Oxidative stress and endothelial dysfunction in pulmonary arteries of aged rats

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1Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma; and 2Departments of Cell Biology, Anatomy, and Pediatrics, New York Medical College, Valhalla, New York

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Podlutsky A, Ballabh P, Csiszar A. Oxidative stress and endothelial dysfunction in pulmonary arteries of aged rats. Am J Physiol Heart Circ Physiol 298; H346–H351, 2010. First published December 4, 2009; doi:10.1152/ajpheart.00972.2009.—Aging in the systemic circulation is associated with generalized endothelial dysfunction and increased oxidative stress, which are thought to contribute to the increased morbidity and mortality of cardiovascular diseases in the elderly. Previous studies have shown that pulmonary artery pressure and vascular resistance increase with normal aging in humans, yet age-related functional and phenotypic changes in the pulmonary arteries have not been characterized. To determine whether in the pulmonary circulation aging elicits endothelial dysfunction and oxidative stress, isolated pulmonary arteries of young (3 mo old) and aged (28 mo old) F344 rats were compared. We found that aging in rat pulmonary arteries is associated with impaired acetylcholine-induced relaxation and vascular oxidative stress [assessed by dihydroethidium and 5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-ester fluorescence assays]. Endothelial dysfunction in the aged pulmonary vessels is reversed by the inhibition of NAD(P)H oxidase. The expressions of gp91phox (both mRNA and protein), NAD(P)H oxidase isofrom type 1 (Nox-1; mRNA), and Nox-4 (mRNA) tend to increase in aged vessels; however, only changes in Nox-4 reached statistical significance. In pulmonary arteries of aged rats, the protein expression of endothelial nitric oxide synthase, Cu,Zn-SOD, Mn-SOD, and glutathione peroxidase is unaltered, whereas the expression of catalase is significantly decreased. Our results suggest that aging is associated with oxidative stress and endothelial dysfunction in the pulmonary arteries, which may contribute to the age-related functional alterations in the pulmonary circulation.

free radical; senescence; reactive oxygen species; pulmonary circulation; lung

PREVIOUS STUDIES by this and other laboratories have demonstrated that in arteries within the systemic circulation, aging is associated with endothelial oxidative stress and vasodilator dysfunction (5, 9, 13, 16, 20, 27, 53). These alterations are generally considered to underlie the increased risk of atherosclerosis in the systemic vessels, which is responsible for the increased incidence of cardiovascular diseases, including stroke, myocardial infarction, and aortic aneurysm, in aging. Atherosclerosis selectively affects the systemic vessels, whereas the pulmonary circulation usually remains free of atherosclerotic plaques even in advanced aging. On the basis of these observations, hypotheses were put forward that aging selectively impairs the function and phenotype of arteries in the systemic circulation (57).

Previous studies have documented age-related changes in the human pulmonary vascular system and right ventricular function as well (22, 23, 35). For example, pulmonary artery pressure and vascular resistance increase with normal aging in humans (17, 19, 34). In the presence of risk factors for systemic hypertension, age-related increases in pulmonary artery pressures and pulmonary vascular resistance appear to be even more pronounced (21). However, there is a paucity of data on age-related phenotypic alterations in endothelial function in the pulmonary arteries.

Aging-induced oxidative stress in the systemic arteries appears to be associated with increased activity of gp91phox-containing NAD(P)H oxidases (1, 13, 20, 60). There is growing evidence that pulmonary arteries abundantly express gp91phox-containing NAD(P)H oxidases (42, 44, 45, 61, 62) and that under steady-state conditions, they exhibit more pronounced NAD(P)H oxidase activity than systemic arteries (including coronary vessels) (6, 25, 43). Previous studies suggest that the induction of NAD(P)H oxidases in the systemic arteries of aged rodents is due, at least in part, to age-related alterations of humoral factors, including the up-regulation of TNF-α (9) and angiotensin II (41). These circulating factors may also affect the pulmonary circulation and were shown to be able to induce NAD(P)H oxidases in pulmonary arteries (46). Thus, in the present study, we tested the hypothesis that aging in the pulmonary arteries is associated with endothelial dysfunction and oxidative stress, similar to the systemic circulation. F344 rats were used as a model system because they exhibit marked age-related activation of NAD(P)H oxidase associated with significant endothelial dysfunction in virtually all vascular beds studies in the systemic circulation (1, 9, 13, 27, 31).

METHODS

Animal models. Animal use protocols were approved by the Institutional Animal Care and Use Committee of the New York Medical College (Valhalla, NY). Male 3-mo-old (young) and 28-mo-old (aged) Fisher 344 rats were purchased from the National Institute of Aging and kept under pathogen-free conditions. All animals were disease free and exhibited no signs of systemic inflammation and/or neoplastic alterations. Upon euthanasia, small intrapulmonary arteries were isolated as reported previously (7).

Functional studies. Endothelial function was assessed as previously described (7). In brief, small intrapulmonary arteries of each animal were cut into ring segments 1.5 mm in length and mounted on 40-μm stainless steel wires in the myographs chambers (Danish Myo Technology) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution containing (in mM) 118 NaCl,
4.7 KCl, 1.5 CaCl₂, 25 NaHCO₃, 1.1 MgSO₄, 1.2 KH₂PO₄, and 5.6 glucose at 37°C, gassed with 21% O₂-5% CO₂-74% N₂. After an equilibration period of 1 h during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), relaxations of precontracted (by 10⁻⁶ mol/l phenylephrine) vessels to acetylcholine (ACh; from 10⁻⁹ to 10⁻⁴ mol/l) and the nitric oxide (NO) donor S-nitroso-N-acetylpenicillamine (from 10⁻⁹ to 3 × 10⁻⁵ mol/l) were obtained. The effects of the NAD(P)H oxidase inhibitor apocynin (3 × 10⁻⁴ mol/l) on ACh-induced responses of pulmonary vessels were also tested.

Measurement of cellular reactive oxygen species production. The production of O₂⁻ in segments of the same pulmonary arteries that were used for functional studies was assessed using dihydroethidine, an oxidative fluorescent dye, as we previously reported (8, 10). In brief, arterial segments were incubated with dihydroethidine (3 × 10⁻⁶ mol/l; at 37°C). For quantitative comparison of vascular O₂⁻ generation, the time course of the buildup of ethidium (the fluorescent reaction product of dihydroethidine and O₂⁻) fluorescence in en face preparations was recorded for 30 min by a Tecan Infinite M200 plate reader (excitation, 520 nm; and emission, 620 nm). The slope factor, representing cellular O₂⁻ production, was calculated and normalized to Hoechst 33258 fluorescence (excitation, 352 nm; and emission, 461 nm), representing DNA content/cell mass (Hoechst 33258 loading was performed after the dihydroethidine loading to avoid any interference with the ethidium signal).

The cell-permec oxidative fluorescent indicator dye 5 (and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate-acetyl ester (C₂H₂DCFDA; Invitrogen, Carlsbad, CA) was used to assess H₂O₂ production in isolated pulmonary arteries as we previously reported (33). C₂H₂DCFDA is a 2',7'-dichlorofluorescein derivative that has a long retention within the cells. In brief, the vessel segments were treated with C₂H₂DCFDA (10⁻⁵ mol/l at 37°C). The time course of the buildup of green dichlorofluorescein fluorescence (excitation, 485 nm; and emission, 530 nm) in en face preparations was recorded for 30 min by a Tecan Infinite M200 plate reader. The slope factor, representing cellular H₂O₂ production, was calculated and normalized to Hoechst 33258 fluorescence (excitation, 352 nm; and emission, 461 nm), representing DNA content/cell mass.

Western blot analysis. Western blot analysis was performed as described (4, 7, 12) to assess the protein expression of gp91phox, endothelial NO synthase (eNOS), Cu,Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase in small intrapulmonary arteries.

Quantitative real-time RT-PCR. We have used a quantitative real-time RT-PCR technique to analyze the mRNA expression of the NAD(P)H oxidase subunits gp91phox, NAD(P)H oxidase isoform type 1 (Nox-1), and Nox-4 in pulmonary arterial samples, as previously described (4, 7, 12) to assess the protein expression of gp91phox, endothelial NO synthase (eNOS), Cu,Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase in small intrapulmonary arteries.

RESULTS

Impaired endothelial function in aging. ACh-induced relaxation was impaired in small pulmonary arteries of aged rats compared with those of young rats (Fig. 1A). Endothelium-dependent relaxation in pulmonary arteries of aged rats was restored following apocynin treatment (Fig. 1A). Vascular relaxations to the NO donor S-nitroso-N-acetylpenicillamine did not differ between arteries of young and aged rats (Fig. 1B).

Increased vascular reactive oxygen species production in aging. Dihydroethidine fluorescence (Fig. 2A) measurements showed that O₂⁻ production was increased in pulmonary arteries of aged rats compared with the arteries from young rats. Consistent with the presence of age-related oxidative stress, vascular H₂O₂ generation (measured by the C₂H₂DCFDA fluorescence method) was also significantly increased in aged rats (Fig. 2B).

Expression of eNOS, NAD(P)H oxidases, and antioxidant enzymes. The protein expression of eNOS in small intrapulmonary arteries was unaffected by aging (Fig. 3A). The expression of the NAD(P)H oxidase subunit gp91phox protein (Fig. 3B) and mRNA (Fig. 4A) as well as Nox-1 (Fig. 4B) and mRNA expression using the Strategen MX3000, as reported (11). The real-time RT-PCR technique was used to analyze the mRNA expression of the reference genes hypoxanthine guanine phosphoribosyl transferase (HPRT), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ-polypeptide (YWHAZ), and GAPDH were determined, and a normalization factor was calculated based on their geometric mean for internal normalization. Fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Data analysis. Data were normalized to the respective control mean values and are expressed as means ± SE. 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.
In this study, we have documented a significant age-related decline in endothelial function (Fig. 1) associated with significant oxidative stress (Fig. 2) in the pulmonary arteries. These findings suggest that the functional consequences of aging are similar both in the systemic and pulmonary circulation and refute the hypothesis that the pulmonary endothelial cells are protected from the deleterious effects of aging. Endothelial dysfunction in the pulmonary circulation likely contributes to the age-related increases in pulmonary vascular resistance and pulmonary arterial pressure in elderly humans (17, 19, 21), which may compromise right ventricular function (26), worsening the outcome of cardiovascular diseases.

Age-related endothelial dysfunction and oxidative stress are known to promote chronic low-grade inflammation in the systemic conduit arteries, facilitating the development of atherosclerosis. Although pulmonary arteries also exhibit endothelial dysfunction and oxidative stress in aging, there are only a few reports on atherosclerosis in the pulmonary circulation. Atherosclerosis typically develops only in vascular segments, which are exposed to high intraluminal pressure, whereas the pulmonary circulation is characterized by low blood pressure. Recent reports suggest that severe atherosclerosis can develop in pulmonary arteries of old patients in the presence of pulmonary hypertension (e.g., due to an atrial septal defect) (47). The findings that age-related endothelial dysfunction is also present in the low-pressure region of the systemic microcirculation argue against the hypothesis that age-related endothelial oxidative stress is due to the increased systolic blood pressure (39, 52).
We found that the expression of eNOS in the pulmonary arteries does not change during aging (Fig. 3A). Our results extend the findings of Challah et al. (3), demonstrating that the expression of eNOS is unchanged in lung homogenates from aged rats. Impaired NO production is associated with the downregulation of eNOS in some [coronary arteries and aorta (3, 13)] but not all (56) systemic arteries of aged F344 rats. Thus it is likely that the downregulation of eNOS is not a prerequisite for generalized aging-induced endothelial dysfunction. There are also data suggesting that eNOS may be uncoupled in aging (18). Increased circulating asymmetric dimethylarginine levels may also negatively affect eNOS activity in the aged pulmonary arteries (32). It is significant that endothelial dysfunction in the aged pulmonary vessels is reversed by the inhibition of NAD(P)H oxidase (Fig. 1A). This observation is in line with findings from previous studies demonstrating that the upregulation and/or increased activation of NAD(P)H oxidases contribute significantly to aging-induced vascular oxidative stress and endothelial dysfunction in the systemic circulation as well. The mechanism by which increased NAD(P)H oxidase activity impairs endothelial function in both vascular beds is likely an inactivation of NO by increased levels of $O_2^-$ (16). Indeed, earlier studies reported that aging significantly attenuates the rate of NO release after calcium ionophore administration in the aorta of aged RORO rats, eliminating the difference between NO production in the pulmonary arteries and the aorta of aged rats (57). NO reacts with the superoxide anion to form the peroxynitrite anion (ONOO$^-\$). There is abundant evidence that oxidative stress in aging is associated with nitrative stress, which likely represents an important mechanism contributing to macromolecular damage and ultimately to cell death (48, 60). The findings of the aforementioned study that young pulmonary arteries produce less NO than young aortas are consistent with the view that pulmonary arterial endothelial cells exhibit higher NAD(P)H oxidase-dependent $O_2^-$ production than endothelial cells derived from the systemic circulation (6, 25). Because NAD(P)H oxidase-derived reactive oxygen species play a key role in chronic hypoxia-induced pulmonary hypertension (37), one may expect that an increased NAD(P)H oxidase activity in the pulmonary arteries will aggravate the symptoms of concomitant chronic obstructive pulmonary diseases in the elderly. Previous studies have shown that in the systemic circulation, oxidative/nitrosative stress-induced activation of poly(ADP-ribose) polymerase-1 (PARP-1) may contribute to the pathogenesis of endothelial dysfunction associated with aging (49–51). PARP-1 is functional in the pulmonary arteries, and there is evidence that the inhibition of PARP-1 exerts beneficial effects on pulmonary oxidative stress and inflammation in various pathophysiological conditions (36). Endothelial oxidative stress may also underlie the age-related ultrastructural alterations in the pulmonary endothelial cells demonstrated by earlier studies (55) and may increase the risk for pulmonary embolism and/or the development of acute respiratory distress syndrome (2).

There are several known mechanisms by which aging may increase NAD(P)H oxidase activity in vascular cells. Because the expression of gp91$^{phox}$, the dominant NAD(P)H oxidase catalytic subunit in endothelial cells (Figs. 3B and 4A), and that of Nox-1 (Fig. 4B) and Nox-4 (Fig. 4C) tended to increase in pulmonary arteries with age, it is possible that the transcriptional induction of NAD(P)H oxidase contributes to the increased NAD(P)H oxidase activity in aged vessels. The age-related upregulation of gp91$^{phox}$ and Nox-4 subunits have been previously reported in systemic arteries of aged rats as well (1, 9, 38). A recent study demonstrated that Nox-4 contributes importantly to lung fibrogenic responses (29), thus the idea that Nox-4 contributes to age-related pulmonary alterations warrants further studies. In addition, several humoral factors, including TNF-$\alpha$ and angiotensin II, are known to directly activate vascular NAD(P)H oxidases via facilitating PKC-dependent phosphorylation and association of its regulatory subunits (24, 58). Because aging both significantly increases circulating TNF-$\alpha$ levels and upregulates angiotensin II (9, 41), these humoral changes may represent a possible mechanism.
that may simultaneously activate NAD(P)H oxidases in the systemic and pulmonary arteries. Increased plasma levels of advanced glycation end products, acting via stimulating receptor for advanced glycation end product, may also contribute to the generalized endothelial alterations in the systemic and pulmonary circulations during aging. Previous studies have demonstrated that left ventricular function is impaired in the aged F344 rat, which may lead to increases in pulmonary arterial pressure affecting vascular redox homeostasis. We cannot exclude the possibility that endothelial oxidative stress in aging, at least in part, also involve cell autonomous effects, as senescent pulmonary artery endothelial cells in culture also exhibit significant increases in O$_3^-$ and H$_2$O$_2$ production (63).

There is increasing evidence that cytosolic NADPH levels regulate NAD(P)H oxidase-derived superoxide production in pulmonary arteries (25, 62). Thus the possibility that aging may affect the supply of NADH/NADPH, which results in an increased NAD(P)H oxidase activity even in the absence of a change in NAD(P)H oxidase expression, cannot be excluded. In theory, a decrease in antioxidant capacity associated with senescence may also contribute to vascular oxidative stress in aging. However, among the antioxidant enzymes investigated, the expressions of Cu,Zn-SOD (Fig. 5A), Mn-SOD (Fig. 5B), and glutathione peroxidase (Fig. 5D) do not change significantly with age in the pulmonary arteries, extending previous findings in lung homogenates (28, 30, 54). The expression of catalase was downregulated in aged vessels (Fig. 5C), which may contribute to the increase peroxide levels present in aged pulmonary arteries (Fig. 2B).

In conclusion, aging in the pulmonary arteries is associated with endothelial dysfunction and oxidative stress, and these age-related functional alterations are similar to those present in the systemic circulation. There is increasing evidence for a close relationship between cellular oxidative stress and chronic low-grade vascular inflammation in aging (14–16, 59). Moreover, clinical evidence suggests that aging is associated with low-grade inflammation in the lungs of elderly patients (40). Because oxidative stress and chronic inflammation are involved in the pathogenesis and progression of chronic obstructive pulmonary disease, aging-induced oxidative stress and inflammation in the lung may accelerate or worsen the effects of environmental pollutants. Thus further studies on the role of age-related oxidative stress and proinflammatory phenotypic alterations in the pulmonary vasculature are warranted.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


PULMONARY ARTERIAL AGING


