Mechanism of Gender-Specific Differences in Aortic Stiffness With Aging in Nonhuman Primates

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Background—Our hypothesis was that the changes in vascular properties responsible for aortic stiffness with aging would be greater in old male monkeys than old female monkeys.

Methods and Results—We analyzed the effects of gender differences in aging on in vivo measurements of aortic pressure and diameter and on extracellular matrix of the thoracic aorta in young adult (age, 6.6±0.5 years) versus old adult (age, 21.2±0.2 years) monkeys (Macaca fascicularis). Aortic stiffness, as represented by the pressure strain elastic modulus (Ep), increased more in old male monkeys (5.08±0.81; P<0.01) than in old females (3.06±0.52). In both genders, collagen density was maintained, collagen-bound glycation end products increased, and collagen type 1 decreased. However, elastin density decreased significantly (from 22±1.5% to 15±1.2%) with aging (P<0.05) only in males. Furthermore, only old males were characterized by a decrease (P<0.05) in collagen type 3 (an isoform that promotes elasticity) and an increase in collagen type 8 (an isoform that promotes the neointimal migration of vascular smooth muscle cells). In contrast to the data in monkeys, collagen types 1 and 3 both increased significantly in aging rats.

Conclusions—There are major species differences in the effects of aging on aortic collagen types 1 and 3. Furthermore, because alterations in collagen density, collagen content, hydroxyproline, and collagen advanced glycation end products were similar in both old male and female monkeys, these factors cannot be responsible for the greater increase in stiffness in old males. However, changes in collagen isoforms and the decrease in elastin observed only in old males likely account for the greater increase in aortic stiffness. (Circulation. 2007;116:669-676.)

Key Words: aging ■ collagen ■ elastin ■ gender ■ primates

Increased vascular stiffness with aging represents an independent risk factor of cardiac dysfunction. It has been suggested that changes in vascular stiffness are not apparent in most animal models, potentially because of the relatively short lifespan of traditional laboratory models, ie, rodents. We reasoned that a nonhuman primate model of aging would be unique for understanding the biology of the aging aorta because monkeys live >20 years but do not have the vascular complications of aging characteristic of humans, eg, diabetes, atherosclerosis, and hypertension. Studies in humans indicate that there are gender differences in aging vessels, with stiffness increasing more in men than women. However, the changes in vascular properties responsible for these differences are not known. In fact, there are no other experimental studies comparing the changes in genes and proteins of the extracellular matrix in old male and female animals, which can be attributed in part to the limitations alluded to above in traditional laboratory animal models. Our hypothesis was that the changes in vascular properties responsible for aortic stiffness with aging would be greater in old male monkeys than old female monkeys. Accordingly, we compared the physiological, histological, biochemical, and molecular differences in old and young male aortas to identify the changes responsible for the greater increases in aortic stiffness in old males.

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The first goal of the present investigation was to compare young and old adult males. We examined aortic stiffness by direct and continuous measurement of aortic pressure and diameter in conscious, chronically instrumented monkeys; performed a molecular analysis of the aorta for collagen and elastin, the genes and proteins of collagen subtypes, elastin, and collagen-bound glycation end products; and finally ex-
amined the aortas histologically for collagen and elastin. A second goal was to search for differences in these measurements in old females. A third goal was to examine interactions between age and gender. After we found that the changes in vascular properties we identified in old males seemed to differ from those described in aging rats, a fourth goal was to compare these novel findings in aortas from old male monkeys and old rats.

Methods

Animals
Young male (age, 6.6±0.5 years), old male (age, 20.0±0.2 years), young female (age, 7.2±0.5 years), and old female (age, 21.2±0.2 years) monkeys (Macaca fascicularis) were studied. The old females were either premenopausal (still cycling) or perimenopausal (irregular cycles), which is consistent with prior reports in this species. The numbers of animals are noted in the tables. Young monkeys were second generation, and old monkeys were feral animals captured at the age of 5 to 7 years old and kept in captivity for 12 to 15 years. All the animals come from the same species of Philippine monkey and were bred in captivity at the Simian Conservation Breeding and Research Center, Inc (Manilla, Philippines). The monkeys were fed a primate diet containing 5% to 6% fat, 18% to 20% protein, and 0.2% to 0.3% sodium chloride. The animals used in the present study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health 83–23, revised 1996).

Hemodynamics
For implantation of transducers, monkeys were tranquilized with ketamine hydrochloride (2 to 3 mg · kg−1 IM), anesthetized with thiamylal sodium (5 to 10 mg · kg−1 IV), and maintained with isoflurane (0.5 to 1.5 vol · 100 mL−1 in oxygen). Catheters and a miniaturized pressure gauge were implanted in the descending thoracic aorta and left atrium, and a pressure gauge was inserted into the left ventricle through the apex. Two piezoelectric crystals were sutured to opposing surfaces of the descending aorta to measure aortic diameter, and an ultrasonic flow probe was implanted around the ascending thoracic aorta to measure stroke volume and cardiac output. In 1 young female, this flow probe did not function properly. Measurements were made in the conscious state, recorded (PC216Ax, Sony Precision Technology, Inc., Tokyo, Japan), and analyzed by computer (Notocord, Croissy, France). Aortic pressure was measured with a high-frequency miniature pressure gauge; aortic diameter was measured with piezoelectric ultrasonic dimension crystals. Aortic strain was calculated as follows: systolic aortic diameter–diastolic aortic diameter (DD/ID). Pressure strain (Ep) was calculated as follows: K×(systolic blood pressure–diastolic blood pressure)/aortic strain (K=1333). Aortic stiffness (β) was computed in this manner: ln(Ps/Pd)/aortic strain.

Rat Samples
Segments of thoracic aorta were harvested from male F344xBN rats (6 versus 30 months old; National Institute on Aging, Bethesda, Md) and immediately frozen in liquid nitrogen. Samples were used for immunoblotting as described below.

Histology and Morphometry
Samples preserved in 10% buffered formalin were taken from the thoracic aorta for histology. Medial thickness was calculated from the medial area and a known length. Tissue staining included hematoxylin and eosin, picric acid sirius red (collagen staining), and orcein (elastin staining).

Quantitative Reverse-Transcription Polymerase Chain Reaction
After reverse transcription of the mRNA of interest from 50 ng total RNA (n=6 per group), the cDNA was used for quantitative polymerase chain reaction (40 cycles of a 10-second step at 95°C and a 1-minute step at 60°C) with dual fluorescent-labeled TaqMan probes on an ABI-Prizm Sequence Detector (Applied Biosystems, Foster City, Calif). Values are reported per cyclophilin transcript to correct for sample-to-sample RNA loading variations.

Immunoblotting
Proteins were extracted from 200 mg of tissue (n=5 per group) homogenized in 4 mL of 50 mmol/L Tris buffer (pH 7.4), 1 mol/L NaCl, and 5 mmol/L EDTA with a protease inhibitor cocktail (Sigma, St Louis, Mo). The homogenate was centrifuged at 26 000g. The pellet was extracted with 0.5 mol/L acetic acid for 24 hours, followed by the addition of pepsin (1 mg/mL) for 60 hours at 4°C. The pepsin extraction was repeated twice. Total collagen was precipitated from the extracts after addition of NaCl to a 5% final concentration and centrifuged. Pellets were solubilized in 0.5 mol/L Tris HCl (pH 7.4), separated by PAGE, and transferred to nitrocellulose membranes. The blots were probed with primary antibodies according to the manufacturer’s instructions, and the signal was visualized by chemiluminescence.

Measurement of Collagen and Elastin
Elastin was quantified using a method previously described. Aortic segments (n=5 per group) were defatted in acetone and dried. Proteins were extracted by agitation in 0.3% SDS for 12 hours. The extracellular proteins other than elastin (including collagen) were solubilized by three 15-minute extractions in 0.1 mol/L NaOH performed in a boiling-water bath. Elastin was quantified from the dry weight of the residue. Collagen content was determined by measuring the hydroxyproline content of the NaOH supernatants after evaporation and hydrolysis in 6N HCl for 24 hours at 110°C using a colorimetric assay.

Measurement of Advanced Glycation End Products
Advanced glycation end products (AGEs) were determined as described previously. Briefly, defatted aortic samples (n=5 per group) were minced and washed with cold saline, resuspended in 0.5 mol/L acetic acid, and digested for 24 hours at 4°C in the presence of 1 mg/mL pepsin (Sigma). Samples were centrifuged at 15 000g for 45 minutes. The resulting supernatant was considered the pepsin-soluble fraction of aorta, whereas the pellet was washed with cold saline, resuspended in 0.2 mol/L Tris-HCl (pH 7.4), and incubated for 24 hours at 37°C with 300 U/mL collagenase type VII (Sigma) containing 100 mmol/L CaCl2. After centrifugation at 15 000g for 45 minutes, the supernatant was regarded as the collagenase-soluble fraction. Both the pepsin-soluble and collagenase-soluble fraction samples were subjected to fluorescence-emission spectra at a 370-nm excitation and 440-nm emission wavelengths (Hitachi F-3010, Hitachi High-Technologies, Tokyo, Japan). The quantity of glycation-induced fluorescence was expressed as the relative fluorescence per milligram of dry weight of the tissue.

Statistical Analysis
Data are reported as mean±SEM. Statistically significant differences were calculated by a 2-way ANOVA. A value of P<0.05 was considered significant. The 2-way ANOVA contained an interaction term; no interactions were found significant for any of the data.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Body weights were not statistically different between old and young male and female monkeys. Biochemical characteristics (total cholesterol, triglycerides, and fasting plasma glucose) were similar among the 4 groups of monkeys, similar to data reported previously.
Hemodynamic Characteristics
Baseline hemodynamic characteristics of the 4 groups are indicated in Table 1 and Figure 1. Pulse pressure was increased in both male and female old monkeys. However, mean aortic pressure was not different among the 4 groups. Pulse diameter was greater in old females (0.24 ± 0.05 mm) than old males (0.12 ± 0.02 mm; P < 0.05). These differences in pulse diameter accounted for the greater increase in the pressure strain elastic modulus (Ep) and the stiffness index (β) in old male monkeys compared with old female monkeys (Figure 1), which reflects the increased stiffness with aging in male monkeys. Heart rate and total peripheral resistance were not different among the 4 groups.

Morphological Alterations in Collagen and Elastin
Hematoxylin and eosin–stained cross sections of young and old aorta showed no atherosclerotic lesions in the arterial intima (Figure 2). Animals in the old group often had mild intimal thickening, rarely more than a few cell layers thick and not characterized by lipid. Aging increased medial thickness in both genders (Table 2 and Figure 2). There was a decrease in the volume percent elastin of the old male monkeys but not old female monkeys. Although there was no increase in the volume percent collagen in the aorta, calculated collagen content increased significantly (P < 0.01) in the aorta of old monkeys compared with those of young monkeys. The calculated ratio of collagen to elastin was increased only in old male monkeys (Table 2). Thus, the differences in elastin may be crucial to explain the greater increase in stiffness of the old male monkey aortas.

There were also qualitative histological alterations in the aorta (Figure 2). The tunica media of the young aorta was composed of compact, fine fenestrated multiple layers of collagen and elastin separated by alternating layers of smooth muscle. The aortas of the old animals frequently exhibited focal breakdown of the elastic lamellae and focal accumulations of collagen replacing the lost elastic fibers. Electron microscopy of the aorta revealed fragmentation of the elastic tissue in the media of old versus young monkeys and increased lipid droplets in medial smooth muscle cells (data not shown).

Qualitative Changes in Collagen Expression
Measurement of collagen isoform transcripts was performed by quantitative polymerase chain reaction using specific dual-labeled probes. The results are illustrated in Figure 3a. The expression of the collagen type 1 mRNA was downregulated with aging by ~2-fold in both genders. Collagen type 3, an antagonist of vascular stiffness, showed a significant downregulation only in aging males, whereas collagen type 8, which promotes vascular smooth muscle cell migration and neointimal formation, increased with aging only in males. The expression of the corresponding proteins was confirmed by Western blot (Figure 3b).

Changes in Ratio of Collagen to Elastin
The results observed by histology and presented in Table 2 were further validated biochemically. To confirm the maintained collagen density observed by pathology (Table 2), we measured the total expression of collagen by measuring the aortic content of hydroxyproline. With this method, the total content of collagen (measured as microgram of hydroxyproline per milligram of tissue) was comparable among all groups (Figure 4a). In addition, the morphological analysis showed that the elastin density decreased in the aorta in aging males but not females (Table 2), suggesting that the production of elastin decreases in males. This observation also confirmed by measuring the elastin content in the aortas from the 4 groups. As shown in Figure 4b, elastin decreased by 35% in old males compared with young males, whereas no statistically significant difference was observed when young and old females were compared. Therefore, the biochemical measurements show that collagen content (per 1 mg tissue) does not change with aging, whereas the elastin content decreases only in aging males. As a consequence, the ratio of collagen to elastin, an index of stiffness, increases with age in males but does not change in females (Figure 4c), which confirms the morphological and physiological observations (Table 2).
The maintained collagen content in aging monkeys contrasts with the immunoblotting data of collagen 1 (Figure 3b). One possible explanation for this difference is the conjugation of AGEs to the collagen bundles. Not only does the conjugation of AGEs render collagen insoluble and undetectable by Western blotting, but also the production of AGEs increases with aging and is an important factor of increased vascular stiffness. We therefore determined the content of AGEs in aortic samples from the 4 groups of monkeys. As shown in Figure 4d, AGEs accumulated with aging but to a similar extent between genders; therefore, they cannot be held responsible for the difference in stiffness between old males and old females.

**Species Differences**

Because several studies have shown an accumulation of collagen with aging in rodent models, our last goal was to determine whether we could detect this species difference between the nonhuman primate model and a rodent model of aging. Thoracic aortas from young (age, 6 months) and old (age, 30 months) male rats were harvested and homogenized for immunoblotting for collagen types 1 and 3. The expression of both isoforms was increased by ~4-fold in old animals compared with young (Figure 5), which is diametrically opposite to our findings in the monkey.

**Discussion**

The main clinical sign of increased aortic stiffness is divergent changes in systolic and diastolic pressures, which result in increased pulse pressure. High arterial pulse pressure in the face of diminished vessel dimensional excursion is an indicator of stiffness and an independent risk factor for cardiovascular mortality. The current investigation demonstrates that changes in aortic stiffness with aging differ between genders because the increased stiffening is greater in old males than females. Importantly, in both aging males and females, aortic pulse pressure increased similarly, but aortic stiffness increased selectively in old males, indicating that the tools of the clinician (ie, blood pressure measurement) can be misleading in diagnosing the vessel stiffness associated with aging.

![Figure 1. Aortic stiffness. Measurements of pulse aortic pressure (pAoP; A), pulse aortic diameter (pAoD; B), pressure strain elastic modulus (Ep; C), stiffness index (β; D), and ratio of pAoP to pAoD (E) are compared in the 4 groups. pAoP was significantly increased in both male and female old monkeys. However, pAoD was significantly reduced only in old male monkeys vs young monkeys. This is likely responsible for the greater increases in Ep, β, and ratio of pAoP to pAoD in old male monkeys vs old female monkeys. *P<0.05 vs corresponding young animals; †P<0.05 vs corresponding old male monkeys.](http://circ.ahajournals.org/content/circulation/116/7/e146)
Comparing the data in both males and females not only provides some of the first data on gender differences in genes and proteins contributing to vascular stiffness in aging but also permits conclusions to be drawn regarding the changes in vascular properties potentially responsible for significantly greater increases in stiffness in the aging male aorta. Because there were several histological and biochemical changes in matrix proteins in both males and females, these changes cannot be responsible for the greater increases in vascular stiffness observed, which were significantly greater in males. Therefore, it is likely that the changes in vascular architecture and biochemistry that were observed only in old males were responsible for the differences. The greater increases in vascular stiffness with aging in the male nonhuman primate model correlate only with alterations in collagen isoform changes and decreases in elastin.

As recent texts and reviews note, currently used animal models for vascular stiffness have several limitations, not the least of which is the very short lifespan of rodents. The existence of species differences is further confirmed in our study by the diametrically opposite regulation of collagen expression between the monkey and the rat during aging. The nonhuman primate model used in this investigation has several advantages: They have a lifespan of 25 to 30 years and phylogenetic similarity to humans but lack associated vascular diseases of aging, eg, atherosclerosis, hypertension, and diabetes. In older humans, intimal thickening usually is associated with and ascribed to other diseases (such as atherosclerosis, hypertension, diabetes or dyslipidemia), which complicates the determination of the precise mechanism of the increased stiffness. A recent investigation has shown that increased arterial stiffness was already found in normotensive persons who subsequently developed hypertension.

The increased vascular stiffness of aging has been ascribed to numerous factors, including decreased elastin, increased medial thickness, increased collagen content, and increased AGEs. We also observed increased collagen content, medial thickness, and AGEs in the aging aorta, but the increases were similar in old males and females, suggesting that these changes may contribute entirely to the increase in stiffness observed in old female aortas but cannot be the mechanism responsible for the greater increase in stiffness in old male aortas.

A recent study suggested that one major difference observed between the older male and female monkeys was in collagen isoform expression. Collagen is expressed in multiple isoforms that may have very different functions regarding tissue rigidity or elasticity. In the aorta, collagen type 1 represents 80% to 85% of the total collagen content, whereas collagen type 3 represents 5% to 10%. Collagen type 1 is organized in thick bundles, conferring the vessel its rigidity and tensile strength. In contrast, collagen type 3 forms small fibers that mesh with the collagen type 1 and promote vascular elasticity. The rigidity of the aortic vessel is proportional to the ratio of collagen type 1 to type 3. Stretching, for example, increases the production of collagen type 1 to improve the tensile resistance of the vessel.

The decrease in collagen type 3 and increase in collagen type 8, observed only in old males, could partially explain the greater increases in stiffness in the older male aorta. Previous studies have suggested differences in collagen isoform expression with age, but no previous study has observed the changes in collagen type 8 during aging. An interesting observation from our study that relates directly to the mechanism of vascular remodeling is the upregulation in aging males of the transcript encoding collagen type 8 (Figure 3).

Figure 2. Histology of aging aorta. Photomicrographs of the thoracic aorta from young (A, C, and E) and old (B, D, and F) male monkeys stained with hematoxylin and eosin (A and B), picric acid sirius red for collagen (C and D), and orcein for elastic tissue (E and F). Medial thickness is clearly increased in the old animals, and there are focal losses of elastic tissue and increased collagen. The old animals have a mild increase in intimal thickness, illustrated in the inset enlargements of the intimal area. See Table 2 for quantification. A, Bars=250 μm for the low-power and 50 μm for the high-power inset, and the magnifications are the same throughout the figure.

### Table 2. Morphometric Measurements of the Thoracic Aorta

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Mean±SEM</th>
<th>Old</th>
<th>Mean±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial thickness, μm</td>
<td>10</td>
<td>329±1.7</td>
<td>7</td>
<td>464±18*</td>
</tr>
<tr>
<td>Collagen density, %</td>
<td>10</td>
<td>24.5±1.7</td>
<td>7</td>
<td>26.1±0.92</td>
</tr>
<tr>
<td>Collagen content, μm²/100 μm</td>
<td>10</td>
<td>7957±625</td>
<td>7</td>
<td>12,241±606*</td>
</tr>
<tr>
<td>Elastin density, %</td>
<td>10</td>
<td>22.0±1.5</td>
<td>7</td>
<td>15.2±1.2*</td>
</tr>
<tr>
<td>Collagen/elastin</td>
<td>10</td>
<td>1.7±0.13</td>
<td>7</td>
<td>1.79±0.16*</td>
</tr>
</tbody>
</table>

Collagen fibers were stained with picrosirius red staining; elastin was stained with Orcein. *P<0.05 vs young monkeys; †P<0.05 vs corresponding male monkeys.
to vascular injury,\textsuperscript{37} has a role opposite to that of collagen type 1 in that it promotes vascular smooth muscle cell migration.\textsuperscript{38} The upregulation of this transcript, together with the downregulation of other isoforms in aging males, again supports the notion that this group is more susceptible to neointimal proliferation, vascular smooth muscle cell migration, and potentially atherosclerosis.

The second major difference was in elastin density, which decreased selectively in old males. Previous studies also have observed either decreased\textsuperscript{33} or no change\textsuperscript{39,40} in elastin with age, but some studies have observed elastin fragmentation,\textsuperscript{21} as we did in this study.

Our study focused mainly on collagen and elastin because these molecules are particularly responsible for the tensile strength of the arterial vasculature. Other important components of the extracellular matrix that might be involved in stiffness are proteoglycans. Although this possibility has not been studied in detail, it is less likely because variations in the

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Gender-specific regulation of collagen isoform expression with aging. Comparison of the gene expression (A) and protein expression (B) of collagen isoforms among the 4 groups. *P*<0.05 vs corresponding male monkeys.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Content of elastin, collagen, and AGEs in aging aortas from monkeys. Comparison of the expression of collagen (A) and elastin (B), ratio of collagen to elastin (C), and accumulation of AGEs (D) in the aorta from the 4 groups of monkeys. *P*<0.05 vs corresponding young animals; #*P*<0.05 vs corresponding male monkeys.
content of proteoglycans in the vascular wall do not correlate with changes in vascular stiffness. Another important parameter that might account for changes in aortic stiffness with aging was addressed in prior studies from our laboratory on the endothelial dysfunction that accompanies aging. Related to this, soy proteins have been implicated in vascular and endothelial protection. The standard monkey diet used in this study contains a significant percentage of soya, which is rich in isoflavones (phytoestrogens) and therefore could potentially affect vascular reactivity. It should be emphasized that all monkeys from both genders received exactly the same diet.

Conflicting reports have been published regarding the regulation of collagen protein expression during aging. Although most animal studies have shown an increase in collagen density of arteries with aging, some other investigations have demonstrated no change or a decrease. It is also controversial whether elastin content decreases in experimental studies and in human arteries. These discrepancies might result from different factors. First, as mentioned, collagen exists in multiple isoforms that have different and sometimes opposing elastic properties and is important in the formation of AGEs. Therefore a global measurement of collagen content or density might inaccurately reflect vessel stiffness. Second, there can be a major difference between collagen content and collagen density. Third, species differences may be responsible in part for the discrepancy in findings, as we demonstrated in the present study.

The diametrically opposite results for collagen types 1 and 3 in the aging rat aorta and the aging monkey aorta serve to emphasize the problems with traditional laboratory animal models of aging noted by others. More important, these data pose further problems for future mechanistic studies that would ordinarily be planned in genetically altered mouse models.

In summary, because of all the factors noted above, it is not surprising that conflicting results in aging male vessels have been reported. However, a gender comparison of extracellular matrix composition has not been shown before. Our study demonstrates a qualitative and quantitative regulation of collagen at both the mRNA and protein levels that differs between males and females. Because alterations in collagen density, collagen content, hydroxyproline, and collagen AGES were similar in old male and female monkeys, these factors cannot be responsible for the greater increases in vascular stiffness observed in old males; rather, the major differences observed between old males and old females were distinct changes in collagen isoforms and the decrease in elastin observed only in old males.

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Disclosures
None.

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10. Appt SE. Usefulness of the monkey model to investigate the role soy in postmenopausal women’s health. Iar J. 2004;45:200–211.

Figure 5. Species differences with aging. Measurement of collagen types 1 and 3 in the thoracic aorta of old (solid bars) vs young (open bars) male rats. These changes are directionally opposite in the monkey (Figure 3). P<0.01 vs corresponding values in young rats (n=4/group).
Increased vascular stiffness with aging represents an independent risk factor of cardiovascular disease. Studies in patients indicate that there are gender differences in aging vessels, with stiffness increasing more in men than women. To understand the biology of the gender-related differences in aging vessels, most prior work has relied on rodent models. Unfortunately, these animals not only exhibit several species differences from humans but also live only 2 to 3 years, relatively short for developing pathological changes in vessels as a result of aging. Accordingly, we used a nonhuman primate model of aging because monkeys live longer than rodents and exhibit several species differences from humans but also live only 2 to 3 years, relatively short for developing pathological changes in vessels as a result of aging. Circulation. 2001;103:2382–2386.


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