Mechanisms of aging-induced impairment of endothelium-dependent relaxation: role of tetrahydrobiopterin

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AGING IS A RISK FACTOR for vascular disease; however, the role of aging, either as a process or as the result of longer exposure to other risks, is not well defined (16, 17). In murine vascular tissue, the age-dependent changes in vasomotor function have not been characterized. Studies on rats have shown impairment of endothelium-dependent relaxation due to increased production of superoxide anions, but the source of the superoxide anions has also not been characterized (16, 30). Reactive oxygen species (ROS) have been implicated in endothelial dysfunction associated with aging, hypertension, hypercholesteremia, diabetes, and cigarette smoking (3). ROS can interfere with endothelium-dependent relaxation particularly by the scavenging of NO by superoxide anion (O2•−; Refs. 1, 3, 34).

Additionally, the product of this reaction, peroxynitrite, is a potent but selective oxidant in vitro and in vivo and can cause cytotoxic damage, which could contribute to the pathology of vascular disease (1, 28).

One possible target for oxidation by peroxynitrite is tetrahydrobiopterin (BH4), which is an essential cofactor of all NO synthase (NOS) isoforms. During biosynthesis of NO, BH4 prevents the uncoupling of the electron transfer from NADPH to L-arginine and the subsequent production of O2•− (4, 32, 35, 36). Recently BH4 was shown to inhibit O2•− production by endothelial NOS (eNOS), whereas the oxidized analog 7,8-dihydrobiopterin (7,8-BH2) potentiated O2•− production by eNOS (33). In addition, BH4 can autoxidize in a radical chain reaction; this could result in the reduction of available cofactors and the contribution of more oxidants to the milieu (14). Finally, peroxynitrite can oxidize BH4 (18, 21, 22). Therefore, reduced BH4 levels can reflect increased oxidative stress as well as contribute to generation of ROS. In this study, we tested the hypothesis that oxidation of BH4 is responsible for aging-induced endothelial dysfunction.

METHODS

Experimental animals. Male C57BL/6 mice were used for all experiments. Young animals were 20–27 wk, and aged animals were 84–114 wk of age. All mice were fed regular pelleted diet and housed in facilities with a 12:12-h light-dark cycle. Experimental protocols and housing facilities were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

The mice were killed with a 60 mg/kg ip injection of pentobarbital. Blood samples were obtained via puncture of the right ventricle. The blood was mixed with heparin and centrifuged at 4°C for 10 min at 2,000 rpm. The plasma was aspirated and stored at −80°C. Cholesterol levels were determined using a colorimetric-based assay on a Cobras Mira system. The aorta, carotid arteries, and kidney were removed and placed immediately in ice-cold modified Krebs-Ringer solution that contained (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4 (monobasic), 25 NaHCO3, 11.1 dextrose, and 0.028 calcium disodium versenate. Carotid arteries were dissected, and connective tissue was removed immediately in ice-cold Krebs-Ringer solution. The plasma was aspirated and stored at −80°C. Cholesterol levels were determined using a colorimetric-based assay on a Cobras Mira system. The aorta, carotid arteries, and kidney were removed and placed immediately in ice-cold modified Krebs-Ringer solution that contained (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4 (monobasic), 25 NaHCO3, 11.1 dextrose, and 0.028 calcium disodium versenate. Carotid arteries were dissected, and connective tissue was removed under a microscope (Carl Zeiss; Oberkochen, Germany). Aorta and kidneys were prepared similarly using a lighted magnifying glass. All of the assays were performed using tissue from the same animal.

Vasomotor reactivity. Carotid arteries were studied individually using a microcannula technique that has been previously described (8). Briefly, each artery was sutured to two microcannulas and placed in a vessel chamber (Living Systems Instrumentation; Burlington, VT) filled with aerated (94% O2-6% CO2) Krebs-Ringer solution at

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37°C, which flowed from a 250-ml reservoir to the vessel chamber at a rate of 50 ml/min. A pressure of 50 mmHg was maintained in the artery through the microcannulas. The arteries were equilibrated 45 min before each experiment. The arteries were submaximally contracted with the thromboxane analog 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin F₂₅ω (U-46,619, 10⁻⁷ to 10⁻⁶ M), and endothelium-dependent relaxation was then obtained using ACh (10⁻⁹ to 10⁻⁵ M). After washout, equilibration, and submaximal contraction of the arteries with U-46,619, endothelium-independent relations were determined using diethylammonium (Z)-1-((N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate, 10⁻⁹ to 10⁻⁵ M). Relaxation values were determined as percents of relaxation to a high concentration of papaverine (3 × 10⁻⁴ M). In a separate protocol, arteries were preincubated with the SOD mimetic Mn(III)tetratetra-benzoic acidporphyrin chloride (MnTBAP, 10⁻⁵ M) 15 min before contraction (8).

Biopterin measurements. Fresh aortas were used for analysis. Aortas were homogenized in extraction buffer that contained (in mM) 50 Tris·HCl (pH 7.4), 1 EDTA, and 10 1,4-dithiothreitol using a glass mortar and pestle (Kontes; Vineland, NJ; Ref. 7). The homogenate was centrifuged at 4°C and 10,000 rpm for 10 min, and the resulting supernatant was used in the assay. Amounts of reduced BH₄ and total oxidized biopterins including 7,8-BH₄ levels were measured after oxidation in acid and base conditions using reverse-phase HPLC (7, 9).

Detection of vascular O₂⁻ production. O₂⁻ production was measured by lucigenin-enhanced chemiluminescence as previously described (7). Briefly, aortas were opened lengthwise and equilibrated for 30 min at 37°C in modified Krebs-HEPES buffer (pH 7.4). Scintillation vials that contained 2 ml of Krebs-HEPES buffer with 5 μM lucigenin were placed into a scintillation counter (LS 5000; Beckman Instruments) that was switched to the out-of-coincidence mode. Background signals were recorded, and vascular segments were then added to each vial. The results were expressed as counts per minute per milligram of dry weight.

Measurement of GTP cyclohydrolase I activity. GTP cyclohydrolase I (GTPCH-I) activity was determined by standardized enzymatic reaction followed by oxidation as previously described with small modifications (31). Concisely, 100 μl of tissue supernatant homogenate, prepared as in the biopterin assay, was filtered using a Sephadex column (Amersham; Piscataway, NJ) to remove endogenous nate, prepared as in the biopterin assay, was filtered using a Sephadex column (Amersham; Piscataway, NJ) to remove endogenous

RESULTS

Animal characteristics. There were no differences observed between the animals with regard to plasma concentrations of glucose, cholesterol, high-density lipoprotein, or triglycerides (Table 1). Additionally, there were no changes in body weight or in basal diameter and wall thickness of the carotid artery. Senescence-associated β-galactosidase staining of kidney was present in the aged animals (data not shown).

Vascular reactivity. There was no statistical difference between young and aged animals in the submaximal contractions of carotid arteries to U-46,619 (Table 1). Maximum endothelium-dependent relaxations to ACh were reduced in aged mouse carotid arteries compared with young animals. Preincubation with MnTBAP significantly improved relaxation such that the maximum values for both the young and the aged mouse carotids were no longer different (Table 2 and Fig. 1). There was no difference between young mouse carotids treated with and without MnTBAP (Fig. 1A). Similar results were obtained.

Table 1. Comparison of different parameters in young and aged mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young Mice</th>
<th>Aged Mice</th>
</tr>
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<tbody>
<tr>
<td>Age, wk</td>
<td>22.1 ± 0.4 (11)</td>
<td>94.5 ± 2.4 (19)</td>
</tr>
<tr>
<td>Weight, g</td>
<td>28.4 ± 1.4 (6)</td>
<td>31.9 ± 2.8 (5)</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
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<tr>
<td>Glucose, mmol/l</td>
<td>12.1 ± 1.8 (6)</td>
<td>10.7 ± 1.3 (4)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>1.3 ± 0.2 (6)</td>
<td>1.2 ± 0.1 (4)</td>
</tr>
<tr>
<td>HDL, mmol/l</td>
<td>1.0 ± 0.2 (6)</td>
<td>1.0 ± 0.1 (4)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.9 ± 0.1 (6)</td>
<td>0.6 ± 0.1 (4)</td>
</tr>
<tr>
<td>Carotid artery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal diameter, μm</td>
<td>415.0 ± 10.4 (4)</td>
<td>414.5 ± 7.1 (6)</td>
</tr>
<tr>
<td>Diameter of contraction to U-46,619, first exposure, μm</td>
<td>171.3 ± 12.8 (7)</td>
<td>173.2 ± 18.8 (6)</td>
</tr>
<tr>
<td>Diameter of contraction to U-46,619, second exposure, μm</td>
<td>191.3 ± 23.6 (4)</td>
<td>170.2 ± 32.5 (6)</td>
</tr>
<tr>
<td>Wall thickness, μm</td>
<td>32.6 ± 7.6 (4)</td>
<td>36.3 ± 3.0 (6)</td>
</tr>
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</table>

Values except age ranges are means ± SE; n, no. of mice, shown in parentheses. U-46,619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂₅ω. *P < 0.05 compared with young mice.
for the endothelium-independent relaxations to DEA-NONOate (Fig. 2).

The EC25 values of aged mouse arteries in response to both ACh and DEA-NONOate were significantly increased (Table 3).

Fig. 1. Effects of superoxide anion (O2⋅−) scavenging on endothelium-dependent relaxation in response to ACh in the carotid arteries of young and aged C57BL/6 mice. A: treatment with Mn(III)tetra(4-benzoic acid)porphyrin chloride (MnTBAP) on young mouse arteries did not affect relaxation in response to ACh. B: treatment with MnTBAP improved relaxation in aged mouse arteries. Results are means ± SE and are expressed as a percent of maximal relaxation to papaverine (3 × 10−4 M); P < 0.05; two-way repeated-measures ANOVA and Bonferroni’s test.

Fig. 2. Effects of O2⋅− scavenging on endothelium-independent relaxation in response to the NO donor diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate) in the carotid arteries of young and aged C57BL/6 mice. A: in young mouse arteries, there was no difference in relaxation in the presence of MnTBAP compared with control. B: treatment of aged mouse arteries with MnTBAP improved relaxation. P < 0.05.

3. There was no difference observed between the young and the aged mouse arteries with the addition of MnTBAP.

Vascular O2⋅− production. Formation of O2⋅− was increased approximately fivefold in aged mouse aortas (young, 11,208 ± 4,373; aged, 60,239 ± 16,515 counts·min−1·mg of dry weight−1; n = 4; P < 0.05).

Table 3. Comparison of EC25 values for carotid arteries from young and aged C57BL/6 mice in the presence and absence of MnTBAP

<table>
<thead>
<tr>
<th>Drug</th>
<th>Young Mice+</th>
<th>Aged Mice†</th>
<th>Young Mice+ with MnTBAP*</th>
<th>Aged Mice† with MnTBAP*</th>
</tr>
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<tbody>
<tr>
<td>ACh</td>
<td>7.74 ± 0.15</td>
<td>7.64 ± 0.13</td>
<td>7.64 ± 0.07</td>
<td>7.59 ± 0.11</td>
</tr>
<tr>
<td>DEA-NONOate</td>
<td>7.40 ± 0.28</td>
<td>6.60 ± 0.15</td>
<td>6.89 ± 0.06</td>
<td>7.17 ± 0.20</td>
</tr>
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</table>

Values are means ± SE; *n = 4 mice; †n = 6 mice. ‡P < 0.05 vs. aged mice.
Biopterin levels and GTPCH-I activity. There were no significant differences between young and aged mouse aortas in BH4 concentrations (Fig. 3A) or in combined 7,8-BH2 and biopterin concentrations (Fig. 3B). The ratios of BH4 to 7,8-BH2 and biopterin were unchanged (Fig. 3C). There was also no change in GTPCH-I activity in the aorta of aged mice (Fig. 4).

Serum concentration of SAP. Levels of circulating SAP were significantly higher in aged compared with young mice (307 ± 167 vs. 16 ± 7 μg/ml, respectively; n = 6 mice; P < 0.05).

DISCUSSION

This is the first study to examine the effects of aging on endothelium-dependent relaxation in mice. Aging significantly reduced relaxation to ACh mediated by release of NO from endothelial cells. Furthermore, relaxation in response to the NO donor DEA-NONOate was also reduced in aged mouse arteries, which indicates that impaired reactivity of smooth muscle cells to NO is an important component of endothelial dysfunction of aged mouse arteries. The SOD mimetic MnTBAP normalized vasomotor function of carotid arteries, which suggests that chemical antagonism between O2· and NO is responsible for impairment of relaxation in response to ACh and DEA-NONOate. We also provide evidence that in a murine model of aging, alterations of BH4 metabolism do not contribute to impaired vasomotor function.

Vascular endothelium plays an essential role in cardiovascular homeostasis. Consistent with the results of the present experiments, numerous previous studies demonstrated that endothelium becomes dysfunctional in aged mouse arteries, mostly due to loss of NO biological activity and/or biosynthesis (10, 12, 13, 15, 20, 23, 30). Pharmacological analysis of impaired endothelium-dependent relaxation in response to ACh in aged mouse arteries demonstrated that reactivity of smooth muscle cells to NO is also reduced. This reduction is an important mechanism that underlies impairment of relaxation induced by the release of NO from endothelium. The ability of the SOD mimetic MnTBAP to normalize relaxation in response to ACh and DEA-NONOate in aged mouse arteries strongly suggests that increased production of O2· and subsequent chemical inactivation of NO are critical mechanisms of vasomotor dysfunction in aged arteries. Indeed, measurements of O2· production demonstrated increased formation of this...
free radical in aortas of old mice. The selectivity of MnTBAP was indicated by the fact that it did not affect relaxation in response to ACh or DEA-NONOate in young arteries. Consistent with our findings, van der Loo et al. (30) previously demonstrated that endothelial dysfunction in aortas of aged rats was due to increased production of \( O_2^- \). Interestingly, in aged rats, increased production of \( O_2^- \) was detected in endothelial cells but not in smooth muscle cells. In contrast, in the present study, MnTBAP normalized endothelium-independent relaxation induced by NO. This finding suggests that in the carotid arteries of aged mice, \( O_2^- \) is present not only in the endothelium but also in the media. This conclusion is consistent with the fact that in carotid arteries of Cu,Zn-SOD-knockout mice, which have increased \( O_2^- \), throughout the vascular wall, both endothelium-dependent relaxation in response to ACh and endothelium-independent relaxation in response to NO are impaired (6).

Examination of various parameters, including plasma glucose level and lipid profile, demonstrated that there were no differences between young and aged animals, which rules out a possible contribution of age-induced metabolic changes to vasomotor dysfunction. Furthermore, measurements of basal diameter and wall thickness of the carotid artery did not differ between young and aged animals, which excludes major age-induced remodeling of the arterial wall as an explanation for the observed differences in vasomotor function. In aged animals, we detected senescence-associated \( \beta \)-galactosidase staining in the kidneys, which indicates that the mice used in the present study were of an age at which cells were senescent. Interestingly, serum SAP levels were significantly increased in aged mice. SAP is the murine analog of C-reactive protein (33), which is an important proinflammatory marker in humans. This observation is consistent with the concept that aging is associated with increased production of proinflammatory mediators, including \( O_2^- \).

Several in vitro studies demonstrated that \( BH_4 \) can be inactivated by peroxynitrite-induced oxidation (18, 21, 22). Simultaneous production of NO and \( O_2^- \) in aged arteries provides favorable conditions for biosynthesis of peroxynitrite. Indeed, existing evidence suggests that increased production of peroxynitrite is an important mechanism of age-induced oxidative stress (30). If the cellular concentration of \( BH_4 \) is reduced to a level suboptimal to that required for enzymatic activity of NOS, this may have inhibitory effect on NO production. Furthermore, uncoupling of NO synthesis from consumption of NADPH may lead to NO-mediated reduction of oxygen and formation of \( O_2^- \) (4, 32, 36, 37). This has been proposed as an important mechanism underlying endothelial dysfunction caused by hypercholesterolemia, hypertension, diabetes, and smoking (5, 11, 24–27, 29, 37). Based on these considerations, we hypothesized that \( BH_4 \) oxidation may be an important component of endothelial dysfunction that is developed as a result of aging. In contrast with our expectations, we did not detect any major change in \( BH_4 \) metabolism in aged mouse vascular tissue. Levels of both \( BH_4 \) and its oxidation products were not different between young and aged mouse tissues. These findings strongly suggest that \( BH_4 \) is not a molecular target for oxidation by an age-induced increase in oxidative stress. Our conclusion was reinforced by the fact that enzymatic activity of GTPCH-I, which is a rate-limiting enzyme in \( BH_4 \) biosynthesis, was not affected by aging.

The results of the present study suggest that in mouse carotid arteries, aging-induced impairment of reactivity to NO is due to increased formation of \( O_2^- \). Aging apparently does not affect \( BH_4 \) metabolism in vascular tissue. Therefore, oxidation of \( BH_4 \) appears to be an unlikely mechanism responsible for vascular dysfunction of aged carotid arteries.

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GRANTS

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