Intracellular Physiology

Aging-Induced Phenotypic Changes and Oxidative Stress Impair Coronary Arteriolar Function

Anna Csiszar, Zoltan Ungvari, John G. Edwards, Pawel Kaminski, Michael S. Wolin, Akos Koller, Gabor Kaley

Abstract—We aimed to elucidate the possible role of phenotypic alterations and oxidative stress in age-related endothelial dysfunction of coronary arterioles. Arterioles were isolated from the hearts of young adult (Y, 14 weeks) and aged (A, 80 weeks) male Sprague-Dawley rats. For videomicroscopy, pressure-induced tone of Y and A arterioles and their passive diameter did not differ significantly. In A, arterioles L-NAME (a NO synthase blocker)–sensitive flow-induced dilations were significantly impaired (Y: 41±8% versus A: 3±2%), which could be augmented by superoxide dismutase (SOD) or Tiron (but not L-arginine or the TXA₂ receptor antagonist SQ29,548). For lucigenin chemiluminescence, O₂⁻ generation was significantly greater in A than Y vessels and could be inhibited with SOD and diphenylidionium. NADH-driven O₂⁻ generation was also greater in A vessels. Both endothelial and smooth muscle cells of A vessels produced O₂⁻ (shown with ethidium bromide fluorescence). For Western blotting, expression of eNOS and COX-1 was decreased in A compared with Y arterioles, whereas expressions of COX-2, Cu/Zn-SOD, Mn-SOD, xanthine oxidase, and the NAD(P)H oxidase subunits p47phox, p67phox, Mox-1, and p22phox did not differ. Aged arterioles showed an increased expression of iNOS, confined to the endothelium. Decreased eNOS mRNA and increased iNOS mRNA expression in A vessels was shown by quantitative RT-PCR. In vivo formation of peroxynitrite was evidenced by Western blotting, and immunohistochemistry showing increased 3-nitrotyrosine content in A vessels. Thus, aging induces changes in the phenotype of coronary arterioles that could contribute to the development of oxidative stress, which impairs NO-mediated dilations. (Circ Res. 2002;90:1159-1166.)

Key Words: arteriole ▪ endothelium ▪ superoxide ▪ reactive oxygen species ▪ free radical scavenger

Numerous studies suggest that aging is an important risk factor for the development of ischemic heart disease. This may be due to an age-related increase in coronary vascular resistance, leading to a reduction in myocardial blood flow and flow reserve. Studies utilizing pharmacological probes, such as acetylcholine (ACh), suggest that aging is associated with endothelial dysfunction in humans and in laboratory animals. One of the most important mechanisms that contribute to the local regulation of myocardial blood flow is the flow (shear stress)–induced NO–mediated dilation of small coronary arteries and arterioles; however, its age-related alterations have not yet been elucidated. Previous studies demonstrated that pathophysiological conditions that are associated with an increased risk of coronary heart disease, such as hypercholesterolemia, diabetes, hypertension, and hyperhomocysteinemia, are characterized by decreased NO synthesis/release and a significant impairment of flow-induced dilation of arterioles.

Thus, it can be hypothesized that aging may also impair NO synthesis/release in the coronary endothelium by decreasing availability of the eNOS substrate L-arginine or by decreasing the activity of eNOS. Also, there may be an increased breakdown of NO due to an augmented arteriolar production of superoxide (O₂⁻) anions or a loss of antioxidant capacity, which normally provides protection against reactive oxygen species (oxidative stress, reviewed in Beckman and Ames). In addition, age-related dysfunction in some conduit vessels may involve an enhanced synthesis of thromboxane A₂ (TXA₂), suggesting that the underlying mechanisms associated with vascular aging are multifactorial with significant anatomic heterogeneity. Recent studies showing that aging significantly alters regulation of gene expression by hormonal and growth factors raise the possibility that complex phenotypic changes affecting expression of eNOS and/or a shift in the expression of pro- and antioxidant enzymes may elicit age-related decreases in NO bioavailability.

To test the hypothesis that aging is associated with impaired NO-mediation of flow-induced dilation due to an increased production of superoxide (which scavenges NO) or TXA₂ (which may counteract the effect of NO), we characterized in isolated coronary arterioles age-related alterations in flow-induced dilation, O₂⁻ production, peroxynitrite generation, and expression of eNOS and the pro- and antioxid...
dant enzymes known to be involved in the modulation of flow-induced arteriolar dilation (SOD and COX isoforms, NAD(P)H oxidases, xanthine oxidase, iNOS).

Materials and Methods

Animals
Fourteen- to sixteen-week-old (young, Y) and 74- to 82-week-old (aged, A) male Sprague-Dawley rats (n=36; Harlan Sprague-Dawley, Indianapolis, Ind) were used. Protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals.

Functional Studies on Isolated Arterioles
Intramural arteriolar branches of the septal artery were isolated from the hearts of Y and A rats. Changes in diameter of pressurized (at 80 mm Hg) Y and A arterioles in response to step increases in intraluminal flow (from 0 to 60 µL/min) were measured with videomicroscopy.13,18,19 Before and after incubation with N·-nitro-L-arginine-methyl-ester (L-NAME, 3×10⁻⁴ mol/L, for 20 minutes, an inhibitor of NO synthesis). Responses to acetylcholine (ACh), the voltage-operated Ca²⁺ channel inhibitor verapamil, and the NO donor sodium nitroprusside (SNP) were also obtained. In separate experiments, responses of arterioles to flow and ACh (10⁻⁶ mol/L) were measured; the arterioles were also incubated with L-arginine (10⁻⁴ mol/L, the substrate of eNOS), SQ29,548 (10⁻⁶ mol/L, a TXA₂ receptor antagonist), or the superoxide scavengers Tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid, 10 mmol/L),20 or superoxide dismutase (SOD, 120 U/mL),21 and responses were reassessed.

Measurement of Vascular Superoxide Level
Vascular O₂⁻ production was assessed from vascular samples similar to those used for biochemical studies, by the lucigenin chemiluminescence method,13 according to the modified protocol of Mohazzab et al.20 In separate experiments, O₂⁻ production of aged vessels was determined in the absence and presence of rotenone (10⁻⁵ mol/L), aminoguanidine (10⁻⁴ mol/L), indomethacin (10⁻⁵ mol/L, a COX inhibitor), allopurinol (10⁻⁴ mol/L, an inhibitor of xanthine oxidase), diphenyliodonium (DPI, 10⁻⁴ mol/L, an inhibitor of flavoprotein-containing oxidases, including NAD(P)H oxidases), or SOD (200 U/mL). In other experiments, superoxide production by aortic smooth muscle actin.18,20 and responses were reassessed.

Xanthine Oxidase Assay and NADH Oxidase Assay
Pooled coronary vessels of Y and A rats were homogenized in liquid nitrogen and lysed in buffer containing protease inhibitors. Xanthine oxidase and NADH oxidase activity were measured with the lucigemin assay.13,20,23 In 20 µL of the particulate fraction of the homogenate after addition of 10⁻⁷ mol/L xanthine and NADH, respectively. Protein content was measured in an aliquot of the homogenate by the Lowry method.

Ethidium Bromide Fluorescence
Hydroethidine, an oxidative fluorescent dye, was used to localize superoxide production in situ according to the modified protocol of Lund et al.23 This method provides sensitive detection of O₂⁻ levels in situ.24 The number of fluorescent ethidium bromide (EB)-stained nuclei was counted per arterial cross sections in unfixed frozen sections of the left ventricle after hydroethidine (2×10⁻⁴ mol/L) exposure. The sections were also counterstained with hematoxylin and immunostained for α-smooth muscle actin. Samples exposed to hydroethidine in the presence of Tiron (10 mmol/L) served as control.

Detection of Coronary Arteriole Protein Expression With Western Blotting
We have modified a Western blot protocol enabling us to use single arterioles, identical to those used for functional studies. A detailed description and validation of the method can be found in the expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org. Expressions of eNOS, COX-1, COX-2, Mn-SOD and Cu, Zn-SOD, iNOS, the NAD(P)H oxidase subunits p22phox, p47phox, p67phox and Mox-1, and xanthine oxidase were analyzed in coronary vessels isolated from the same Y and A rats that were used for functional studies. Anti–β-actin was used to normalize for loading variations.

RNA Isolation and Real-Time RT-PCR
A quantitative reverse transcription–PCR technique was used to determine relative expression levels of eNOS mRNA in coronary arterioles of Y and A rats. Total RNA from single coronary arterioles was isolated and was reverse transcribed. PCR reactions were performed using a real-time fluorescent determination in the Roche Molecular Biochemicals LightCycler System. The housekeeping gene β-actin was used for internal normalization.

Detection of Coronary 3-Nitrotyrosine Content With Western Blotting
3-Nitrotyrosine content in single coronary arterioles was quantified with Western blotting. Isolated coronary arterioles of control rats incubated with authentic ONOO⁻ (10 µmol/L and 100 mmol/L) served as positive controls.

Immunohistochemical Detection of 3-Nitrotyrosine
Frozen sections from the interventricular septum and the anterior wall of the left ventricle were labeled for 3-nitrotyrosine, stained with Vector-DAB, and counterstained with hematoxylin.

Localization of iNOS With Dual Immunofluorescence Staining and Immunohistochemistry
Immunolabeling was carried out with primary anti-iNOS and anti–α-smooth muscle actin antibody (to visualize smooth muscle) or anti-CD31 antibody (to stain endothelium). Separate tissue sections were immunolabeled for iNOS, stained with Vector-ALP substrate, and counterstained with hematoxylin.

Measurement of Serum Nitrite/Nitrate (NOx)
NOx content was measured in serum samples of Y and A rats as described previously.25

Data Analysis
Arteriolar dilations were expressed as percentage of the maximal dilation in the absence of extracellular Ca²⁺ at 80 mm Hg intraluminal pressure. Lucigenin chemiluminescence data were normalized to the respective control mean values. Densitometric ratios were expressed as a percentage of the control mean value. Data are mean±SEM. Statistical analyses of data were performed by Student’s t test or by 2-way ANOVA followed by the Tukey post hoc test, as appropriate. A value of P<0.05 was considered statistically significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
Isolated coronary arterioles developed active tone in response to intraluminal pressure of 80 mm Hg without the use of any vasoactive agent (Y: 31±2%, A: 33±4%; NS). The passive diameters of Y and A arterioles in the absence of extracellular Ca²⁺ were 200±11 and 229±16 µm (NS), respectively (at 80 mm Hg).
Flow- and Agonist-Induced Dilations of Coronary Arterioles

Step increases in intraluminal flow (from 0 to 60 μL/min, corresponding to a shear stress of ≈20 dyn/cm²) elicited marked dilations of coronary arterioles of Y rats, which were significantly inhibited by L-NAME (Figure 1A). In contrast, in arterioles of aged rats, responses to flow were greatly diminished and were unaffected by L-NAME (Figure 1B).

Administration of the eNOS substrate L-arginine or the presence of SQ29,548 (Figure 1C) did not restore flow-induced arteriolar dilations, excluding the possibility that decreased availability of L-arginine or an increased PGH₂/TXA₂ synthesis affects coronary arteriolar responses in aging. In contrast, administration of the free radical scavengers SOD or Tiron significantly augmented dilations of A arterioles to step increases in intraluminal flow (Figures 1C and 1D). In the presence of SOD, administration of L-NAME diminished flow-induced dilation of A arterioles (Figure 1D). SOD, Tiron, l-arginine, or SQ29548 had no significant effect on responses to Y arterioles.

We also demonstrated that ACh-induced dilation of aged coronary arterioles is impaired (Figure 1E), extending results of previous studies on vessels from other vascular beds. Dilations to the NO donor SNP (Figure 1F) and the Ca²⁺ antagonist verapamil (at 10⁻⁶ mol/L, Y: 75±8%, A: 81±9%; NS, not shown), as well as the passive mechanical properties of arterioles of Y and A rats were similar.

Vascular Superoxide Production

Under basal conditions, levels of O₂⁻ produced by coronary vessels of A rats were significantly higher than by vessels of Y rats (Figure 2A). O₂⁻ level in A vessels was significantly decreased by administration of DPI or SOD, whereas it was unaffected by rotenone, aminoguanidine, indomethacin, or allopurinol (Figure 2B). In aortic segments of A rats, there was also a significantly increased O₂⁻ production (Y: 100±11%, A: 239±16%), confirming results of previous studies.

Xanthine Oxidase and NADH Oxidase Assays

Xanthine-driven O₂⁻ production by coronary vessel homogenates from A and Y rats did not differ significantly (Figure 2C). NADH-driven O₂⁻ production by vascular samples from A rats was significantly increased as compared with Y controls (Figure 2C). Both xanthine- and NADH-induced increases in lucigenin chemiluminescence could be abolished by addition of SOD.

Hydroethidine Fluorescence

Representative fluorescent photomicrographs of EB-stained aged (Figure 2D, left) and young (Figure 2E, left) coronary vessel sections are shown in Figure 2 (brightfield photomicrographs of the same vessel sections: Figures 2D and 2E, right). The relative number of EB-positive nuclei in arterioles and arteries was significantly greater in sections of A hearts than in sections of Y hearts. Overlaying (Figure 2H) of EB-stained fluorescent (Figure 2F) and hematoxylin-stained brightfield (Figure 2G) images of aged vessels or EB-stained fluorescent images with images of the same vessel sections stained for smooth muscle α-actin (Figure 2I, green) showed that increased O₂⁻ levels are present both in the endothelium and the smooth muscle of aged vessels. Incubation of tissue sections with Tiron (10 mmol/L) prevented EB staining of nuclei.

Detection of Coronary 3-Nitrotyrosine Content With Western Blotting and Immunohistochemistry

Aging was associated with an increased prevalence of nitrotyrosine residues of proteins in coronary vessels, whereas 3-nitrotyrosine immunoreactivity was weak or absent in coronary vessels of young rats (Figure 3A, left). In Y arteries pretreated with authentic ONOO⁻ (Figure 3A, lanes 1 and 2), there was a strong 3-nitrotyrosine immunoreactivity. The summary data of the total background-corrected band densities normalized to the vascular β-actin content (Figure 3A, right) show that 3-nitrotyrosine content of coronary vessels of A rats is significantly increased, compared with vessels of Y rats.

In intramural coronary arterioles and arteries of A rats, immunostaining for 3-nitrotyrosine was localized both to the endothelium (Figures 3B through 3F, arrows) and the media/adventitia (arrowheads). In control experiments on consecutive tissue sections, in the absence of the primary antibody or
immunostaining for iNOS (red) did not overlap with smooth muscle α-actin staining (green, Figure 7C) and was colocalized with CD31 in the endothelium (green, Figure 7D). Immunohistochemistry also showed that in coronary vessels of A rats immunostaining for iNOS was predominantly localized to the endothelium (Figures 7E and 7G) whereas the vascular endothelium of Y rats (Figure 7K) was free of immunoreactivity. In control experiments on consecutive tissue sections in the absence of the primary antibody (Figures 7F and 7L), there was no evidence for nonspecific immunostaining.

### Changes in Protein Expression

Arteriolar protein expressions were assessed in single vessels by Western blotting and normalized to arteriolar β-actin content. Original Western blots in Figures 4 through 6 are representative of 3 to 4 separate experiments (each vessel was isolated from a different animal). The bar graphs show summary data of the total background-corrected band densities normalized to the arteriolar β-actin content. β-Actin expression did not change significantly with aging. Figure 4 shows that in arterioles of A rats, expression of eNOS was significantly decreased compared with arterioles of Y rats. Arteriolar expressions of Cu/Zn-SOD and Mn-SOD were not affected significantly by aging (Figure 5A). Expression of COX-1 (Figure 5B) was significantly decreased in A arterioles, whereas expressions of COX-2 (Figure 5B) and xanthine oxidase (Figure 5C) were unaltered. Expression of the NAD(P)H oxidase subunits p22phox and mox-1, p47phox and p67phox (Figure 6) did not differ significantly between Y and A arterioles, whereas expression of iNOS was significantly greater in A than in Y arterioles (Figure 7A).

### Changes in eNOS mRNA Expression

Expression of eNOS mRNA in A coronary arterioles was significantly decreased (Figure 4B), whereas expression of iNOS mRNA was significantly increased (Figure 7B), compared with arterioles of Y rats.

### Localization of iNOS Expression With Dual Immunofluorescence

In intramural coronary arterioles and arteries of A rats, immunostaining for iNOS (red) did not overlap with smooth muscle α-actin staining (green, Figure 7C) and was colocalized with CD31 in the endothelium (green, Figure 7D). Immunohistochemistry also showed that in coronary vessels of A rats immunostaining for iNOS was predominantly localized to the endothelium (Figures 7E and 7G) whereas the vascular endothelium of Y rats (Figure 7K) was free of immunoreactivity. In control experiments on consecutive tissue sections in the absence of the primary antibody (Figures 7F and 7L), there was no evidence for nonspecific immunostaining.

### Measurement of Serum NOx

Serum NOx concentration in Y and A rats was 5.9±0.9×10⁻⁶ mol/L and 3.5±0.3×10⁻⁶ mol/L, respectively (P<0.05).

### Discussion

One of the new findings of the present study is that in arterioles isolated from rats with a biological age corresponding to that of 65- to 70-year-old humans, increases in intraluminal flow did not elicit substantial NO-mediated dilation (Figure 1B), a response that is present in arterioles of young adult animals with a biological age corresponding to that of 18- to 20-year-old humans. Our findings have important clinical implications because healthy elderly humans of similar age frequently have significant coronary endothelial dysfunction, as demonstrated in vivo by vasomotor responses to pharmacological probes. An age-related decreased vascular release of NO is also supported by microelectrode measurements in the aorta and a significant decrease in serum level and urinary excretion of the NO metabolites, nitrate and nitrite. However, the mechanisms responsible for reduced NO release are less clear.

Our finding, that scavenging of superoxide with SOD and Tiron augmented flow-induced NO-mediated dilation in coronary arterioles of aged rats (Figures 1C and 1D), suggests...
that in older vessels, increases in flow do elicit NO synthesis and release, but elevated levels of \( \text{O}_2^- \) react with NO to decrease its bioavailability. This is likely to be the case in human aging because ACh-induced increases in brachial blood flow in elderly patients can be significantly augmented by administration of the antioxidant vitamin C.\(^{28}\) To further support this hypothesis in the present study, increased vascular \( \text{O}_2^- \) production was evidenced by showing increased lucigenin chemiluminescence in aged coronary vessels (Figure 3A). We also identified NAD(P)H oxidase(s) as major sources of vascular \( \text{O}_2^- \) generation in aging because increased lucigenin chemiluminescence of aged vessels could be substantially inhibited by DPI (as well as by SOD), but not by rotenone or inhibitors of iNOS, COX, and xanthine oxidase (Figure 2B). A primary role for increased NAD(P)H oxidase activity in aging is further supported by the findings that in aged coronary vessels NADH-driven (but not xanthine-driven) \( \text{O}_2^- \) generation was substantially enhanced (Figure 2C). Similar findings have also been reported recently on aged rat aorta and carotid artery.\(^{13}\) Localization of EB-stained nuclei suggests that in aging both endothelial and smooth muscle cells generate substantial amounts of \( \text{O}_2^- \) (Figures 2H and 2I). This finding also accords with results of recent studies showing that NAD(P)H oxidases are expressed and active both in vascular endothelium\(^{20,29}\) and smooth muscle.\(^{30}\) Importantly, an increased activity of vascular NAD(P)H oxidase(s) has been shown recently to be associated with several pathophysiological conditions with increased risk for coronary heart disease (eg, hypercholesterolemia, hypertension, and diabetes).\(^{13,29,31}\)

It is known that interaction of superoxide with NO results in the formation of peroxynitrite (ONOO\(^-\)).\(^{32}\) Because in coronary vessels of A rats there is an increased protein 3-nitrotyrosine content (a biomarker of in vivo production of ONOO\(^-\))\(^{32,33}\); Figure 3A), we assume that in aging the increased levels of \( \text{O}_2^- \) in vivo scavenge endothelium-derived NO, resulting in increased formation of ONOO\(^-\). 3-Nitrotyrosine immunoreactivity was present both in the endothelium and smooth muscle of coronary arteries and arterioles of aged rats (Figures 3B through 3E), consistent
with the idea that NO released from the endothelium to flow reacts with \( \text{O}_2^- \) generated in both endothelial and smooth muscle cells (Figures 2D through 2K). A recent study also demonstrated enhanced 3-nitrotyrosine staining in aged rat aortas.17 Because flow-induced dilation of aged arterioles could be improved by scavenging \( \text{O}_2^- \), it is likely that ONOO\(^-\) did not inactivate eNOS, yet ONOO\(^-\)-induced protein modification (eg, tyrosine nitration or cysteine oxidation) is likely to affect the function of other enzymes,17,33–35 further promoting coronary vessel dysfunction in aging.

We also sought to characterize age-related changes in arteriolar expression of some of the pro- and antioxidant enzymes that are likely to be involved in the maintenance of normal flow-induced arteriolar dilation. In coronary arterioles of aged rats, expression of eNOS protein was significantly decreased (Figure 4A). Because levels of eNOS mRNA were also decreased in A arterioles, as determined by QRT-PCR (Figure 4B), transcription of the eNOS gene is likely to be downregulated in aging. Similar age-related decreases in eNOS mRNA and/or protein expression have also been reported in some other, but not all,17 vascular beds.36 Interestingly, a recent study also reported a time-dependent decline in eNOS expression in cultured endothelial cells in vitro,37 suggesting that this decline may contribute to the development of aging-induced oxidative stress promoting endothelial apoptosis.37

Aging was shown to decrease the activity/expression of superoxide dismutases, which may impair elimination of \( \text{O}_2^- \) in certain organs, such as the liver, of aged rats (reviewed by Matsumoto38). Because in coronary arterioles the expression of neither Cu, Zn-SOD, nor Mn-SOD (which are the major SOD isoforms in the cytoplasm and the mitochondria, respectively, whereas the function of extracellular SOD may be somewhat less pronounced in the rat39) were affected by aging (Figure 5A), it is likely that aging-induced oxidative stress in these vessels is not due to a decreased presence of SOD. Previous studies also revealed important tissue- and species-dependent differences in SOD expression and demonstrated no significant changes in SOD expression in aged rat hearts.38 Yet, one
cannot exclude the possibility that vascular SOD activity declines with age due to posttranslational protein modification\(^\text{38}\) or that vascular antioxidant systems distinct from SOD are altered by the aging process.\(^\text{38}\)

The findings that expression of COX-1 decreased, whereas that of COX-2 did not change significantly with aging (Figure 5B), together with the lack of an effect of SQ29,548 and indomethacin on flow-induced responses and superoxide production (Figures 2A and 3B, respectively), suggest that in coronary arterioles endothelial dysfunction is unlikely to be due to COX-related production of oxygen free radicals and/or TXA\(_2\).\(^\text{5}\) Arteriolar expression of xanthine oxidase (Figure 5C), similar to xanthine oxidase activity (Figures 2B and 2C), was also unchanged in aging.

Because lucigenin chemiluminescence measurements showed that increased NAD(P)H oxidase activity contributes importantly to the development of oxidative stress in aged coronary vessels (Figures 2B and 2C), we investigated the expression of the 2 membrane-bound and the 2 cytosolic subunits of NAD(P)H oxidases known to be involved in superoxide production. Although in coronary arterioles of aged rats expression of the membrane-associated subunits (p22\(^\text{phox}\), which is essential for the oxidative activity in endothelial and smooth muscle cells\(^\text{20}\) and Mox-1,\(^\text{20}\) an analog of the leukocyte-type gp91\(^\text{phox}\) subunit) tended to increase, this change did not reach statistical significance (Figures 6A and 6B). Interestingly, a previous study using semiquantitative immunohistochemical analysis showed an age-dependent up-regulation of p22\(^\text{phox}\) in the rat aorta.\(^\text{13}\) Aging also did not seem to affect the arteriolar expressions of p47\(^\text{phox}\) and p67\(^\text{phox}\) (Figures 6C and 6D), cytosolic subunits that are known to regulate the function of the membrane oxidase complex. Nevertheless, it is known that activity of NAD(P)H oxidases is regulated by nontranslational mechanisms (eg, phosphorylation by protein kinases). Recent studies demonstrated that vascular NAD(P)H oxidase activity is regulated by cytokines, including TNF-\(\alpha\),\(^\text{21}\) the plasma level\(^\text{41}\) and vascular expression\(^\text{42}\) (see also the online data supplement) of which is increased in aging.

One could speculate that increased vascular production of cytokines\(^\text{42}\) and/or an increased NF-\(\kappa B\)-dependent sensitivity of vascular cells to cytokine stimulation\(^\text{43}\) may also be responsible for the increased iNOS expression found in aged arterioles (Figure 7A), aorta,\(^\text{12}\) and other vascular tissues.\(^\text{44}\) QRT-PCR analysis also showed increased levels of iNOS mRNA in arterioles of aged rats (Figure 7B). Because expression of iNOS was confined predominantly to the endothelium (Figures 7C through 7J), it seems likely that basal NO production by iNOS may contribute to the increased endothelial ONOO\(^-\) formation (Figure 3).

In conclusion, flow-induced NO-mediated dilation of coronary arterioles is significantly diminished in aged rats due to increased O\(_2\)\(^-\) production. We propose that a shift in arteriolar phenotype, including decreased expression of eNOS, increased expression of iNOS, and increased activity of NAD(P)H oxidases, underlies these changes and contributes to ONOO\(^-\) generation and the impairment of the vasoactive function of the coronary endothelium in aging, which could limit or reduce myocardial perfusion thereby promoting ischemic heart disease.

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References


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Expanded Materials and Methods

Animals

Fourteen to sixteen week old ("young", n=15) and 74-82 week old ("aged", n=15) male Sprague-Dawley rats\(^1\) were used.

Isolation of arterioles, Videomicroscopy

Functional studies were conducted on isolated intramural coronary arterioles (diameter: \(\sim 150 \, \mu m\)), as described previously\(^2\)-\(^4\). In brief, the heart was excised, the septum was exposed and arteriolar branches of the septal artery\(^5\) running intramuscularly were isolated. The arterioles were canulated on both sides in an organ chamber containing physiological salt solution (in mmol/L: 110 NaCl, 5.0 KCl, 2.5 CaCl\(_2\), 1.0 MgSO\(_4\), 1.0 KH\(_2\)PO\(_4\), 5.0 glucose and 24.0 NaHCO\(_3\), equilibrated with 10% O\(_2\), 5% CO\(_2\), 85% N\(_2\) at pH 7.4, 37\(^\circ\)C). Pressure on both sides was adjusted by a pressure servocontrol system. Internal diameter at the midpoint of the arteriolar segment was measured with videomicroscopy. Intraluminal flow (0 to 60 \(\mu L/min\), corresponding to \(\sim 20 \, \text{dyn/cm}^2\) wall shear stress) was established at a constant intravascular pressure (80 mmHg) by changing the inflow and outflow pressure to an equal degree, but in opposite directions, to keep midpoint intraluminal pressure constant\(^2\). Wall shear stress (\(\tau\)) was calculated as follows: \(\tau = 4\eta Q/\pi r^3\), where \(\eta\) is the viscosity of the perfusate (\(\sim 0.007 \, \text{poise, at 37}\,^\circ\text{C}\)), \(Q\) is perfusate flow, and \(r\) is the vessel radius. Isolated coronary arterioles developed active tone in response to intraluminal pressure of 80 mmHg without the use of any vasoactive agent (Y: 31\(\pm\)2%, A: 33\(\pm\)4%, n.s.). The passive diameter of Y and A arterioles in the absence of extracellular Ca\(^{2+}\) was 200\(\pm\)11 \(\mu m\), and 229\(\pm\)16 \(\mu m\) (n.s.), respectively (at 80
mmHg). Distensibility of Y and A arterioles - calculated by normalizing pressure-dependent increases in passive diameter to the passive diameter obtained at 20 mmHg - did not differ significantly (Y: 37±6%, A: 42±4%, n.s.).

Functional studies

Dilations of Y and A arterioles were measured in response to step increases in intraluminal flow (from 0 to 60 µL/min) before and after incubation with N°-nitro-L-arginine-methyl-ester (L-NAME, 3x10⁻⁴ mol/L, for 20 min), an inhibitor of nitric oxide synthesis.

Arteriolar responses to acetylcholine (ACh, 10⁻⁹ to 10⁻⁵ mol/L), the voltage-operated Ca²⁺ channel inhibitor verapamil and the NO donor sodium nitroprusside (SNP, 10⁻⁹ to 10⁻⁵ mol/L) were also assessed. In separate experiments responses of A arterioles to flow and ACh (10⁻⁶ mol/L) were measured; the arterioles were also incubated with L-arginine (10⁻⁴ mol/L, for 30 min, the substrate of eNOS), SQ29,548 (10⁻⁶ mol/L, for 15 min, a TxA₂ receptor antagonist), the superoxide scavengers Tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid, 10 mmol/L, for 30 min, a spin trap known to eliminate superoxide in coronary endothelium) or SOD (120 U/mL, for 30 min, a potent scavenger of superoxide in coronary arteries and isolated arterioles) and responses were reassessed. The effective concentrations of inhibitors and receptor antagonists used in the present study have been previously determined in functional studies on isolated arterioles. In coronary arterioles of A (n=4) and Y (n=4) rats 10⁻⁶ mol/L U46619 elicited 60±7% and 58±5% constriction, respectively, and these responses were abolished by 10⁻⁶ mol/L SQ29,548. Basal tone of both Y and A arterioles was not significantly
affected by administration of L-arginine, SOD or SQ29,548, whereas it was increased by ~10% by L-NAME and reduced by ~10% in the presence of Tiron.

At the conclusion of each experiment in order to obtain the maximum passive diameter, the suffusion solution was changed to a Ca\textsuperscript{2+} free solution, which contained EGTA (ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N\,'N\,-tetraacetic acid, 10\textsuperscript{-3} mol/L), and the vessel was incubated for 10 min and changes in passive arteriolar diameter in response to changes in intraluminal pressure (20 to 140 mmHg) were obtained.

All drugs were added to the vessel chamber, and final concentrations are reported. Salts and chemicals were obtained from Sigma-Aldrich Co. Solutions were prepared on the day of the experiment. Data are expressed as means ± S.E.M. Statistical analyses were performed by two-way ANOVA followed by the Tukey post hoc test or Student’s t-test, as appropriate. \(P<0.05\) was considered statistically significant.

Measurement of vascular superoxide level

Vascular O\(_2\)\textsuperscript{-} production was assessed from similar coronary vascular samples that were used for biochemical studies by the lucigenin chemiluminescence method\textsuperscript{11, 12} according to the modified protocol of Mohazzab et al.\textsuperscript{7}. In brief, coronary vessels were pooled from each young and aged rat, and placed in scintillation vials containing 1 mL physiological buffer solution and 10 \(\mu\)mol/L lucigenin. Lucigenin chemiluminescence was measured in a liquid scintillation counter (Beckman-Coulter) with a single active photomultiplier tube positioned in out-of-coincidence mode in the darkroom. Scintillation counts were obtained 15 to 20 minutes after addition of vessels (averaged) and background-corrected values were normalized to tissue weight. In separate experiments O\(_2\)\textsuperscript{-} production of aged vessels was determined in the absence or presence of rotenone.
(10^{-5} \text{ mol/L, an inhibitor of proximal respiratory chain in the mitochondrion}), or
aminoguanidine (10^{-4} \text{ mol/L, a selective inhibitor of iNOS}), or indomethacin (10^{-5} \text{ mol/L,}
 a COX inhibitor), or allopurinol (10^{-4} \text{ mol/L, an inhibitor of xanthine oxidase}) or
diphenyliodonium [DPI, 10^{-4} \text{ mol/L, an inhibitor of flavin-containing enzymes, including
NAD(P)H oxidase}] or superoxide dismutase (SOD, 200 U/mL). In other experiments
superoxide production by aortic segments (~4 mm) from young and aged rats was
measured.

Xanthine oxidase assay and NADH oxidase assay

Xanthine oxidase and NADH oxidase activity was measured with a lucigenin
assay according to the modified protocol of Mohazzab et al. 7,12,13. Briefly, isolated
pooled coronary vessels of young and aged rats were homogenized in liquid nitrogen and
lysed in buffer containing protease inhibitors [10 \mu g/ml aprotinin, 0.5 \mu g, ml leupeptin,
0.7 \mu g/ml pepstatin, 0.5 mmol/L PMSF]. 20 \mu l of the particulate fraction of the
homogenate was used for lucigenin chemiluminescence measurements. Xanthine oxidase
activity and NADH oxidase activity was measured by the addition of 10^{-4} \text{ mol/L xanthine
and NADH, respectively. Protein content was measured in an aliquot of the homogenate
by the Lowry method.}

Ethidium bromide fluorescence

Hydroethidium, an oxidative fluorescent dye, was used to localize superoxide
production in situ according to the modified protocol of Lund et al. 14 and Rey et al. 15.
This method provides sensitive detection of \mathbf{O}_2^- \text{ levels in situ}^{16,17}. In brief, cells are
permeable to hydroethidium, which in the presence of \mathbf{O}_2^- is oxidized to fluorescent
ethidium bromide (EB), which form is trapped by intercalation with DNA. Unfixed
frozen left ventricular samples were cut into sections (10 μm thick) and placed on glass slides. Hydroethidine (2×10^{-6} mol/L) was applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37℃ for 30 minutes. Fluorescent images were collected using a 585-nm long-pass filter. Then, the sections were counterstained with hematoxylin, washed and incubated with an anti-α-smooth muscle actin antibody (Sigma, 1:50, overnight, 4 ℃) then with a FITC-labeled anti-mouse antibody (Jackson Laboratories, 1:50). Brightfield and fluorescent images of the same vessel sections were captured. Young and aged tissues were processed and imaged in parallel. Samples incubated with hydroethidine in the presence of Tiron (10 mmol/L) served as negative control. The number of EB-stained nuclei was counted and the percentage of EB-positive negative nuclei was averaged for each vascular cross section. To maximize consistency of scoring, only nuclei showing moderate or strong staining were regarded as positive.

Detection of coronary arteriole protein expression with Western blotting

In order to investigate aging-induced alterations in protein expression in the coronary microcirculation we have developed a Western blot protocol using single arterioles, identical to those used for functional studies. Isolated single coronary arterioles from the same young and aged rats that were used for functional studies were snap-frozen in liquid nitrogen and stored at −80 ℃. Samples were homogenized by sonification in Laemmli buffer, boiled for 5 min, electrophoresed on 10% SDS-PAGE gel and transferred to Hybond-P (Amersham-Life Science, Arlington Heights, IL) membrane at 1 mA/cm² for 60 min with a semidry blotting system (Biorad). The membranes were blocked in TBS buffer containing 5% nonfat milk and 0.05% Tween 20 for overnight at 4℃. Primary
polyclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY, DF=1:1500), monoclonal anti-β-actin antibody (Abcam, UK, DF=1:5000), monoclonal anti-COX-1 or polyclonal anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI, DF=1:500), polyclonal anti-Cu,Zn-SOD or anti-Mn-SOD antibody (StressGen Biotechnologies Corp. Victoria, BC, DF=1:2000), polyclonal anti-p22\(^{phox}\) (DF=1:250), anti-p47\(^{phox}\) (DF=1:250), anti-p67\(^{phox}\) (DF=1:250), anti-Mox-1 (DF=1:250)(Santa Cruz Biotechnology, Inc, Santa Cruz, CA) or anti-xanthine oxidase antibody (DF=1:200) was added to the membrane for 1 h at room temperature. The membranes were washed with PBS and incubated for 1 h with sheep anti-rabbit IgG-horseradish peroxidase, donkey anti-mouse IgG-horseradish peroxidase (Amersham) or donkey anti-goat IgG-horseradish peroxidase(Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at the final titer of 1:4,000. The membranes were developed with ECL-Plus (Amersham) and analyzed with densitometry. Anti-β-actin (Abcam, UK) was used to normalize for loading variations.

*Validation of single arteriole Western blotting protocols*

In preliminary experiments isolated arterioles (4-6 from each animal) were pooled, homogenized and protein concentration in the lysate was determined by the Lowry method. On 10% SDS-PAGE gel known amounts of proteins (2.5 to 10 μg) were loaded. Membranes were labeled with anti-β-actin primary antibody and HRP-labeled anti-mouse secondary antibody (Figure 2 in the Online Data Supplement shows a typical Western blot). Band densities showed good correlation with the loaded protein amount (R\(^2\)=0.94, n=3). Comparison of band densities confirmed that total protein amounts extracted from single arterioles (in average ~6 μg) fall in the linear range of the protein concentration-density curves. In separate experiments arterioles were pooled (4x4 vessels from each
group) and homogenized. From the samples 10 μg protein was loaded for Western blotting. Analysis of band densities showed that there was no significant difference between β-actin concentrations in samples from Y and A rats.

**RNA isolation and real-time QRT-PCR**

Real time reverse transcription QRT-PCR technique was used to determine relative expression levels of eNOS mRNA in coronary arterioles of A and Y rats. In brief, total RNA from single coronary arterioles was isolated with Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript II RT (Life Technologies, Gaithersburg, MD) at 42 °C for 60 min in the presence of 3 mmol/L MgCl₂, 5mmol/L DTT, 0.5 mmol/L dNTP, 8U RNasin (Promega, Corp., Madison, WI) and 5 μmol/L oligo(dT). PCR reactions were performed in the Roche Molecular Biochemicals LightCycler System. PCR amplification was performed in the total volume of 20 μL with the LightCycler DNA Master SYBR Green I kit. The housekeeping gene β-actin was used for internal normalization. Oligonucleotides used for real-time QRT-PCR are listed in additional Table 1. Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

**Detection of vascular TNFα mRNA expression with multiplex PCR**

Vascular expression of IL-6, TNF-α, TGF-β, IL-1 and GM-CSF mRNA was investigated with a multiplex RT-PCR method using the BioSource Rat Inflammatory Cytokine Set1 (rNFIG-MPCR, Biosource International, Camarillo, CA). The PCR primers have similar Tm and no obvious 3'-end overlap to enhance multiple amplification. Cytokine gene expression was normalized against GAPDH expression. RT-MPCR reaction was performed according to the manufacturer's instruction: 5 μl
antibody against nitrated tyrosine residues of proteins (Cell Signaling Technology Co, Beverly, MA, 1:100). To label bound primary antibody, sections were incubated for 45 min at 25 °C with a 1:100 dilution of goat anti-rabbit secondary antibody conjugated to biotin (Vector Laboratories). Between each step of the labeling protocol sections were rinsed in 0.1% Triton-X in 0.05 mol/L Tris-saline solution. Sections were then incubated with avidin-biotinylated enzyme complex (ABC Vectastain, Vector Laboratories), stained with Vector-DAB (diaminobenzidine tetrahydrochloride) substrate and counterstained with hematoxyline. Sections were then rinsed in distilled water, permanently mounted with VectaMount medium, and covered with a coverslip. The specificity of the immunolabeling was evaluated by omitting the primary antibody or omitting both primary and secondary antibodies in control experiments. Further specificity controls were made by immunostaining sections after overnight incubation of the primary antibody with the homologous antigen for nitrotyrosine (10 mmol/L). Images of the sections were collected with a CoolSnap-CF CCD camera (Roper Scientific, Tucson, AZ) connected to an Olympus BX60 microscope.

*Localization of iNOS with dual immunofluorescence staining and immunohistochemistry*

Immunolabeling was carried out as described previously with a 1:5 dilution of polyclonal anti-iNOS antibody (Transduction Laboratories) and an anti-α-smooth muscle actin antibody (Sigma, 1:50, overnight, 4 °C) then with a Texas red-conjugated goat anti-rabbit antibody (1:50) and a FITC-labeled goat anti-mouse antibody (Jackson Laboratories, 1:50). In other experiments instead of anti-α-smooth muscle actin antibody a monoclonal antibody against CD31 (a specific endothelial marker) with a dilution of 1:25 was used. All incubations were at room temperature and washes in PBS were
cDNA was used in 50 µl rat IFN Cytokine multiplex PCR solution with dNTP, MPCR buffer, MPCR primers and 2.5 U Taq DNA polymerase. PCR products were detected on 2% agarose gel.

Detection of coronary nitrotyrosine content with Western blotting

It is known that interaction of superoxide with NO results in the formation of peroxynitrite (ONOO⁻)\textsuperscript{18} that reacts with tyrosine residues of proteins forming 3-nitrotyrosine. Thus, we quantified protein 3-nitrotyrosine content [a stable biomarker of \textit{in vivo} production of ONOO⁻ \textsuperscript{18,19}] in single coronary arterioles with Western blotting by measuring whole band densities after labeling with a primary polyclonal anti-3-nitrotyrosine antibody (Cell Signaling Technology Co, Beverly, MA, 1:750, 1 h at room temperature). Isolated coronary arterioles of control rats incubated with authentic ONOO⁻ (10 µmol/L and 10 mmol/L) served as positive controls.

Immunohistochemical detection of 3-nitrotyrosine

Tissue samples from the interventricular septum and the anterior wall of the left ventricle were embedded in OTC 4583 medium (Sakura, Finetek, Torrance, CA), snap-frozen in liquid nitrogen and sections (thickness: 4 µm) cut by using a cryostat were collected on Probe-on-Plus (Fisher Scientific) microscope slides. Sections were fixed with cold (4 °C) acetone and exposed to 3% hydrogen peroxide (5 min, 25 °C, in methanol) to quench endogenous peroxidase activity. Slides were washed three times for 5 min with phosphate-buffered saline (PBS). To block nonspecific binding of antibodies, sections were incubated for 4 h (25 °C) with 2.5% normal horse serum, which was then aspirated from each section. Then sections were incubated overnight at 4 °C with optimally diluted (in PBS containing 0.1% BSA and 0.2% Triton X-100) primary
performed in between. The slides were then coverslipped and mounted using an anti-fading medium. Red (Texas red) and green (FITC) fluorescent images were captured using appropriate filter cubes (Chroma Technologies). Separate tissue sections were immunolabeled for iNOS, stained with Vector-ALP substrate and counterstained with hematoxylin. In intramural coronary arterioles and arteries of A rats immunostaining for iNOS was predominantly localized to the endothelium. In control experiments on consecutive tissue sections in the absence of the primary antibody or both primary and secondary antibodies there was no evidence for nonspecific immunostaining.

Measurement of serum nitrite/nitrate (NOx)

NOx content in serum samples of Y and A rats were measured as described previously\textsuperscript{20,21}. Serum nitrite was measured by acidifying serum to pH \textless 2.0 to convert nitrite to NO. Serum nitrate was measured by incubating plasma with \textit{Aspergillus} nitrate reductase (Boehringer Mannheim) to reduce nitrate into nitrite and then converting nitrite into NO by the addition of hydrochloric acid. The NO produced was then injected into the NO analyzer (Sievers, Inc), and the NO content of the sample was determined by measuring the luminescence generated in the presence of ozone. Each assay was run in duplicate. We found that serum NOx concentration was significantly decreased in aged rats as compared to young rats (Y: 5.9±0.9 x 10\textsuperscript{-6} mol/L, A: 3.5±0.3 x 10\textsuperscript{-6} mol/L, p<0.05), confirming recent findings\textsuperscript{22}.

Data analysis

Arteriolar dilations are expressed as percentage of the maximal dilation in the absence of extracellular Ca\textsuperscript{2+} at 80 mmHg intraluminal pressure. Distensibility was calculated by normalizing pressure-dependent increases in passive diameter to the passive
diameter obtained at 20 mmHg. Lucigenin chemiluminescence data were normalized to
the respective control mean values. Densitometric ratios are expressed as a percentage of
the control mean value. Data are expressed as means ± S.E.M. Statistical analyses of data
were performed by Student’s t-test or by two-way ANOVA followed by the Tukey post
hoc test, as appropriate. P<0.05 was considered statistically significant.

Extended Results

Isolated coronary arterioles developed active tone in response to intraluminal
pressure of 80 mmHg without the use of any vasoactive agent (Y: 31±2%, A: 33±4%,
n.s.). The passive diameters of Y and A arterioles in the absence of extracellular Ca^{2+}
were 200±11 μm, and 229±16 μm (n.s.), respectively (at 80 mmHg).

Flow- and agonist-induced dilations of coronary arterioles

Step increases in intraluminal flow (from 0 to 60 μL/min, corresponding to a
shear stress of ≈20 dyn/cm²) elicited marked dilations of coronary arterioles of Y rats,
which were significantly inhibited by L-NAME (Fig. 1). In contrast, in arterioles of aged
rats responses to flow were significantly decreased and were unaffected by L-NAME
(Fig. 1).

We also demonstrated that ACh-induced dilation of aged coronary arterioles is
impaired (Fig. 1), extending results of previous studies on vessels from other vascular
beds. Because dilations to the endothelium-independent NO donor SNP (Fig.
1D) and the NO-independent Ca^{2+} antagonist verapamil (at 10^{-6} mol/L, Y: 75±8%,
A: 81±9%, n.s., not shown), as well as the passive mechanical properties of arterioles of
Y and A rats were similar, it is likely that neither the sensitivity to NO nor the dilator capacity of the smooth muscle is affected significantly by aging.

In arterioles of A (Fig. 1) and Y (not shown) rats flow-induced responses were unaffected by the presence of extracellular L-arginine or the PGH₂/TxA₂ receptor antagonist SQ 29,548. Administration of the free radical scavengers SOD or Tiron significantly augmented dilations of A arterioles to step increases in intraluminal flow (Fig. 1). In contrast, SOD had no significant effect on maximal flow-induced dilation of Y arterioles (Y: 22±3 μm, Y+SOD: 23±6 μm, n=3). Although maximal flow-induced dilations of aged arterioles in the presence of SOD tended to be smaller than dilations of young arterioles in the presence of SOD, this difference was not statistically significant.

In the presence of SOD the time course of dilation of A arterioles to a step increase in intraluminal flow (from 0 to 60 μL/min) was characterized by a ~30 s lag phase after initiation of flow, followed by a gradual dilation reaching a maximum at ~60 s and a sustained plateau phase (Fig. 1). In the presence of L-NAME both phases of dilation were significantly decreased. The time course of responses of A arterioles to ACh (10⁻⁶ mol/L) were characterized by a rapid peak dilation followed by a plateau phase. The presence of SOD significantly augmented the plateau phase of ACh-induced dilations of A arterioles, an effect that was reversed by L-NAME (additional Fig. 1 in Online Data Supplement).

*Vascular superoxide production*

Under basal conditions, levels of O₂⁻ produced by coronary vessels of A rats were significantly higher than in vessels of Y rats (as detected by lucigenin-enhanced chemiluminescence; Fig. 2A). O₂⁻ level in A vessels was significantly decreased by administration of DPI or SOD, whereas it was unaffected by rotenone, aminoguanidine,
indomethacin or allopurinol (Fig. 3B). In aortic segments of A rats there was also a significantly increased $O_2^-$ production (Y: 100±11%, A: 239±16%), confirming results of previous studies.\textsuperscript{12,27}

\textit{Xanthine oxidase and NADH oxidase assays}

Xanthine-driven $O_2^-$ production by coronary vessel homogenates from A and Y rats did not differ significantly (Fig. 2C). NADH-driven $O_2^-$ production by vascular samples from A rats was significantly increased as compared to Y controls (Fig. 2C). Both xanthine- and NADH-induced increases in lucigenin chemiluminescence could be abolished by addition of SOD.

\textit{Hydroethidium fluorescence}

In sections of A hearts (n=3) the relative number of EB-positive nuclei in intramural coronary arterioles and arteries was significantly greater than in sections of Y hearts (Fig. 2D-G). Consecutive staining of sections for smooth muscle actin showed that EB-positive nuclei were localized both in the media and the intima of coronary vessels (Fig. 2I-H). Incubation of tissue sections with Tiron (10 mmol/L) prevented EB staining of nuclei.

\textit{Detection of coronary 3-nitrotyrosine content with Western blotting and immunohistochemistry}

Aging was associated with an increased prevalence of nitrated tyrosine residues of proteins in coronary vessels, whereas 3-nitrotyrosine immunoreactivity was weak or absent in coronary vessels of young rats (Fig. 3A, left). In Y arteries pretreated with authentic ONOO$^-$ (Fig. 3A, Lanes 1-2) there was a strong 3-nitrotyrosine immunoreactivity. The summary data of the total background-corrected band densities
normalized to the vascular β-actin content (Fig. 3A, right) show that 3-nitrotyrosine content of coronary vessels of A rats is significantly increased, compared to vessels of Y rats.

Samples from the hearts of A and Y animals were analyzed with immunohistochemistry. In intramural coronary arterioles and arteries of A rats immunostaining for 3-nitrotyrosine was localized both to the endothelium (Fig. 3B-F, arrows) and the media/adventitia (arrowheads). In control experiments on consecutive tissue sections, in the absence of the primary antibody or after absorption of the primary antibody with the homologous antigen for 3-nitrotyrosine (Fig. 3G-H), there was no evidence of nonspecific immunostaining.

Changes in protein expression

Arteriolar protein expressions were assessed in single vessels by Western blotting and normalized to arteriolar β-actin content. Original Western blots on Fig. 4-6 are representative of three to four separate experiments (each vessel was isolated from a different animal). The bar graphs show summary data of the total background-corrected band densities normalized to the arteriolar β-actin content. Fig. 4 shows that in arterioles of A rats expression of eNOS was significantly decreased compared to arterioles of Y rats. Arteriolar expressions of Cu/Zn-SOD and Mn-SOD were not affected significantly by aging (Fig. Fig. 5A). Expression of COX-1 (Fig. 5B) was significantly decreased in A arterioles, whereas expressions of COX-2 (Fig. 5B) and xanthine oxidase (Fig. 5C) were unaltered. Expression of the NAD(P)H oxidase subunits p22phox and mox-1, p47phox and p67phox (Fig.6) did not differ significantly between Y and A arterioles, whereas expression of iNOS was significantly greater in A than in Y arterioles (Fig. 7A).
Changes in eNOS mRNA expression

Expression of eNOS mRNA in A coronary arterioles was significantly decreased (Fig. 4B), whereas expression of iNOS mRNA was significantly increased (Fig. 7B), compared to arterioles of Y rats.

Localization of iNOS expression with dual immunofluorescence

In intramural coronary arterioles and arteries of A rats immunostaining for iNOS (red) did not overlap with smooth muscle α-actin staining (green, Fig. 7C) and was co-localized with CD31 in the endothelium (green, Fig. 7D). Immunohistochemistry also showed that in coronary vessels of A rats immunostaining for iNOS (red) was predominantly localized to the endothelium (Fig. 7E,G-J), whereas the vascular endothelium of Y rats (Fig. 7K) was free of immunoreactivity. In control experiments on consecutive tissue sections in the absence of the primary antibody (Fig. 7F,L) there was no evidence for nonspecific immunostaining.

Vascular expression of TNFα mRNA

Expression of IL-6, TNF-α and TGF-β could be detected in samples from isolated coronary microvessels of A and Y rats with multiplex RT-PCR. Densitometric analysis showed an increased TNF-α and TGF-β mRNA expression in A compared to Y vessels (Fig. 3 in Online Data Supplement). IL-6 mRNA expression did not differ significantly between the two groups. Expression of IL-1 and GM-CSF mRNA in A and Y arterioles could not be detected with this method.
References for Online Data Supplement


### Additional Table

**Table 1.** Oligonucleotides for QRT-PCR

<table>
<thead>
<tr>
<th>mRNA targets</th>
<th>Sense</th>
<th>Antisense</th>
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<td>β-actin</td>
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<td>3'-CTGGCGCCTCAACACCT-5'</td>
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</table>
Figure 1. Time course of dilations of coronary arterioles from aged rats in response to acetylcholine (ACh, $10^{-6}$ mol/L) before and after administration of SOD or SOD plus L-NAME. Data are mean ± SE. *p<0.05 (n=7)
Figure 2. Western blot β-actin band densities as a function of total loaded protein amount from homogenates of pooled rat coronary arterioles (representative to 3 separate experiments). Dotted line shows average protein amount loaded in single vessel Western blotting protocols.
Figure 3. Cytokine mRNA expression in coronary arterioles of young (n=3) and aged (n=3) rats (determined with multiplex RT-PCR). *<0.05