Circulating and cellular markers of endothelial dysfunction with aging in rats

M. CHALLAH,1 S. NADAUD,2 M. PHILIPPE,1 T. BATTLE,1 F. SOUBRIER,2 B. CORMAN,3 AND J. B. MICHEL1

1Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 460, 75870 Paris; 2INSERM Unité 358, 75010 Paris; and 3Department of Cell and Molecular Biology, Centre d’Étude Nucléaire, 91191 Gif sur Yvette, France

There is much evidence now of endothelial function impairment associated with aging (31). The endothelium-dependent vasodilator response to acetylcholine decreases with aging in different species (16, 39), including humans (41). Bradykinin-induced endothelium-dependent vasodilation is changed to a vasoconstrictor response in vessels of aged rats (30). Endothelium-dependent relaxation to catecholamines is impaired (6) with aging as is the endothelial release of hyperpolarizing factor (19, 35). Recently, Tschudi et al. (43) showed an alteration of nitric oxide (NO) release by the aorta of aged rats. These dysfunctions are observed in different territories including not only conductance arteries, such as the aorta (9, 26), carotid arteries (37), and coronary arteries (16), but also resistance arteries, such as the mesenteric vascular bed (7). Moreover, in the rat, age differently altered NO release in the systemic arterial system and the pulmonary vascular bed (43). Nevertheless, most of these studies involve a pharmacological approach of the ex vivo sensitivity of arterial rings to endothelium-dependent and -independent vasodilatator and vasoconstrictor agents. The in vivo biological correspondences of such endothelial dysfunctions are less well documented.

Angiotensin I-converting enzyme (ACE) is one of the major peptidases expressed by endothelial cells and plays a major role in the regulation of vascular tone. It circulates in the plasma as a soluble enzyme, whereas in tissue it is present as a membrane-bound enzyme. A shift in ACE expression from endothelium to smooth muscle cells has been recently reported in hypertension (2) and after deendothelialization (18, 40).

NO accounts for the biological activity of the endothelium-derived relaxing factor. NO causes vasodilatation and platelet inhibition and thereby prevents vasoconstriction and thrombus formation. NO is generated from a terminal guanidino nitrogen of L-arginine, and its formation is catalyzed by a family of enzymes called NO synthases (NOS). Endothelial NOS (eNOS) is Ca2+ dependent, constitutively present in endothelial cells, and is reputed to functionally adapt the vascular wall to shear stress (27). Inducible NOS (iNOS) is classically cytokine inducible but has recently been shown to be constitutively implicated in vascular wall modeling during development (10). Therefore, the respective roles of eNOS and iNOS in vascular lumen modeling remain to be clarified.

We showed earlier (32) that aortic and carotid lumen diameter increased progressively and regularly with age, independently of the level of blood pressure in rats. These data confirmed previous findings in rats (13) and humans (42) that showed a similar, pressure-independent increase in arterial lumen diameter and wall thickness with aging. Therefore, aging modified hemodynamic stresses and both endothelial and smooth muscle cell functions and phenotypes.

In the present study, we investigate different endothelial markers in old normotensive rats with in vivo molecular and cellular approaches. We report a decrease in ACE expression in pulmonary endothelial cells. Until now, no data on the expression of the different NO synthases in old animals have been available; we show that in the aorta aging is associated with a decrease in eNOS and iNOS expressions without changes in neuronal NOS (nNOS) expression. Finally,
we show an increase in the number of endothelial cells circulating in the blood.

MATERIAL AND METHODS

Experimental Design

Two series of 36 normotensive male WAG/Rij rats were used to assess the effects of aging on three different markers of endothelial biology. Rats were maintained in the specific pathogen-free husbandry of the Centre d’Etudes de Saclay (Gif sur Yvette, France) (32). The 50% survival age was 27 mo, and 37% of the animals were still alive at 30 mo (22). Twelve rats of 10, 20, and 30 mo of age were anesthetized, and blood was collected in heparinized tubes. The aortas and lungs were quickly removed and frozen in liquid nitrogen. The procedure followed for the care and euthanasia of the study animals was in accordance with the European Community Standards on the care and use of laboratory animals (Ministère de l’Agriculture, France; authorization no. 00577).

ACE Measurement

ACE activity. ACE activity was assayed by measuring the hydrolysis of a radiolabeled synthetic substrate (glycine-hippuryl-L-histidyl-L-leucine) in the presence or absence of hydrolysis of a radiolabeled synthetic substrate (glycine-

Aortic segments and lungs were homogenized in 10 vol of cold tris(hydroxymethyl)aminomethane-HCl buffer (0.05 M, pH 7.4) containing the detergent 3-[cholamidopropyl]dimethylammonio]-1-propane sulfonate (CHAPS; 8 mM, Sigma Chemical, St. Louis, MO) and using a glass-glass (aorta) or a Teflon-glass homogenizer (lungs). The crude homogenate was centrifuged at 1,000 g for 15 min at 4°C. The supernatant was recovered, sonicated twice for 10 s, then assayed for ACE activity. Membrane ACE activity was expressed as picomoles of hippuryl-histidyl leucine per milligram per minute. Protein concentration was determined by the Bio-Rad Coomassie brilliant blue G-250 method using bovine serum albumin as standard.

ACE mRNA expression. Forty micrograms of total lung RNA were used for Northern blotting. RNA blots were probed with a 20°C until assayed. One aliquot was used to determine the protein concentration. The cGMP content was determined radioimmunologically (23).

eNOS mRNA Expression

Quantitative reverse transcription-polymerase chain reaction. eNOS mRNA was quantified by comparing rat aorta or lung RNA with defined amounts of internal standard complementary RNA (cDNA). The standard preparation, the reverse transcription (RT), and the polymerase chain reaction (PCR) protocols are described in detail elsewhere (34). Briefly, the standard was obtained by amplifying a 616-base pair (bp) fragment of the rat eNOS cDNA using PCR. The amplified fragment was purified on an agarose gel, phosphorylated, filled in with T4 DNA polymerase, and subcloned in the EcoRV site of pBluescript. The plasmid pReNOS1 obtained was then used to prepare the internal standard construct by adding a 64-bp double-stranded oligonucleotide corresponding to a fragment of polylinker inserted in the SacII site (526 bp) of the rat eNOS cDNA plasmid pReNOS1, resulting in pReNIS5. A cDNA was synthesized in vitro as a sense probe from the EcoRI linearized plasmid using T3 RNA polymerase and the mRNA capping kit (Stratagene). The cDNA concentration was measured by spectrophotometry at 260 nm.

The two RNA (assay and standard) were reverse transcribed in the same reaction to avoid variations in the RT. Two primers, one sense (5’-TTCCGCGTCCACCTGATCTAA-3’) and one antisense (5’-AACATGTGTCCTTGCGAGGCA-3’), surrounding the 46-bp fragment insertion site were designed to allow the distinct amplification of eNOS mRNA (340 bp) and of the internal standard cRNA (404 bp). Furthermore, these primers were chosen to encompass several introns to avoid amplification of contaminating genomic DNA. A negative control was used for each set of samples to check the RT and PCR amplification reagents for any contamination.

Quantification of the PCR products was performed by using radiolabeled primers and counting the radioactivity of the amplified fragments. The quantitative assay was performed according to the method developed by Gilliland and co-workers (21). For each sample, a defined quantity of total RNA was reverse transcribed with five different concentrations of internal standard (competitor) cRNA. The results for one sample were plotted as the logarithm of the ratio to the quantity of competitor cRNA at each point. When the quantity of eNOS mRNA in the sample is equivalent to the quantity of competitor cRNA, the PCR values are equal and log ratio is zero.

iNOS mRNA Expression

Quantitative RT-PCR. iNOS mRNA expression was quantified in rat aortas and lungs. Rat iNOS internal standard was produced according to the same method used for rat eNOS standard. Rat iNOS cDNA was amplified from rat heart RNA, subcloned, and sequenced. A polylinker fragment (47 bp) was inserted in a unique Bsu 361 site between the two primers used for the amplification (36). After Hind III linearization, iNOS standard plasmid was treated as that for eNOS. Two primers, one sense (5’-GTGTTTTGTGGAGTGTCAGT-3’) and one antisense (5’-CGGACCATCTCCTGTACC-3’) surrounding the 47-bp fragment insertion site, were designed to allow the distinct amplification of iNOS mRNA (227 bp) and of the internal standard cRNA (274 bp). Comparative RT-PCR. Because the data obtained with quantitative RT-PCR were unexpected, they have been verified by comparative RT-PCR. cDNA from the aortas and lungs of 10-, 20-, and 30-mo-old rats were primed with 1 μg of oligo(dT)(12–18) and reverse transcribed. Primers were the same as those used for quantitative PCR. Primers for GAPDH include 5’-GTGAAAGGGAGTCAGCTCAAGC-3’ (sense) and 5’-TTTCTTTGTTGGGAGTGTCAGT-3’ (antisense).
and 5’-GCTGCTCAACAGAATACTAGGCT-3’ (antisense), which amplify a 299-bp mRNA region. Radiolabeled primers were used, and the quantification of PCR products was performed by counting the radioactivity of the amplified fragment. PCR amplification was verified to be exponential, and the iNOS product was proportional to sample input. iNOS mRNA expression was calculated by normalizing iNOS mRNA to GAPDH mRNA.

nNOS mRNA Expression

Comparative RT-PCR. nNOS mRNA expression was quantified in the rat aorta by comparative RT-PCR using the same method as that used for rat iNOS mRNA. Primers for nNOS include 5’-CTGGCTCAACAGAATACTAGGCT-3’ (sense) and 5’-GCAGTGTACAGCTCTCAGAAGA-3’ (antisense), which amplify a 293-bp mRNA region. Primers for GAPDH were the same as those described above. PCR amplification was verified to be exponential, and the nNOS product was proportional to sample input. nNOS mRNA expression was calculated by normalizing nNOS mRNA to GAPDH mRNA.

Quantification of Circulating Endothelial Cells

The method is derived from the one described by George et al. (20) in human blood. Monodispersed magnetizable particles (Dynabeads M-450) were obtained from Dynal (Oslo, Norway). They were 4.5-µm-diameter polystyrene beads coated with affinity-purified sheep anti-mouse immunoglobulin (Medac, Hamburg, Germany) diluted 1:10 in phosphate-buffered saline (PBS). One milliliter of total blood collected on heparin was added to the samples, and the total volume of rosetted cell suspension stained with acridine orange was examined per fraction of 20 µl in a hemacytometer under a fluorescent microscope.

Preliminary experiments with injection of cultured cells in rats and the publication by George et al. (20) indicated that positively rosetted endothelial cells consisted of more than three rosetted beads per target cell (Fig. 1B). This criteria was kept for the current experiment. For each rat in all groups, the total volume of rosetted cell suspension stained with acridine orange was examined per fraction of 20 µl in a hemacytometer under a fluorescence microscope.

Statistics

Results are expressed as means ± SE. One-way analysis of variance (ANOVA) was used to test the effect of age on the different parameters. A two-way factorial ANOVA was performed to test the effect of age and NOS isoforms on NOS expressions in the aorta and in the lung. When the F value indicated overall significance, specific comparisons were performed using Scheffé’s F-test. Statistical significance was assessed for P < 0.05.

RESULTS

ACE

Results of determination of ACE activity in plasma, lung, and aorta are summarized in Table 1. Plasma ACE activity significantly decreased with age (F = 7.1, P < 0.01). Values were significantly lower in 30-mo-old than in 10- and 20-mo-old rats. Similarly, lung ACE
Tables 1 and 2 provide data on the age-related changes in ACE activity and NOS expression, respectively.

### Table 1. ACE activity in plasma and tissues

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>ACE activity (nmol HHL·ml⁻¹·min⁻¹)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>252 ± 6</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>243 ± 8</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>210 ± 10*</td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± SE; n = 12 rats for each group. ACE, ANG I-converting enzyme; ANOVA, analysis of variance; HHL, hippuryl-histidyl leucine; prot, protein. *Significant difference compared with 10-mo-old rats; †significant difference compared with 20-mo-old rats.

### Table 2. NOS expressions

<table>
<thead>
<tr>
<th>NOS mRNA</th>
<th>Age, mo</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic cGMP, fmol/mg prot</td>
<td>1,164 ± 35</td>
<td>1,121 ± 72</td>
</tr>
<tr>
<td>eNOS mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR, fg/400 ng total RNA</td>
<td>57.6 ± 2.6</td>
<td>59.7 ± 4.4</td>
</tr>
<tr>
<td>Lung</td>
<td>150.8 ± 17</td>
<td>130.7 ± 6.9</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR, fg/400 ng total RNA</td>
<td>42.5 ± 2.6</td>
<td>32.9 ± 2.3*</td>
</tr>
<tr>
<td>Lung</td>
<td>4.6 ± 1.1</td>
<td>1.6 ± 0.2*</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comparative PCR, iNOS/GAPDH</td>
<td>1.6 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>1.39 ± 0.29</td>
<td>0.58 ± 0.06*</td>
</tr>
<tr>
<td>Aorta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nNOS mRNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comparative PCR, nNOS/GAPDH</td>
<td>0.10 ± 0.02</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

Results are means ± SE; n, no. of rats. NOS, nitric oxide synthase; cGMP, guanosine 3’5’-cyclic monophosphate; eNOS, endothelial NOS; PCR, polymerase chain reaction; iNOS, inducible NOS; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nNOS, neuronal NOS; NS, not significant. *Significant difference compared with 10-mo-old rats; †significant difference compared with 20-mo-old rats.
comparative RT-PCR and $F = 6.5, P < 0.01$ for quantitative RT-PCR; Fig. 4). In contrast to the aorta, iNOS mRNA expression significantly increased with age in the lung ($F = 10.8, P < 0.01$). In lung eNOS, mRNA was only slightly ($+30\%$) more expressed than iNOS ($F = 23, P < 0.001$). No changes were observed in nNOS mRNA expression in rat aortas during aging ($F = 1.0, P = \text{NS}$).

Circulating Endothelial Cells

The number of circulating endothelial cells in 100 µl of blood significantly increased with age ($F = 40, P < 0.001$; Fig. 5).

DISCUSSION

The present data demonstrate in vivo considerable modulation of endothelial phenotype and NOS expression with aging in normotensive Wistar WAG/Rij rats: ACE endothelial expression in the lung significantly decreased, aortic cGMP content also decreased (partly in relationship to changes in NOS expression), and endothelial cell turnover probably increased, as suggested by the increase in the number of circulating endothelial cells.

In the present study, we have investigated both aortic and pulmonary tissue as two different sites of possible endothelial dysfunction. The lung is rich in endothelial cells but contains fewer smooth muscle cells. Conversely, the aorta has a high smooth muscle cell content but contains many fewer endothelial cells compared with the lung. Moreover, the endothelial functions and phenotype probably differ between large arteries, such as the aorta, and capillaries, such as in lung.

ACE expression has already been demonstrated to be modulated by the activation and the density of vascular cells. For example, we and others recently showed that endothelial ACE expression decreases in the lung of rats with congestive heart failure (24) or with pulmonary hypertension (33). In pathological situations, such as arterial wall injury (18, 40) or hypertension (2) corresponding to different levels of smooth muscle cell activation, expression of ACE can shift from a predominantly endothelial to a predominantly nonendothelial...
ACE expression in endothelial cell cultures is dependent on the cell confluency; ACE activity is minimal in preconfluent conditions and increases after confluency (15). In vivo, the lung is an organ particularly rich in endothelial cells and therefore in ACE expression, and the circulating, soluble, plasma form of ACE is reputed to originate from pulmonary endothelium in physiological conditions. We observed a small but clear decrease in lung and plasma ACE activity associated with a decrease in pulmonary ACE mRNA expression in the oldest rats, suggesting a decrease in endothelial cell functional activity with aging as proposed in congestive heart failure (24) and pulmonary hypertension (33). In contrast, ACE activity paradoxically increased with age in the aortic wall, suggesting an increased ACE expression in the activated smooth muscle cells as observed with hypertension (2) and vascular wall injury (40). These data probably reflect smooth muscle cell activation associated with the hypertrophy and intimal proliferation (32) observed with aging.

There is clear pharmacological evidence in the literature that arterial endothelium-dependent relaxation is perturbed with aging (16, 39). Our present physiological results extend these previous pharmacological data to the analysis of eNOS and iNOS expression in the vascular wall. We (3, 4) have already shown that NO interaction with soluble guanylate cyclase was the main determinant of the cGMP concentration within the aortic wall. The present data demonstrate a very significant decrease in cGMP level within the arterial wall with aging. In a first step, we explored the constitutive eNOS expression, because we (34) and others (28) recently showed that eNOS was regulated not only at the level of its activity but also at the level of its expression. Chronic changes in shear stress modulated eNOS expression in the endothelium (25, 44). Because shear stress probably decreases with aging due to arterial lumen enlargement (11), we hypothesized a decrease in eNOS expression with aging in the aorta. In the present study, we demonstrate a downregulation of eNOS expression in the aorta, but eNOS expression was not modified in the lung, an endothelium-rich organ. We can speculate that this could be due to the increase in endothelial cell turnover as suggested by the increase in circulating endothelial cells. It has recently been demonstrated that, in contrast to ACE expression, eNOS expression was higher in growing than in confluent endothelial cells in vitro (5). Therefore, the endothelial regrowth could maintain a stable eNOS expression within the lung during aging. In contrast, we observed a low level of iNOS expression in the aortic wall in the youngest animals. This expression decreased with aging. Because iNOS expression is usually considered to be a pathological phenomenon associated with inflammatory and infectious processes, the regulation of iNOS expression within the arterial wall in physiological conditions remains to be explored. Wu et al. (45) could detect iNOS expression in the aorta of spontaneously hypertensive rats. Moreover, it has recently been proposed that iNOS rather than eNOS was involved in the developmental modeling of the arterial lumen (10). Therefore, the observed decrease in NOS expressions could appear as secondary to the age-dependent arterial lumen enlargement. Another unexpected result was that, although the lung is richer in endothelial cells than the aorta, the level of eNOS mRNA was three times higher in the aorta than in the lung. This suggests that the regulation of eNOS mRNA...
expression in aortic endothelial cells is different from its regulation in endothelial cells of pulmonary capillaries.

In contrast to the aorta, iNOS expression significantly increased in the lung with aging. Nevertheless, we do not know which cell type (endothelial cells, resident macrophages, or epithelial cells) is responsible for this overexpression of iNOS in the lung. Our molecular data, showing a decrease in NOS expressions within the aorta and an increase in the lung, fit well with the functional data recently reported by Tshudi et al. (43), showing a decreased detection of NO with aging in the aorta but a simultaneous increase in NO production in the lung. The decrease in NOS expressions with aging in the aorta does not exclude a simultaneous increase in NO interaction with free radicals (1) or with glycated proteins (29). It has been proposed that glycation of the extracellular matrix increases with aging. Therefore, the role of such interactions with NO efficiency in aging remains to be explored.

To evaluate in vivo endothelial cell sloughing, we adapted a method of detection of circulating endothelial cells, described in humans (20), to the rat. We showed that circulating endothelial cells increased in the oldest rats, suggesting an increase in endothelial turnover with aging. The reason why endothelial sloughing increased, whether due to a decrease in endothelial cell adherence or to an increase in endothelial cell proliferation, remains to be determined. Phillips et al. (38) recently described that primary cultures of endothelial cells proliferated more when cells came from old rats than when they came from young rats. Nevertheless, this method to measure circulating endothelial cells is probably semiquantitative: the absolute number of circulating endothelial cells detected by each experimenter depended mainly on the threshold definition of intact endothelial cells. Some membrane skeletons of circulating endothelial cells could bind RECA-1 antibody and therefore give intermediate images between background and intact endothelial cells. In the present study, only apparently endothelial cells have been taken into account. This is probably why the level of circulating endothelial cells appeared low compared with earlier data.

In conclusion, our study has demonstrated physiological modifications of endothelial markers with aging, involving a probable increase in endothelial cell turnover, a decrease in endothelial ACE expression, and a decrease in arterial wall cGMP content, which could be related to a significant decrease in expression of iNOS and eNOS in the aorta. These data do not exclude other phenomena such as NO interactions with free radicals or glycated residues that could probably contribute to the observed decrease in cGMP content. Nevertheless, the respective roles of eNOS and iNOS are probably more complex than a simple decrease in endothelial capacity to produce NO with aging.

The authors thank Mary Osborne-Pellegrin for editing the manuscript.

This study was supported by a grant from the French Ministère de l’Education Nationale de l’Enseignement Superieur, de la Recherche et de l’Insertion professionnelle. ACC no. 86/96. M. Challah was supported by a grant from Synthelabo, France.

Address for reprint requests: J. B. Michel, INSERM U460, UFR de Medecine X. Bichat, 18, rue Henri Huchard, 75870 Paris Cedex 18, France.

Received 18 March 1997; accepted in final form 30 June 1997.

REFERENCES

18. Fishel, R. S., V. Thourani, S. J. Eisenberg, S. Y. Shai, M. A. Corson, E. G. Nabel, K. E. Bernstein, and B. C. Berk. Fibroblast growth factor stimulates angiotensin converting en-
zyme expression in vascular smooth muscle cells. Possible media-


29. Michalc, J. B., D. Heudes, O. Michel, P. Poitevin, M. Philip-


36. Ragazzi, E., A. Chinningi, F. Gandolfo, G. Morbi, L. Caparrotta, G. Aliev, M. Prosdocimi, and F. Bassina. Endothelial nucleotide-mediated aorta relaxation in aged Watanabe heri-


