dose responses were assessed by Repeated Measures ANOVA. Maximal vasodilation was determined for each dose response curve and a two-way ANOVA with Tukey post hoc was used to determine differences. Data are means±SEM, P 0.05

Figure 2. Endothelium dependent dilation in normal chow and western diet fed mice after antioxidant treatment

Dose responses to acetylcholine in the absence or presence of the superoxide dismutase mimetic, TEMPOL, in young (Y, N=9/diet) (A) and old (O, N=8/diet) (B) normal (NC) and western diet (WD) fed mice. Dose responses to acetylcholine (ACh) in the absence or presence of the superoxide dismutase mimetic, TEMPOL, with and without treatment with the nitric oxide synthase inhibitor, L-NAME in young (C) and old (D) NC and WD fed mice. Maximal vasodilation to acetylcholine in young (E) and old (F) NC and WD fed mice. * Denotes difference from NC, † Denotes difference from WD § Denotes difference with L-NAME from ACh alone. Differences in dose responses were assessed by Repeated Measures ANOVA. Maximal vasodilation was determined for each dose response curve and a two-way ANOVA with Tukey post hoc was used to determine differences. Data are means $\pm$ SEM, P 0.05

Figure 3. Endothelium dependent dilation in normal chow and western diet fed mice after wheel running

Dose responses to acetylcholine (A) in the absence or presence of the nitric oxide synthase (NOS) inhibitor, L-NAME, in carotid arteries from young (Y) western diet (WD) voluntary wheel running (VR) (N=6) and O WD-VR (N=6) mice. Y normal chow (NC) data from Figure 1 are presented as a reference. § denotes difference with L-NAME from ACh alone. Maximal vasodilation (B) to acetylcholine (ACh) in carotid arteries from young (N=6–9/group) and old (N=6–8/group) normal chow (NC) and western diet fed cage control (WD) and WD fed mice allowed access to voluntary wheel running wheels (WD-VR). * Denotes difference from age-matched NC, † Denotes difference from age-matched WD, ‡ Denotes difference between young and old within treatment. Dose responses to acetylcholine in the presence of the superoxide dismutase mimetic, TEMPOL (C), with and without combined treatment with L-NAME in carotid arteries from young (Y) western diet (WD) voluntary wheel running (VR) (N=5) and O WD-VR (N=5) mice. Y normal chow (NC) data are presented as a reference. § denotes difference with L-NAME from ACh alone. Maximal vasodilation (D) to acetylcholine (ACh) in carotid arteries from young WD (N=7–8/age group) and WD-VR (N=5/age group) in the absence or presence of TEMPOL and L-NAME. Maximal vasodilation (E) to acetylcholine (ACh) in carotid arteries from old WD (N=7–8/age group) and WD-VR (N=5/age group) in the absence or presence of TEMPOL and L-NAME. * Denotes difference from age-matched WD group, § denotes difference with L-NAME from ACh alone. Differences in dose responses were assessed by Repeated Measures ANOVA. Maximal vasodilation was determined for each dose response curve and a two-way ANOVA with Tukey post hoc was used to determine differences. Data are means $\pm$ SEM, P 0.05. No Tx: No treatment.

Body mass, food intake and tissue masses in young and old normal chow (NC) and western diet (WD) fed cage control and voluntary running (VR) mice.

Table 1

N	Y NC	O NC	Y WD	O WD	Y WD-VR	O WD-VR
	9	8	9	8	6	6
Body Mass (g)	32.8 ± 0.7	34.4 ± 0.6	36.3 ± 1.5	40.4 ± 2.5 ^{*†}	29.0 ± 0.8 [‡]	30.7 ± 1.3 [‡]
Food Intake (kcal/day)	14.6 ± 0.3	15.1 ± 0.2	14.1 ± 0.2	15.2 ± 0.9	16.0 ± 0.7	15.8 ± 0.9
Food Intake (g/day)	4.9 ± 0.1	5.0 ± 0.1	3.1 ± 0.1 [*]	3.4 ± 0.2 ^{*†}	3.6 ± 0.1	3.5 ± 0.2
Heart Mass (g)	0.19 ± 0.01	0.23 ± 0.01 [*]	0.17 ± 0.01	0.21 ± 0.01	0.17 ± 0.01	0.26 ± 0.03 [§]
Heart:Body Mass (%)	0.54 ± 0.03	0.66 ± 0.03 [*]	0.57 ± 0.04	0.57 ± 0.04 [†]	0.58 ± 0.04	0.84 ± 0.10 ^{*†}
Liver Mass (g)	1.9 ± 0.1	2.1 ± 0.2	1.7 ± 0.1	2.1 ± 0.1	1.5 ± 0.1	2.1 ± 0.2
Liver:Body Mass (%)	5.4 ± 0.2	5.4 ± 0.2	4.8 ± 0.2	5.8 ± 0.4	5.2 ± 0.2	6.4 ± 0.5 [§]
WAT Mass (g)	1.1 ± 0.2	0.6 ± 0.1	1.6 ± 0.2	1.9 ± 0.5 ^{*†}	0.7 ± 0.1 [‡]	0.4 ± 0.1 ^{‡§}
WAT:Body Mass (%)	2.9 ± 0.4	1.7 ± 0.2	4.5 ± 0.3 [*]	4.7 ± 1.1 ^{*†}	2.6 ± 0.3 [‡]	1.4 ± 0.2 ^{‡§}
Gast Mass (g)	0.19 ± 0.01	0.15 ± 0.01 [*]	0.19 ± 0.01	0.15 ± 0.01 [*]	0.18 ± 0.01	0.14 ± 0.01 [§]
Gast:Body Mass (%)	0.56 ± 0.04	0.43 ± 0.02 [*]	0.53 ± 0.03	0.37 ± 0.02 [*]	0.63 ± 0.03 [‡]	0.41 ± 0.07 [§]

Y: young; O: old; NC: normal chow; WD: western diet; WAT: epididymal white adipose tissue; Gast: gastrocnemius muscle;

^{*} Denotes difference from Y NC,

[†] denotes difference from O NC.

[‡]denotes difference from age-matched cage-control WD fed mice;

[§]denotes difference from YWD-VR, Data presented as mean ± SEM, P<0.05

Carotid artery maximal diameter, incremental stiffness, phenylephrine-induced pre-contraction and sensitivity (IC₅₀) to acetylcholine in the absence or presence of the nitric oxide inhibitor, L-NAME and/or the superoxide dismutase mimetic, TEMPOL. Dose responses in the presence of TEMPOL were assessed in the contralateral arteries of the mice.

Table 2

	Y NC	O NC	Y WD	O WD	Y WD-VR	O WD-VR
Maximal Diameter (µm)	408 ± 5	432 ± 8 *	423 ± 5	439 ± 6 *	423 ± 12	433 ± 12
Incremental Stiffness (ΔUs)	5.7 ± 0.6	8.5 ± 0.9 *	7.6 ± 0.8 *	9.5 ± 0.8 *	8.1 ± 1.6	6.8 ± 0.5 #
Pre-Constriction (%)	18 ± 1	22 ± 3	18 ± 2	18 ± 2	23 ± 3	23 ± 4
A+L	29 ± 3 ‡	27 ± 3	28 ± 2 ‡	24 ± 2	32 ± 5	25 ± 5
T+A	15 ± 2	14 ± 2 §	13 ± 2 §	14 ± 1 §	17 ± 4	12 ± 2 §
T+A+L	21 ± 3	17 ± 2	20 ± 2 ‡	19 ± 2 ‡	14 ± 1	18 ± 3 ‡
IC₅₀ (log M)	A	-8.2 ± 0.4	-8.0 ± 0.2	-7.4 ± 0.3	-7.6 ± 0.3	-8.0 ± 0.2
A+L	-6.8 ± 0.3 ‡	-6.6 ± 0.3 ‡	-6.5 ± 0.4 ‡	-6.7 ± 0.2	-6.5 ± 0.5	-7.2 ± 0.6
T+A	-7.9 ± 0.2	-8.6 ± 0.2	-7.3 ± 0.3	-7.7 ± 0.3	-8.0 ± 0.2	-8.0 ± 0.3
T+A+L	-7.1 ± 0.4	-6.8 ± 0.4 ‡	-7.0 ± 0.4	-7.2 ± 0.3	-8.0 ± 0.3	-7.0 ± 0.5

Y: young; O: old; NC: normal chow; WD: western diet; VR: voluntary running; A: acetylcholine; L: L-NAME; T: TEMPOL;

* Denotes difference from Y NC,

‡ denotes difference from O NC,

‡ denotes difference after L-NAME,

§ denotes difference after TEMPOL,

denotes difference from age-matched cage-control WD fed mice, WD fed data presented as mean ± SEM, P<0.05