Mitochondrial Oxidative Stress in Aortic Stiffening With Age

The Role of Smooth Muscle Cell Function


Objective—Age-related aortic stiffness is an independent risk factor for cardiovascular diseases. Although oxidative stress is implicated in aortic stiffness, the underlying molecular mechanisms remain unelucidated. Here, we examined the source of oxidative stress in aging and its effect on smooth muscle cell (SMC) function and aortic compliance using mutant mouse models.

Methods and Results—Pulse wave velocity, determined using Doppler, increased with age in superoxide dismutase 2 (SOD2)+/− but not in wild-type p47phox−/− and SOD1+/− mice. Echocardiography showed impaired cardiac function in these mice. Increased collagen I expression, impaired elastic lamellae integrity, and increased medial SMC apoptosis were observed in the aortic wall of aged SOD2+/− versus wild-type (16-month-old) mice. Aortic SMCs from aged SOD2+/− mice showed increased collagen I and decreased elastin expression, increased matrix metalloproteinase-2 expression and activity, and increased sensitivity to staurosporine-induced apoptosis versus aged wild-type and young (4-month-old) SOD2+/− mice. Smooth muscle α-actin levels were increased with age in SOD2+/− versus wild-type SMCs. Aged SOD2+/− SMCs had attenuated insulin-like growth factor-1-induced Akt and Forkhead box O3a phosphorylation and prolonged tumor necrosis factor-α-induced Jun N-terminal kinase 1 activation. Aged SOD2+/− SMCs had increased mitochondrial superoxide but decreased hydrogen peroxide levels. Finally, dominant-negative Forkhead box O3a overexpression attenuated staurosporine-induced apoptosis in aged SOD2+/− SMCs.

Conclusion—Mitochondrial oxidative stress over a lifetime causes aortic stiffening, in part by inducing vascular wall remodeling, intrinsic changes in SMC stiffness, and aortic SMC apoptosis. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: aging ■ blood pressure ■ reactive oxygen species ■ signal transduction ■ vasodilation

Advancing age is the major risk factor for cardiovascular disease (CVD) morbidity and mortality. With aging, central arteries stiffen (and dilate) as a result of physiological remodeling arising from the fracture of elastin lamellae from repetitive pulsations and also from endothelial dysfunction, chronic low-grade inflammation, and altered vascular smooth muscle tone.1,2 Aortic stiffening is the principal cause of CVD with age in people without atherosclerosis,1 including increased systolic and pulse pressures, increased left ventricular hypertrophy and diastolic dysfunction, and congestive heart failure.3 Carotid-femoral pulse wave velocity (PWV), a direct noninvasive measure of the thoracic and abdominal aortic stiffness, is correlated with higher CVD events and is an independent predictor of coronary heart disease and stroke.4 Despite the strong epidemiological and biological connection of age to CVD risk, the molecular mechanisms responsible for age-related vascular dysfunction have yet to be elucidated. Although advancing age is an unmodifiable risk factor for CVD, it might be possible to target specific molecular signals as an approach to limiting age-related CVD risk.

Oxidative stress has been implicated in vascular dysfunction, whether as a result of CVD or aging or both.5–7 The free radical theory of aging, first proposed by Harman more than 50 years ago,8 suggested that increased reactive oxygen species (ROS) generation underlies many features of aging. Prior studies have indicated that increased vascular ROS generation results in decreased compliance, as measured by PWV.9,10 Recent studies suggest that mitochondrial dysfunction plays an important role in aging and impairing vascular function.11,12

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Many pro- and antioxidant enzymes regulate ROS levels in cells. Of these, the superoxide dismutase (SOD) family is the most studied antioxidant system and has been previously implicated in CVD. SODs convert superoxide to produce hydrogen peroxide (H₂O₂), which is further degraded by either catalase or glutathione peroxidase. One member of the SOD family, manganese SOD (SOD2), is present in mitochondria. Deletion of the SOD2 gene results in early postnatal lethality in mice. SOD2-deficient (SOD2<sup>−/−</sup>) mice are viable but demonstrate increased susceptibility to oxidative stress, diminished mitochondrial function, and enhanced sensitivity to apoptosis. In an atherosclerotic background (apolipoprotein E knockout), SOD2 deficiency results in accelerated atherosclerosis and endothelial dysfunction in mice. In addition, decreased expression/activity of SOD2 with age is implicated in vascular aging.

In the present study, we investigated the effect of oxidative stress in aging-associated increase in aortic stiffness using mutant mouse models. Our data indicate that prolonged exposure to increased mitochondrial oxidative stress decreases aortic compliance and induces cardiac dysfunction. Specifically, we elucidate the significance of lifelong SOD2 deficiency on the phenotype, function, and molecular signaling pathways in aortic smooth muscle cells (SMCs) and how these events regulate aortic wall homeostasis and aortic stiffening.

Materials and Methods

**Aortic PWV**
Arterial compliance was determined as described by Hartley et al. In brief, mice were anesthetized with inhaled isoflurane (1% in O₂) and fixed in a supine position on the temperature-controlled ECG board (THM100, Indus Instruments). Body temperature was maintained at 37°C and monitored with a rectal probe. Blood flow velocity was recorded using a 20-MHz pulsed Doppler probe at the levels of aortic arch and at the abdominal aorta. Data were analyzed using an Indus Instruments Doppler Signal Processing Workstation. Aortic PWV was calculated by dividing separation distance (40 mm) by difference in pulse wave arrival time in respect to ECG R-peaks.

**Echocardiography**
Mice were anesthetized with inhaled isoflurane (1% in O₂) and fixed in a supine position on the ECG temperature-controlled board. Ultrasound biomicroscopy was performed using VisualSonics Vevo 660 equipped with a 30-MHz probe. Ultrasound images of left ventricle were acquired at the long axis using M-mode. Measurements of interventricular septum, posterior wall thickness, and ventricle internal diameter at systole and diastole were taken. Values of ejection fraction, end diastolic volume, and myocardial mass were derived using VisualSonics Vevo 660 software.

**Blood Pressure**
Systolic and diastolic blood pressure was measured as described in the online-only Data Supplement.

**Vascular Relaxation in Isolated Mouse Aortic Rings**
Relaxation of isolated mouse aortic rings was measured as described in the online-only Data Supplement.

**Cell Culture and Materials**
Mouse aortic SMCs were isolated from young (4 months) and aged (16 months) wild-type and SOD2<sup>−/−</sup> mice (C57BL/6J) as described previously (see online-only Data Supplement).

**Histology, Immunohistochemistry, and Immunofluorescence**
Immunohistochemistry and immunofluorescence studies were performed as previously described.

**Western Blot Analysis**
Preparation of cell extracts and Western blot analysis was performed as described previously.

**Quantitative Real-Time Polymerase Chain Reaction**
Quantitative analysis of mRNA expression of target genes was performed using total RNA extracted from cells and tissues. Reverse transcription was performed using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). Real-time polymerase chain reaction was performed in quadruplicate with TaqMan Gene Expression Assays for mouse collagen I (Mm01302043_g1), elastin (Mm00439508_m1), and 18S rRNA (Hs99999901_s1) using an ABI Prism 7900 HT Sequence Detection System according to manufacturer’s recommended protocol. Target gene mRNA expression was normalized to 18S rRNA expression. Individual gene expression in SOD2<sup>−/−</sup> aortic SMCs was calculated relative to that in wild-type using REST2008 (Relative Expression Software Tool).

**Gelatin Zymography**
MMP-2 activity was assayed by gelatin zymography (see Methods in the online-only Data Supplement).

**Adenovirus Infection of Aortic SMCs**
A replication-defective adenoviral vector expressing dominant-negative Forkhead box O3a (DN-FOXO3a) was obtained from Vector Biolabs. DN-FOXO3a, constructed by deletion of the transactivation domain from the C-terminus, had an HA tag at the N terminus and expressed green fluorescent protein. Adenovirus expressing only green fluorescent protein was used as a negative control. Mouse aortic SMCs were cultured to 80% to 90% confluence before adenoviral infection. Infections were performed using a multiplicity of infection of 100, and the infection efficiency was typically greater than 90%. Measurement of proteins of interest was made in cells harvested 36 hours after viral infection.

**Detection of Mitochondrial Superoxide**
Mitochondrial superoxide levels in aortic SMCs were detected as described in the Methods in the online-only Data Supplement.

**H₂O₂ Measurement**
Aortic SMCs extracellular H₂O₂ levels were determined using the Amplex Red assay (Invitrogen) (see Methods in the online-only Data Supplement).

**Statistical Analysis**
Data presented graphically are shown as mean±SE from at least 3 independent experiments. All data were tested for normality using the Kolmogorov-Smirnov test and were analyzed by 1-way ANOVA, and post hoc analysis was performed using the Newman-Keuls test. To account for multiple comparisons, arterial compliance and cardiac function data were analyzed by 1-way ANOVA followed by the Ryan-Einot-Gabriel-Welsch multiple-range test with an overall α=0.05 (SSPS software, version 19.0).

**Results**
Aortic Compliance, Cardiac Function, and Vasorelaxation Are Decreased With Age in SOD2<sup>−/−</sup> Mice
To examine the interactive effect of oxidative stress, diet, and aging on vascular health, we measured aortic compliance in...
young (4 months) and aged (16 months) mice fed normal chow or Western diet. We used wild-type mice, as well as mice with decreased (p47phox<sup>-/-</sup>) or increased (SOD1<sup>+/+</sup>) cytosolic and increased mitochondrial (SOD2<sup>+/+</sup>) oxidative stress. There was no difference in central aortic compliance, as measured by PWV, between young and aged wild-type (Figure 1A), p47phox<sup>-/-</sup>, and SOD1<sup>+/+</sup> mice (data not shown), either on normal chow or Western diet. PWV was also not significantly different between young wild-type and SOD2<sup>+/+</sup> mice, whether on a normal chow or Western diet. However, aged SOD2<sup>+/+</sup> fed normal chow had significant increase in PWV compared with aged wild-type or young SOD2<sup>+/+</sup> mice (P<0.05 in each case; Figure 1A) on a normal chow diet. Similarly, aged SOD2<sup>+/+</sup> on Western diet had significantly increased PWV compared with aged wild-type or young SOD2<sup>+/+</sup> mice on Western diet (P<0.05 in each case). These data indicate that prolonged mitochondrial oxidative stress is sufficient to induce aortic stiffening.
To determine whether prolonged mitochondrial oxidative stress also affects cardiac function, we examined the above mentioned mice by echocardiography. Aged SOD2+/− had impaired left ventricular function, as indicated by significantly decreased ejection fraction (Figure 1B) compared with aged wild-type mice, whether on normal chow (P<0.01) or Western diet (P<0.05). In consonance with decreased ejection fraction, aged SOD2+/− had increased left ventricle end-diatolic volume compared with aged wild-type mice, whether on normal chow (P<0.05) or Western diet (P<0.05) (Figure 1C). Ejection fraction and left ventricle end-diatolic volume in aged SOD2+/− were significantly different from young SOD2+/− mice, irrespective of the diet. Left ventricle posterior wall thickness (Figure 1D) and left ventricle mass (Figure 1E) also increased in aged SOD2+/− compared with aged wild-type and young SOD2+/− mice, independent of diet. Together, these data suggest that long-term exposure to increased mitochondrial oxidative stress causes adverse effects on vascular health, as evidenced by increased arterial stiffening and impaired cardiac function.

Because blood pressure is an important determinant of PWV,29 we measured changes in blood pressure with aging. No significant difference was observed in systolic blood pressure between wild-type and SOD2+/− mice (Table I in the online-only Data Supplement). Diet and age had no effect; however, SOD2 deficiency significantly increased diastolic blood pressure (Table I in the online-only Data Supplement), indicating that enhanced diastolic blood pressure associated with prolonged mitochondrial oxidative stress may contribute to aortic stiffening. To determine the interaction of age and SOD2 deficiency on SMC function, we measured nitroglycerine-induced relaxation of phenylephrine-preconstricted thoracic aortic rings. Wild-type mice had decreased vascular relaxation with age at 10−5 mol/L nitroglycerine (P<0.01 versus young) (Figure 1F in the online-only Data Supplement). At this concentration, young and aged SOD2+/− had impaired vascular relaxation compared with young wild-type mice (P<0.001). No significant difference was observed in nitroglycerine-induced relaxation between SOD2+/− and aged wild-type mice. However, at 10−6 mol/L nitroglycerine, SOD2+/− had impaired aortic relaxation compared with aged wild-type mice (P<0.01, young SOD2+/− versus aged wild-type; P<0.05, aged SOD2+/− versus aged wild-type). SOD2 deficiency had impaired vascular relaxation independent of age. These data indicate that aging in general and increased mitochondrial oxidative stress in particular impair vascular SMC function and hence vascular relaxation.

Collagen Levels Are Increased and Elastin Levels and Integrity Are Decreased With Age in the Aortic Wall and SMCs of SOD2+/− Mice

Because a decrease in the elastin/collagen ratio is associated with increase in aortic stiffness30 and increased aortic oxidative stress is correlated with extensive collagen deposition and elastin degradation and decline in aortic compliance,31 we examined aortic collagen and elastin expression in the aortic wall of wild-type and SOD2+/− mice by immunohistochemistry. Collagen I expression was increased in the media of aged SOD2+/− compared with aged wild-type mice (Figure 2A). The elastic laminae in the media were normal in aged wild-type mice, but their integrity was compromised with ruptures in aged SOD2+/− mice (Figure 2A). No perceptible increase in collagen I or ruptures in elastic lamina were observed in the aortas of young SOD2+/− mice (data not shown). Because increased calcification is implicated in aortic stiffening,32 we examined calcium deposition in the aortic sections. We did not detect any calcium deposition or focal calcification in aged wild-type or SOD2+/− mice.

To determine whether the changes in aortic collagen expression and elastin integrity represent the intrinsic effect of SOD2 deficiency, we examined collagen I and elastin expression in SMCs. Real-time reverse transcription–polymerase chain reaction analysis showed a significant increase in collagen I mRNA levels in aged SOD2+/− compared with aged wild-type aortic SMCs (1.7±0.1-fold increase; P<0.01). In contrast, elastin mRNA levels were significantly lower in aged SOD2+/− SMCs (2.5±0.1-fold decrease versus aged wild-type; P<0.001). Increase in collagen I mRNA levels was followed by a significant increase in collagen I protein levels in aged SOD2+/− SMCs (2.2-fold increase versus aged wild-type; P<0.01; Figure 2B). Similarly, elastin protein levels were decreased nearly 7-fold in aged SOD2+/− SMCs compared with aged wild-type SMCs (P<0.05; Figure 2B). Taken together, these data suggest that prolonged exposure to mitochondrial oxidative stress during aging induces structural changes in the arterial wall by regulating collagen levels, as well as elastin synthesis and degradation.

MMP-2 Expression and Activity Are Increased in SOD2+/− Aortic SMCs

MMP-2 is a critical regulator of extracellular matrix degradation and age-associated vascular remodeling33 and has been implicated in arterial stiffening.34 A 3.2±0.8-fold increase in MMP-2 mRNA expression was observed in aged SOD2+/− compared with aged wild-type SMCs (P<0.001) as determined by real-time reverse transcription–polymerase chain reaction. MMP-2 activity was significantly increased (P<0.001) in both young and aged SOD2+/− compared with wild-type SMCs (Figure 2C). These data suggest that mitochondrial oxidative stress activates signaling pathways involved in MMP-2 expression and activity.

Prolonged SOD2 Deficiency Renders Aortic SMCs Susceptible to Apoptosis and Impairs Antiapoptotic Akt Pathway

A decrease in arterial medial SMC number and vascular remodeling with aging has been attributed to increased apoptosis,35 and we and others have shown that increased mitochondrial oxidative stress is an important regulator of SMC apoptosis.36,37 As shown in Figure 3A, immunofluorescence staining for the cleaved form of caspase-3, a member of the caspase superfamily that initiates apoptotic events, is increased in medial SMCs of aged SOD2+/− mice. Cleaved caspase-3 was barely detectable in young SOD2+/− mice (data not shown) and not observed in the aortic walls of either young or aged wild-type mice. Similarly, we did not find any apoptosis in the hearts of either aged wild-type or SOD2+/− mice (data not shown).
Figure 2. Superoxide dismutase 2 (SOD2) deficiency increased collagen I synthesis and disrupted elastic laminae in aortas of aged mice, increased collagen levels and decreased elastin levels in aged aortic smooth muscle cells (SMCs), and enhanced matrix metalloproteinase-2 (MMP-2) activity in the SMCs of young and aged mice. A, Representative sections from fresh frozen aortas were stained for collagen I and elastin. B, Aortic SMC lysates were analyzed by Western blotting with anti-collagen I, anti-elastin, and anti-GAPDH antibodies. Densitometric analysis of collagen I and elastin levels is shown in the lower panels (mean±SE, n=3). C, Representative gelatin zymogram showing MMP-2 activity in aortic SMC lysates. Densitometric analysis of MMP-2 activity is shown in the lower panel (mean±SE, n=3).

To determine whether the increased apoptosis of medial SMCs in aged SOD2+/− mice reflects the intrinsic effect of SOD2 deficiency, we examined cleaved caspase-3 levels in aortic SMCs of young and aged wild-type and SOD2+/− mice exposed to staurosporine, a well-known inducer of apoptosis in a wide spectrum of cells, by Western analysis. Although not observed in untreated cells, cleaved caspase-3 levels were significantly increased in aged SOD2+/− compared with aged wild-type SMCs following staurosporine treatment (Figure 3B). Cleaved caspase-3 was not detected in young wild-type and barely detectable in young SOD2+/− SMCs treated with staurosporine. Activated caspase-3 proteolytically cleaves and inactivates many proteins, including the nuclear enzyme poly(ADP-ribose) polymerase (PARP), involved in cell viability, and cleaved PARP is a more specific marker of apoptosis. A significant increase in cleaved PARP levels in response to staurosporine treatment was observed in SOD2+/− compared with wild-type SMCs (Figure 3B). Consistent with this, aged SOD2+/− SMCs treated with staurosporine had significantly higher number of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cells compared with aged wild-type (Figure 3C).

To determine whether prolonged exposure to mitochondrial oxidative stress also impairs other cell survival pathways, we investigated the activation of protein kinase B/Akt, which preserves mitochondrial integrity and protects against apoptosis, in aged wild-type and SOD2+/− SMCs treated with and without insulin-like growth factor-1 (IGF-1). Akt phosphorylation increased significantly at 3 hours (3.7-fold, \(P<0.001\)) and remained elevated at 6 hours after IGF-1 treatment in aged wild-type SMCs (Figure 4A). The increase in Akt phosphorylation in aged SOD2+/− compared with untreated cells was much less robust at both 3 and 6 hours (2-fold increase) and significantly less than in aged wild-type (\(P<0.01\) versus aged wild-type at 3 hours). Forkhead box O (FoxO) transcription factors are important downstream targets of Akt, and FoxO3a has been implicated in SMC apoptosis. IGF-1 significantly increased FoxO3a phosphorylation at both 3 hours (3.7-fold increase) and 6 hours (4.1-fold increase) after treatment in aged wild-type SMCs (Figure 4B). In contrast, the increase in FoxO3a phosphorylation following IGF-1 treatment was significantly less in aged SOD2+/− SMCs (\(P<0.001\) versus aged wild-type at both 3 and 6 hours). Attenuation of FoxO3a phosphorylation in aged SOD2+/− SMCs was also observed in cells treated with angiotensin II, thrombin, and platelet-derived growth factor, indicating the intrinsic effect of SOD2 deficiency on SMC apoptosis (data not shown). Less robust stimulation of Akt in aged SOD2+/− SMCs was not associated with a decreased proliferative response to IGF-1 (data not shown), indicating that SMCs of various phenotypes can coexist in the arterial wall during remodeling. Together, these data suggest that...
prolonged exposure to increased mitochondrial oxidative stress during aging affects cell viability by impairing survival and activating apoptotic signaling pathways.

Stress-Activated Protein Kinase 1/c-Jun N-terminal Kinase 1 Activity and α-Smooth Muscle Actin Levels Are Decreased With Aging in SOD2+/− Aortic SMCs

An inhibition of Akt and increase in caspase activity result in c-Jun N-terminal kinase 1 (JNK1) activity,40 which induces stress-activated protein kinase 1/c-Jun and activating apoptotic signaling pathways.

Figure 3. Superoxide dismutase 2 (SOD2) deficiency enhanced medial smooth muscle cell (SMC) apoptosis in the aorta of aged mice and sensitized aortic SMCs from aged mice to staurosporine-induced apoptosis. A, Dual immunofluorescent staining of cleaved caspase-3 (red) and α-smooth muscle actin (green) demonstrated colocalization (yellow) in SMCs. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) (blue). B, Lysates from aortic SMCs treated or without 1.0 μmol/L staurosporine for 6 hours were analyzed by Western blotting with anti-caspase-3, anti-poly[ADP-ribose] polymerase (anti-PARP), and anti-β-actin antibodies. Data shown represent an experiment that was repeated at least twice with similar results. C, Aged SMCs treated with staurosporine (0.1 μmol/L) for 12 hours were analyzed by fluorescent (green) terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Nuclei were stained with DAPI (blue). Quantitation of apoptotic cells is presented as the percentage of TUNEL-positive cells in each field of view (mean±SE, n=3).

Increased smooth muscle α-actin levels and intrinsic SMC stiffness are a mechanism for increased aortic stiffening with aging.43 Consistent with the data shown in Figure 3A, α-actin levels were increased 2.8-fold in aging young wild-type and 3.5-fold in young SOD2+/− compared with young wild-type SMCs (Figure 4D). However, aged SOD2+/− had significantly higher α-actin levels compared with young SOD2+/− (5.4-fold versus 3.5-fold) and aged wild-type SMCs (5.4-fold versus 2.8-fold). Collectively, these data indicate concurrent activation of apoptotic signaling pathways and alterations in arterial wall structure and SMC cytoskeleton.

Basal Mitochondrial Superoxide Levels Are Increased and Basal and IGF-1-Induced H$_2$O$_2$ Levels Are Decreased With Aging in SOD2+/− Aortic SMCs

Recent evidence indicates that H$_2$O$_2$ activates the phosphatidylinositol-3-kinase/Akt pathway and promotes cell survival.44 To determine whether impaired cell survival pathways in aged SOD2+/− SMCs are mediated by changes in ROS levels, we measured superoxide and H$_2$O$_2$ levels in young and aged wild-type and SOD2+/− cells. First, we investigated colocalization of MitoTracker Green FM, a mitochondria-selective dye, with MitoSOX Red, a superoxide-sensitive fluorescent dye using confocal microscopy (Figure 5A). Compared with young wild-type, young SOD2+/− SMCs showed bright yellow fluorescence in mitochondria because of colocalization of MitoTracker Green and MitoSOX Red, indicating increased mitochondrial superox-
Figure 4. Superoxide dismutase 2 (SOD2) deficiency decreased insulin-like growth factor-1 (IGF-1)–induced Akt and FoxO3a phosphorylation and increased tumor necrosis factor-α (TNF-α)–induced c-Jun N-terminal kinase 1 (JNK1) phosphorylation in aortic smooth
ide production. Similarly, aged SOD2+/− had more mitochondrial superoxide levels, as shown by yellow/orange fluorescence in mitochondria compared with that in aged wild-type and young SOD2+/− SMCs.

To determine the effect of prolonged SOD2 deficiency on H2O2 levels, we measured basal and IGF-1-induced H2O2 levels in aged wild-type and SOD2+/− SMCs by Amplex Red assay (Figure 5B). H2O2 levels were significantly lower (31% decrease, P<0.001) in aged SOD2+/− SMCs compared with aged wild-type SMCs. IGF-1 treatment significantly increased H2O2 levels in wild-type cells (30% increase, P<0.001) but had no such effect in SOD2+/− SMCs. These results indicate that decreased H2O2 levels, caused by SOD2 deficiency, impair Akt activity and aortic SMC survival in aged mice via enhanced FoxO3a activation.

Downregulation of FoxO3a Activity Decreases Staurosporine-Induced Apoptosis in Aged Aortic SMCs

Because staurosporine, which inhibits Akt45 and activates FoxO3a,46 increased cleaved caspase-3 and PARP levels, we investigated whether alteration in Akt/FoxO3a signaling pathway contributes to increased apoptosis in aged SOD2+/− SMCs. Adenoviral overexpression of DN-FoxO3a significantly decreased (58%, P<0.001) cleaved PARP levels in aged SOD2+/− compared with cells transfected with control virus (Figure 6). Collectively, our data suggest that prolonged exposure to increased mitochondrial oxidative stress during aging in SOD2+/− SMCs increases apoptosis by modulating Akt/FoxO3a signaling pathway.

Discussion

In this study, we provide evidence that (1) SOD2 deficiency over a lifetime is sufficient to induce aortic stiffening, decrease aortic compliance, and cause cardiac dysfunction; (2) aortic stiffening with aging in SOD2+/− mice is associated with structural changes in the aortic wall, with increased collagen content and ruptures in elastin laminae; (3) SOD2 deficiency increases collagen I expression, decreases elastin expression, and increases MMP-2 expression and activity in aged SMCs; (4) SOD2 deficiency over a lifetime increases medial SMC apoptosis in aged mice and sensitizes SMCs to stauarosporine-induced increase in cleaved caspase-3 and cleaved PARP levels; (5) prolonged SOD2 deficiency in SMCs activates JNK1 in response to TNF-α treatment; (6) prolonged SOD2 deficiency impairs cell survival as observed by decreased Akt and increased FoxO3a activation in response to IGF-1 treatment; and (7) increased α-actin levels in SOD2+/− SMCs are integral to increased aortic stiffness with aging. It was previously established that SOD2+/− mice have an ≈50% reduction in SOD2 activity in all tissues compared with the wild-type mice, and the decrease in enzyme activity does not cause any compensatory upregulation of other major components of mitochondrial antioxidant defense system.18 Although impairment of cardiac function was reported in 6-month-old TRE/SOD2+/− mice,47 our finding is the first to implicate increased mitochondrial oxidative stress over a lifetime as the source of aortic stiffening and cardiac dysfunction in SOD2+/− mice. Specifically, we provide evidence of how molecular signaling pathways initiated by increased mitochondrial oxidative stress in aortic SMCs contribute to aortic stiffening.

Aortic stiffness is a complex phenomenon that arises from structural alterations in the aortic wall, impaired endothelial function, increased smooth muscle tone, phenotypic modulation of adventitial fibroblasts to myofibroblasts, and chronic low-grade inflammation.2 The scaffolding proteins collagen and elastin provide the structural integrity of the aortic wall, and our results show that increased mitochondrial oxidative stress over a lifetime increases the collagen content and ruptures and decreases the elastin in the aorta. The changes in aortic collagen and elastin levels were accompanied by increased expression and activity of MMP-2 in aged SOD2+/− aortic SMCs. Similar to this, Dasgupta et al48 and others33 reported both age- and redox-regulated increase in MMP expression and activity. Although the increase in MMP-2 expression and activity should decrease collagen levels at first glance, increased collagen I levels are observed in SMCs under oxidative stress conditions.49 In fact, an increase in interstitial and perivascular collagen is observed in cardiac MMP-2 transgenic mice.50 Nevertheless, activation of MMP-2 is strongly correlated with elastic fiber fragmentation, disorganization, and increased stiffness of the arterial vasculature.51 Endothelial dysfunction and inflammation may contribute to increased aortic stiffening in aged SOD2+/−, as endothelial dysfunction is increased in apolipoprotein E−/− mice that are deficient in SOD2,21 and proinflammatory cytokine production is upregulated with increased mitochondrial ROS levels.51

Our results showing increased aortic stiffness in aged SOD2+/− mice accompanied by ventricular dysfunction are supported by several cross-sectional studies that reported a positive association between age-related aortic stiffness and ventricular dysfunction.52 Aortic stiffening increases left ventricular afterload by inducing earlier return of reflected waves in the late systole and causes left ventricle hypertrophy and ventricular dysfunction. Interestingly, the impairment of aortic relaxation and increased diastolic blood pressure in SOD2+/− mice precede increased PWV and Doppler abnormalities in heart function. Furthermore, mitochondrial oxidative stress–induced coupling of vascular-ventricular dysfunction is supported by the observation of impaired heart function with lifelong reduction of SOD2.47

Increased apoptosis of SMCs in the aortic media and increased sensitivity to stauarosporine-induced apoptosis in
aged SOD2<sup>+/−</sup> mouse SMCs observed in the present investigation are consistent with the concept that medial SMC apoptosis is an important contributor to age-associated vascular remodeling and loss of aortic elasticity. The propensity of aged SOD2<sup>+/−</sup> aortic SMCs to apoptosis is underlined by impaired activation of Akt and increased activation of FoxO3a in response to IGF-1 treatment. Akt is a negative regulator of FoxO3a transcription factor, which in the absence of Akt-mediated phosphorylation induces the expression of genes involved in apoptosis. Interestingly, an increase in MMP-2 and MMP-9 activities was observed in vascular cells following FoxO3a activation. Because these MMPs do not contain a consensus binding site for forkhead factors, activated FoxO3a may regulate MMP-2 activity indirectly, including via activation of MMP-3.

Activated MMP-2 induces apoptosis by stimulating JNK activity, as well as cytochrome c release. Inhibition of Akt signaling has been shown to induce JNK activity and promote the cleavage of caspase-3 in SMCs. JNK activation, in turn, initiates mitochondrial apoptotic pathway via Bax-dependent release of cytochrome c. Alternatively, aged SOD2<sup>+/−</sup> aortic SMCs could undergo apoptosis in the absence of Akt-mediated phosphorylation of apoptosis regulatory proteins Bad and Bax, which suggests that Akt-JNK cross-talk is an important determinant of aged SMC apoptosis. Our observation that DN-FoxO3a overexpression attenuates cleaved PARP levels is consistent with the regulatory role of Akt/FoxO3a signaling in aged SOD2<sup>+/−</sup> aortic SMC apoptosis.

Calcium channel blockers and angiotensin II receptor antagonists are used to treat large artery stiffening. These drugs affect vascular SMC tone, which suggests that age-associated vascular stiffening is partly regulated by intrinsic mechanical properties of these cells. Our data showing significantly increased α-actin levels in aged SOD2<sup>+/−</sup> compared with aged wild-type SMCs are in agreement with the report of Qiu et al that smooth muscle α-actin is a key determinant of vascular SMC stiffness during aging. Increases in α-actin levels and MMP-2 activity were observed in young SOD2<sup>−/−</sup> compared with young wild-type SMCs, and yet the aortic stiffening and cardiac dysfunction are evident only in aged SOD2<sup>−/−</sup> mice, which suggests a threshold for mitochondrial oxidative stress to affect structural and biochemical changes in the SMC and aorta and to cause a phenotypic effect. Our observation that H<sub>2</sub>O<sub>2</sub> levels are decreased in SOD2<sup>+/−</sup> SMCs is consistent with similar findings in SOD2-deficient and knockout mice. Exogenous H<sub>2</sub>O<sub>2</sub> stimulates Akt phosphorylation in many cell types, including vascular SMCs. Therefore, it is conceivable that low H<sub>2</sub>O<sub>2</sub> levels in aged SOD2<sup>−/−</sup> SMCs impair cell survival and promote apoptosis by downregulating Akt signaling and activating FoxO3a.

In summary, our data provide insight into the molecular mechanisms by which increased mitochondrial oxidative stress promotes aortic stiffening associated with aging. Altered ROS metabolism in the mitochondria over a lifetime not only enhances collagen secretion and intrinsic stiffness of aortic medial SMCs but also affects redox signaling to induce SMC apoptosis, all of which contribute to aortic stiffening. It would be worth determining whether strategies aimed at...
regulating mitochondrial oxidative stress have a therapeutic effect against aortic stiffening and its pathophysiological sequelae.

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Disclosures

None.

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SUPPLEMENTAL METHODS

Mice and Diet
All animal experimental procedures were performed in compliance with protocols approved by University of North Carolina IACUC according to NIH guidelines. Mice were maintained at 22°C with a 12-hour light/dark cycle and given free access to food and water. Mice on high-fat diet were maintained on standard rodent chow until 1 (young) or 13 months (old) of age and then fed a Western-type diet (TD88137, Harlan Teklad, Indianapolis) for 12 weeks.

Blood pressure
Systolic and diastolic blood pressure was measured in conscious mice daily for 5 days using volume-pressure recording system (CODA 6, Kent Scientific). Mice were acclimated to the warmed (32°C) restrainer for 10-15 min and blood pressure was recorded during 10 acclimation followed by 20 measurement cycles.

Vascular relaxation in isolated mouse aortic rings
Mouse aortas were dissected and cleaned from adventitia. Segments of mouse aortas approximately 2-3 mm in length were placed in Krebs-Hensleit buffer (in mmol/L; 120 NaCl, 25 NaHCO₃, 11 glucose, 1.8 CaCl₂, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄). The aorta segments were then mounted in a Radnoti 4-unit organ bath system (Radnoti Glass Technology Inc.). Each reservoir held 20 mL of buffer and was aerated constantly with 95% O₂ + 5% CO₂ gas mixture. After 15-20 min stabilization, the baseline tension of the rings was adjusted to 700 mg. All rings were pre-constricted with 1 µmol/L phenylephrine and then response to nitroglycerine (NTG); 10⁻³ to 10⁻⁵ mol/L) was recorded. The degree of relaxation was expressed as a percentage of maximum constriction induced by phenylephrine.

Cell Culture and Materials
Aortic smooth muscle cells (SMC) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS). Primary aortic SMC cultures from 4-6 mice were pooled and used at passages 5-10 for all experiments. Antibodies were obtained from the following commercial sources: Akt, phosphorylated Akt, FoxO3a, caspase-3 and cleaved caspase-3 (Asp¹⁷⁵) (5A1E) antibodies were purchased from Cell Signaling; phosphorylated FoxO3α, collagen type I and elastin from Abcam; β-actin, α-tubulin, and hemagglutinin (HA) antibodies were obtained from Sigma. HRP-conjugated secondary antibodies against mouse or rabbit IgG were also purchased from Sigma. Rhodamine-conjugated AffiniPure donkey anti-rabbit IgG, normal mouse sera and donkey sera were purchased from Jackson ImmunoResearch. Phenylephrine was purchased from Sigma and nitroglycerin was supplied by Dupont.

Histology, Immunohistochemistry, and Immunofluorescence
Abdominal aortas from WT and SOD2⁺⁻ mice were embedded in OCT compound (Tissue-Tek), and snap-frozen in liquid nitrogen. Aortic sections were stained using rabbit anti-collagen type I antibody while rabbit IgG was used as a negative control. Immunostaining was performed using the Vector VIP peroxidase substrate kit (Vector Laboratories) following manufacturer recommendations. Sections were counterstained with methylgreen. Aortic cross-sections were stained with Verhoeff's stain for delineating elastic laminae. For immunofluorescence studies, aortic sections were stained with rabbit anti-cleaved caspase-3 antibody followed by rhodamine-conjugated donkey anti-rabbit secondary antibody. Nuclei were counter-stained with DAPI and permanently mounted with VectaMount Mounting Medium (Vector Laboratories). Photographs were taken using Nikon Eclipse E800 microscope. Fresh frozen aortic sections were stained by von Kossa method for visualization of calcium deposits.
Gelatin Zymography
Cell lysates were diluted in Tris-glycine SDS sample buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS (w/v), and 0.025% bromophenol blue for gel electrophoresis. Samples were resolved on 10% denaturing polyacrylamide gels containing 0.1% gelatin (Bio-Rad) with Tris-glycine SDS running buffer (0.29% Tris base, 1.44% glycine and 0.1% SDS). Gels were then incubated in zymogram renaturing buffer containing 2.5% (v/v) Triton X-100 with gentle agitation for 30 min at room temperature. Renaturing buffer was then removed and replaced with zymogram developing buffer containing 0.121% Tris base, 0.63%, Tris-HCl, 1.17% NaCl, 0.074% CaCl₂ and 0.02% Brij 35. After incubation for 30 min at room temperature with gentle agitation, gels were then placed in fresh zymogram developing buffer and incubated overnight at 37°C. Gels were stained with 0.5% (w/v) Coomassie Blue R-250 for 30 min and then destained with solution containing methanol:acetic acid:water (5:1:4). Areas of protease activity appeared as clear bands – signifying protease digestion of substrate – set against a dark blue background. The gels were scanned and the bands densities were measured using ImageJ.

Detection of Mitochondrial Superoxide
Aortic SMC grown in glass bottom dishes were washed with Hanks' balanced salt solution and incubated with 5 μmol/L MitoSOX Red and 1 μmol/L MitoTracker Green FM (Molecular Probes) at 37°C for 10 min. Excess stains were removed, and cells were imaged using Olympus FV500 confocal laser-scanning microscopy. MitoTracker Green FM was visualized at an excitation of 490 nm and an emission of 516 nm, whereas MitoSOX Red was visualized at an excitation of 560 nm and an emission of 600 nm. MitoTracker Green FM preferentially translocates to the mitochondria. MitoSOX Red accumulates in mitochondria and exhibits bright red fluorescence upon oxidation and subsequent binding to mitochondrial DNA.

H₂O₂ Measurement
Extracellular H₂O₂ levels were determined using Amplex Red assay (Invitrogen). In the presence of horseradish peroxidase, N-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) reacts with H₂O₂ to produce the fluorescent molecule resorufin. Mouse aortic SMC were grown on 24-well plates to subconfluent condition and were starved in 0.5 ml OPTI-MEM for 24 h. To measure H₂O₂ production, IGF-1 was added to a final concentration of 100 ng/ml. After 30 min incubation, 50 μL of media were transferred to a 96-well plate containing Amplex Red reaction mixture (50 μmol/L Amplex Red and 0.1 U/mL horseradish peroxidase). After 30 min incubation, fluorescence was measured at an excitation wavelength of 560 nm and emission wavelength of 590 nm.
Suppl Table I. Systolic and diastolic blood pressure was measured in 4- and 16-month-old wild-type and SOD2+/− mice fed normal chow (ND) or Western diet (WD) (mean±SE, n=15).

<table>
<thead>
<tr>
<th></th>
<th>Systolic BP</th>
<th></th>
<th>Diastolic BP</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ND-4 mon</td>
<td>HF-4 mon</td>
<td>ND-16 mon</td>
<td>HF-16 mon</td>
</tr>
<tr>
<td>Wild-type</td>
<td>136.7±2.7</td>
<td>130.0±2.8</td>
<td>129.0±3.1</td>
<td>132.0±4.9</td>
</tr>
<tr>
<td>SOD2+/−</td>
<td>138.4±3.6</td>
<td>133.4±3.0</td>
<td>131.7±3.4</td>
<td>132.6±3.5</td>
</tr>
</tbody>
</table>

|                     | ND-4 mon    | HF-4 mon        | ND-16 mon    | HF-16 mon       |
| Wild-type           | 102.8±2.1   | 99.6±2.4        | 101.5±1.3    | 100.1±2.7       |
| SOD2+/−             | 115.7±3.2*  | 109.5±3.2*      | 109.4±2.7*   | 111.1±3.2*      |

*P<0.01 *P<0.05 *P<0.05 *P<0.05

Suppl Figure I: Vascular relaxation is impaired with age and SOD2 deficiency in mice. Relaxation (% maximum response) of aortic rings, precontracted with 1 µmol/L phenylephrine (PE), to NTG treatment (6-8 mice and 12-15 rings were used per each group). *P<0.01 aged vs young wild-type; **P<0.001 young and aged SOD2+/− vs young wild-type; ***P<0.01 young SOD2+/− vs aged wild-type; ****P<0.05 aged SOD2+/− vs aged wild-type mice.
산화 스트레스가 혈관노화의 특징적 소견인 대동맥 경직도 증가의 주범이다.

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Summary

배경
연령증가에 따른 대동맥 경직도 증가는 심혈관질환의 독립적인 위험인자이다. 산화 스트레스가 대동맥 경직도 증가와 관련되어 있지만 이를 뒷받침하는 분자생물학적 기전에 대해서는 밝혀져 있지 않다. 우리는 노화에 의한 산화 스트레스의 유발원인을 찾고, 산화 스트레스가 혈관 평활근세포의 기능과 대동맥 순응도에 미치는 영향을 유전자변형 생쥐모델로 연구하였다.

방법 및 결과

결론
미토콘드리아의 산화 스트레스가 혈관벽의 재형성과 혈관 평활근세포의 경직도 증가, 그리고 대동맥 혈관 평활근세포의 세포고사를 초래하여 대동맥 경직도를 증가시킨다.
연령이 증가함에 따라 혈관벽의 특징적인 소견이 관찰되며 이러한 혈관노화는 고혈압, 당뇨병, 고지혈증 등의 다른 위험인자와 독립적으로 심혈관질환의 위험을 증가시키는 위험인자임이 잘 알려져 있다. 노화에 의해 혈관에서 나타나는 가장 현저한 변화는 혈관 내경의 증가와 혈관벽의 비후이다. 혈관의 내막과 중막(intima-medial thickness, IMT) 두께는 연령에 비례하여 증가하며 이러한 변화는 심혈관뇌혈관질환 발생과 유의한 상관관계가 있다. 또한 혈관벽에서 탄성섬유(elastin fiber)가 감소되고 콜라겐(collagen)이 침착하여 혈관의 탄성이 감소된다. 한편, 연령이 증가함에 따라 혈관 평활근세포의 증식이 증가하고 세포외기질 생성이 증가하는 것도 혈관벽의 경직도 증가의 원인이 된다. 이러한 혈관노화에 의해 특히 대동맥과 같은 큰 혈관의 경직도가 증가하게 되어 좌심실의 후부하가 증가된다.

한편, 노화에 의해 혈관의 구조적인 변화뿐 아니라 혈관내피세포의 기능이 저하되며 혈관내피세포에서 생성되는 산화질소(nitric oxide, NO)의 감소로 혈관의 장기적 조절이 저하되어 혈관의 경직도 증가의 원인이 된다. 이러한 혈관노화에 의해 특히 대동맥과 같은 큰 혈관의 경직도가 증가하게 되어 좌심실의 후부하가 증가된다.

혈관벽의 비후와 혈관내피세포의 기능저하로 인한 대동맥의 경직도 증가는 동맥경화와는 무관하게 노화에 의해 진행하며 이러한 변화는 좌심실의 후부하를 증가시키며 심비대를 유발하고 고혈압과 죽상동맥경화의 진행을 초래한다. 즉, 노화에 따른 혈관의 변화는 그 자체로 심혈관질환의 원인이 되며 진행을 촉진하는 위험인자로서 심혈관질환의 발생을 줄이기 위해서는 혈관노화의 진행을 막는 새로운 치료법에 대한 관심을 기울여야 할 것으로 생각된다.

이와 같이 혈관노화의 특징적인 소견에 대해서는 잘 알려져 있으나 혈관노화의 기전에 대한 연구는 아직까지 많이 진행되지 못해 혈관노화의 근본적인 원인과 기전에 대해 잘 밝혀져 있지 못하였다. 본 논문은 유전자변형 생쥐모델을 이용하여 미토콘드리아의 산화스트레스 증가가 혈관노화의 특징적인 소견을 발현하는 것을 확인하였다. 또한 미토콘드리아의 산화스트레스 증가가 클라렌 생성 증가, 혈관 평활근세포의 세포고사 촉진 등의 기전에 의해 혈관노화를 발현하는 것을 확인하여 향후 혈관노화에 대한 근본적인 치료적 접근을 위해서는 미토콘드리아의 산화스트레스를 감소시키는 전략적 방법을 개발해야 한다는 점을 제시하였다.

산화스트레스에 의한 세포 손상은 지난 50년간 노화 이론에서 중요하게 생각되고 있는 기전으로 산화스트레스의 생성이 증가하거나 방어 기제에 장애가 있는 경우 지속적으로 산화스트레스에 의해 세포가 손상되어 세포의 수명이 단축되고 궁극적으로 개체의 수명이 줄어들게 된다는 이론이다. 따라서 산화스트레스에 대한 방어 기제에 관여하는 유전자 영향에 대한 많은 관심이 있었다. 즉, 산소를 매개로 한 에너지 대사를 이용하는 개체에서는 필연적으로 생성되는 산소라디칼의 효과적인 제거가 중요하며 따라서 산화스트레스에 대한 효율적인 방어 시스템이 에너지 대사 과정 중 지속적으로 생성되는 산소라디칼에 의한 세포 손상과 이에 기인한 노화에 중요한 역할을 할 것으로 생각된다.
단순히 얇은 라디칼의 효과적인 제거를 위해서는 과산화 이온을 제거하는 SOD (superoxide dismutase), catalase, glutathione peroxidase 등의 항산화 효소계와 항산화 물질인 vitamin C, E, uric acid 등이 중요한 역할을 한다. 이중 SOD는 대표적인 항산화 효소로로서 3개의 isoform이 존재하며 주로 세포질과 핵 내에 존재하는 Cu/ZnSOD (SOD1), 미토콘드리아에 존재하는 MnSOD (SOD2), 세포 밖으로 분비되어 세포 표면에 heparan sulfate proteoglycan과 결합하여 존재하는 EcSOD (SOD3)로 구분된다.

그러나 산화 스트레스에 대한 방어 기제를 향상시킴으로써 노화를 역전할 수 있는가에 대해서는 논란이 있어 왔다. 초파리에서 Cu/ZnSOD를 과발현시킴으로써 산화 스트레스가 감소하였다고 하는 초기의 보고가 있었으나 이후 Cu/ZnSOD 발현을 조절한 생쥐 모델에서 산화 스트레스가 증가하였다는 결과를 고려하면 산화 스트레스에 의한 노화 현상은 결국 세포 내에서 산화 스트레스에 가장 취약한 곳에서 증가된 산화 스트레스에 대해 적절하게 대처하지 못해 발생하는 것으로 생각되며 특히 세포의 노화에 있어 미토콘드리아의 손상이 중요하다는 점을 시사한다. 특허 미토콘드리아는 그 자체의 유전 정보를 가지고 있으며 산화 스트레스가 많이 생성되는 장소에 존재한다는 특성과 함께 손상에 대한 복구 시스템이 핵 내 유전 정보에 비교적 완전히 유전 정보에 비해 불완전하여 유전 정보의 손상에 취약하다. 따라서 미토콘드리아의 산화 스트레스를 감소시키는 것이 향후 혈관노화의 진행을 억제할 수 있는 새로운 치료전략으로 고려될 수 있을 것으로 기대된다.

REFERENCES
Mitochondrial Oxidative Stress in Aortic Stiffening With Age
The Role of Smooth Muscle Cell Function


Objective—Age-related aortic stiffness is an independent risk factor for cardiovascular diseases. Although oxidative stress is implicated in aortic stiffness, the underlying molecular mechanisms remain unelucidated. Here, we examined the source of oxidative stress in aging and its effect on smooth muscle cell (SMC) function and aortic compliance using mutant mouse models.

Methods and Results—Pulse wave velocity, determined using Doppler, increased with age in superoxide dismutase 2 (SOD2)\textsuperscript{+/−} but not in wild-type, p47phox\textsuperscript{−/−} and SOD1\textsuperscript{+/−} mice. Echocardiography showed impaired cardiac function in these mice. Increased collagen I expression, impaired elastic lamellae integrity, and increased medial SMC apoptosis were observed in the aortic wall of aged SOD2\textsuperscript{+/−} versus wild-type (16-month-old) mice. Aortic SMCs from aged SOD2\textsuperscript{+/−} mice showed increased collagen I and decreased elastin expression, increased matrix metalloproteinase-2 expression and activity, and increased sensitivity to staurosporine-induced apoptosis versus aged wild-type and young (4-month-old) SOD2\textsuperscript{+/−} mice. Smooth muscle α-actin levels were increased in aged SOD2\textsuperscript{+/−} versus wild-type SMCs. Aged SOD2\textsuperscript{+/−} SMCs had attenuated insulin-like growth factor-1-induced Akt and Forkhead box O3a phosphorylation and prolonged tumor necrosis factor-α–induced Jun N-terminal kinase 1 activation. Aged SOD2\textsuperscript{+/−} SMCs had increased mitochondrial superoxide but decreased hydrogen peroxide levels. Finally, dominant-negative Forkhead box O3a overexpression attenuated staurosporine-induced apoptosis in aged SOD2\textsuperscript{+/−} SMCs.


Key Words: aging ■ blood pressure ■ reactive oxygen species ■ signal transduction ■ vasodilation

Advancing age is the major risk factor for cardiovascular disease (CVD) morbidity and mortality. With aging, central arteries stiffen (and dilate) as a result of physiological remodeling arising from the fracture of elastin lamellae from repetitive pulsations and also from endothelial dysfunction, chronic low-grade inflammation, and altered vascular smooth muscle tone.\textsuperscript{1,2} Aortic stiffening is the principal cause of CVD with age in people without atherosclerosis,\textsuperscript{1} including increased systolic and pulse pressures, increased left ventricular hypertrophy and diastolic dysfunction, and congestive heart failure.\textsuperscript{3} Carotid-femoral pulse wave velocity (PWV), a direct noninvasive measure of the thoracic and abdominal aortic stiffness, is correlated with higher CVD events and is an independent predictor of coronary heart disease and stroke.\textsuperscript{4} Despite the strong epidemiological and biological connection of age to CVD risk, the molecular mechanisms responsible for age-related vascular dysfunction have yet to be elucidated. Although advancing age is an unmodifiable risk factor for CVD, it might be possible to target specific molecular signals as an approach to limiting age-related CVD risk.

Oxidative stress has been implicated in vascular dysfunction, whether as a result of CVD or aging or both.\textsuperscript{5-7} The free radical theory of aging, first proposed by Harman more than 50 years ago,\textsuperscript{8} suggested that increased reactive oxygen species (ROS) generation underlies many features of aging. Prior studies have indicated that increased vascular ROS generation results in decreased compliance, as measured by PWV.\textsuperscript{9,10} Recent studies suggest that mitochondrial dysfunction plays an important role in aging and impairing vascular function.\textsuperscript{11,12}

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Many pro- and antioxidant enzymes regulate ROS levels in cells. Of these, the superoxide dismutase (SOD) family is the most studied antioxidant system and has been previously implicated in CVD. SODs convert superoxide to produce hydrogen peroxide (H₂O₂), which is further degraded by either catalase or glutathione peroxidase. One member of the SOD family, manganese SOD (SOD2), is present in mitochondria. Deletion of the SOD2 gene results in early postnatal lethality in mice, SOD2-deficient (SOD2−/−) mice are viable but demonstrate increased susceptibility to oxidative stress, diminished mitochondrial function, and enhanced sensitivity to apoptosis. In an atherosclerotic background (apolipoprotein E knockout), SOD2 deficiency results in accelerated atherosclerosis and endothelial dysfunction in mice. In addition, decreased expression/activity of SOD2 with age was implicated in vascular aging.

In the present study, we investigated the effect of oxidative stress in aging-associated increase in aortic stiffness using mutant mouse models. Our data indicate that prolonged exposure to increased mitochondrial oxidative stress decreases aortic compliance and induces cardiac dysfunction. Specifically, we elucidate the significance of lifelong SOD2 deficiency on the phenotype, function, and molecular signaling pathways in aortic smooth muscle cells (SMCs) and how these events regulate aortic wall homeostasis and aortic stiffening.

**Materials and Methods**

**Aortic PWV**
Arterial compliance was determined as described by Hartley et al. In brief, mice were anesthetized with inhaled isoflurane (1% in O₂) and fixed in a supine position on the temperature-controlled ECG board (THM100, Indus Instruments). Body temperature was maintained at 37°C and monitored with a rectal probe. Blood flow velocity was recorded using a 20-MHz pulsed Doppler probe at the levels of aortic arch and at the abdominal aorta. Data were analyzed using an Indus Instruments Doppler Signal Processing Workstation. Aortic PWV was calculated by dividing separation distance (40 mm) by difference in pulse wave arrival time in respect to ECG R-peaks.

**Blood Pressure**
Systolic and diastolic blood pressure was measured as described in the online-only Data Supplement.

**Vascular Relaxation in Isolated Mouse Aortic Rings**
Relaxation of isolated mouse aortic rings was measured as described in the online-only Data Supplement.

**Cell Culture and Materials**
Mouse aortic SMCs were isolated from young (4 months) and aged (16 months) wild-type and SOD2−/− mice (C57BL/6J) as described previously (see online-only Data Supplement).

**Histology, Immunohistochemistry, and Immunofluorescence**
Immunohistochemistry and immunofluorescence studies were performed as previously described.

**Western Blot Analysis**
Preparation of cell extracts and Western blot analysis was performed as described previously.

**Quantitative Real-Time Polymerase Chain Reaction**
Quantitative analysis of mRNA expression of target genes was performed using total RNA extracted from cells and tissues. Reverse transcription was performed using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). Real-time polymerase chain reaction was performed in quadruplicate with TaqMan Gene Expression Assays for mouse collagen I (Mm01302043_g1), elastin (Mm00514670_ml), matrix metalloproteinase-2 (MMP-2) (Mm00439508_m1), and 18S rRNA (Hs99999901_sl) using an ABI Prism 7900 HT Sequence Detection System according to manufacturer’s recommended protocol. Target gene mRNA expression was normalized to 18S rRNA expression. Individual gene expression in SOD2−/− aortic SMCs was calculated relative to that in wild-type using REST2008 (Relative Expression Software Tool).

**Gelatin Zymography**
MMP-2 activity was assayed by gelatin zymography (see Methods in the online-only Data Supplement).

**Adenovirus Infection of Aortic SMCs**
A replication-defective adenoviral vector expressing dominant-negative Forkhead box O3a (DN-FOXO3a) was obtained from Vector BioLabs. DN-FoxO3a, constructed by deletion of the transactivation domain from the C-terminus, had a heme agglutinin tag at the N-terminus and expressed green fluorescent protein. Adenovirus expressing only green fluorescent protein was used as a negative control. Mouse aortic SMCs were cultured to 80% to 90% confluence before adenoviral infection. Infections were performed using a multiplicity of infection of 100, and the infection efficiency was typically greater than 90%. Measurement of proteins of interest was made in cells harvested 36 hours after viral infection.

**Detection of Mitochondrial Superoxide**
Mitochondrial superoxide levels in aortic SMCs were detected as described in the Methods in the online-only Data Supplement.

**H₂O₂ Measurement**
Aortic SMCs extracellular H₂O₂ levels were determined using the Amplex Red assay (Invitrogen) (see Methods in the online-only Data Supplement).

**Statistical Analysis**
Data presented graphically are shown as mean±SE from at least 3 independent experiments. All data were tested for normality using the Kolmogorov-Smirnov test and were analyzed by 1-way ANOVA, and post hoc analysis was performed using the Newman-Keuls test. To account for multiple comparisons, arterial compliance and cardiac function data were analyzed by 1-way ANOVA followed by the Ryan-Einot-Gabriel-Welsch multiple-range test with an overall α=0.05 (SSPS software, version 19.0).

**Results**
Aortic Compliance, Cardiac Function, and Vasorelaxation Are Decreased With Age in SOD2−/− Mice
To examine the interactive effect of oxidative stress, diet, and aging on vascular health, we measured aortic compliance in...
young (4 months) and aged (16 months) mice fed normal chow (ND) or Western diet (WD). We used wild-type mice, as well as mice with decreased (p47phox−/−) or increased (SOD1+/−) cytosolic and increased mitochondrial (SOD2+/−) oxidative stress. There was no difference in central aortic compliance, as measured by PWV, between young and aged wild-type (Figure 1A), p47phox−/−, and SOD1+/− mice (data not shown), either on normal chow or Western diet. PWV was also not significantly different between young wild-type and SOD2+/− mice, whether on a normal chow or Western diet. However, aged SOD2+/− fed normal chow had significant increase in PWV compared with aged wild-type or young SOD2+/− mice (P<0.05 in each case; Figure 1A) on a normal chow diet. Similarly, aged SOD2+/− on Western diet had significantly increased PWV compared with aged wild-type or young SOD2+/− mice on Western diet (P<0.05 in each case). These data indicate that prolonged mitochondrial oxidative stress is sufficient to induce aortic stiffening.

To determine whether prolonged mitochondrial oxidative stress also affects cardiac function, we examined the above mentioned mice by echocardiography. Aged SOD2+/− had impaired left ventricular function, as indicated by significantly decreased ejection fraction (Figure 1B) compared with aged wild-type mice, whether on normal chow (P<0.01) or Western diet (P<0.05). In consonance with decreased ejection fraction, aged SOD2+/− had increased left ventricle end-diastolic volume compared with aged wild-type mice, whether on normal chow (P<0.05) or Western diet (P<0.05).
Superoxide dismutase 2 (SOD2) deficiency increased collagen I synthesis and disrupted elastic laminae in aortas of aged mice, increased collagen levels and decreased elastin levels in aged aortic smooth muscle cells (SMCs), and enhanced matrix metalloproteinase-2 (MMP-2) activity in the SMCs of young and aged mice. A, Representative sections from fresh frozen aortas were stained for collagen I and elastin. B, Aortic SMC lysates were analyzed by Western blotting with anti-collagen I, anti-elastin, and anti-GAPDH antibodies. Densitometric analysis of collagen I and elastin levels is shown in the lower panels (mean ± SE, n = 3). C, Representative gelatin zymogram showing MMP-2 activity in aortic SMC lysates. Densitometric analysis of MMP-2 activity is shown in the lower panel (mean ± SE, n = 3).

Collagen Levels Are Increased and Elastin Levels and Integrity Are Decreased With Age in the Aortic Wall and SMCs of SOD2+/− Mice

Because a decrease in the elastin/collagen ratio is associated with increase in aortic stiffness and increased aortic oxidative stress is correlated with extensive collagen deposition and elastin degradation and decline in aortic compliance, we examined aortic collagen and elastin expression in the aortic wall of wild-type and SOD2+/− mice by immunohistochemistry. Collagen I expression was increased in the media of aged SOD2+/− compared with aged wild-type mice (Figure 2A). The elastic laminae in the media were normal in aged wild-type mice, but their integrity was compromised with ruptures in aged SOD2+/− mice (Figure 2A). No perceptible...
Superoxide dismutase 2 (SOD2) deficiency enhanced medial smooth muscle cell (SMC) apoptosis in the aorta of aged mice and sensitized aortic SMCs from aged mice to staurosporine-induced apoptosis. A, Dual immunofluorescent staining of cleaved caspase-3 (red) and α-smooth muscle actin (green) demonstrated colocalization (yellow) in SMCs. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). B, Lysates from aortic SMCs treated or without 1.0 μmol/L staurosporine for 6 hours were analyzed by Western blotting with anti-caspase-3, anti-poly(ADP-ribose) polymerase (anti-PARP), and anti-β-actin antibodies. Data shown represent an experiment that was repeated at least twice with similar results. C, Aged SMCs treated with staurosporine (0.1 μmol/L) for 12 hours were analyzed by fluorescent (green) terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Nuclei were stained with DAPI (blue). Quantitation of apoptotic cells is presented as the percentage of TUNEL-positive cells in each field of view (mean±SE, n=3).

Figure 3. Superoxide dismutase 2 (SOD2) deficiency enhanced medial smooth muscle cell (SMC) apoptosis in the aorta of aged mice and sensitized aortic SMCs from aged mice to staurosporine-induced apoptosis.

MMP-2 Expression and Activity Are Increased in SOD2+/− Aortic SMCs
MMP-2 is a critical regulator of extracellular matrix degradation and age-associated vascular remodeling and has been implicated in arterial stiffening. A 3.2±0.8-fold increase in MMP-2 mRNA expression was observed in aged SOD2+/− compared with aged wild-type SMCs (P<0.001) as determined by real-time reverse transcription–polymerase chain reaction. MMP-2 activity was significantly increased (P<0.01) in both young and aged SOD2+/− compared with wild-type SMCs (Figure 2C). These data suggest that mitochondrial oxidative stress activates signaling pathways involved in MMP-2 expression and activity.

Prolonged SOD2 Deficiency Renders Aortic SMCs Susceptible to Apoptosis and Impairs Antiapoptotic Akt Pathway
A decrease in arterial medial SMC number and vascular remodeling with aging has been attributed to increased apoptosis, and we and others have shown that increased mitochondrial oxidative stress is an important regulator of SMC apoptosis. As shown in Figure 3A, immunofluorescence staining for the cleaved form of caspase-3, a member of the caspase superfamily that initiates apoptotic events, was increased in medial SMCs of aged SOD2+/− mice. Cleaved caspase-3 was barely detectable in young SOD2+/− mice (data not shown) and not observed in the aortic walls of either
young or aged wild-type mice. Similarly, we did not find any apoptosis in the hearts of either aged wild-type or SOD2+/− mice (data not shown).

To determine whether the increased apoptosis of medial SMCs in aged SOD2+/− reflects the intrinsic effect of SOD2 deficiency, we examined cleaved caspase-3 levels in aortic SMCs of young and aged wild-type and SOD2+/− mice exposed to staurosporine, a well-known inducer of apoptosis in a wide spectrum of cells, by Western analysis. Although not observed in untreated cells, cleaved caspase-3 levels were significantly increased in aged SOD2+/− compared with aged wild-type SMCs following staurosporine treatment (Figure 3B). Cleaved caspase-3 was not detected in young wild-type and barely detectable in young SOD2+/− SMCs treated with staurosporine. Activated caspase-3 proteolytically cleaves and inactivates many proteins, including the nuclear enzyme poly(ADP-ribose) polymerase (PARP), involved in cell viability, and cleaved PARP is a more specific marker of apoptosis. A significant increase in cleaved PARP levels in response to staurosporine treatment was observed in SOD2+/− compared with wild-type SMCs (Figure 3B). Consistent with this, aged SOD2+/− SMCs treated with staurosporine had significantly higher number of terminal deoxynucleotid transferase dUTP nick-end labeling–positive cells compared with aged wild-type (Figure 3C).

To determine whether prolonged exposure to mitochondrial oxidative stress also impairs other cell survival pathways, we investigated the activation of protein kinase B/Akt, which preserves mitochondrial integrity and protects against apoptosis,34 in aged wild-type and SOD2+/− SMCs treated with and without insulin-like growth factor-1 (IGF-1). Akt phosphorylation increased significantly at 3 hours (3.7-fold, P<0.001) and remained elevated at 6 hours after IGF-1 treatment in aged wild-type SMCs (Figure 4A). The increase in Akt phosphorylation in aged SOD2+/− compared with untreated cells was much less robust at both 3 and 6 hours (2-fold increase) and significantly less than in aged wild-type (P<0.01 versus aged wild-type at 3 hours). Forkhead box O (FoxO) transcription factors are important downstream targets of Akt, and FoxO3α has been implicated in SMC apoptosis.35 IGF-1 significantly increased FoxO3α phosphorylation at both 3 hours (3.7-fold increase) and 6 hours (4.1-fold increase) after treatment in aged wild-type SMCs (Figure 4B). In contrast, the increase in FoxO3α phosphorylation following IGF-1 treatment was significantly less in aged SOD2+/− SMCs (P<0.001 versus aged wild-type at both 3 and 6 hours). Attenuation of FoxO3α phosphorylation in aged SOD2+/− SMCs was also observed in cells treated with angiotensin II, thrombin, and platelet-derived growth factor, indicating the intrinsic effect of SOD2 deficiency on SMC apoptosis (data not shown). Less robust stimulation of Akt in aged SOD2+/− SMCs was not associated with a decreased proliferative response to IGF-1 (data not shown), indicating that SMCs of various phenotypes can coexist in the arterial wall during remodeling. Together, these data suggest that prolonged exposure to increased mitochondrial oxidative stress during aging affects cell viability by impairing survival and activating apoptotic signaling pathways.

**Stress-Activated Protein Kinase I/c-Jun N-terminal Kinase 1 Activity and α-Smooth Muscle Actin Levels Are Increased in Aged SOD2+/− Aortic SMCs**

An inhibition of Akt and increase in caspase activity result in c-Jun N-terminal kinase 1 (JNK1) activity,40 which induces the mitochondrial death pathway leading to apoptosis.41 It is known that circulating tumor necrosis factor-α (TNF-α) levels are increased with aging in both animals and humans.42 Therefore, we investigated TNF-α-induced activation of JNK1 in young and aged wild-type and SOD2+/− SMCs. As shown in Figure 4C, JNK1 phosphorylation was significantly increased in young SOD2+/− compared with young wild-type SMCs after 3 hours of TNF-α treatment (P<0.01). In aged wild-type SMCs, JNK1 phosphorylation was increased significantly at 3 hours after TNF-α treatment (P<0.05). In contrast to young SOD2+/− and aged wild-type SMCs, aged SOD2+/− SMCs had sustained JNK1 activation throughout the TNF-α treatment (P<0.001). TNF-α-induced JNK1 phosphorylation was also significantly higher in aged SOD2+/− compared with young SOD2+/− (P<0.05) and aged wild-type (P<0.01) SMCs 3 hours after the treatment.

Increased smooth muscle α-actin levels and intrinsic SMC stiffness are a mechanism for increased aortic stiffening with aging.33 Consistent with the data shown in Figure 3A, α-actin levels were increased 2.8-fold with aging in wild-type and 3.5-fold in young SOD2+/− compared with young wild-type SMCs (Figure 4D). However, aged SOD2+/− had significantly higher α-actin levels compared with young SOD2+/− (5.4-fold versus 3.5-fold) and aged wild-type SMCs (5.4-fold versus 2.8-fold). Collectively, these data indicate concurrent activation of apoptotic signaling pathways and alterations in arterial wall structure and SMC cytoskeleton.

**Basal Mitochondrial Superoxide Levels Are Increased and Basal and IGF-1-Induced H2O2 Levels Are Decreased With Aging in SOD2+/− Aortic SMCs**

Recent evidence indicates that H2O2 activates the phosphatidylinositol-3-kinase/Akt pathway and promotes cell survival.44 To determine whether impaired cell survival pathways in aged SOD2+/− SMCs are mediated by changes in ROS levels, we measured superoxide and H2O2 levels in young and aged wild-type and SOD2+/− cells. First, we investigated colocalization of MitoTracker Green FM, a mitochondria-selective dye, with MitoSOX Red, a superoxide-sensitive fluorescent dye using confocal microscopy (Figure 5A). Compared with young wild-type, young SOD2+/− SMCs showed bright yellow fluorescence in mitochondria because of colocalization of MitoTracker Green and MitoSOX Red, indicating increased mitochondrial superoxide production. Similarly, aged SOD2+/− had more mitochondrial superoxide levels, as shown by yellow/orange fluorescence in mitochondria compared with that in aged wild-type and young SOD2+/− SMCs.

To determine the effect of prolonged SOD2 deficiency on H2O2 levels, we measured basal and IGF-1-induced H2O2 levels in aged wild-type and SOD2+/− SMCs by Amplex Red assay (Figure 5B). H2O2 levels were significantly lower (31%
Figure 4. Superoxide dismutase 2 (SOD2) deficiency decreased insulin-like growth factor-1 (IGF-1)--induced Akt and FoxO3a phosphorylation and increased tumor necrosis factor-α (TNF-α)--induced c-Jun N-terminal kinase 1 (JNK1) phosphorylation in aortic smooth muscle cells (SMCs) from aged mice and α-actin levels in SMCs from young and aged mice. A and B, Lysates from growth-arrested and IGF-1 (100 ng/mL)--treated aged SMCs were analyzed by Western blotting with anti-phosphospecific Akt or Akt (A) or anti-phosphospecific FoxO3a or FoxO3a antibodies (B). C, Lysates from growth-arrested and TNF-α (100 ng/mL)--treated SMCs were analyzed by Western blotting with anti-phosphospecific JNK1 or JNK1 antibodies. A to C, Densitometric analysis of phosphorylated proteins is shown in the lower panels (mean±SE, n=3). D, Representative Western blot of SMC lysates probed with anti-α-actin or GAPDH antibodies. Densitometric analysis of α-actin levels is shown in the lower panel (mean±SE, n=3).
GAPDH antibodies. Densitometric analysis of phosphospecific FoxO3a or FoxO3a antibodies (and IGF-1 (100 ng/mL)–treated aged SMCs were analyzed by Western blotting with anti-phosphospecific Akt or Akt (ylation and increased tumor necrosis factor-)

**Figure 4.**

muscle cells (SMCs) from aged mice and lysed by Western blotting with anti-phosphospecific JNK1 or JNK1 antibodies.

**A**

**B**

![Graph showing H2O2 levels](image)

**Figure 5.** Mitochondrial superoxide generation was increased and extracellular H2O2 levels were decreased with age in aortic smooth muscle cells (SMCs) from superoxide dismutase 2 (SOD2)+/− mice. A. Confocal laser-scanning microscopy showing colocalization of mitochondria-targeting fluorescent probe MitoSOX Red with the mitochondria-selective dye MitoTracker Green. Yellow fluorescence indicates localization of superoxide in mitochondria. B. H2O2 production was measured using the Amplex Red fluorescence assay (mean±SE, n=9).

The decrease, *P*<0.001) in aged SOD2+/− SMCs compared with aged wild-type SMCs. IGF-1 treatment significantly increased H2O2 levels in wild-type cells (30% increase, *P*<0.001) but had no such effect in SOD2+/− SMCs. These results indicate that decreased H2O2 levels, caused by SOD2 deficiency, impair Akt activity and aortic SMC survival in aged mice via enhanced FoxO3a activation.

**Downregulation of FoxO3a Activity Decreases Staurosporine-Induced Apoptosis in Aged Aortic SMCs**

Because staurosporine, which inhibits Akt and activates FoxO3a, increased cleaved caspase-3 and PARP levels, we investigated whether alteration in Akt/FoxO3a signaling pathway contributes to increased apoptosis in aged SOD2+/− SMCs. Adenoviral overexpression of DN-FoxO3a significantly decreased (58%, *P*<0.001) cleaved PARP levels in aged SOD2+/− compared with cells transfected with control virus (Figure 6). Collectively, our data suggest that prolonged exposure to increased mitochondrial oxidative stress during aging in SOD2+/− SMCs increases apoptosis by modulating Akt/FoxO3a signaling pathway.

**Discussion**

In this study, we provide evidence that (1) SOD2 deficiency over a lifetime is sufficient to induce aortic stiffening, decrease aortic compliance, and cause cardiac dysfunction; (2) aortic stiffening with aging in SOD2+/− mice is associated with structural changes in the aortic wall, with increased collagen content and ruptures in elastin laminae; (3) SOD2 deficiency increases collagen I expression, decreases elastin expression, and increases MMP-2 expression and activity in aged SMCs; (4) SOD2 deficiency over a lifetime increases medial SMC apoptosis in aged mice and sensitizes SMCs to staurosporine-induced increase in cleaved caspase-3 and cleaved PARP levels; (5) prolonged SOD2 deficiency in SMCs activates JNK1 in response to TNF-α treatment; (6) prolonged SOD2 deficiency impairs cell survival as observed by decreased Akt and increased FoxO3a activation in response to IGF-1 treatment; and (7) increased α-actin levels in SOD2+/− SMCs are integral to increased aortic stiffness with aging. It was previously established that SOD2+/− mice have an −50% reduction in SOD2 activity in all tissues compared with the wild-type mice, and the decrease in enzyme activity does not cause any compensatory upregulation of other major components of mitochondrial antioxidant defense system. Although impairment of cardiac function was reported in 6-month-old TRE/SOD2−/− mice, our finding is the first to implicate increased mitochondrial oxidative stress over a lifetime as the source of aortic stiffening and cardiac dysfunction in SOD2−/− mice. Specifically, we provide evidence of how molecular signaling pathways initiated by increased mitochondrial oxidative stress in aortic SMCs contribute to aortic stiffening.

Aortic stiffness is a complex phenomenon that arises from structural alterations in the aortic wall, impaired endothelial function, increased smooth muscle tone, phenotypic modulation of adventitial fibroblasts to myofibroblasts, and chronic low-grade inflammation. The scaffolding proteins collagen
and elastin provide the structural integrity of the aortic wall, and our results show that increased mitochondrial oxidative stress over a lifetime increases the collagen content and ruptures and decreases the elastin in the aorta. The changes in aortic collagen and elastin levels were accompanied by increased expression and activity of MMP-2 in aged SOD2+/− aortic SMCs. Similar to this, Dasgupta et al.48 and others33 reported both age- and redox-regulated increase in MMP expression and activity. Although the increase in MMP-2 expression and activity should decrease collagen levels at first glance, increased collagen I levels are observed in SMCs under oxidative stress conditions.49 In fact, an increase in interstitial and perivascular collagen is observed in cardiac MMP-2 transgenic mice.50 Nevertheless, activation of MMP-2 is strongly correlated with elastic fiber fragmentation, disorganization, and increased stiffness of the arterial vasculature.34 Endothelial dysfunction and inflammation may contribute to increased aortic stiffening in aged SOD2+/−, as endothelial dysfunction is increased in apolipoprotein E−/− mice that are deficient in SOD2,21 and proinflammatory cytokine production is upregulated with increased mitochondrial ROS levels.51

Our results showing increased aortic stiffness in aged SOD2+/− mice accompanied by ventricular dysfunction are supported by several cross-sectional studies that reported a positive association between age-related aortic stiffness and ventricular dysfunction.52 Aortic stiffening increases left ventricular afterload by inducing earlier return of reflected waves in the late systole and causes left ventricle hypertrophy and ventricular dysfunction. Interestingly, the impairment of aortic relaxation and increased diastolic blood pressure in SOD2+/− mice precede increased PWV and Doppler abnormalities in heart function. Furthermore, mitochondrial oxidative stress–induced coupling of vascular-ventricular dysfunction is supported by the observation of impaired heart function with lifelong reduction of SOD2.47

Increased apoptosis of SMCs in the aortic media and increased sensitivity to staurosporine-induced apoptosis in aged SOD2+/− mouse SMCs observed in the present investigation are consistent with the concept that medial SMC apoptosis is an important contributor to age-associated vascular remodeling and loss of aortic elasticity.35 The propensity of aged SOD2+/− aortic SMCs to apoptosis is underlined by impaired activation of Akt and increased activation of FoxO3a in response to IGF-1 treatment. Akt is a negative regulator of FoxO3a transcription factor, which in the absence of Akt-mediated phosphorylation induces the expression of genes involved in apoptosis.53 Interestingly, an increase in MMP-2 and MMP-9 activities was observed in vascular cells following FoxO3a activation.54 Because these MMPs do not contain a consensus binding site for forkhead factors, activated FoxO3a may regulate MMP-2 activity indirectly, including via activation of MMP-3.

Activated MMP-2 induces apoptosis by stimulating JNK activity, as well as cytochrome c release.41 Inhibition of Akt signaling has been shown to induce JNK activity and promote the cleavage of caspase-3 in SMCs.40 JNK activation, in turn, initiates mitochondrial apoptotic pathway via Bax-dependent release of cytochrome c.55 Alternatively, aged SOD2+/− aortic SMCs could undergo apoptosis in the absence of Akt-mediated phosphorylation of apoptosis regulatory proteins Bad and Bax, which suggests that Akt-JNK cross-talk is an important determinant of aged SMC apoptosis.40 Our observation that DN-FoxO3a overexpression attenuates cleaved PARP levels is consistent with the regulatory role of Akt/FoxO3a signaling in aged SOD2+/− aortic SMC apoptosis.

Calcium channel blockers and angiotensin II receptor antagonists are used to treat large artery stiffening.43 These drugs affect vascular SMC tone, which suggests that age-associated vascular stiffening is partly regulated by intrinsic mechanical properties of these cells. Our data showing significantly increased α-actin levels in aged SOD2+/− compared with aged wild-type SMCs are in agreement with the report of Qiu et al.43 that smooth muscle α-actin is a key determinant of vascular SMC stiffness during aging. Increases in α-actin levels and MMP-2 activity were observed in young SOD2+/− compared with young wild-type SMCs, and yet the aortic stiffening and cardiac dysfunction are evident only in aged SOD2+/− mice, which suggests a threshold for mitochondrial oxidative stress to affect structural and biochemical changes in the SMC and aorta and to cause a phenotypic effect. Our observation that H2O2 levels are decreased in SOD2+/− SMCs is consistent with similar findings in SOD2-deficient and knockout mice.56,57 Exogenous H2O2 stimulates Akt phosphorylation in many cell types, including vascular SMCs.4458 Therefore, it is conceivable that low H2O2 levels in aged SOD2+/− SMCs impair cell survival and promote apoptosis by downregulating Akt signaling and activating FoxO3a.

In summary, our data provide insight into the molecular mechanisms by which increased mitochondrial oxidative stress promotes aortic stiffening associated with aging. Altered ROS metabolism in the mitochondria over a lifetime not only enhances collagen secretion and intrinsic stiffness of aortic medial SMCs but also affects redox signaling to induce SMC apoptosis, all of which contribute to aortic stiffening. It would be worth determining whether strategies aimed at regulating mitochondrial oxidative stress have a therapeutic effect against aortic stiffening and its pathophysiological sequelae.

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Disclosures
None.

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