Role of oxidative stress and AT<sub>1</sub> receptors in cerebral vascular dysfunction with aging

Mary L. Modrick, Sean P. Didion, Curt D. Sigmund, and Frank M. Faraci

Departments of Internal Medicine, Physiology, and Pharmacology, Cardiovascular Center, Carver College of Medicine, University of Iowa, Iowa City, Iowa

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Modrick ML, Didion SP, Sigmund CD, Faraci FM. Role of oxidative stress and AT<sub>1</sub> receptors in cerebral vascular dysfunction with aging. Am J Physiol Heart Circ Physiol 296: H1914–H1919, 2009. First published April 24, 2009; doi:10.1152/ajpheart.00300.2009.—Vascular dysfunction occurs with aging. We hypothesized that oxidative stress and ANG II [acting via ANG II type 1 (AT<sub>1</sub>) receptors] promotes cerebral vascular dysfunction with aging. We studied young (5–6 mo), old (17–19 mo), and very old (23 ± 1 mo) mice. In basilar arteries in vitro, acetylcholine (an endothelium-dependent agonist) produced dilation in young wild-type mice that was reduced by ~60 and 90% (P < 0.05) in old and very old mice, respectively. Similar effects were seen using A23187, a second endothelium-dependent agonist. The vascular response to acetylcholine in very old mice was almost completely restored with tempol (a scavenger of superoxide) and partly restored by PJ34, an inhibitor of poly(ADP-ribose) polymerase (PARP). We used mice deficient in Mn-SOD (Mn-SOD<sup>−/−</sup>) to test whether this form of SOD protected during aging but found that age-induced endothelial dysfunction was not altered by Mn-SOD deficiency. Cerebral vascular responses were similar in young mice lacking AT<sub>1</sub> receptors (AT<sub>1</sub><sup>−/−</sup>) and wild-type mice. Vascular responses to acetylcholine and A23187 were reduced by ~50% in old wild-type mice (P < 0.05) but were normal in old AT<sub>1</sub>-deficient mice. Thus, aging produces marked endothelial dysfunction in the cerebral artery that is mediated by ROS, may involve the activation of PARP, but was not enhanced by Mn-SOD deficiency. Our findings suggest a novel and fundamental role for ANG II and AT<sub>1</sub> receptors in age-induced vascular dysfunction.

basilar artery; endothelium; genetically altered mice; acetylcholine; angiotensin II; A23187

DIVERSE VASCULAR CHANGES OCCUR WITH AGING (4, 17, 28). In the cerebral circulation, for example, aging produces both structural and functional changes (10, 20, 21, 29, 34). Aging has an enormous negative impact on cerebral blood vessels and is the major risk factor for cerebral vascular disease, stroke, Alzheimer’s disease, and vascular cognitive impairment (19, 36). Despite its prominence as a cardiovascular risk factor, the impact of aging is one of least studied areas in vascular biology. Few studies have attempted to define mechanisms that produce changes in the cerebral circulation with aging. Most studies in models of vascular disease and stroke are performed in adult, not aged, animals. Because cerebral vascular disease in humans occurs primarily in older individuals, this focus may limit our understanding of the vascular disease process.

There has been considerable interest in the role of ROS in aging (16, 25). The causes and impact of oxidative stress are a major focus in studies of vascular biology. Oxidative stress plays a fundamental role in cerebral vascular dysfunction in diverse models of disease, but little is known regarding its role in aging (5). The first goal of this study was to define the effect of age on the cerebral artery and to examine the hypothesis that age-related dysfunction is mediated by ROS. As part of this effort, we examined the role of a downstream target of ROS, poly(ADP-ribose) polymerase (PARP), a potential mediator of vascular dysfunction (24, 33).

Vessels are protected from oxidative stress by an array of antioxidant enzymes including SOD (11). In relation to aging, the form of SOD localized in mitochondria (Mn-SOD) (11) is of particular interest as mitochondria are a focus in studies of aging (16, 25). The functional importance of Mn-SOD in cerebral blood vessels with aging is unknown. Thus, our second goal was to examine the hypothesis that age-induced vascular dysfunction is enhanced in Mn-SOD-deficient mice.

The renin-angiotensin system plays a major role in vascular disease in part by promoting oxidative stress (5, 8, 32). Most of the detrimental effects of ANG II on blood vessels are mediated by ANG II type 1 (AT<sub>1</sub>) receptors (8, 30, 32). To further examine the mechanisms that promote cerebral vascular dysfunction, our third goal was to examine the hypothesis that a deficiency in the expression of AT<sub>1</sub> receptors protects against cerebral vascular dysfunction during aging.

Our findings suggest that aging produces marked endothelial dysfunction in the cerebral artery that is mediated by ROS and activation of PARP but is not enhanced by Mn-SOD deficiency. Our findings also implicate a novel and fundamental role for ANG II and AT<sub>1</sub> receptors in age-related cerebral vascular dysfunction.

METHODS

Experimental animals. The source, genetic background, and breeding protocols for the mice used in these experiments have been previously described (1, 14). Mn-SOD deficient and AT<sub>1</sub><sub>a</sub> receptor-deficient mice were on a CD-1 and mixed genetic background of 129P3/J and C57BL/6J, respectively (1, 14). For both strains of mice, wild-type littermates were used as controls. Male and female mice were studied at the ages listed below. Mice were fed regular chow, and water was available ad libitum. All breeding was performed in a virus- and pathogen-free barrier facility at the University of Iowa. All protocols and procedures conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Experiments on cerebral arteries in vitro. After an overdose of anesthesia (150 mg/kg ip pentobarbital), the brain was rapidly removed and placed in ice-cold Krebs buffer. As previously described (14, 42), the basilar artery was isolated, cannulated onto glass micropipettes filled with Krebs buffer in an organ chamber, and secured with nylon monofilament. Arteries were pressurized to 60 mmHg. Using a microscope and video camera, vessel images were projected...
on a video monitor. An electronic dimension analyzer was used to measure lumen diameter.

Once prepared, basilar arteries were allowed to equilibrate for at least 30 min at a distending pressure of 60 mmHg before protocols were initiated. We examined changes in the diameter of the basilar artery in response to KCl (50 mM). For experiments that tested vasodilator responses, basilar arteries were constricted by ~30% (~60% of the response to 50 mM KCl) with U-46619, a thromboxane A2 mimetic. Acetylcholine was used as an endothelium-dependent agonist (9). In some experiments, A23187 [which produces receptor-independent endothelium-dependent relaxation (6, 9)] was also used. After the development of a stable baseline diameter, cumulative dose-response curves were obtained. At the end of the protocols, papaverine (an endothelium-independent vasodilator, 100 μM) was used to produce maximal vasodilation.

Statistical analysis. For the basilar artery, constriction in response to KCl was determined by calculating the percent reduction in vessel diameter from the baseline control level. For vasodilator responses, results are expressed as percent dilation (percentages of induced tone), with 100% representing the difference between the resting value under basal conditions and the constricted value with U-46619. As appropriate, comparisons were made using paired or unpaired t-tests or ANOVA with repeated measures followed by Student-Newman-Keuls test to detect individual differences. P values of <0.05 were defined as significant.

RESULTS

Aging produces marked endothelial dysfunction in the cerebral artery. Baseline diameters of the basilar artery were similar in young (6 ± 1 mm) and old (18 ± 1 mm) wild-type mice but were increased in very old (23 ± 1 mm) mice (Fig. 1). The increase in resting diameter before constriction in very old mice (~12%) may reflect a structural change in the vessel wall with aging. Body weights were similar in the different groups (44 ± 2, 41 ± 2, and 37 ± 3 g in young, old, and very old mice, respectively). Dilation of the basilar artery to acetylcholine was markedly reduced in old mice and almost abolished in very old mice (Fig. 2). For example, increases in vessel diameter in response to the maximum concentration of acetylcholine in young, old, and very old mice were 80 ± 4%, 31 ± 6% (P < 0.05 vs. young mice), and 11 ± 1% (P < 0.05 vs. old mice), respectively. Baseline responses and effects of age were generally not affected by gender in wild-type mice. For example, vasodilation to the maximum concentration of acetylcholine was similar in young male and female mice [76 ± 5% (n = 7) vs. 90 ± 3% (n = 3), P > 0.05] as well as in very old male and female mice [10 ± 3% (n = 3) vs. 11 ± 2% (n = 6), P > 0.05]. However, vasodilation to acetylcholine in old female mice [44 ± 7% (n = 6)] was greater than in old male mice [14 ± 4% (n = 5), P < 0.05 vs. old females], suggesting that age-induced endothelial dysfunction may occur earlier in male mice.

Vasodilation to A23187 was markedly impaired in old mice (P < 0.05 vs. young; Fig. 3). These inhibitory effects of aging on endothelial function in the basilar artery were selective as dilation to papaverine and constriction to KCl were not altered in old or very old mice (Fig. 1).

Vascular dysfunction with aging is mediated by ROS. To examine the mechanisms that produce endothelial dysfunction with aging, we first tested the role of superoxide in arteries from very old mice. Acute treatment with tempol (1 mM), a scavenger of superoxide, restored responses to acetylcholine to almost normal in arteries from very old wild-type mice (n = 7; Fig. 2). Responses of the basilar artery to acetylcholine in the presence of tempol and L-NNA in very old mice was 8 ± 4% (n = 3), demonstrating that the protective effect of tempol was mediated by NO. In a separate time control group in which vascular responses were examined twice in the absence of any blocker, impaired vasodilation to acetylcholine in very old mice was reproducible.

![Fig. 1. Baseline diameter (A) and changes in diameter of the basilar artery in response to KCl (50 mM; B) and papaverine (100 μM; C) in young (n = 10), old (n = 11), and very old (VO; n = 9) wild-type (WT; Mn-SOD+/+) and Mn-SOD−/− mice [knockout (KO); n = 7 young, 6 old, and 16 VO mice]. WT mice were littermates of Mn-SOD−/− mice and were on a CD-1 genetic background (1). Values are means ± SE. *P < 0.05 vs. young WT mice; #P < 0.05 vs. young Mn-SOD−/− mice.](http://ajpheart.physiology.org/Downloadedfrom.jpg)
Papaverine produced similar dilation in arteries from young, SOD responses to acetylcholine in arteries from very old Mn-involved superoxide as treatment with tempol fully restored means ± SE. *P < 0.05 vs. young mice; †P < 0.05 vs. old mice; #P < 0.05 vs. VO mice.

Effect of Mn-SOD deficiency. Baseline diameters of the basilar artery in young (5 ± 1 mo), old (17 ± 1 mo), and very old (23 ± 1 mo) Mn-SOD+/− mice were similar to those observed in wild-type mice (Fig. 1). Body weights were similar in the different groups (45 ± 2, 40 ± 4, and 41 ± 2 g in young, old, and very old mice, respectively). In young Mn-SOD+/− mice, the response to acetylcholine was similar to that observed in young wild-type mice (Fig. 2), suggesting that heterozygous Mn-SOD deficiency per se did not alter endothelial function in cerebral arteries. Dilation of the basilar artery to acetylcholine and A23187 was impaired with aging in Mn-SOD+/− mice, and the degree of impairment was very similar to that seen in wild-type littermates (P > 0.05 vs. wild-type; Figs. 2 and 3). Like wild-type mice, the mechanism of impaired endothelial function with age in Mn-SOD+/− mice involved superoxide as treatment with tempol fully restored responses to acetylcholine in arteries from very old Mn-SOD+/− mice (n = 8; Fig. 2). In contrast to acetylcholine, papaverine produced similar dilation in arteries from young, old, and very old Mn-SOD+/− mice (Fig. 1).

To examine the mechanisms that produce endothelial dysfunction with aging in greater detail, we tested the role of PARP in very old wild-type and Mn-SOD+/− mice. Treatment with PJ34 [3 μM, an inhibitor of PARP (24)] improved endothelial function in both strains. For example, the maximum increase in vessel diameter in response to 100 μM acetylcholine in very old mice increased from 12 ± 3% to 40 ± 6% in wild-type mice (n = 3) and from 10 ± 3% to 29 ± 7% in Mn-SOD deficient mice (n = 3, P < 0.05).

Deficiency in AT1 receptors prevents endothelial dysfunction with aging. To further examine the mechanisms that may promote age-induced vascular dysfunction, we examined responses in arteries from young (5 ± 1 mo) and old (19 ± 1 mo) mice deficient in AT1 receptors (AT1−/−) and their wild-type littermates. Body weights were similar in the different groups (27 ± 2 and 27 ± 1 g in young wild-type and AT1−/− mice and 29 ± 1 and 30 ± 2 g in old wild-type and AT1−/− mice, respectively). Baseline diameters of the basilar artery in wild-type and AT1 receptor-deficient mice were similar and averaged 108 ± 5 μm (n = 8) and 115 ± 4 μm (n = 9) in young mice and 120 ± 4 μm (n = 8) and 118 ± 6 μm (n = 9) in old mice, respectively.

In young AT1 receptor-deficient mice, the response to acetylcholine was similar to that observed in young wild-type mice (Fig. 4), indicating that AT1 receptor deficiency did not alter endothelial function in cerebral arteries. Dilation of the basilar artery to acetylcholine was impaired by >50% in old wild-type mice (Fig. 4). However, arteries from old wild-type mice relaxed normally to papaverine (91 ± 2%). In contrast, maximum vasodilation to acetylcholine in old AT1 receptor-deficient mice was similar (P > 0.05) to that observed in young wild-type and young AT1 receptor-deficient mice (Fig. 4). In experiments using A23187 as a second endothelium-dependent agonist (6, 9), we obtained similar findings: impaired vasodi-

Fig. 2. Responses of the basilar artery to acetylcholine in young (n = 10), old (n = 11), and VO (n = 9) Mn-SOD+/− and Mn-SOD+/− mice (n = 7 young, 6 old, and 16 VO mice). Responses in very old mice were examined in the absence and presence of tempol (T). Values are means ± SE. *P < 0.05 vs. young mice; †P < 0.05 vs. old mice; #P < 0.05 vs. VO mice.

Fig. 3. Dilation of the basilar artery in response to A23187 in young and old Mn-SOD+/− and Mn-SOD+/− mice. Values are means ± SE. n = 7 young WT and Mn-SOD+/− mice and 6 old WT and Mn-SOD+/− mice. *P < 0.05 vs. young mice.
lation in old wild-type mice but normal responses to A23187 in old AT1 receptor-deficient mice (Fig. 4).

**DISCUSSION**

There are several novel findings in this study. First, aging produced dysfunction in the basilar artery that became progressively worse with age. Although the extent of vascular dysfunction at the oldest time point studied was very large, this change was selective and appeared to primarily involve the endothelium. Importantly, we observed similar endothelial dysfunction in wild-type animals on two different genetic backgrounds. Second, in relation to the mechanisms that mediate this dysfunction, our data suggest a key role for oxidative stress and ROS and a partial role for the activation of PARP. Even though endothelial function was greatly impaired in very old mice, acute treatment with a scavenger of superoxide almost completely restored the response in wild-type mice. Third, our findings with Mn-SOD mice suggest that the age-induced impairment of function in cerebral arteries is mediated by ROS but not enhanced by partial deficiency in Mn-SOD. Finally, endothelial dysfunction did not occur in old mice lacking AT1 receptors, suggesting a novel and fundamental role for ANG II in age-related cerebral vascular dysfunction.

**Impact of aging on the cerebral vasculature.** Aging is associated with diverse vascular changes (4, 10, 17, 20, 21, 28, 29, 34). For example, resting cerebral blood flow (10), endothelial function (10, 21, 29, 34), neurovascular coupling (34, 38), and vascular responses to hypercapnia all decrease with aging (10, 34). Despite the enormous potential impact of these changes, few studies have attempted to define the mechanisms that produce cerebral vascular dysfunction with aging.

We have previously shown that endothelial function in the mouse aorta and carotid artery is not impaired or is impaired only modestly at 22–24 mo of age (4, 7). In the present study, we examined mice at three ages and found marked endothelial dysfunction in the cerebral artery at ~18 mo of age and an almost complete loss of endothelial function in very old mice (~23 mo). Thus, one of the key concepts that emerges from this work is that age-induced endothelial dysfunction occurs earlier and perhaps to a greater extent in the cerebral circulation than in blood vessels outside of the brain.

A previous study (37) has shown that genetic factors (such as genetic background) can have a great impact on vascular function. The Mn-SOD-deficient and AT1 receptor-deficient mice used in these experiments were from different genetic backgrounds (1, 14). The fact that we observed similar endothelial dysfunction in old wild-type mice on two genetic backgrounds suggests that the effect of age is similar across strains, thus strengthening our overall findings.

The basilar artery was used in these experiments, and we assume that changes observed in this vessel are representative of other segments of the brain vasculature. The brain is relatively unique compared with other organs in that cerebral arteries are important resistance vessels (12). Because endothelial dysfunction with aging has been described previously in the basilar artery, in other cerebral arteries, and in the brain microcirculation (18, 21, 29, 34), our basic assumption appears valid.

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**Fig. 4.** Dilation of the basilar artery in response to acetylcholine and A23187 in young and old WT [ANG II type 1 receptor (AT1) ] and AT1 receptor-deficient (AT1 ) mice. WT mice were littermates of the AT1 receptor-deficient mice and were on a mixed genetic background of 129P3/J and C57BL/6J (14). Values are means ± SE; n = 8 young and old WT mice and 9 young and old AT1 mice. *P < 0.05 vs. young mice.
Role of ROS in vascular dysfunction with aging. ROS are thought to be key mediators of changes in vascular structure and function with disease (5, 23). Although endothelial dysfunction occurs in animal and human cerebral blood vessels during aging (10, 21, 29, 34), little is known regarding the mechanisms that account for these changes. In the present experiments, endothelial function in the basilar artery was nearly abolished in very old mice but was restored to almost normal with a scavenger of superoxide. Thus, our study suggests a major role for ROS in mechanisms that produce dysfunction in the cerebral artery with aging. While the present study was in progress, evidence that superoxide is produced by NADPH oxidase and contributes to cerebral vascular dysfunction with aging appeared (29, 34).

The interaction of NO with superoxide results in the formation of peroxynitrite, and increases in superoxide and peroxynitrite in the vasculature, including in cerebral vessels, have been described in models of aging (3, 4, 7, 34, 39). Peroxynitrite may be an important mediator of cellular injury through several effects, including the activation of PARP (24, 26, 27, 33). Excessive activation of PARP can deplete cellular energy pools and thus produce cellular dysfunction (24, 33). Our finding that an inhibitor of PARP activity (PJ34) partially restored endothelial function in very old wild-type and Mn-SOD-deficient mice suggests that the activation of PARP may contribute to cerebral vascular dysfunction with aging. PJ34 is thought to be a selective inhibitor of PARP activity (24) and does not act as an antioxidant (7). Considering the diverse effects that increased PARP activity can have (33), it is unclear at this point how PARP activation may affect cerebral blood vessels during aging.

Impact of Mn-SOD in the cerebral vasculature with aging. In relation to aging and the cerebral circulation, a protective role for Mn-SOD seemed likely. Mn-SOD is expressed in relatively high levels in endothelial cells (11, 15) and may be present in higher levels in cerebral arteries than in arteries outside of the brain (31).

We hypothesized that cerebral arteries from old Mn-SOD-deficient mice would exhibit greater dysfunction compared with old wild-type mice. In contrast, the degree of age-induced endothelial dysfunction was not enhanced in these mice. In a similar study on the aorta, endothelial function was only modestly reduced in old wild-type animals, but the dysfunction was increased by deficiency in Mn-SOD (4). Because endothelial dysfunction in the aorta is modest in old mice, it may have been easier to detect additional impairment as a result of the combination of aging and Mn-SOD deficiency. In contrast, because we found marked impairment of endothelial function in the basilar artery at ~18 mo of age in wild-type mice, it may be more difficult to detect additional dysfunction in Mn-SOD-deficient animals. Our data do not exclude the possibility that Mn-SOD plays a key role earlier in the aging process in the cerebral circulation. We also recognize the possibility that other forms of SOD (CuZn-SOD or extracellular SOD) may protect the cerebral vasculature with aging. In this regard, we have previously shown that CuZn-SOD protects the carotid artery from age-induced oxidative stress and endothelial function.

Role of AT1 receptors in aged-induced vascular dysfunction. The renin-angiotensin system plays a major role in vascular biology, contributing importantly to changes in the vascular structure and function in diseases, including hypertension and atherosclerosis (5, 30, 32). This system may also contribute to vascular disease with aging. For example, the expression of angiotensin-converting enzyme, tissue levels of ANG II, and AT1 receptors are increased in the aorta in old monkeys and humans (40, 41). In the aorta, chronic pharmacological inhibition of the renin-angiotensin system attenuates normal growth (2), and genetic deficiency in AT1 receptors attenuated increases in peroxynitrite during aging (3). Vascular function was not examined in these previous studies. Before this study, the role of ANG II as a mediator of changes in the cerebral circulation with aging were unknown.

While it is well established that ANG II produces oxidative stress in blood vessels (5, 30, 32), the relative importance of ANG II and ANG II-induced signaling in producing vascular dysfunction with age is unclear. The effects of ANG II occur via the activation of two receptor subtypes: AT1 and ANG II type 2 receptors (22). In this study, we used AT1 receptor-deficient mice to examine the role of this receptor in cerebral vascular dysfunction during aging. We were particularly interested in the role of AT1 receptors as ANG II is a major stimulus for the activation of NADPH oxidase (5, 30), which has recently been implicated in cerebral vascular abnormalities in aging (29, 34). Importantly, a major new finding was that while endothelial function was impaired in arteries from old wild-type mice, this same dysfunction was absent in old AT1 receptor-deficient mice. These findings suggest a novel role for AT1 receptors in the accelerated dysfunction that occurs in the cerebral circulation with aging. Because the activation of superoxide formation via NADPH oxidase is a prominent effect of ANG II in vascular cells, we speculate that the increases in circulating or local levels of ANG II with aging increase superoxide levels via AT1 receptor-dependent activation of this source of superoxide. Increased formation of peroxynitrite and the subsequent activation of PARP may then occur. Endothelial dysfunction with aging may result from both the loss of normal NO-mediated signaling as well as the activation of additional mechanisms including PARP.

Summary and implications. Aging has an enormous negative impact on the cerebral circulation. The incidence of cerebral vascular disease, stroke, Alzheimer’s disease, and vascular cognitive impairment increase markedly with age (19, 36). A better understanding of the mechanisms that promote vascular disease may provide insight into approaches that could delay the progression of cerebral vascular disease. In this study, we used physiological, pharmacological, and genetic approaches to define the mechanisms that promote oxidative stress and vascular dysfunction during aging. Our data suggest that aging produces greater endothelial dysfunction in cerebral blood vessels than in blood vessel outside of the brain and that this dysfunction is mediated by ROS and may involve the activation of PARP. Our findings also suggest a novel and fundamental role for ANG II and AT1 receptors in age-related vascular dysfunction. The impact of endothelial dysfunction is particularly far reaching in the brain as the endothelium affects vascular muscle and blood elements as well as neuronal signaling and neurogenesis (5). The loss of these effects may contribute to cognitive decline (35) and impair recovery from injury.
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