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Mitochondrial Oxidative Stress in Aortic Stiffening With Age:

The Role of Smooth Muscle Cell Function

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Abstract

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 $SOD2^{+/-}$, 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 $SOD2^{+/-}$ vs wild-type (16-month-old) mice. Aortic SMC 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 vs aged wild-type and young (4-month-old) $SOD2^{+/-}$ mice. SM α-actin levels were increased with age in $SOD2^{+/-}$ vs wild-type SMC. Aged $SOD2^{+/-}$ SMC had attenuated insulin-like growth factor-1-induced Akt and FoxO3a phosphorylation and prolonged tumornecrosis factor-α-induced Jun N-terminal kinase 1 activation. Aged $SOD2^{+/-}$ SMC had increased mitochondrial superoxide but decreased hydrogen peroxide levels. Finally, dominant negative FoxO3a overexpression attenuated staurosporine-induced apoptosis in aged $SOD2^{+/-}$ SMC.

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.

Keywords

superoxide dismutase 2; arterial compliance; vascular wall remodeling; apoptosis; Akt; signal transduction; matrix metalloproteinases

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Disclosures
None

Introduction

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 epidemiologic and biologic connection of age to CVD risk, the molecular mechanisms responsible for age-related vascular dysfunction have yet to be elucidated. While advancing age is an unmodifiable risk factor for CVD, it might be possible to target specific molecular signals as an approach to limit 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 fifty 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]

Many pro- and anti-oxidant 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.[13–15] SODs convert superoxide to produce hydrogen peroxide (H_2O_2), 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.[16,17] SOD2-deficient ($SOD2^{+/-}$) mice are viable but demonstrate increased susceptibility to oxidative stress, diminished mitochondrial function and enhanced sensitivity to apoptosis.[18,19] In an atherosclerotic background (apoE knockout), SOD2 deficiency results in accelerated atherosclerosis[20] and endothelial dysfunction in mice.[21] In addition, decreased expression/activity of SOD2 with age was implicated in vascular aging.[22]

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 (SMC) and how these events regulate aortic wall homeostasis and aortic stiffening.

Materials and Methods

Aortic Pulse Wave Velocity

Arterial compliance was determined as described by Hartley et al.[23] In brief, mice were anesthetized with inhaled isoflurane (1% in O_2) and fixed in 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 20 MHz pulsed Doppler probe at the levels of aortic arch and at the abdominal aorta.

Data were analyzed using 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 EKG R-peaks.

Echocardiography

Mice were anesthetized with inhaled isoflurane (1% in O_2) and fixed in supine position on the ECG temperature-controlled board. Ultrasound biomicroscopy was performed using VisualSonics Vevo 660™ equipped with a 30 MHz probe. Ultrasonic images of left ventricle were acquired at 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 data supplement.

Vascular relaxation in isolated mouse aortic rings

Relaxation of isolated mouse aortic rings was measured as described in the online 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[24] (see online data supplement).

Histology, Immunohistochemistry, and Immunofluorescence

Immunohistochemistry and immunofluorescence studies were performed as previously described.[25]

Western Blot Analysis

Preparation of cell extracts and Western blot analysis was performed as described previously.[26]

Quantitative Real-time PCR

Quantitative analysis of mRNA expression of target genes was performed using total RNA extracted from cells and tissues. Reverse transcription was performed using TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). Real-time PCR was performed in quadruplicate with TaqMan Gene Expression Assays for mouse collagen I (Mm01302043_gl), elastin (Mm00514670_ml), MMP-2 (Mm00439508_ml), 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 SMC was calculated relative to that in wild-type using REST2008 (Relative Expression Software Tool).[27]

Gelatin Zymography

MMP-2 activity was assayed by gelatin zymography (see supplemental methods).

Adenovirus Infection of Aortic SMC

A replication-defective adenoviral vector expressing dominantnegative Forkhead Box O3a (DN-FOXO3a) was obtained from Vector Biolabs. DN-FoxO3a, constructed by deletion of

the transactivation domain from the C-terminus,[28] had a HA-tag at the N-terminus andexpressed GFP. Adenovirus expressing only GFP was used as a negative control. Mouse aortic SMC were cultured to 80–90% confluence prior to adenoviral infection. Infections were performed using 100 MOI and the infection efficiency was typically greater than 90%. Measurement of proteins of interest was made in cells harvested 36 h after viral infection.

Detection of Mitochondrial Superoxide

Mitochondrial superoxide levels in aortic SMC were detected as described online in the supplemental methods.

H_2O_2 Measurement

Aortic SMC extracellular H_2O_2 levels were determined using Amplex Red assay (Invitrogen) (see supplemental methods).

Statistical Analysis

Data presented graphically are shown as mean±SE from at least 3 independent experiments. All data were tested for normality using Kolmogorov-Smirnov test and were analyzed by one-way ANOVA, and post hoc analysis was performed using Newman-Keuls test. To account for multiple comparisons, arterial compliance and cardiac function data were analyzed by one-way ANOVA followed by Ryan-Einot-Gabriel-Welsch (REGWF) multiple-range test with an overall α=0.05 (SSPS Software; v. 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 normal chow or Western diet fed young (4 months) and aged (16 months) mice. We used wild-type 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 a 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 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 (EF; 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 EF, aged $SOD2^{+/-}$ had increased left ventricle end-diastolic volume (LVEDV) compared with aged wild-type mice, whether on a normal chow (P<0.05) or Western diet (P<0.05) (Figure 1C). EF and LVEDV in aged $SOD2^{+/-}$ were significantly different from young $SOD2^{+/-}$ mice, irrespective of the diet. Left ventricle posterior wall thickness (LVPW) (Figure 1D) and LV 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 (Suppl Table 1). Diet and age had no effect; however, SOD2 deficiency significantly increased diastolic blood pressure (Suppl Table 1) 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 (NTG)-induced relaxation of phenylephrine-preconstricted thoracic aortic rings. Wild-type mice had decreased vascular relaxation with age at 10^{-7} mol/L NTG ($P<0.01$ vs young) (Suppl Figure 1). 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 NTG-induced relaxation between $SOD2^{+/-}$ and aged wild-type mice. However, at 10^{-6} mol/L NTG, $SOD2^{+/-}$ had impaired aortic relaxation compared with aged wild-type mice ($P<0.01$, young $SOD2^{+/-}$ vs aged wild-type; $P<0.05$, aged $SOD2^{+/-}$ vs 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 SMC of $SOD2^{+/-}$ Mice

Because decrease in elastin/collagen ratio is associated with increase in aortic stiffness[30] 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, 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 SMC. Real time RT-PCR analysis showed a significant increase in collagen I mRNA levels in aged $SOD2^{+/-}$ compared with aged wild-type aortic SMC (1.7±0.1-fold increase; $P<0.01$). In contrast, elastin mRNA levels were significantly lower in aged $SOD2^{+/-}$ SMC (2.5±0.1-fold decrease *vs* 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 $SOD2^{+/-}$ SMC (2.2-fold increase *vs* aged wild-type; $P<0.01$; Figure 2B). Similarly, elastin protein levels were decreased nearly 7-fold in aged $SOD2^{+/-}$ compared with aged wild-type SMC ($P<0.05$; 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.

Matrix Metalloproteinase-2 (MMP-2) Expression and Activity Are Increased in SOD2$^{+/-}$ Aortic SMC

MMP-2 is a critical regulator of extracellular matrix degradation and age-associated vascular remodeling[33] 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 SMC (P<0.001) as determined by real time RT-PCR. MMP-2 activity was significantly increased (P<0.01) in both young and aged SOD2$^{+/-}$ compared with wild-type SMC (Figure 2C). These data suggest that mitochondrial oxidative stress activates signaling pathways involved in MMP-2 expression and activity.

Prolonged SOD2 Deficiency Renders Aortic SMC Susceptible to Apoptosis and Impairs Anti-apoptotic Akt Pathway

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 SMC of aged SOD2$^{+/-}$ mice. Cleaved caspase-3 was barely detectable in young SOD2$^{+/-}$ (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 SMC in aged SOD2$^{+/-}$ reflects the intrinsic effect of SOD2 deficiency, we examined cleaved caspase-3 levels in aortic SMC 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 SMC following staurosporine treatment (Figure 3B). Cleaved caspase-3 was not detected in young wild-type and barely detectable in young SOD2$^{+/-}$ SMC 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. Significant increase in cleaved PARP levels in response to staurosporine treatment was observed in SOD2$^{+/-}$ compared with wild-type SMC (Figure 3B). Consistent with this, aged SOD2$^{+/-}$ SMC treated with staurosporine had significantly higher number of TUNEL-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,[38] in aged wild-type and SOD2$^{+/-}$ SMC treated with and without insulin-like growth factor-1 (IGF-1). Akt phosphorylation increased significantly at 3 h (3.7-fold, P<0.001) and remained elevated at 6 h after IGF-1 treatment in aged wild-type SMC (Figure 4A). Increase in Akt phosphorylation in aged SOD2$^{+/-}$ over untreated cells was much less robust at both 3 and 6 h (2-fold increase) and significantly less than in aged wild-type (P<0.01 vs aged wild-type at 3 h). Forkhead box O (FoxO) transcription factors are important downstream targets of Akt and FoxO3a has been implicated in SMC apoptosis.[39] IGF-1 significantly increased FoxO3a phosphorylation at both 3 (3.7-fold increase) and 6 h (4.1-fold increase) after treatment in aged wild-type SMC (Figure 4B). In contrast, the increase in FoxO3a phosphorylation following IGF-1 treatment was significantly less in aged SOD2$^{+/-}$ SMC (P<0.001 vs aged wild-type at both 3 and 6 h). Attenuation of FoxO3a phosphorylation in aged SOD2$^{+/-}$ SMC 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 $SOD2^{+/-}$ SMC was not associated with decreased proliferative response to IGF-1 (data not shown), indicating that SMC 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(SAPK1/JNK1) Activity and α-Smooth Muscle Actin Levels Are Increased in Aged $SOD2^{+/-}$ Aortic SMC

Inhibition of Akt and increase in caspase activity result in JNK1 activity,[40] which induces mitochondrial death pathway leading to apoptosis.[41] It is known that circulating 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 $SOD2^{+/-}$ SMC. As shown in Figure 4C, JNK1 phosphorylation was significantly increased in young $SOD2^{+/-}$ compared with young wild-type SMC after 3 h TNF-α treatment ($P<0.01$). In aged wild-type, JNK1 phosphorylation was increased significantly at 3 h after TNF-α treatment ($P<0.05$). In contrast to young $SOD2^{+/-}$ and aged wild-type, aged $SOD2^{+/-}$ SMC 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 ($P<0.01$) SMC 3 h after the treatment.

Increased SM 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 with aging in wild-type and 3.5-fold in young $SOD2^{+/-}$ compared with young wild-type SMC (Figure 4D). However, aged $SOD2^{+/-}$ had significantly higher α-actin levels compared with young $SOD2^{+/-}$ (5.4-fold vs 3.5-fold) and aged wild-type SMC (5.4-fold vs 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_2O_2 Levels Are Decreased With Aging in $SOD2^{+/-}$ Aortic SMC

Recent evidence indicates that H_2O_2 activates phosphatidylinositol-3-kinase (PI3K)/Akt pathway and promotes cell survival.[44] To determine whether impaired cell survival pathways in aged $SOD2^{+/-}$ SMC are mediated by changes in ROS levels, we measured superoxide and H_2O_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 $SOD2^{+/-}$ SMC showed bright yellow fluorescence in mitochondria, due to colocalization of MitoTracker Green and MitoSOX Red, indicating increased mitochondrial superoxide production. Similarly, aged $SOD2^{+/-}$ had more mitochondrial superoxide levels as seen by yellow/orange fluorescence in mitochondria compared with that in aged wild-type and young $SOD2^{+/-}$ SMC.

To determine the effect of prolonged SOD2 deficiency on H_2O_2 levels, we measured basal and IGF-1-induced H_2O_2 levels in aged wild-type and $SOD2^{+/-}$ SMC by Amplex Red assay (Figure 5B). H_2O_2 levels were significantly lower (31% decrease, $P<0.001$) in aged $SOD2^{+/-}$ compared with aged wild-type SMC. IGF-1 treatment significantly increased H_2O_2 levels in wild-type cells (30% increase, $P<0.001$), but had no such effect in $SOD2^{+/-}$ SMC. These results indicate that decreased H_2O_2 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 SMC

Because staurosporine, which inhibits Akt[45] 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 $SOD2^{+/-}$ SMC. 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 $SOD2^{+/-}$ SMC 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 and decreases elastin expression and increases MMP-2 expression and activity in aged SMC; 4) SOD2 deficiency over a lifetime increases medial SMC apoptosis in aged mice and sensitizes SMC to staurosporine-induced increase in cleaved caspase-3 and cleaved PARP levels; 5) prolonged SOD2 deficiency in SMC 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 $SOD2^{+/-}$ SMC are integral to increased aortic stiffness with aging. It was previously established that $SOD2^{+/-}$ 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] Though 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 SMC 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 SMC. Similar to this, Dasgupta et al.[48] and others[33] reported both age and redox-regulated increase in MMP expression and activity. Although increase in MMP-2 expression and activity should decrease collagen levels at first glance, increased collagen I levels were observed in SMC under oxidative stress conditions.[49] In fact, increase in interstitial and perivascular collagen was observed in cardiac MMP-2 transgenic mice.[50] Nevertheless, activation of MMP-2 was strongly correlated with elastic fiber fragmentation, disorganization and increased stiffness of the arterial vasculature.[34] Endothelial dysfunction and inflammation may have contributed to increased aortic stiffening in aged $SOD2^{+/-}$ as endothelial dysfunction was increased in $apoE^{-/-}$ mice that are deficient in SOD2[21] and proinflammatory cytokine production was 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 LV 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 SMC in the aortic media and increased sensitivity to staurosporine-induced apoptosis in aged $SOD2^{+/-}$ mouse SMC 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 SMC 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, increase in MMP-2 and MMP-9 activities were 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 SMC.[40] JNK activation, in turn, initiates mitochondrial apoptotic pathway via Bax-dependent release of cytochrome *c*.[55] Alternatively, aged $SOD2^{+/-}$ aortic SMC 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 SMC is in agreement with the report of Qiu et al.[43] that SM 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 SMC, 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 H_2O_2 levels are decreased in $SOD2^{+/-}$ SMC is consistent with similar findings in SOD2 deficient and knockout mice.[56,57] Exogenous H_2O_2 stimulates Akt phosphorylation in many cell types, including vascular SMC.[44,58] Therefore, it is conceivable that low H_2O_2 levels in aged $SOD2^{+/-}$ SMC 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 SMC, 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 therapeutic effect against aortic stiffening and its pathophysiological sequelae.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. O'Rourke MF, Hashimoto J. Mechanical factors in arterial aging: a clinical perspective. J Am Coll Cardiol. 2007; 50:1–13. [PubMed: 17601538]
2. Laurent S, Boutouyrie P. Recent advances in arterial stiffness and wave reflection in human hypertension. Hypertension. 2007; 49:1202–1206. [PubMed: 17452508]
3. Chue CD, Townend JN, Steeds RP, Ferro CJ. Arterial stiffness in chronic kidney disease: causes and consequences. Heart. 2010; 96:817–823. [PubMed: 20406771]
4. Mitchell GF, Hwang SJ, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA, Levy D, Benjamin EJ. Arterial stiffness and cardiovascular events: the Framingham Heart Study. Circulation. 2010; 121:505–511. [PubMed: 20083680]
5. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. Circulation. 2003; 108:2034–2040. [PubMed: 14581381]
6. Sindler AL, Fleenor BS, Calvert JW, Marshall KD, Zigler ML, Lefer DJ, Seals DR. Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging. Aging Cell. 201110.1111/j.1474-9726.2011.00679.x
7. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Münzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation. 2001; 104:2673–2678. [PubMed: 11723017]
8. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1956; 11:298–300. [PubMed: 13332224]
9. Delles C, Zimmerli LU, McGrane DJ, Koh-Tan CH, Pathi VL, McKay AJ, Steedman T, Dargie HJ, Hamilton CA, Dominiczak AF. Vascular stiffness is related to superoxide generation in the vessel wall. J Hypertens. 2008; 26:946–955. [PubMed: 18398337]
10. Noma K, Goto C, Nishioka K, Jitsuiki D, Umemura T, Ueda K, Kimura M, Nakagawa K, Oshima T, Chayama K, Yoshizumi M, Liao JK, Higashi Y. Roles of rho-associated kinase and oxidative stress in the pathogenesis of aortic stiffness. J Am Coll Cardiol. 2007; 49:698–705. [PubMed: 17291936]
11. Edgar D, Shabalina I, Camara Y, Wredenberg A, Calvaruso MA, Nijtmans L, Nedergaard J, Cannon B, Larsson NG, Trifunovic A. Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice. Cell Metab. 2009; 10:131–138. [PubMed: 19656491]
12. Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. Circ Res. 2008; 102:488–496. [PubMed: 18096818]
13. Kinouchi H, Epstein CJ, Mizui T, Carlson E, Chen SF, Chan PH. Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. Proc Natl Acad Sci U S A. 1991; 88:11158–11162. [PubMed: 1763030]

14. Harrison CM, Pompilius M, Pinkerton KE, Ballinger SW. Mitochondrial Oxidative Stress Significantly Influences Atherogenic Risk and Cytokine Induced Oxidant Production. Environ Health Perspect. 2010 Dec 17.
15. Van Deel ED, Lu Z, Xu X, Zhu G, Hu X, Oury TD, Bache RJ, Duncker DJ, Chen Y. Extracellular superoxide dismutase protects the heart against oxidative stress and hypertrophy after myocardial infarction. Free Radic Biol Med. 2008; 44:1305–1313. [PubMed: 18206658]
16. Li Y, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat Genet. 1995; 11:376–381. [PubMed: 7493016]
17. Lebovitz RM, Zhang H, Vogel H, Cartwright J Jr, Dionne L, Lu N, Huang S, Matzuk MM. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Natl Acad Sci U S A. 1996; 93:9782–9787. [PubMed: 8790408]
18. Van Remmen H, Williams MD, Guo Z, Estlack L, Yang H, Carlson EJ, Epstein CJ, Huang TT, Richardson A. Knockout mice heterozygous for Sod2 show alterations in cardiac mitochondrial function and apoptosis. Am J Physiol Heart Circ Physiol. 2001; 281:H1422–H1432. [PubMed: 11514315]
19. Williams MD, Van Remmen H, Conrad CC, Huang TT, Epstein CJ, Richardson A. Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. J Biol Chem. 1998; 273:28510–28515. [PubMed: 9774481]
20. Ballinger SW, Patterson C, Knight-Lozano CA, Burow DL, Conklin CA, Hu Z, Reuf J, Horaist C, Lebovitz R, Hunter GC, McIntyre K, Runge MS. Mitochondrial integrity and function in atherogenesis. Circulation. 2002; 106:544–549. [PubMed: 12147534]
21. Ohashi M, Runge MS, Faraci FM, Heistad DD. MnSOD deficiency increases endothelial dysfunction in ApoE-deficient mice. Arterioscler Thromb Vasc Biol. 2006; 26:2331–2336. [PubMed: 16873728]
22. Li M, Chiu JF, Mossman BT, Fukagawa NK. Down-regulation of manganese-superoxide dismutase through phosphorylation of FOXO3a by Akt in explanted vascular actin muscle cells from old rats. J Biol Chem. 2006; 281:40429–40439. [PubMed: 17079231]
23. Hartley CJ, Taffet GE, Michael LH, Pham TT, Entman ML. Noninvasive determination of pulse-wave velocity in mice. Am J Physiol. 1997; 273:H494–H500. [PubMed: 9249523]
24. Moon SK, Thompson LJ, Madamanchi N, Ballinger S, Papaconstantinou J, Horaist C, Runge MS, Patterson C. Aging, oxidative responses, and proliferative capacity in cultured mouse aortic smooth muscle cells. Am J Physiol Heart Circ Physiol. 2001; 280:H2779–H288. [PubMed: 11356636]
25. Zhou RH, Pesant S, Cohn HI, Eckhart AD. Enhanced sterol response element-binding protein in postintervention restenotic blood vessels plays an important role in vascular smooth muscle proliferation. Life Sci. 2008; 82:174–181. [PubMed: 18068195]
26. Madamanchi NR, Li S, Patterson C, Runge MS. Thrombin regulates vascular smooth muscle cell growth and heat shock proteins via the JAK-STAT pathway. J Biol Chem. 2001; 276:18915–18924. [PubMed: 11278437]
27. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for groupwise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002; 30:e36. [PubMed: 11972351]
28. Skurk C, Maatz H, Kim HS, Yang J, Abid MR, Aird WC, Walsh K. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. J Biol Chem. 2004; 279:1513–1525. [PubMed: 14551207]
29. Nürnberger J, Dammer S, Opazo Saez A, Philipp T, Schäfers RF. Diastolic blood pressure is an important determinant of augmentation index and pulse wave velocity in young, healthy males. J Hum Hypertens. 2003; 17:153–158. [PubMed: 12624604]
30. Osborne-Pellegrin M, Labat C, Mercier N, Challande P, Lacolley P. Changes in aortic stiffness related to elastic fiber network anomalies in the Brown Norway rat during maturation and aging. Am J Physiol Heart Circ Physiol. 2010; 299:H144–H152. [PubMed: 20435849]
31. Steed MM, Tyagi N, Sen U, Schuschke DA, Joshua IG, Tyagi SC. Functional consequences of the collagen/elastin switch in vascular remodeling in hyperhomocysteinemic wild-type, eNOS−/−,

and iNOS−/− mice. Am J Physiol Lung Cell Mol Physiol. 2010; 299:L301–L311. [PubMed: 20581102]

32. Ng K, Hildreth CM, Phillips JK, Avolio AP. Aortic stiffness is associated with vascular calcification and remodeling in a chronic kidney disease rat model. Am J Physiol Renal Physiol. 2011; 300:F1431–F1436. [PubMed: 21478483]
33. Wang M, Lakatta EG. Altered regulation of matrix metalloproteinase-2 in aortic remodeling during aging. Hypertension. 2002; 39:865–873. [PubMed: 11967241]
34. Chung AW, Yang HH, Kim JM, Sigrist MK, Chum E, Gourlay WA, Levin A. Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease. Circulation. 2009; 120:792–801. [PubMed: 19687355]
35. Sawabe M. Vascular aging: from molecular mechanism to clinical significance. Geriatr Gerontol Int. 2010; 10 (Suppl 1):S213–S220. [PubMed: 20590836]
36. Tchivilev I, Madamanchi NR, Vendrov AE, Niu XL, Runge MS. Identification of a protective role for protein phosphatase 1cgamma1 against oxidative stress-induced vascular smooth muscle cell apoptosis. J Biol Chem. 2008; 283:22193–205. [PubMed: 18540044]
37. Lee JY, Jung GY, Heo HJ, Yun MR, Park JY, Bae SS, Hong KW, Lee WS, Kim CD. 4-Hydroxynonenal induces vascular smooth muscle cell apoptosis through mitochondrial generation of reactive oxygen species. Toxicol Lett. 2006; 166:212–221. [PubMed: 16919899]
38. Miyamoto S, Murphy AN, Brown JH. Akt mediated mitochondrial protection in the heart: metabolic and survival pathways to the rescue. J Bioenerg Biomembr. 2009; 41:169–180. [PubMed: 19377835]
39. Park KW, Kim DH, You HJ, Sir JJ, Jeon SI, Youn SW, Yang HM, Skurk C, Park YB, Walsh K, Kim HS. Activated forkhead transcription factor inhibits neointimal hyperplasia after angioplasty through induction of p27. Arterioscler Thromb Vasc Biol. 2005; 25:742–747. [PubMed: 15662024]
40. Suhara T, Kim HS, Kirshenbaum LA, Walsh K. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. Mol Cell Biol. 2002; 22:680–691. [PubMed: 11756562]
41. Singh BM, Ross RS, Johnson JN, Singh K. beta-Adrenergic receptor-stimulated apoptosis in adult cardiac myocytes involves MMP-2-mediated disruption of β1 integrin signaling and mitochondrial pathway. Am J Physiol Cell Physiol. 2006; 290:C254–C261. [PubMed: 16148033]
42. Csiszar A, Wang M, Lakatta EG, Ungvari Z. Inflammation and endothelial dysfunction during aging: role of NF-kappaB. J Appl Physiol. 2008; 105:1333–1341. [PubMed: 18599677]
43. Qiu H, Zhu Y, Sun Z, Trzeciakowski JP, Gansner M, Depre C, Resuello RR, Natividad FF, Hunter WC, Genin GM, Elson EL, Vatner DE, Meininger GA, Vatner SF. Short communication: vascular smooth muscle cell stiffness as a mechanism for increased aortic stiffness with aging. Circ Res. 2010; 107:615–619. [PubMed: 20634486]
44. Sadidi M, Lentz SI, Feldman EL. Hydrogen peroxide-induced Akt phosphorylation regulates Bax activation. Biochimie. 2009; 91:577–585. [PubMed: 19278624]
45. Hill MM, Andjelkovic M, Brazil DP, Ferrari S, Fabbro D, Hemmings BA. Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. J Biol Chem. 2001; 276:25643–25646. [PubMed: 11373274]
46. Zhang Y, Xing D. PUMA promotes Bax activation in a FoxO3a-dependent manner in STS-induced apoptosis. J Innov Opt Health Sci. 2010; 3:31–38.
47. Loch T, Vakhrusheva O, Piotrowska I, Ziolkowski W, Ebelt H, Braun T, Bober E. Different extent of cardiac malfunction and resistance to oxidative stress in heterozygous and homozygous manganese-dependent superoxide dismutase-mutant mice. Cardiovasc Res. 2009; 82:448–457. [PubMed: 19293248]
48. Dasgupta J, Kar S, Van Remmen H, Melendez JA. Age-dependent increases in interstitial collagenase and MAP Kinase levels are exacerbated by superoxide dismutase deficiencies. Exp Gerontol. 2009; 44:503–510. [PubMed: 19409972]
49. Bachem MG, Wendelin D, Schneiderhan W, Haug C, Zorn U, Gross HJ, Schmid-Kotsas A, Grünert A. Depending on their concentration oxidized low density lipoproteins stimulate

extracellular matrix synthesis or induce apoptosis in human coronary artery smooth muscle cells. Clin Chem Lab Med. 1999; 37:319–326. [PubMed: 10353478]

50. Bergman MR, Teerlink JR, Mahimkar R, Li L, Zhu BQ, Nguyen A, Dahi S, Karliner JS, Lovett DH. Cardiac matrix metalloproteinase-2 expression independently induces marked ventricular remodeling and systolic dysfunction. Am J Physiol Heart Circ Physiol. 2007; 292:H1847–H1860. [PubMed: 17158653]
51. Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, Sack MN, Kastner DL, Siegel RM. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J Exp Med. 2011; 208:519–533. [PubMed: 21282379]
52. Abhayaratna WP, Srikusalanukul W, Budge MM. Aortic stiffness for the detection of preclinical left ventricular diastolic dysfunction: pulse wave velocity versus pulse pressure. J Hypertens. 2008; 26:758–764. [PubMed: 18327086]
53. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999; 96:857–868. [PubMed: 10102273]
54. Lee HY, You HJ, Won JY, Youn SW, Cho HJ, Park KW, Park WY, Seo JS, Park YB, Walsh K, Oh BH, Kim HS. Forkhead factor, FOXO3a, induces apoptosis of endothelial cells through activation of matrix metalloproteinases. Arterioscler Thromb Vasc Biol. 2008; 28:302–308. [PubMed: 18063811]
55. Papadakis ES, Finegan KG, Wang X, Robinson AC, Guo C, Kayahara M, Tournier C. The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway. FEBS Lett. 2006; 580:1320–1326. [PubMed: 16458303]
56. Lee S, Van Remmen H, Csete M. Sod2 overexpression preserves myoblast mitochondrial mass and function, but not muscle mass with aging. Aging Cell. 2009; 8:296–310. [PubMed: 19627269]
57. Morten KJ, Ackrell BA, Melov S. Mitochondrial reactive oxygen species in mice lacking superoxide dismutase 2: attenuation via antioxidant treatment. J Biol Chem. 2006; 281:3354–3359. [PubMed: 16326710]
58. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem. 1999; 274:22699–22704. [PubMed: 10428852]

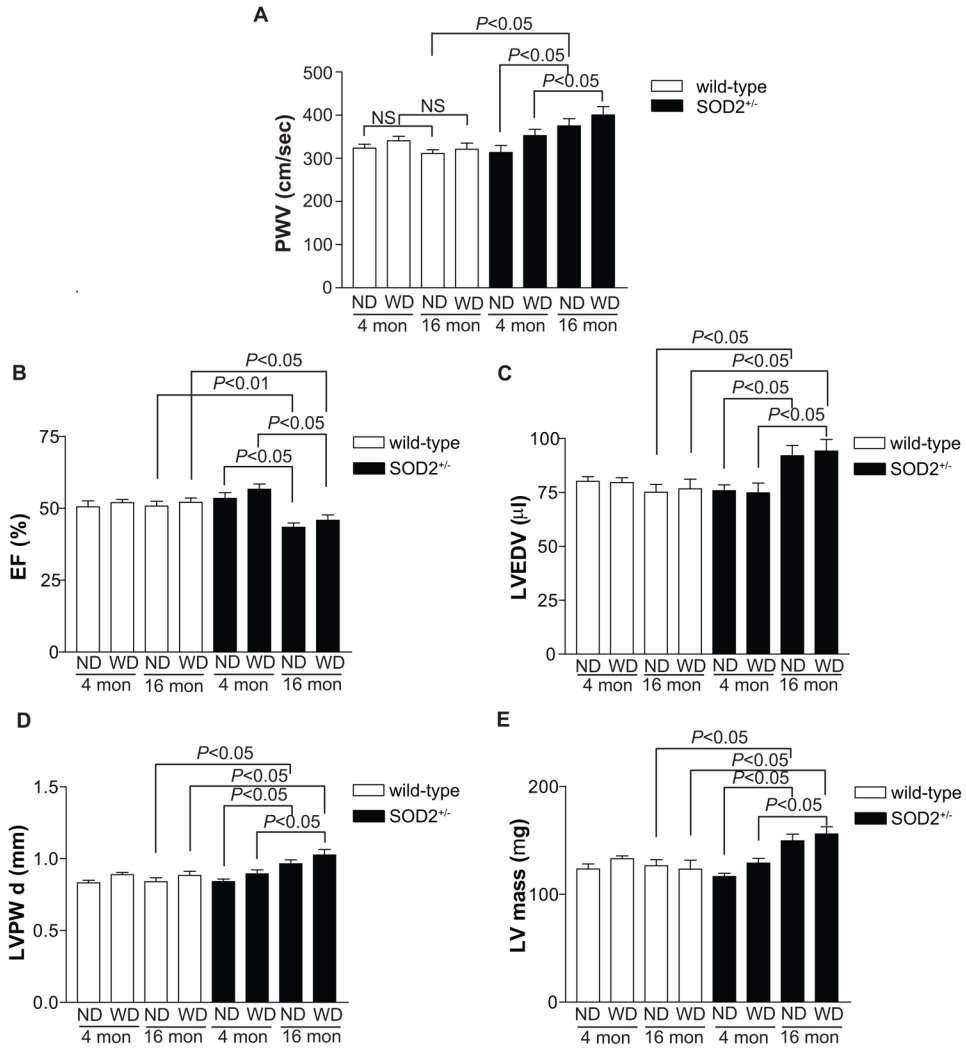

Figure 1.

Age-dependent changes in arterial compliance and cardiac function in wild-type and SOD2$^{+/-}$ mice fed normal chow (ND) or Western diet (WD). Aortic pulse wave velocity (PWV, A), ejection fraction (EF, B), left ventricle end diastolic volume (LVEDV, C), left ventricle posterior wall thickness (LVPW, D) and calculated left ventricle mass (LV mass, E) are presented as mean±SE (n=10).

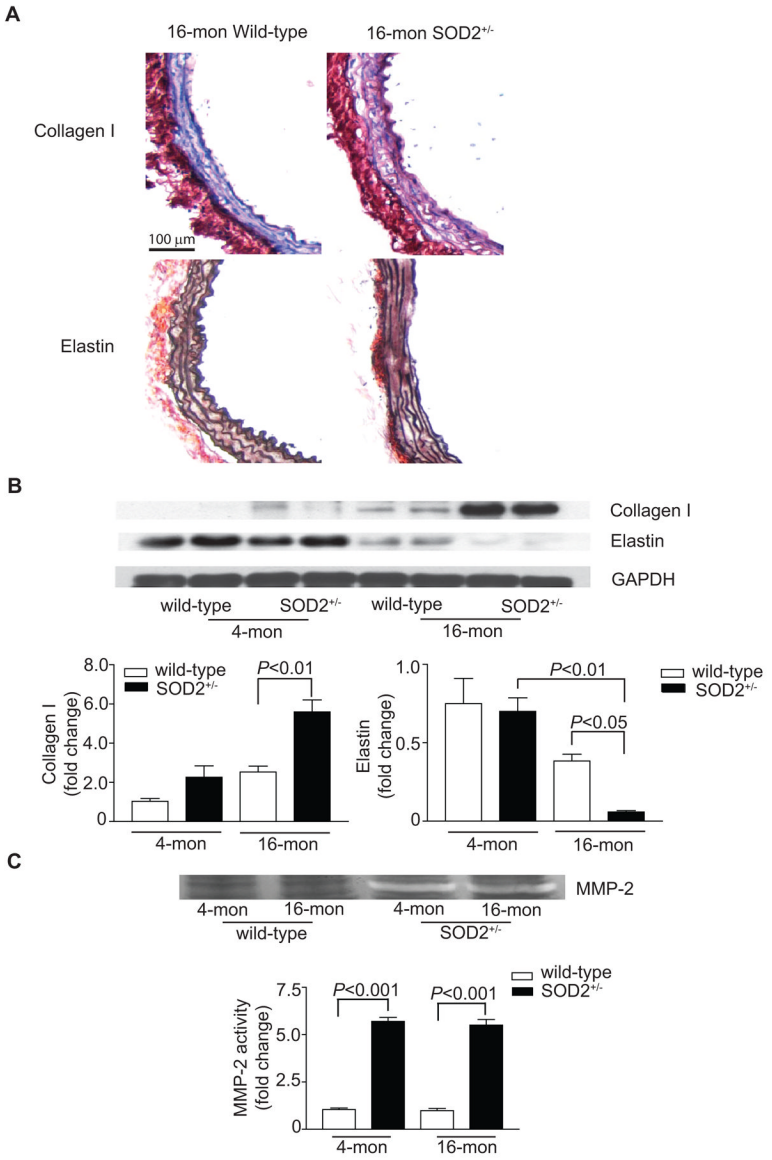

Figure 2.

SOD2 deficiency increases collagen I synthesis and disrupts elastic laminae in aortas of aged mice, increases collagen and decreases elastin levels in aged aortic SMC and enhances MMP-2 activity in SMC 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 was shown in the lower panel (mean ±SE, n=3). C, A representative gelatin zymogram showing MMP-2 activity in aortic SMC lysates. Densitometric analysis of MMP-2 activity was shown in the lower panel (mean±SE, n=3).

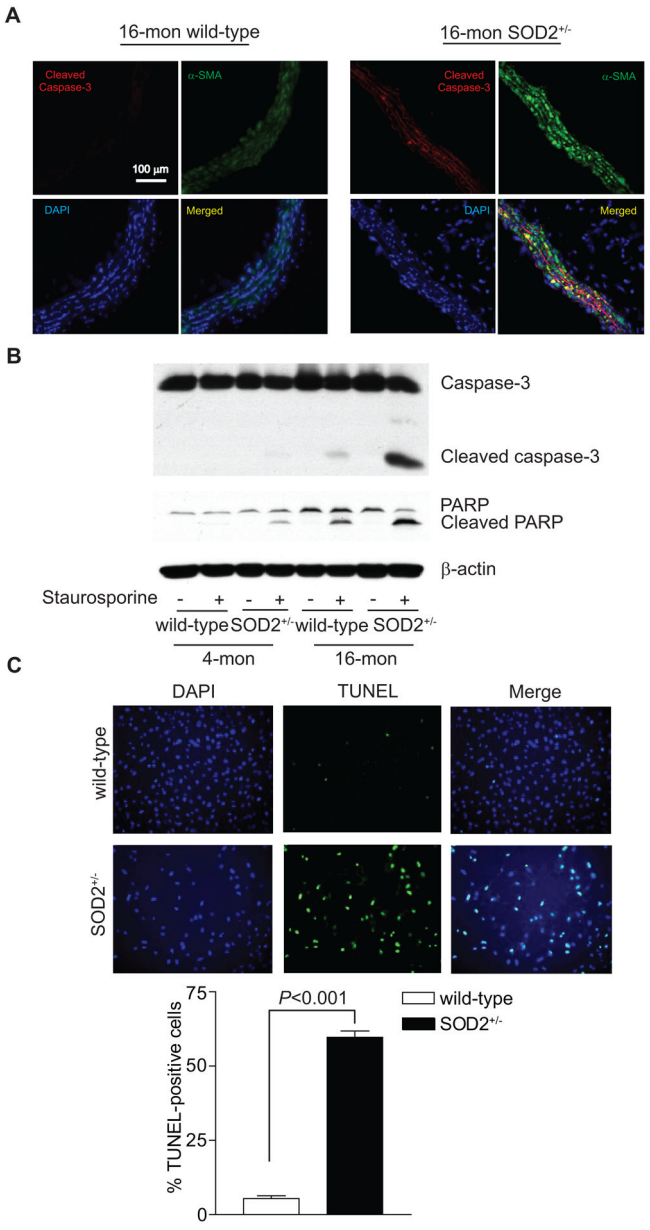

Figure 3.

SOD2 deficiency enhances medial SMC apoptosis in the aorta of aged mice and sensitizes aortic SMC 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 SMC. Nuclei were counterstained with DAPI (blue). B, Lysates from aortic SMC treated or without 1.0 μmol/L staurosporine for 6 h were analyzed by Western blotting with anti-caspase-3, anti-PARP and anti-β-actin antibodies. Data shown represent an experiment that was repeated at least twice with similar results. C, Aged SMC treated with staurosporine (0.1 μmol/L) for 12 h were analyzed by fluorescent (green) TUNEL staining. Nuclei were stained with DAPI (blue). Quantitation of apoptotic cells presented as % TUNEL-positive cells in each field of view (mean ± SE, n=3).

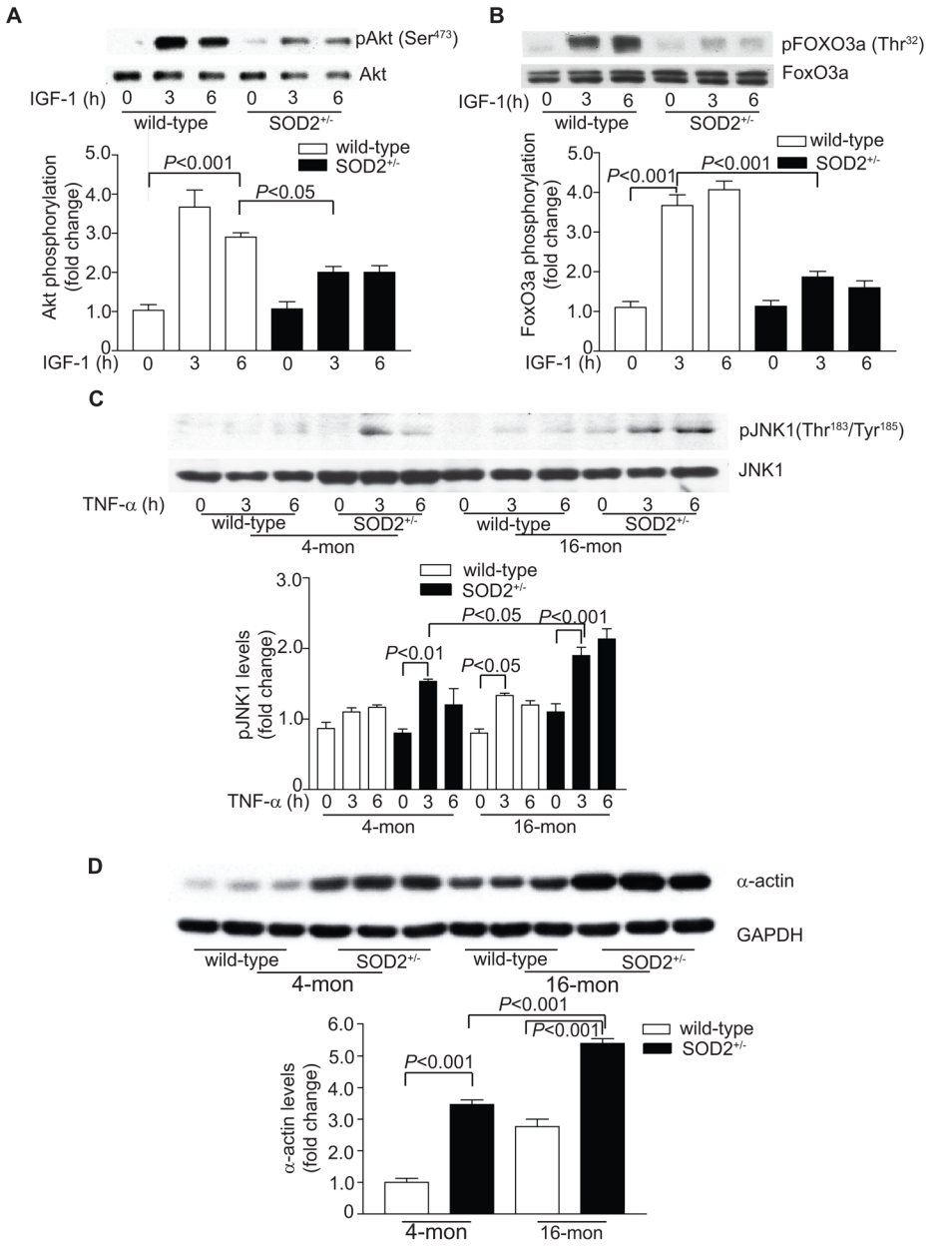

Figure 4.

SOD2 deficiency decreases IGF-1-induced Akt and FoxO3a phosphorylation and increases TNF-α-induced JNK1 phosphorylation in aortic SMC from aged mice and α-actin levels in SMC from young and aged mice. Lysates from growth-arrested and IGF-1 (100 ng/ml) treated aged SMC were analyzed by Western blotting with anti-phosphospecific Akt or Akt (A) or anti-phosphospecific FoxO3a or FoxO3a antibodies (B). Lysates from growth-arrested and TNF-α (100 ng/ml) treated SMC were analyzed by Western blotting with anti-phosphospecific JNK1 or JNK1 antibodies (C). Densitometric analysis of phosphorylated proteins was shown in the lower panel of each figure (mean±SE, n=3). A representative Western blot of SMC lysates probed with anti-α-actin or GAPDH antibodies (D). Densitometric analysis of α-actin levels in the lower panel (mean±SE, n=3).

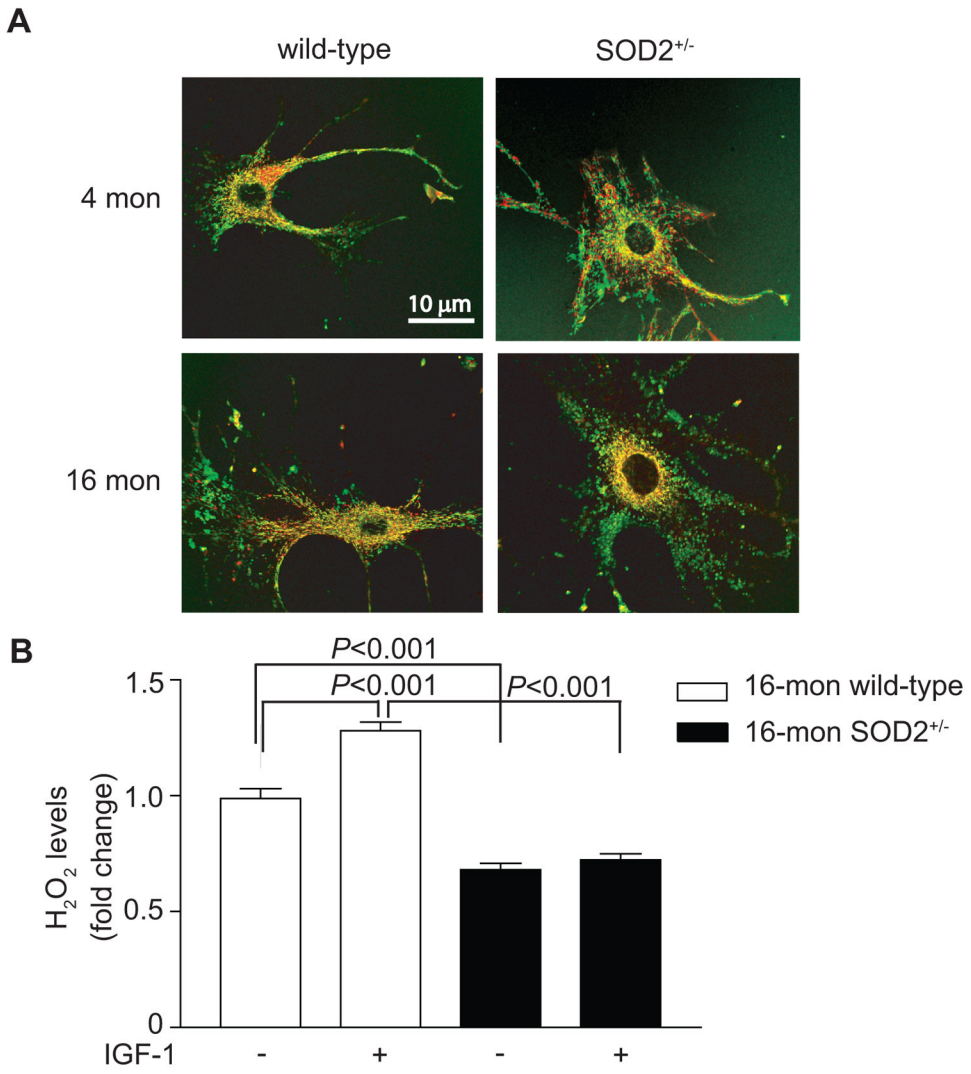

Figure 5.

Mitochondrial superoxide generation is increased whereas extracellular H_2O_2 levels are decreased with age in aortic SMC from 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, H_2O_2 production was measured using Amplex Red fluorescence assay (mean±SE, n=9).

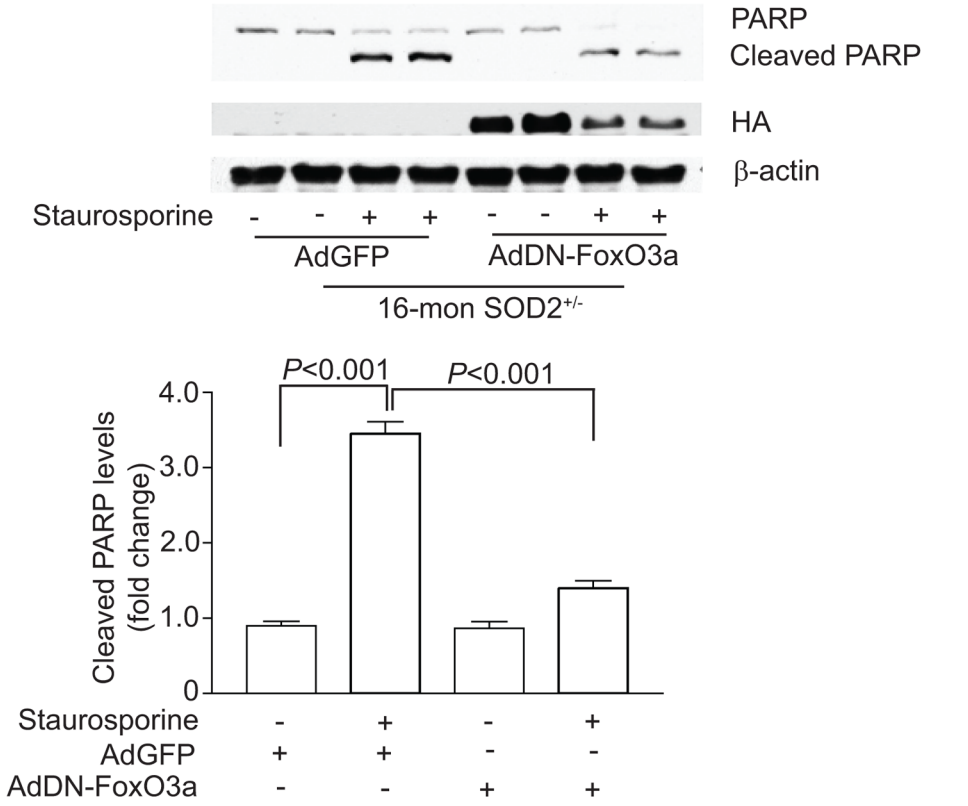

Figure 6.

DN-FoxO3a overexpression attenuates increase in cleaved PARP levels induced by staurosporine in aortic SMC from aged $SOD2^{+/-}$ mice. A, Aged aortic SMC infected with AdGFP or AdDN-FoxO3a were either untreated or treated with 1.0 μmol/L staurosporine for 6 h and cell lysates were analyzed by Western blotting with anti-PARP, anti-HA or anti-β-actin antibodies. Densitometric analysis of β-actin levels in the lower panel (mean±SE, n=3).