Arterial stiffening with ageing is associated with transforming growth factor-β1-related changes in adventitial collagen: reversal by aerobic exercise

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We tested the hypothesis that carotid artery stiffening with ageing is associated with transforming growth factor-β1 (TGF-β1)-related increases in adventitial collagen and reductions in medial elastin, which would be reversed by voluntary aerobic exercise. *Ex vivo* carotid artery incremental stiffness was greater in old (29–32 months, $n = 11$) *vs.* young (4–7 months, $n = 8$) cage control B6D2F1 mice (8.84 ± 1.80 *vs.* 4.54 ± 1.18 AU, $P < 0.05$), and was associated with selective increases in collagen I and III and TGF-β1 protein expression in the adventitia ($P < 0.05$), related to an increase in smooth muscle α-actin (SMαA) (myofibroblast phenotype) ($P < 0.05$). In cultured adventitial fibroblasts, TGF-β1 induced increases in superoxide and collagen I protein ($P < 0.05$), which were inhibited by Tempol, a superoxide dismutase. Medial elastin was reduced with ageing, accompanied by decreases in the pro-synthetic elastin enzyme, lysyl oxidase, and increases in the elastin-degrading enzyme, matrix metalloproteinase 2. Fibronectin was unchanged with ageing, but there was a small increase in calcification ($P < 0.05$). Increased incremental stiffness in old mice was completely reversed (3.98 ± 0.34 AU, $n = 5$) by 10–14 weeks of modest voluntary wheel running (1.13 ± 0.29 km day^{-1}), whereas greater voluntary wheel running (10.62 ± 0.49 km day^{-1}) had no effect on young mice. The amelioration of carotid artery stiffness by wheel running in old mice was associated with reductions in collagen I and III and TGF-β1, partial reversal of the myofibroblast phenotype (reduced SMαA) and reduced calcification (all $P < 0.05$ *vs.* old controls), whereas elastin and its modulating enzymes were unaffected. Adventitial TGF-β1-related oxidative stress may play a key role in collagen deposition and large elastic artery stiffening with ageing and the efficacious effects of voluntary aerobic exercise.

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Abbreviations CVD, cardiovascular disease; MMP-2, matrix metalloproteinase 2; SMαA, smooth muscle α-actin; TGF-β1, transforming growth factor-β1.

Cardiovascular diseases (CVDs) remain the leading cause of death in modern societies and much of this mortality is caused by dysfunction of arteries (Lloyd-Jones *et al.* 2010). Advancing age is the major risk factor for CVD and this is attributable in part to the development of large elastic artery stiffening, which can lead to numerous CV pathologies including systolic hypertension, stroke and heart failure (Lakatta & Levy, 2003). Thus, understanding the mechanisms by which large elastic arteries stiffen with age and interventions that reverse this stiffening are of major physiological and biomedical importance.

Increases in the deposition of the major load-bearing isoforms of collagen (I and III) and reductions in elastin are believed to be important mechanisms mediating large elastic artery stiffening with ageing (Zieman *et al.* 2005; Diez, 2007). Increases in the extracellular matrix glycoprotein fibronectin and calcification also may contribute to arterial stiffness with ageing (Boumaza *et al.* 2001; Atkinson, 2008). However, several aspects of these processes are poorly understood. For example, it is unknown if the changes in these collagens and elastin with ageing occur in the medial layer of arteries, the adventitial layer or both; nor do we understand the mechanisms by which such region-specific changes could be mediated.

Habitual aerobic exercise is a first-line therapeutic strategy for reducing the risk of CVD with ageing (Blair *et al.* 1989). Middle-aged and older adults who regularly perform aerobic exercise demonstrate less age-associated stiffening of large elastic arteries compared with their sedentary peers (Vaitkevicius *et al.* 1993; Tanaka *et al.*

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1998; Tanaka *et al.* 2000; Seals *et al.* 2008, 2009). However, the mechanisms by which regular aerobic exercise exerts its favourable effects on large elastic artery stiffening with ageing have not been established, partly because of lack of access to these tissues in humans. The limited available data in experimental animals (forced swimming in rats) do not support an influence of voluntary exercise on whole artery collagen or elastin (Matsuda *et al.* 1993; Nosaka *et al.* 2003).

In the present study we hypothesized that stiffening of the carotid artery with ageing would be associated with increased deposition of collagen primarily in the adventitia because cultured fibroblasts synthesize more collagen than vascular smooth muscle cells (Patel *et al.* 2000), and that this would be related to increased expression of the profibrotic cytokine transforming growth factor-β1 (TGF-β1) and a shift to a myofibroblast (i.e. 'secretory' or collagen synthesizing) phenotype. We further hypothesized that because cultured vascular smooth muscle cells produce more elastin than fibroblasts (Ruckman *et al.* 1994), age-associated reductions in elastin would occur primarily in the medial layer of the carotid artery and be related to changes in the elastin-modulating enzymes lysyl oxidase and matrix metalloproteinase 2 (MMP-2). We also hypothesized that increases in fibronectin and/or calcification may be associated with arterial stiffening with ageing. Finally, we hypothesized that regular aerobic exercise would reverse some or all of the age-associated stiffening of large elastic arteries by reducing adventitial collagen, increasing medial elastin, or both. We postulated that exercise would produce these respective effects in old mice by inhibiting expression of TGF-β1 and reversing the shift to a myofibroblast phenotype, and by ameliorating the age-related changes in lysyl oxidase and MMP. We also assessed the possibility that exercise could normalize the increase in fibronectin and calcification of large elastic arteries observed in old mice.

To test these hypotheses, we performed *ex vivo* analyses of carotid arteries using a recently established B6D2F1 mouse model of arterial ageing (Lesniewski *et al.* 2009), combined with selective *in vitro* experiments on adventitial fibroblasts. As is the case in humans (Kawasaki *et al.* 1987; Tanaka *et al.* 1998), the B6D2F1 mouse demonstrates stiffening of the carotid artery with ageing in the absence of increases in peripheral artery stiffness (Lesniewski *et al.* 2009). Voluntary wheel running was used as a model of voluntary aerobic exercise in humans (Bradley *et al.* 2008; Durrant *et al.* 2009).

Methods

Animals

B6D2F1 mice were obtained from the National Institute on Ageing rodent colony. All mice were housed in an animal care facility at the University of Colorado at Boulder on a 12:12 light:dark cycle. Twenty young (4–7 months) and 20 old (29–32 months) male B6D2F1 mice were fed normal rodent chow *ad libitum* and housed in standard mouse cages or in cages with running wheels 10–14 weeks prior to killing. The 10–14 weeks of voluntary running was based on prior work from our laboratory demonstrating that exercise interventions of this length were sufficient to induce improvements in vascular function in both older mice (Durrant *et al.* 2009) and middle-aged and older adult humans (DeSouza *et al.* 2000; Tanaka *et al.* 2000). Running distance was measured as reported recently (Durrant *et al.* 2009). All procedures were approved by University of Colorado at Boulder Animal Care and Use Committee and conformed to the US National Institutes of Health guidelines set out in the *Guide to the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996).

Carotid artery stiffness measurements

Mice were killed in random order by exsanguinations via cardiac puncture while under isoflurane anaesthesia. Carotid arteries were excised and incubated in a myograph chamber (DMT, Inc., Atlanta, GA, USA) containing Ca^{2+}-free PSS for 1 h at 37°C. Intraluminal pressure was increased (5 and 20–200 mmHg, in 20 mmHg increments) and lumen diameter and medial wall thickness were measured. To account for potential individual or group differences in baseline diameter, passive-pressure relations were expressed relative to lumen diameter measured at 100 mmHg. Circumferential stress, stretch and incremental stiffness were calculated as previously described (Muller-Delp *et al.* 2002; Lesniewski *et al.* 2009). Wall thickness was measured at each pressure increment and used in the calculation of circumferential stress.

Immunohistochemistry

Carotid arteries were excised and frozen in optimal cutting temperature compound (Fisher Scientific, Pittsburgh, PA, USA) in liquid nitrogen cooled isopentane. Sections (7 μm) were fixed with acetone and washed in Tris buffer. All slides were stained with the Dako EnVision+ System-HRP-DAB kit according to the manufacturer's protocol (Dako North America Inc., Carpinteria, CA, USA). Briefly, primary antibodies for collagen type I (1:4000, Millipore), collagen type III (1:2000, Millipore), α-elastin (1:25, Abcam Inc., Cambridge, MA, USA), lysyl oxidase (1:1000, Imgenex Corp., San Diego, CA, USA), MMP-2 (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), TGF-β1 (1:1000, Santa Cruz), fibronectin (1:4000, Sigma) and smooth muscle α-actin (1:1000, Epitomics Inc., Burlingame, CA, USA) were incubated for 1 h at 4°C. Labelled polymer secondary was applied for 30 min and staining was visualized after a 2-min

exposure to diaminobenzidine. Slides were counterstained with haematoxylin to visualize the nuclei. Slides were dehydrated and coverslipped.

Picro Sirius Red was used to stain for collagen fibres. Slides were stained with direct red (0.5 g, Sigma) in a saturated aqueous solution of picric acid (500 ml, Ricca Chemical Co., Stamford, CT, USA) for 30 min. Slides were washed twice in acidified water (5 ml acetic acid, glacial in 1 litre distilled water), dehydrated in three changes of 100% ethanol and coverslipped.

The Von Kossa stain (Polysciencs, Inc., Warrington, PA, USA) was used to stain calcification. Three per cent silver nitrate was applied to samples and exposed to UV light for 60 min. The slides were rinsed in three changes of distilled water, place in 5% sodium thiosulfate for 2 min and again rinsed in three changes of distilled water. Slides were counterstained with nuclear fast red for 5 min, dehydrated and coverslipped.

Quantification

Digital photomicrographs were obtained using a Nikon Eclipse TS100 photomicroscope, and quantification was performed with Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). To determine density, images were converted to a grey scale in the green channel. The entire region of interest, either media or adventitia, was quantified. A pixel-by-pixel analysis was performed with a predetermined and optimized model (Image-Pro Plus software). The data are presented as relative density (sample density/positive control density stained on the same day). To determine the percentage of smooth muscle α-actin cells in the adventitia, positive stained cells (brown) were divided by total cells (blue nuclei) and multiplied by 100. Percentage staining was determined by taking the area stained divided by the total area for the same region of interest.

Primary adventitial fibroblast cell culture

Two male Sprague–Dawley rats (6 months of age) were obtained from the University of Colorado at Boulder breeding colony. Rats were killed with an overdose of carbon dioxide. Adventitial fibroblasts were isolated as previously described (Pagano, 1997). Briefly, thoracic aortas were excised and cleared of perivascular adipose tissue. The vessel was cut longitudinally, endothelial cells scraped off and the medial smooth muscle cells were peeled from the adventitia. The adventitia was placed in Dulbecco's modified Eagle's medium (DMEM)–F12 containing 1 mg ml^{-1} collagenase for 4 h at 37°C. The digested tissue was centrifuged for 5 min at 675 *g* and the re-suspended pellet was plated in a T-75 flask and cells allowed to grow for 4–5 days. Cells were passaged using 1:1 phosphate-buffered saline (PBS)–trypsin–EDTA, plated at a density of 30% and 90–95% confluent cells were studied between passages 3 and 6.

To determine the effects of TGF-β1 (R&D Systems, Minneapolis, MN, USA) on collagen production, cells were grown to 90–95% confluence and serum starved for 24 h prior to experimentation. Cells were treated with TGF-β1 (10 ng ml^{-1}) and/or Tempol (100 μM) for 24 h and subsequently lysed in ice-cold RIPA buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride) containing protease (Protease Inhibitor Cocktail Tablet, Roche) and phosphatase (0.01% phoshatase inhibitor cocktail, Sigma) inhibitors. Protein concentrations were determined using a BCA colorimetric protein assay (Pierce (Thermo Scientific), Rockford, IL, USA). Five micrograms of protein containing 1 M DTT was loaded into a polyacrylamide gel, separated by electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked in Tris-buffered saline (TBS)–0.05% Tween (TBST) containing 5% non-fat milk for 1 h. The membrane was washed for 30–60 min with frequent changes of TBST. Membranes were incubated overnight at 4°C on a rocker with anti-type I collagen (1:2000, Millipore, Billerica, MA, USA). The membrane was washed for 30–60 min in TBST and a secondary HRP-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was applied and incubated on a rocker at room temperature for 1 h. Blots were developed using ECL (Pierce/Thermo Scientific) and bands were visualized using a digital acquisition system (ChemiDoc-It, UVP Inc., Upland, CA, USA). The blot was stripped and re-probed for glyceraldehyde phosphate dehydrogenase (GAPDH) (1:2000, Cell Signaling Technology, Inc., Danvers, MA, USA). Bands were analysed with ImageJ software (NIH). Collagen type I bands were normalized to GAPDH protein expression and the control group.

Superoxide production was assessed in control and TGF-β1 (10 ng ml^{-1}) treated cells via electron paramagnetic resonance spectroscopy using modified procedures that have been described previously (Rippe *et al.* 2010). Cells were grown to 90–95% confluence in a six-well plate and serum starved for 24 h before treatment. After the 24 h treatment period, the medium was removed and cells were washed once with oxygenated Krebs–Henseleit bicarbonate (KHB) buffer. Cells were lysed in 60 μl per well deoxygenated KHB buffer, the lysate was centrifuged at 500 *g* for 10 min and supernatant was removed. The pellet was resuspended in 90 μl of deoxygenated KHB buffer and 10 μl of 10 mM 1-hydroxy-3-carboxy-pyrrolidine (CPH) spin trap and incubated at 37°C for 1 h. Electron paramagnetic resonance (EPR) was then performed on each sample using a capillary tube.

Table 1. Animal characteristics

	YC	OC	YVR	OVR
Body weight (g)	39.2 ± 1.4	34.5 ± 0.7*	31.1 ± 0.5*	31.2 ± 1.3*†
Heart weight (mg)	188 ± 5	232 ± 12**	186 ± 4	205 ± 7**
Heart:body ratio (g/g × 100)	0.50 ± 0.03	0.65 ± 0.03*	0.60 ± 0.02*	0.65 ± 0.02*
Gastrocnemius (mg)	183 ± 9	138 ± 5**	204 ± 13	153 ± 11**
Gastrocnemius:body ratio (g/g × 100)	0.47 ± 0.03	0.41 ± 0.02**	0.65 ± 0.04††	0.48 ± 0.03**††
Carotid artery diameter (μm)	395 ± 5	430 ± 6**	398 ± 5	416 ± 6**
Average running distance (km)			10.6 ± 0.49	1.13 ± 0.29*

Values and means ± S.E.M.; *$P < 0.05$ *vs.* YC; †$P < 0.05$ *vs.* OC; **$P < 0.05$ main effect of age; ††$P < 0.05$ main effect of voluntary running. YC, young control; OC, old control; YVR, young voluntary running; OVR, old voluntary running.

Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). A two-way ANOVA was used to analyse stiffness and animal characterization data. All histology and Western blot data were analysed with a one-way ANOVA and EPR data were analysed with Student's *t* test. Least squared difference *post hoc* tests were used where appropriate. Significance was set at $P < 0.05$.

Results

Animal characteristics are shown in Table 1. Body mass was lower in the old compared with the young cage control mice, and in the wheel running compared with the cage control groups (both $P < 0.05$). Heart weight was greater in the old compared with the young groups ($P < 0.05$), and the heart to body weight ratio was greater in all groups compared with the young cage controls ($P < 0.05$). Gastrocnemius muscle weight (absolute and the ratio to body weight) was lower in the old compared with the young groups ($P < 0.05$), and increased (young, $P < 0.05$) or tended to increase (old, $P = 0.06$) with wheel running.

Maximal carotid artery diameter was greater in the old compared with the young groups ($P < 0.05$). Both young and old mice ran voluntarily when placed in cages equipped with running wheels; however the daily running distance of the old mice was only ∼10% of that observed in the young mice ($P < 0.05$), as described previously (Durrant *et al.* 2009).

Carotid artery stiffness

Incremental stiffness (Fig. 1*A*) was greater ($P < 0.05$) in old compared with young cage control mice due to a combination of reduced passive distention to high intraluminal pressures (Fig. 1*B*) and increased circumferential stretch to low intraluminal pressures (Fig. 1*C*) (both $P < 0.05$). The resultant reductions in passive distention and increased circumferential stress in aged arteries demonstrate an inability of the vessels to adequately dilate at high pressures and relax at low pressures, respectively, both of which contribute to arterial stiffening. Wheel running had no effect on incremental stiffness in young mice, but completely reversed carotid artery stiffening with ageing ($P < 0.05$). The normalization of carotid artery stiffness by wheel running in old mice was mediated by the combination of greater distention and circumferential stretch compared with old cage control animals (both $P < 0.05$). Because wheel running affected carotid artery stiffness only in the old mice, histochemical analyses are presented below for young and old cage control and old wheel running groups, with one noted exception.

Carotid artery collagen and related proteins

As assessed by Picro Sirius Red staining, total collagen did not differ with age in the whole artery, media or adventitia in the cage control mice, nor were there differences between the old cage control and wheel running groups (Fig. 2*A*). Total collagen in the whole artery was slightly (∼5%) lower ($P < 0.05$) in the old wheel running mice than in the young cage controls, but mean values in the media and adventitia were not significantly different among the three groups (Fig. 2*A*).

In contrast to total collagen, collagen I (Fig. 2*B*) was greater ($P < 0.05$) and collagen III (Fig. 2*C*) tended to be greater ($P = 0.19$) in the whole artery of old compared with young cage control mice, solely as a result of greater expression in the adventitia (both $P < 0.05$). Wheel running reversed the age-associated increases in collagen I and III in the adventitia, and also reduced the expression of these proteins in the media and whole artery (all $P < 0.05$). Because expression of collagen I and III in old wheel running mice was below that of young cage control mice, we also assessed these proteins in the carotid arteries of the young wheel running mice (Table 2). Although collagen I and III were lower in medial layer of young running *vs.* cage control mice ($P < 0.05$), there were no differences in the adventitia of the two groups, coinciding with the stiffness measurements.

Given that the increases in collagen I and III with ageing were confined to the adventitia, we first determined if

TGF-β1, a profibrotic cytokine implicated in stimulating collagen deposition, was altered in the adventitial layer of the carotid artery. Similar to collagen I and III, expression of TGF-β1 protein was greater ($P < 0.05$) in the adventitia of carotid arteries from old compared with young cage control mice, and this was reversed by wheel running (Fig. 3*A*). The greater collagen I and III and TGF-β1 in adventitia of old compared with young cage control animals was associated with a substantial increase in smooth muscle α-actin, a marker of adventitial fibroblast transformation to a secretory (i.e. myofibroblast or collagen-producing) phenotype (Fig. 3*B*). Wheel running reduced the age-associated increase in smooth muscle α-actin by ∼50% ($P < 0.05$ *vs.* old cage control).

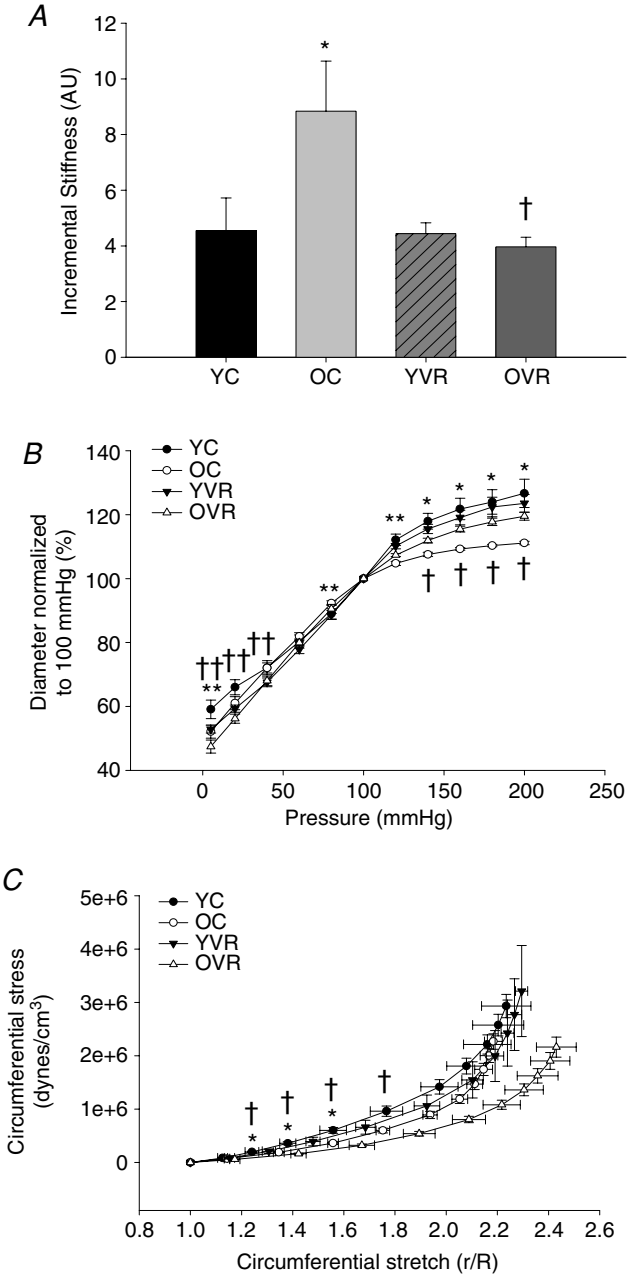

Figure 1. Carotid artery stiffness
Carotid artery incremental stiffness (*A*), passive pressure–diameter relations (*B*) and circumferential stress and stretch relations (*C*) from young control (YC), old control (OC), young voluntary running (YVR) and old voluntary running (OVR) mice ($n = 8$–11/group); values are means ± S.E.M. $^{*}P < 0.05$ *vs.* YC; †$P < 0.05$ *vs.* OC; $^{**}P < 0.05$ main effect of age; ††$P < 0.05$ main effect of training.

TGF-β1 stimulation of collagen I expression *in vitro*: role of oxidative stress

To obtain further evidence that the increases in TGF-β1 may have contributed to increased collagen deposition in carotid arteries with ageing (and normalization of collagen with wheel running) and that superoxide signalling may be involved, we performed additional experiments in cultured fibroblasts. TGF-β1 increased superoxide production (Fig. 4*A*) and collagen I protein expression (Fig. 4*B*) in fibroblasts (both $P < 0.05$). The TGF-β1 induced increase in collagen I was abolished by Tempol, a superoxide dismutase mimetic (Fig. 4*B*), suggesting that the increase was mediated by superoxide-associated oxidative stress.

Carotid artery elastin and related proteins

Elastin was lower ($P < 0.05$) in the whole carotid artery in the old compared with young cage control mice, and this was solely the result of lower expression in the media ($P < 0.05$), as adventitial elastin was not different in the two groups (Fig. 5*A*). The lower elastin in the whole carotid artery and media in the old cage control mice was associated with lower expression of the pro-synthetic elastin enzyme lysyl oxidase (Fig. 5*B*), and greater expression of the elastin degrading enzyme MMP-2 (Fig. 5*C*). In general, wheel running did not affect the age-associated changes in elastin, lysyl oxidase and MMP-2 (Fig. 5*A*–*C*).

Carotid artery fibronectin and calcification

Fibronectin did not differ with age in the cage control mice and was unaffected by wheel running (Table 3). There was only modest staining for calcification in the carotid arteries in all groups. However, calcification was greater in the old compared with the young cage control animals, and this effect of ageing was abolished in the old exercising mice (Table 3, all $P < 0.05$).

Table 2. Collagens I and III in carotid arteries

	Collagen I density (AU)			Collagen III density (AU)		
	Whole vessel	Media	Adventitia	Whole vessel	Media	Adventitia
YC	2.45 ± 0.23	1.42 ± 0.29	1.27 ± 0.18	2.06 ± 0.18	1.28 ± 0.12	0.77 ± 0.10
OC	3.79 ± 0.41*	1.48 ± 0.26	2.32 ± 0.17*	2.36 ± 0.12	1.29 ± 0.07	1.07 ± 0.09*
YVR	1.90 ± 0.42	0.98 ± 0.35††	0.91 ± 0.14	1.71 ± 0.32	0.97 ± 0.24††	0.74 ± 0.10
OVR	1.39 ± 0.29†	0.54 ± 0.16††	0.84 ± 0.22†	1.18 ± 0.17†	0.65 ± 0.10††	0.53 ± 0.10†

Values and means ± S.E.M.; *$P < 0.05$ *vs.* YC; †$P < 0.05$ *vs.* OC; ††$P < 0.05$ main effect of voluntary running. YC, young control; OC, old control; YVR, young voluntary running; OVR, old voluntary running.

Discussion

The present study is the first to assess the possible cellular and molecular mechanisms by which voluntary aerobic exercise reduces large elastic artery stiffening with ageing. To our knowledge, these also are the first data on mechanisms of age-associated arterial stiffening using a mouse model.

We found that the increase in carotid artery stiffness with ageing in B6D2F1 mice was associated with increases in collagen I and III in the adventitia, which were, in turn, related to increases in adventitial TGF-β1 and evidence for a shift to a myofibroblast (secretory) phenotype. *In vitro* experiments in fibroblasts confirmed the collagen-stimulating effects of TGF-β1 in these cells by a mechanism dependent on superoxide. Carotid stiffening with age also was associated with reductions in elastin in the media, which were related to decreases in lysyl oxidase and increases in MMP-2. Fibronectin was not altered with ageing, but a small degree of calcification was observed. Importantly, voluntary aerobic exercise

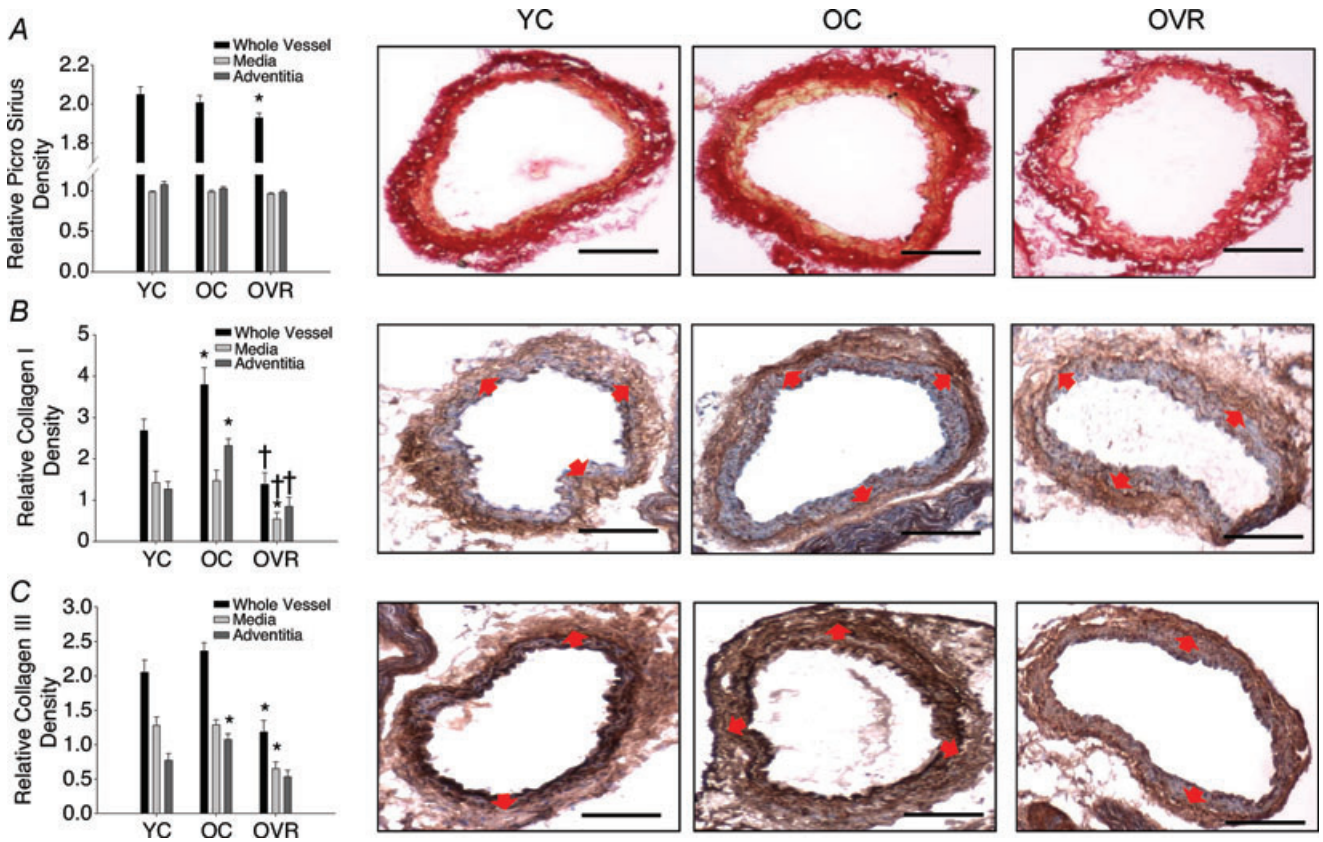

Figure 2. Carotid artery collagen protein expression
Carotid artery staining for total collagen (Picro Sirius Red) (*A*), collagen type I (*B*) and collagen type III (*C*) from young control (YC), old control (OC) and old voluntary running (OVR) mice ($n = 5$–7/group); values are means ± S.E.M. *$P < 0.05$ *vs.* YC, †$P < 0.05$ *vs.* OC. Representative images are presented and red arrows demarcate the medial–adventitial border; bar = 100 μm.

ameliorated carotid artery stiffening in old mice. This was associated with reductions in collagen I and III, decreased TGF-β1 and a partial reversal of the myofibroblast phenotype. Voluntary exercise also reduced calcification in old mice, but did not affect elastin or its modulating enzymes.

Carotid artery stiffness

In the present study, *ex vivo* carotid artery stiffness was increased with ageing in cage control B6D2F1 mice as reported previously (Cox, 1983; Simon & Danneman, 2005; Lesniewski *et al.* 2009), and observed *in vivo* in older healthy adult humans (Tanaka *et al.* 2000; Moreau *et al.* 2003). The present investigation extends earlier findings on ageing by demonstrating that 10–14 weeks of voluntary wheel running completely reverses carotid artery stiffening in old mice, restoring levels to those observed in young adult animals. Remarkably, this occurred despite the fact that old mice ran only ∼10% as much as the young mice. Thus, even small amounts of voluntary wheel running exert a potent physiological stimulus on carotid artery stiffness. Wheel running had no effect on the already normal levels of stiffness in young mice, as noted previously in humans (Tanaka *et al.* 2000). The present results are in partial agreement with earlier observations in rats in which forced swimming reduced the incremental elastic modulus in aorta by ∼40% in old animals (Nosaka *et al.* 2003). It is not clear if the different degrees of adaptation in the two studies are due to differences in species, type or voluntariness of the exercise (Niederhoffer *et al.* 2000), or other factors.

Collagen, myofibroblast phenotype and TGF-β1

Collagen I and III are the primary load-bearing proteins that comprise ∼90% of all collagens in the arterial wall (Diez, 2007). Increases in collagen expression and cross-linking are believed to contribute importantly to increases in large-elastic artery stiffness with ageing (Lakatta & Levy, 2003). Although results of previous studies vary (Cox, 1983; Maurel *et al.* 1987; Brüel & Oxlund, 1996; Wang & Lakatta, 2002; Qiu *et al.* 2007*a*,*b*; Wang *et al.* 2007), increased expression of both collagen I and collagen III is found in aorta of older compared with young adult human donors in the absence of clinical CVD (Wang *et al.* 2007). In agreement with this, in the present study we found increased abundance of collagens I and III in whole carotid arteries of old compared with young cage control mice as a result of greater expression in the adventitia, whereas total and medial collagen were not increased. Carotid artery stiffness also was selectively associated with adventitial collagen in the young cage control and wheel running groups. These observations are consistent with results from a recent *ex vivo* analysis of human carotid arteries suggesting that the adventitia is the key load-bearing component of the wall under physiological conditions (Sommer *et al.* 2010).

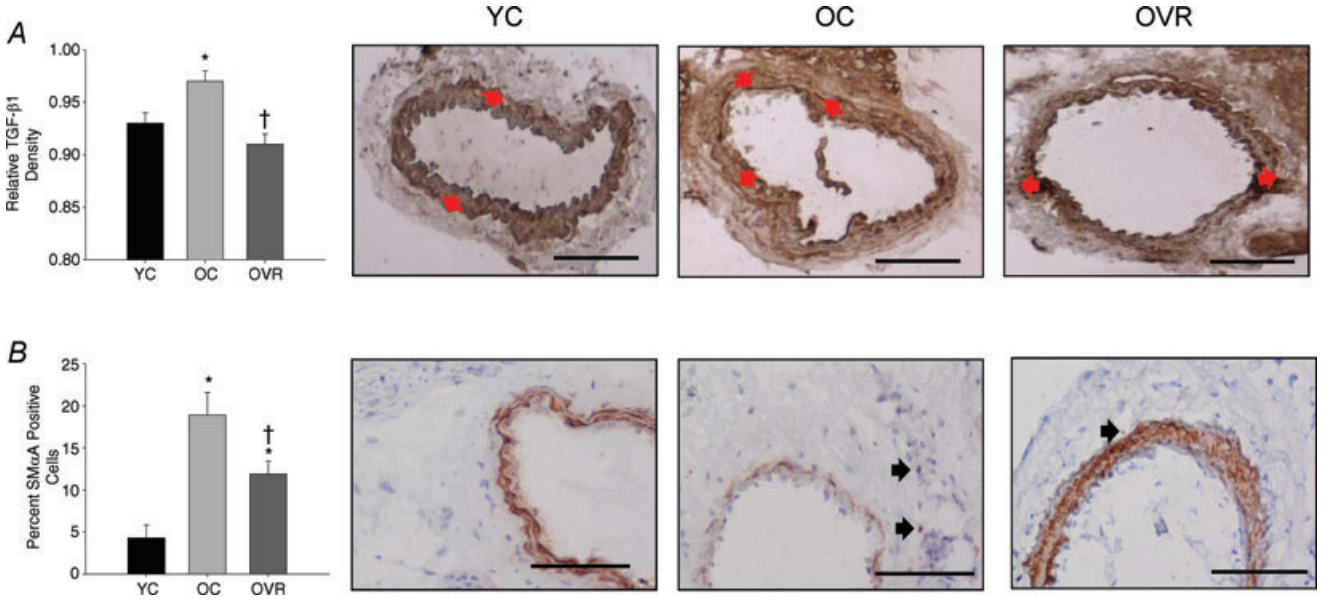

Figure 3. Carotid artery transforming growth factor-β1 and smooth muscle α-actin protein expression
Adventitial transforming growth factor-β1 (TGF-β1) density (*A*) and the percentage smooth muscle α-actin (SMαA) positive cells (*B*) in the adventitia from young control (YC), old control (OC) and old voluntary running (OVR) mice ($n = 4$–11/group); values are means ± S.E.M. $^*P < 0.05$ *vs.* YC, †$P < 0.05$ *vs.* OC. Representative images are presented and black arrows denote smooth muscle alpha actin positive cells; bar = 100 μm.

The mechanisms by which ageing causes greater accumulation of these collagens in the adventitia of carotid arteries is unknown. We hypothesized that increases in TGF-β1, a profibrotic cytokine implicated in both general and vascular-specific fibrosis (Blobe *et al.* 2000; Ruiz-Ortega *et al.* 2007), could be involved, perhaps in part by stimulating a change in adventitial fibroblasts to a myofibroblast (collagen secreting) phenotype. In agreement with this, we found that carotid adventitia of old mice demonstrate greater expression of TGF-β1 and smooth muscle α-actin, a myofibroblast marker (Siow & Churchman, 2007). Additional experiments in cultured fibroblasts demonstrated that TGF-β1 stimulates superoxide production and collagen I deposition. The latter was dependent on superoxide, as it was blocked by administration of Tempol, a superoxide dismutase mimetic. It also is possible that increases in nitric oxide bioavailability with voluntary running in old mice (Durrant *et al.* 2009) was involved in the exercise-related inhibition of adventitial TGF-β1 expression observed, as demonstrated in other models (Koyanagi *et al.* 2000). Taken together, our results provide new evidence that TGF-β1 may stimulate collagen deposition in the adventitia, which, in turn, contributes to the stiffening of carotid arteries with ageing, perhaps in part by an oxidative stress-related transformation of adventitial fibroblasts into collagen synthesizing/secreting myofibroblasts.

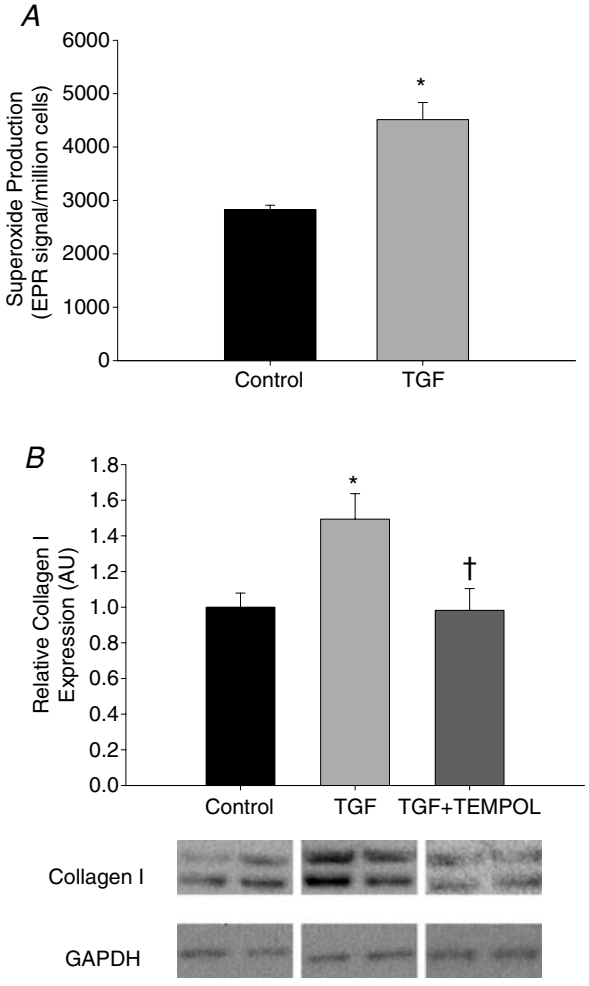

Figure 4. Effects of transforming growth factor-β1 on superoxide and collagen I protein production
Superoxide production (*A*) and collagen I expression (*B*) in cultured adventitial fibroblasts treated with transforming growth factor-β1 (TGF-β1, 10 ng ml^{-1}) or TGF-β1 + Tempol for 24 h (n = 4–8/group); values are means ± S.E.M. $^*P < 0.05$ *vs.* Control, †$P < 0.05$ *vs.* TGF-β1 treatment.

A previous study reported no effects of forced swimming on total collagen in aorta of old rats, despite improvements in stiffness (Nosaka *et al.* 2003). In the present study, we observed that wheel running had only a small influence on total collagen in the carotid artery. However, we found that normalization of carotid artery stiffness by wheel running in old mice was associated with reductions in collagen I and III to, or even below, levels observed in young control mice. Unlike ageing, the effects of exercise were observed in both the adventitia and media. Wheel running also completely reversed the increase in carotid adventitial TGF-β1 observed with ageing and reduced smooth muscle α-actin. Collectively, these findings support the possibility that voluntary aerobic exercise may 'de-stiffen' the carotid artery of old B6D2F1 mice by reversing the age-associated increases in collagen I and III. The latter may be mediated in part by reversing the age-related transformation of fibroblasts into myofibroblasts in the adventitia, a layer implicated in arterial stiffening with ageing (Schulze-Bauer *et al.* 2002; Sommer *et al.* 2010). However, the fact that wheel running reduced collagen I and III even in the media of carotid arteries suggests that other, yet to be identified, mechanisms may have been acting on vascular smooth muscle cells.

Elastin and related enzymes

Consistent with data from several previous investigations (Cox, 1983; Sauvage *et al.* 1998; Wang *et al.* 2002; Qiu *et al.* 2007*a*,*b*), we found that elastin was lower, albeit only modestly, in whole carotid arteries of old compared with young cage control mice. In agreement with an earlier report in aorta of rats (Wang & Lakatta, 2002), this was the result of a small reduction in expression of elastin in the media. The lower whole artery and medial elastin in old cage control mice was associated with reduced expression of lysyl oxidase, an enzyme involved in elastin maturation (Bedell-Hogan *et al.* 1993; Kothapalli & Ramamurthi, 2009), and increased expression of MMP-2, an enzyme involved in elastin fibre degradation and remodelling (Emonard & Hornebeck, 1997; Sternlicht & Werb, 2001).

Table 3. Fibronectin and calcification in carotid arteries

	Fibronectin density (AU)			Calcification (% stained)		
	Whole vessel	Media	Adventitia	Whole vessel	Media	Adventitia
YC	3.78 ± 0.30	2.50 ± 0.07	1.29 ± 0.05	0.22 ± 0.06	0.70 ± 0.36	0.07 ± 0.05
OC	3.86 ± 0.33	2.48 ± 0.08	1.38 ± 0.05	2.39 ± 0.87*	3.54 ± 1.70	1.24 ± 0.45*
OVR	3.57 ± 0.30	2.35 ± 0.11	1.22 ± 0.05	0.64 ± 0.30†	1.24 ± 0.55	0.22 ± 0.06†

Values and means ± S.E.M.; *$P < 0.05$ *vs.* YC; †$P < 0.05$ *vs.* OC. YC, young control; OC, old control; OVR, old voluntary running.

Although MMP-2 has been shown to increase with age in the whole carotid (Wang *et al.* 2005), to our knowledge, this is the first report of reduced lysyl oxidase expression with large elastic artery ageing and the first evidence that the changes in expression of both enzymes in the whole artery are primarily the result of changes in the media.

Importantly, here we show that neither elastin nor these two elastin-modulating enzymes appear to be key mechanisms involved in the de-stiffening effects of wheel running in carotid arteries of B6D2F1 mice, as they were not different in old exercising compared with cage control animals. The observation of unaltered whole artery elastin in old mice with wheel running in the present study is in agreement with previous observations in aorta of old rats in response to swimming (Nosaka *et al.* 2003). Collectively, our results and those from the previous study (Nosaka *et al.* 2003) suggest that regular aerobic exercise does not reduce large elastic artery stiffness via changes in elastin.

Fibronectin and calcification

Expression of fibronectin, a glycoprotein that influences stiffness by binding to extracellular matrix proteins including integrins, collagen and proteoglycans (Hynes

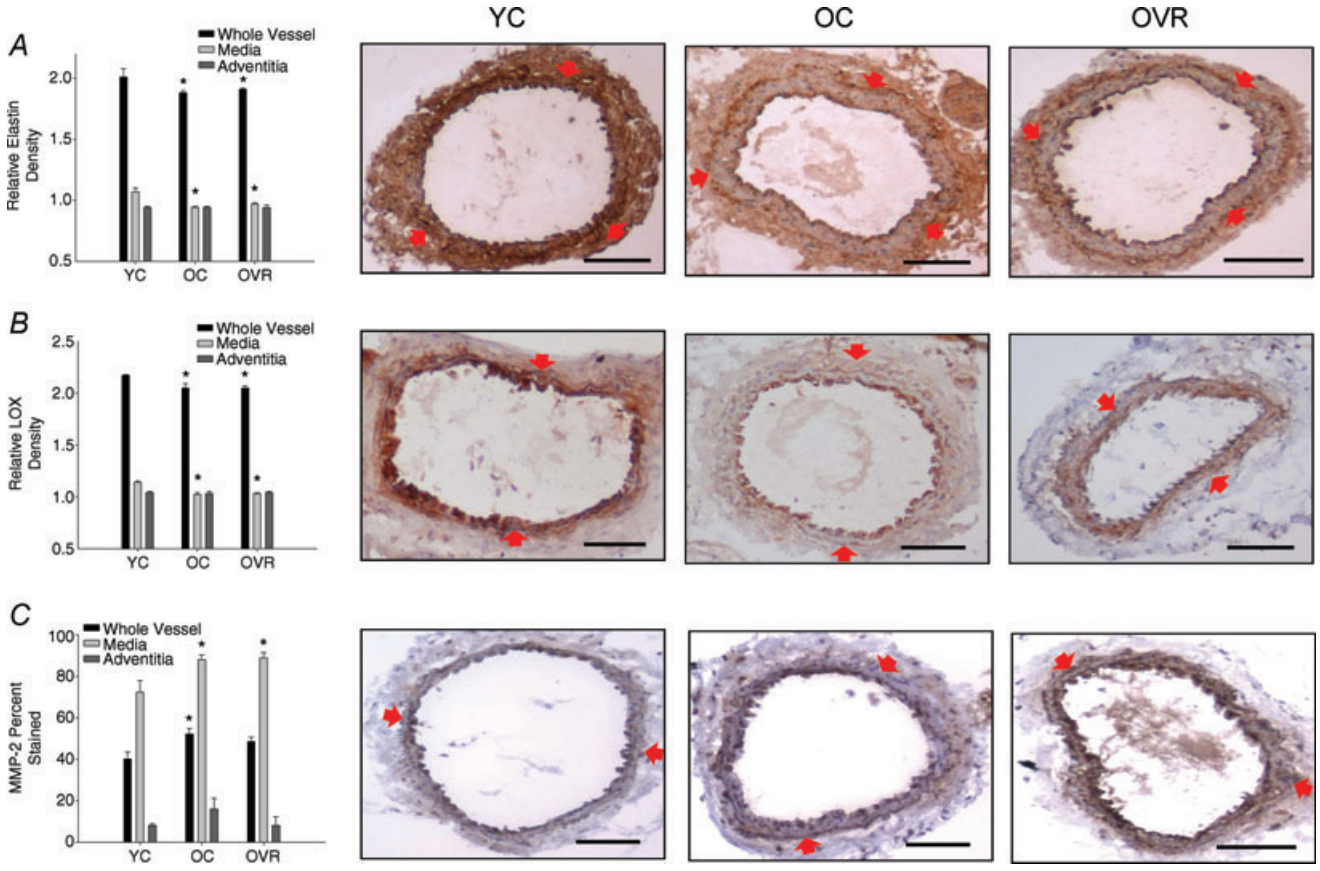

Figure 5. Carotid artery elastin, lysyl oxidase and matrix metalloproteinase 2 protein expression
Elastin (*A*), lysyl oxidase (LOX) (*B*) and matrix metalloproteinase 2 (MMP-2) (*C*) densities from young control (YC), old control (OC) and old voluntary running (OVR) mice ($n = 4$–8/group; values are means ± S.E.M. *$P < 0.05$ *vs.* YC. Representative images are presented and red arrows demarcate the medial–adventitial border; bar = 100 μm.

& Yamada, 1982), has been reported to increase in whole aorta with ageing in rats (Li *et al.* 1999; Wang *et al.* 2006). However, in the present study we found no influence of either age or wheel running on fibronectin in the whole carotid artery or in the media or adventitia. As such, age-related increases in carotid artery stiffness are not obviously associated with increased fibronectin, nor does it appear that wheel running de-stiffens the carotid arteries of old mice by reducing this protein.

Calcification of aorta in rats is reported to either increase (Kieffer *et al.* 2000) or not change (Brüel & Oxlund, 1996) with ageing in the absence of disease. Here we found modest, but greater calcification of the carotid artery with ageing in B6D2F1 mice. We also found that wheel running reversed this calcification in old mice to levels not significantly different from that observed in young mice. It is uncertain if such small changes in calcification could contribute to increases in carotid artery stiffness with ageing or play a role in the stiffness-reducing influence of wheel running. Our findings are in contrast to a previous report in rats that observed no consistent associations between age or forced swimming and aortic calcification (Nosaka *et al.* 2003), but in agreement with an earlier study in young rats (Matsuda *et al.* 1993).

Calcification is mediated by several cellular events that include oxidant stress, inflammation and elastolysis. In our aged B6D2F1 mice, elastin content was reduced in the medial layer of the carotid artery. Such elastolysis can produce elastin-derived peptides that, in turn, promote an osteogenic phenotype in smooth muscle cells (Simionescu *et al.* 2005). However, elastin content was not altered with voluntary running. On the other hand, adventitial TGF-β1 was increased with ageing and reduced with voluntary wheel running. TGF-β1 can induce an osteogenic phenotype in fibroblasts (Simionescu *et al.* 2007), suggesting this molecule could have contributed to altered calcification in the adventitia. Changes in other proteins also could be involved. For example, bone morphogenetic proteins 2 and 7 are members of the TGF-β superfamily that are implicated in promoting (Hruska *et al.* 2005) and reversing (Kang *et al.* 2010) calcification, respectively, and are additional potential mechanisms of action for modulation of vascular calcification with ageing and voluntary wheel running in the present study.

Limitations

The present study has several limitations that should be noted. Cross-linking of extracellular proteins contributes to large elastic artery stiffening with ageing (Semba *et al.* 2009), and reductions in cross-linking reduce stiffness (Kass *et al.* 2001). However, protein cross-linking was not assessed in the present study. Moreover, only male mice were studied. Although in humans, large elastic arteries stiffen with ageing and this is reduced with regular aerobic exercise in both men and women (Tanaka *et al.* 1998; Tanaka *et al.* 2000; Moreau *et al.* 2003), it is possible that the effects of ageing and exercise differ in female mice. Finally, our results regarding the mechanisms by which ageing and regular exercise modulate carotid artery stiffness in mice are association-based. Direct cause and effect evidence linking these events is needed to more definitively establish the mechanisms involved. In this context, the ageing and exercise training of conditional knockout mice may be required to demonstrate the role of TGF-β1 in arterial stiffening.

Conclusions

The results of this study provide evidence that increases in carotid artery stiffness with ageing are associated with TGF-β1-stimulated increases in adventitial collagen I and III related to changes in fibroblasts to the more secretory myofibroblast phenotype. Our findings also indicate that reductions in elastin in the medial layer of the carotid may contribute to stiffening, in part as a result of alterations in the elastin-modulating proteins lysyl oxidase and MMP-2. A small degree of calcification also occurs with ageing and could contribute to stiffening.

Importantly, even modest levels of regular voluntary aerobic exercise, a first-line therapy for preventing CVD in humans, completely reverse carotid artery stiffening with ageing in B6D2F1 mice. The de-stiffening effects of aerobic exercise on carotid arteries of old mice are associated with reductions in collagen I and III in both the adventitia and the media, as well as adventitial TGF-β1, and a partial reversal of the age-related shift to a myofibroblast phenotype. Voluntary aerobic exercise also may contribute to reductions in stiffness by reversing age-associated calcification of arteries.

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Author contributions

B.S.F., D.R.S. and L.A.L. contributed to the conception and design of the studies. All authors contributed to analysis and interpretation of data and the drafting and revision of the article, and provided final approval of the version to be published. All experiments were carried out at the University of Colorado at Boulder.

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