Aging-Associated Vascular Phenotype in Mutant Mice With Low Levels of BubR1

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Background and Purpose—Aging is a major risk for stroke and a highly complex biological process believed to involve multiple mechanisms. Mutant mice that express low levels of the spindle assembly checkpoint protein BubR1 are known to develop several aging-associated phenotypes at a very young age, including cataracts, lordokyphosis, loss of subcutaneous fat, and impaired wound healing. However, whether BubR1 acts to prevent vascular aging has not yet been established. The present study was designed to investigate the vascular phenotype of mutant mice with low levels of BubR1.

Methods—Morphological, functional, and biochemical analyses were performed on aortas and carotid arteries of 3- to 5-month-old BubR1 mutant mice and wild-type littermates.

Results—Arterial wall thickness and inner diameter were significantly reduced in BubR1 mutant mice. Arterial walls of BubR1 mutant mice had low numbers of medial smooth muscle cells. Masson trichrome staining showed profound fibrosis in arterial walls of BubR1 mutant. In agreement with these morphological changes, functional analysis of pressurized isolated carotid arteries of BubR1 mutant mice demonstrated reduced elastic properties. Endothelium-dependent relaxations to acetylcholine and endothelium-independent relaxations to the nitric oxide donor DEA-NONOate were significantly reduced in carotid arteries of BubR1 mutant mice. Furthermore, enzymatic activity of nitric oxide synthase and levels of cyclic GMP were significantly reduced in aortas of mutant mice, but production of superoxide anions was significantly increased.

Conclusions—These findings demonstrate that BubR1 insufficiency in mice results in phenotypic changes reminiscent of vascular aging in humans and suggest a role for BubR1 in suppressing the vascular aging process. (Stroke. 2007; 38:1050-1056.)

Key Words: aging ■ carotid arteries ■ endothelium ■ nitric oxide ■ nitric oxide synthase

Evidence continues to accumulate on the importance of aging as a risk factor for development of cardiovascular disease and stroke.1 A large number of published studies demonstrated that endothelial dysfunction is one of the major phenotypic changes in aged arteries.2–8 Loss of nitric oxide (NO) biological activity is a central mechanism responsible for dysfunction of the vascular endothelium. Aging-induced increase in formation of oxygen-derived free radicals, including superoxide anion, appears to be the most likely explanation for the loss of NO.9 Indeed, superoxide anion chemically inactivates NO, thereby decreasing local bioavailability of NO in the vascular wall.7,8,10 This in turn may stimulate smooth muscle cells proliferation, thereby contributing in part to lesion formation in early stages of atherosclerosis.11–13 However, the proliferative capacity of smooth muscle cells declines with aging, which ultimately contributes to plaque destabilization and subsequent vessel occlusion.14–16

Previous studies have demonstrated that mouse mutants defective in genome maintenance mechanisms display symptoms of accelerated aging.17 Most notably, commonly described human syndromes of accelerated aging are also caused by genome maintenance defects.17 Thus, it seems that DNA maintenance and repair pathways play an important role in aging processes. Consistent with this concept, a study by Baker et al18 demonstrated that mice that express only 10% of normal levels of the mitotic spindle assembly checkpoint protein BubR1 leads to progressive aneuploidy and development of specific early aging-associated phenotypes, including short life span, cachectic dwarfism, lordokyphosis, cataracts, loss of subcutaneous fat, and impaired wound healing.18 In addition, natural aging of wild-type mice is marked by decreased BubR1 expression is multiple tissues, further suggesting that this protein may be a regulator of normal aging.18 The physiological relevance of BubR1 to the
vascular system has not yet been studied. Here we present the major characteristics of vascular phenotype caused by BubR1 insufficiency.

Materials and Methods

Experimental Animals

Hypomorphic BubR1 mutant (BubR1<sup>H/H</sup>) mice were generated in Dr van Deursen’s laboratory as previously described. Male BubR1<sup>H/H</sup> mice (3 to 5 months of age) and their age-matched wild-type littermates on mixed 129 and C57BL/6 background (BubR1<sup>+/+</sup>) were used for all experiments. The mice were fed regular pellet diet and housed in facilities with a 12:12 hour light–dark cycle. Experimental protocols and housing facilities were approved by the Institutional Animal Care and Use Committee.

The mice were killed with a 60 mg/kg intraperitoneal injection of pentobarbital. Blood samples were obtained through puncture of the right ventricle. The blood was mixed with heparin and centrifuged at 80°C. The aorta and carotid arteries were removed and placed immediately in ice-cold modified Krebs-Ringer solution stored at 4°C for 10 minutes at 2000 rpm. The plasma was aspirated and the arterial samples were washed twice by centrifugation at 900g for 10 minutes.

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Morphological Analyses

Thoracic aortas were carefully dissected, fixed in 4% formalin, and embedded in paraffin. Serial sections (5-μm-thick) were cut for further analysis by hematoxylin-eosin, elastica van Gieson, and Masson trichrome staining. The arterial segments were cut into 5 serial sections at 5-mm intervals. The medial area was quantified using Image-Pro PLUS (Media Cybernetics).

Vasomotor Reactivity

Carotid arteries were studied as previously described. Briefly, both sites of the artery were sutured onto microcannaules and placed in a vessel chamber (Living Systems Instrumentation) filled with aerated (94% O₂, 6% CO₂) Krebs-Ringer solution (37°C). A pressure of 50 mm Hg was maintained in the artery through the microcannulae. The arteries were equilibrated and submaximal contraction with U46619, endothelial-dependent relaxation was obtained by cumulatively adding acetylcholine (10⁻⁹ to 10⁻⁵ mol/L). After washout, equilibration, and submaximal contraction with U46619, endothelin-1-independent relations were determined using diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-IM1.2-diolate (DEA-NONOate; 10⁻⁹ to 10⁻⁵ mol/L). Relaxation was determined as a percent of relaxation to a high concentration of papaverine (3×10⁻⁴ mol/L). In a separate protocol, diameter changes were measured during stepwise increments in intraluminal pressure from 25 to 150 mm Hg. At the end of each experiment, arteries were incubated in Ca²⁺-free control solution containing EGTA (2×10⁻⁴ mol/L) and sodium nitroprusside (10⁻⁴ mol/L) for 45 minutes. Stepwise increase in intraluminal pressure was repeated to determine the passive diameter of the arteries.

Mechanical Properties of Arterial Wall

Passive diameter is defined as the inner diameter of carotid artery measured in Ca²⁺-free control solution. Cross-sectional compliance (C) is defined by the change in luminal cross-sectional area (ΔS) for a given change in intravascular pressure (ΔP), i.e., C = ΔS/ΔP (μm²/mm Hg). Distensibility is the compliance value normalized for the luminal cross-sectional area and defined by distensibility = (ΔS)/(ΔS/ΔP) (mm Hg⁻¹×1000). Western Blot Analysis

We performed Western blot analysis as previously described. Aortas from wild-type and BubR1 hypomorphs were isolated and all surrounding material was removed. After preparing lysates, blots were probed with antibody for BubR1 as previously described. Equal loading was confirmed by using α-tubulin (T-9026, Sigma; 1:2000 dilution). Results are representative for 3 independent males of each genotype at each age.

Nitric Oxide Synthase Enzymatic Activity

Total enzymatic activity of nitric oxide synthase (NOS) was assayed in aorta with L-arginine to L-citrulline conversion as described previously. Briefly, 2 aortas (n=1 experiment) were homogenized on ice in lysis buffer (pH 7.5). Total protein was determined using the BioRad DC Protein assay kit. Equal amounts of total protein homogenates prepared as described were applied. [¹⁴C]-L-citrulline was measured in a scintillation counter (LS 5000TD; Beckman Instruments). NOS activity was expressed as fmol of [¹⁴C]-citrulline produced per mg of protein per min.

Measurement of cGMP Levels in Aorta

A radioimmunoassay technique was used to determine the levels of cGMP as reported previously. Briefly, aortic tissue was initially incubated in minimal essential media with 0.1% albumin and 1% penicillin-streptomycin (GIBCO) in a 5% CO₂ incubator at 37°C for 30 minutes. Then, 3-isobutyl-1-methylxanthine (10⁻⁴ mol/L; Sigma) was added and aortas were incubated for additional 30 minutes to inhibit the degradation of cyclic nucleotides by phosphodiesterases. The aortic tissue was then removed from the media and quickly frozen in N₂. cGMP levels were measured by a radioimmunoassay kit (Amersham). The results were expressed as pmol/mg protein.

Detection of Vascular Superoxide Anion Production

Superoxide anion production was measured by lucigenin-enhanced chemiluminescence as previously described. Briefly, aortas were opened length-wise and equilibrated for 30 minutes at 37°C in the modified Krebs-HEPES buffer (pH 7.4, composition in mmol/L: NaCl 118.3, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.1, Ca²⁺/Na² 2 versenate 0.026, glucose 11.1). Arteries were dissected and connective tissue was removed under a microscope (Carl Zeiss). Because of the small size of the BubR1 arteries, we used carotid artery for analysis of vasomotor function and mechanical properties, whereas the largest conduit vessel, aorta, was used for biochemical and morphological analysis.

Statistical Analysis

Results are expresses as means±SEM for number of animals used for each experimental protocol. An unpaired Student t test was used to detect significant differences when 2 groups were compared. Factorial ANOVA followed by a Bonferroni/Dunn hoc test was used to detect significant differences in multiple comparisons. The concentration-response curves were analyzed by ANOVA for repeated measures followed by a Bonferroni/Dunn hoc test. Statistical significance was accepted at a level of P<0.05.
Mechanical Properties of the Vascular Wall

The average internal diameter of common carotid arteries was stepwise increased, but BubR1<sup>H/H</sup> arteries barely expanded under these conditions (Figure 2a). In addition, compliance and distensibility of BubR1<sup>H/H</sup> carotid arteries were significantly reduced as compared with carotid arteries of BubR1<sup>+/+</sup> mice (Figure 2b and 2c).

Vascular Reactivity

Absolute values of vasoconstriction to thromboxane analog U46619 were reduced in BubR1<sup>H/H</sup> mice carotid arteries (Table). However, when normalized to percent of maximal contractions to U46619, contractions were not significantly different between the 2 groups of mice (Figure 3a). During submaximal contractions to U46619 (10<sup>-7</sup>–3×10<sup>-7</sup> mol/L) endothelium-dependent relaxations to acetylcholine were significantly impaired in the carotid artery of BubR1<sup>H/H</sup> mice (Figure 3b). Endothelium-independent relaxations to DEA-NO were also significantly impaired in carotid arteries of BubR1<sup>H/H</sup> mice as compared with BubR1<sup>+/+</sup> mice (Figure 3c).

Expression of BubR1 Protein

Expression of BubR1 protein was detectable in aortas of wild type mice but not in aortas of BubR1<sup>H/H</sup> mice (Figure 4). Aging caused significant reduction in BubR1 protein expression in aortas of wild type mice (Figure 4).

cGMP Levels

Basal cGMP levels were significantly lower in aortas of BubR1<sup>H/H</sup> mice than in those of BubR1<sup>+/+</sup> mice (Figure 5a).

NOS Enzyme Activity

Aortas from BubR1<sup>H/H</sup> mice had significantly lower NOS enzyme activity than aortas from BubR1<sup>+/+</sup> mice (Figure 5b).

Vascular Superoxide Anion Production

Formation of superoxide anion was increased =5-fold in BubR1<sup>H/H</sup> aortas (199 653±33 637 counts/min per mg versus BubR1<sup>+/+</sup> mice: 41 928±17 504 counts/min per mg; P<0.05; n=3 to 7).

Discussion

Cells are equipped with surveillance mechanisms that detect various kinds of genome-destabilizing events (such as incompletely replicated or damaged DNA or unaligned metaphase chromosomes) and prevent cell cycle progression until DNA damage has been repaired. The spindle checkpoint protein BubR1 plays an essential role in the maintenance of genetic stability by ensuring proper microtubule–kinetochore attachment and sister chromatid segregation during mitosis.26 Efforts designed to characterize the physiological relevance of BubR1 have been hindered by the fact that null mutant mice die during embryogenesis soon after implantation. To bypass this problem, hypomorphic BubR1 mice were created.18 These animals are viable despite having only ~10% of normal levels of BubR1.18 As anticipated, reduced expression of BubR1 caused chromosome number instability but no other genome maintenance defects.18 The results of our morphological, biochemical, and functional analyses described here demonstrate that BubR1 insufficiency in mice results in phenotypic changes reminiscent of vascular aging in humans and rodents, and suggest a role for BubR1 in...
suppressing vascular aging. Reduced expression of BubR1 did not affect circulating levels of cholesterol or glucose, ruling out the possibility that hypercholesterolemia or hyperglycemia contributes to alterations of vascular phenotype in BubR1^{H/H} mice.

Morphological analyses of arteries from BubR1^{H/H} mice demonstrated reduced numbers of smooth muscle cells and diffuse medial fibrosis. This observation is consistent with reported age-related decline in proliferative capacity of vascular smooth muscle cells derived from humans and mice. As the number of smooth muscle cells in arterial media decreases, major changes may occur in extracellular matrix, including increased content of collagen and decreased levels of elastin leading to fibrosis of vascular wall, a phenomenon observed in aged arteries of humans and rats. Most notably, changes in vascular structure of BubR1^{H/H} mice were in agreement with alterations in mechanical properties of arterial wall. Aortas of BubR1^{H/H} mice exhibited impaired elasticity and compliance, a typical characteristic of aged arteries. The molecular mechanism underlying loss of smooth muscle cells and increased rigidity of vascular wall in BubR1^{H/H} mice is unknown and remains to be determined.

A large number of published studies demonstrated that endothelial dysfunction in aged arteries is caused by the loss of NO biological activity. In a recent mouse study, we have shown that aging impairs NO-mediated endothelium-dependent relaxations in carotid artery. This phenomenon was explained by an age-induced increase in production of superoxide anion and subsequent chemical inactivation of NO. Consistent with these reports, endothelium-dependent relaxations to acetylcholine were impaired in carotid arteries of genetically altered mice suggesting that consumption of NO by superoxide anion plays a role in the impairment of endothelial function. In agreement with elevated superoxide anion production, relax-
ations of BubR1H/H mice carotid arteries to exogenous NO were also reduced.

Biochemical analysis of BubR1H/H mice aortas demonstrated significantly reduced levels of cyclic GMP, a second messenger for NO. Decrease in arterial cGMP content is most likely caused by reduced availability of NO. In addition to chemical inactivation of NO by superoxide anion, measurements of NOS enzymatic activity indicated that reduced production of NO contributes to loss of NO in BubR1H/H mice. Previous studies detected decreased enzymatic activity of eNOS in aged rats, rabbits, and humans, further supporting our conclusion that reduced expression of BubR1 causes phenotypic alterations resembling vascular aging. The

Figure 2. Passive arterial diameter (a), cross-sectional compliance (b), and distensibility (c) of isolated common carotid arteries of wild-type (BubR1+/+) and BubR1H/H mice. *P<0.05 vs wild-type mice (n=6).

Figure 3. Concentration-dependent contractions to U46619 (a) obtained in common carotid arteries of BubR1+/+ and BubR1H/H mice. Contractions to U46619 (normalized as %) were not significantly different between both groups (n=7 to 9). Concentration-dependent relaxations to acetylcholine (b) and DEA-NONOate (c) obtained in common carotid arteries of BubR1+/+ and BubR1H/H mice. Endothelium-dependent relaxations to acetylcholine were reduced in BubR1H/H mice as compared with wild-type mice (*P<0.05; n=6 to 9). Concentration-dependent relaxations to DEA-NONOate were reduced in BubR1H/H mice as compared with BubR1+/+ mice (*P<0.05; n=7 to 12).
molecular mechanisms responsible for downregulation of NO production and its biological activity in BubR1\(^{+/+}\) mice. It is important to determine whether mouse strains in which DNA damage repair or telomere maintenance defects cause premature aging also exhibit early vascular aging or whether this phenotype is specific for deficiency of BubR1.

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**Disclosures**

None.

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