Different Impact of Essential Hypertension on Structural and Functional Age-Related Vascular Changes

Rosa Maria Bruno, Emiliano Duranti, Chiara Ippolito, Cristina Segnani, Nunzia Bernardini, Giulio Di Candio, Massimo Chiarugi, Stefano Taddei, Agostino Virdis

Abstract—We evaluated whether vascular remodeling is present in physiological aging and whether hypertension accelerates the aging process for vascular function and structure. Small arteries from 42 essential hypertensive patients (HT) and 41 normotensive individuals (NT) were dissected after subcutaneous biopsy. Endothelium-dependent vasodilatation (pressurized myograph) was assessed by acetylcholine, repeated under the nitric oxide synthase inhibitor \(\text{N}-\text{nitro-L-arginine methylester}\) or the antioxidant tempol. Structure was evaluated by media–lumen ratio (M/L). Intravascular oxidative generation and collagen deposition were assessed. Inhibition by \(\text{N}-\text{nitro-L-arginine methylester}\) on ACh was inversely related to age in both groups (\(P<0.0001\)) and blunted in HT versus NT for each age range. In NT, tempol enhanced endothelial function in the oldest subgroup; in HT, the potentiating effect started earlier. HT showed an increased M/L (\(P<0.001\)) versus control. In both groups, M/L was directly related to age (\(P<0.0001\)). M/L was greater in HT, starting from 31 to 45 years range. A significant age–hypertension interaction occurred (\(P=0.0009\)). In NT, intravascular superoxide emerged in the oldest subgroup, whereas it appeared earlier among HT. Among NT, aged group displayed an increment of collagen fibers versus young group. In HT, collagen deposition was already evident in youngest, with a further enhancement in the aged group. In small arteries, aging shows a eutrophic vascular remodeling and a reduced nitric oxide availability. Oxidative stress and fibrosis emerge in advanced age. In HT, nitric oxide availability is early reduced, but the progression rate with age is similar. Structural alterations include wide collagen deposition and intravascular reactive oxygen species, and the progression rate with age is steeper. (Hypertension. 2017;69:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.08041.) • Online Data Supplement

Key Words: endothelial dysfunction ■ microcirculation ■ nitric oxide ■ oxidant stress ■ remodeling

The endothelium plays a crucial role in acute regulation of vascular tone and in long-term vascular remodeling. In healthy conditions, nitric oxide (NO) is the most important endothelium-derived vasodilator molecule able to inhibit the major key mechanisms promoting the development of atherosclerosis, thus, promoting vascular health.

NO breakdown by reactive oxygen species (ROS) is the main cause of reduced NO availability and endothelial dysfunction, both in physiological aging and in many pathological conditions, including arterial hypertension. Hypertension is supposed to induce early vascular aging in different arterial districts. However, vascular features of physiological aging and hypertension are not necessarily similar. With respect to endothelial function, essential hypertensive patients (HT) show a reduced response to acetylcholine (Ach) as compared with normotensive individuals (NT) in each age range. However, with increasing age, vascular response to Ach is similarly reduced among HT and NT.

Vascular structure changes in the microcirculation represent another hallmark of essential hypertension. Increased media to lumen ratio (M/L), which characterizes vascular remodeling, can result from a reduced outer diameter that narrows the lumen without net growth (eutrophic remodeling) or from a thicker media encroaching on the lumen (hypertrophic remodeling). Eutrophic remodeling is most often found in essential hypertension. M/L is considered the most reproducible index of small resistance artery structure, with a relevant prognostic value, being associated with increased prevalence of cardiovascular events in a high-risk population. Vascular fibrosis is critically important in determining vascular remodeling in hypertension, and it involves, among others, changes in collagen deposition. The impact of physiological aging on microvascular structure and the role of vascular fibrosis are still unknown. Therefore, in the present study, we evaluated whether vascular remodeling is physiologically present with advancing age at the level of peripheral small resistance arteries. We also assessed whether the hypertensive disease causes an acceleration of the aging process for vascular function and structure. Collagen deposition as a
contributor of vascular remodeling in ageing and hypertensive disease was also investigated.

Methods
Detailed methods are available in the online-only Data Supplement.

Study Population
In this cross-sectional study, we enrolled 41 NT (age range: 23–65 years) and 42 age- and sex-matched HT (age range: 24–67 years). Part of the study population (14 NT and 14 HT, respectively) was already recruited in previous articles,\(^{15,16}\) while 27 HT and 28 NT were recruited on purpose for this analysis.

The protocol was approved by the local ethical committee, and informed consent was obtained from each participant.

Preparation of Small Arteries and Structural and Functional Parameters
All participants underwent a biopsy of subcutaneous fat from the anterior abdominal region, taken during a surgical procedure. Small arteries were isolated and mounted in a pressurized myograph, as previously described.\(^{3,17}\)

Media thickness and lumen diameter were measured in 3 different points from each small artery for M/L calculation. Media cross-sectional area (MCSA) was obtained by subtraction of the internal cross-sectional area from the external cross-sectional area using external plus lumen diameters, as previously described.\(^{18}\) The remodeling (RI) and growth (GI) indices for each age group were calculated (considering the normotensive group aged <30 years as the reference group) according to Heagerty et al.\(^{19}\)

Endothelium-dependent and endothelium-independent relaxations were assessed by cumulative concentrations of Ach (1 mmol/L to 100 μmol/L; Sigma Chemicals, St Louis, MO) and sodium nitroprusside (0.01–100 mmol/L; Sigma) in vessels precontracted with noradrenaline (1 mmol/L). NO availability and ROS production were investigated by repeating Ach after 30-minute incubation with the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L; Sigma) or the antioxidant and superoxide dismutase mimetic tempol (1 mmol/L; Sigma), respectively.

Detection of Vascular Superoxide Anion Generation
The in situ production of superoxide anion from 30-μm frozen mesenteric vessel sections was evaluated by means of the fluorescent dye dihydroethidium (Sigma), as previously described.\(^{20}\) Three slides per section were analyzed simultaneously and evaluated under a confocal laser scanning microscope (561-nm excitation).

Histochemical Staining
The analysis was conducted in small vessels from youngest (<30 years) and aged (>60 years) NT and HT individuals (n=5 each group). For each patient, formalin-fixed and paraffin-embedded vascular samples were sectioned and then processed for Sirius Red/Fast Green staining to evaluate vascular collagen fibers, which appeared red-stained, and the noncollagen proteins, which were green, as previously reported.\(^{21}\)

Data Analysis
Statistical analysis was performed using NCSS 8 (NCSS, Kaysville, Utah). The results were expressed as means±SD or counts and percentages. Differences in clinical characteristics between NT and HT were compared by 2-sided unpaired Student’s t test for normally distributed continuous variables, Wilcoxon rank-sum test for not normally distributed continuous variables, or \(\chi^2\) for categorical variables, as appropriate. Differences in vasodilation to Ach and Ach+L-NAME between HT and NT were analyzed by repeated measures analysis of variance, considering hypertension (yes/no) as between factor and Ach concentration (10\(^{-6}\) to 10\(^{-5}\)) as within factor. Univariate correlations were assessed by Spearman rank. The population was then classified into 4 age classes (<30, 31–45, 46–60, and >60 years). Differences in functional and structural arterial parameters were analyzed by general linear model analysis of variance, considering hypertension (yes/no) and age classes as factor variables, to explore possible interactions between age and the presence of hypertension. Differences within groups were explored by Tukey–Kramer post hoc multiple comparison test.

Results
Clinical characteristics of the study population and classes of antihypertensive medication previously taken are listed in Table; and Table S2 in the online-only Data Supplement. Sex prevalence, metabolic profile, body mass index, and renal function were similar in NT and HT, either when considered as whole population (Table) or when considered between the different age groups (Table S1).

Structural Alterations: Relation With Age
HT showed a lower resting lumen diameter (214±11 versus 222±10 μm; \(P=0.0006\)) and a higher media thickness (15±2 versus 12±1 μm; \(P<0.0001\)) in comparison to NT, resulting in a significantly higher M/L (6.8±1.0% versus 5.6±0.5%; \(P<0.0001\)). Although M/L increased with age in both groups, the slope of the linear regression line was steeper in HT (Figure 1A). The analysis by age ranges confirmed these results. In the younger age class, M/L was similar between HT and NT. In contrast, M/L resulted significantly higher in HT compared with NT in the remaining older age classes (Figure 1B). A significant age-group interaction (\(P=0.0009\) on general linear model analysis of variance) was shown. In HT, although a tight direct correlation between hypertension duration (years) and M/L occurred (\(r=0.844; P<0.0001\)), this correlation was weakened (\(β=0.077; 95\%\) confidence limits \(-0.017 to 0.171; P=0.11\)) in a multiple linear regression model considering M/L as dependent variable and including age, sex, and hypertension duration as independent variables.

MCSA was higher in HT as compared with NT (222±1073 versus 9249±1076 μm\(^2\); \(P=0.004\)). In parallel to M/L results, although MCA increased with age in both hypertensive and healthy individuals, the slope of the linear regression line was steeper in HT (Figure 2A).

Table. Clinical Characteristics of the Study Populations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normotensive Subjects (n=41)</th>
<th>Essential Hypertensive Patients (n=42)</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>46.1±13.2</td>
<td>45.8±13.6</td>
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<td>Male/female</td>
<td>20/21</td>
<td>18/24</td>
<td>0.50</td>
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<tr>
<td>Body mass index, kg/m(^2)</td>
<td>25.6±1.8</td>
<td>26.1±4.2</td>
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<tr>
<td>Systolic BP, mm Hg</td>
<td>128.5±6.7</td>
<td>156.7±7.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>80.7±4.2</td>
<td>99.6±2.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plasma glucose, mg/dL</td>
<td>82.8±8.7</td>
<td>88.9±7.5</td>
<td>0.56</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>200.5±20.0</td>
<td>203.1±18.0</td>
<td>0.56</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>47.0±9.4</td>
<td>45.2±10.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>137.2±24.6</td>
<td>158.7±35.4</td>
<td>0.17</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>126.1±23.7</td>
<td>126.2±21.5</td>
<td>0.35</td>
</tr>
<tr>
<td>eGFR, ml/min 1.73 m(^2)</td>
<td>85.4±12.4</td>
<td>80.3±14.9</td>
<td>0.52</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.
In HT, MCSA was significantly higher in the 46- to 60-year subgroup, with a further increment in the oldest subgroup (Figure 2B), together with a significant enhancement of the GI, indicating some degree of hypertrophic remodeling (Table S2). Among NT, ≤60 years, the vascular remodeling was largely eutrophic, as suggested by normal MCSA, low GI but a remodeling index close to 100%. In contrast, MCSA and GI appeared significantly increased in the oldest age group, with a toned down remodeling index (Figure 2B; Table S2).

Despite these subtle differences, age-group interaction for MCSA did not reach statistical significance (P = 0.07), indicating a broadly similar behavior of MCSA with age between HT and NT. In HT, a tight direct correlation between hypertension duration and MCSA occurred (r = 0.844; P < 0.0001); this association remained significant (β = 228; 95% confidence limits 43–414; P = 0.02) in a multiple linear regression model considering MCSA as dependent variable and including age, sex, and hypertension duration as independent variables.

**Functional Alterations: Relation With Age**

The whole population of HT showed a significantly (P < 0.001) reduced vasodilation to Ach and a blunted inhibitory effect of L-NAME on response to Ach (P < 0.001) compared with NT (Figure S1). Relaxation to sodium nitroprusside was similar in the 2 groups (data not shown).

Inhibition by L-NAME on response to Ach declined with age in both HT and NT, and the slope of the 2 linear regression lines did not differ significantly (Figure 3A). For each age range, HT showed a reduced inhibition of L-NAME on relaxation to Ach compared with NT (Figure 3B). In both groups, a significant decline in inhibition by L-NAME on Ach in comparison to the youngest age class was evident for the oldest
age classes (46–60 years and >60 years; Figure 3B). There was no significant age-group interaction (P=0.25).

In HT, tempol potentiated the response to Ach starting from 31 to 45 years age range group, an effect that progressively increased with advancing age (Figure S2). Among NT, tempol enhanced the relaxation to Ach only in the oldest subgroup (Figure S2). A significant age-group interaction (P<0.001) emerged.

In HT, an inverse correlation between hypertension duration and inhibition by L-NAME on Ach emerged (r=−0.838; P<0.0001). In a multiple regression model considering M/L as dependent variable and age, sex, and hypertension duration as independent variables, age (β=−0.515; 95% confidence limits −0.762 to −0.268; P=0.0001) but not hypertension duration (β=0.047; 95% confidence limits −0.551 to 0.644; P=0.86) remained independently associated with inhibition by L-NAME.

Analysis of Vascular Superoxide Anion Generation
HT showed globally an increased superoxide anion production versus NT (18.0±6.0 versus 10.3±3.2; P<0.001). A significant age-group interaction was found (P<0.001). In particular, among NT, a significant increase in superoxide production was found only in oldest vessels (Figure 4). Among HT, an increased superoxide generation was already present in vessels from the 31 to 45 years subgroup, with a further progressive increment among the oldest ranges (Figure 4). HT and NT showed similar superoxide anion production only below 30 years, whereas values were greater in HT for each age class.

Correlation with age was tighter in HT than in NT (r=0.95, P<0.001 and r=0.73, P<0.001, respectively). Of note, superoxide production was related to the percent inhibition of L-NAME on Ach, M/L, and MCSA only in HT (r=−0.84, P<0.001; r=0.87, P<0.001; r=0.65, P=0.01, respectively), but not in NT (r=−0.09, P=0.74; r=0.44, P=0.09; r=0.28, P=0.30, respectively). Univariate analysis showed a tight direct correlation between the potentiating effect of tempol on Ach and intravascular superoxide detection (r=0.706, P<0.0001 in NT and r=0.981, P<0.0001 in HT).

Histochemical Analysis of Vascular Collagen Deposition
Among NT, vessels from aged group displayed a significant increment of Sirius Red–stained collagen fibers compared with young counterpart (Figure 5). Among HT, vascular fibrosis was already significantly evident in young HT, with marked, further enhancement of collagen deposition within the whole wall of vessels from aged group (Figure 5).

Discussion
In line with extensive literature, small arteries from our HT population showed a reduced NO availability and a eutrophic vascular remodeling.11,16,23,24

Our first major novel finding consists in the demonstration that vascular structural changes occur with ageing. Previous studies on this issue exclusively focused on intima–media thickness and elasticity/distensibility in large arteries.24–28 We observed that starting from the fourth decade of life (31–45 years), NT exhibited a higher M/L ratio accompanied by a slight increase in MCSA that, however, had not achieved statistical significance, with the exception of individuals >60 years. Accordingly, the GI increased only in the oldest subgroup. This represents the first evidence that a progressive eutrophic vascular remodeling occurs throughout life in isolated small arteries, with a slight switch toward a hypertrophic remodeling in the advanced age. These results reinforce the concept of aging as a major nonmodifiable risk factor in the development of vascular disease. Interestingly, large artery stiffening shows a broadly similar behavior with aging, supporting the hypothesis of a cause–effect relationship between the 2 alterations.29 We observed that starting from the fourth decade of life (31–45 years), NT exhibited a higher M/L ratio accompanied by a slight increase in MCSA that, however, had not achieved statistical significance, with the exception of individuals >60 years. Accordingly, the GI increased only in the oldest subgroup. This represents the first evidence that a progressive eutrophic vascular remodeling occurs throughout life in isolated small arteries, with a slight switch toward a hypertrophic remodeling in the advanced age. These results reinforce the concept of aging as a major nonmodifiable risk factor in the development of vascular disease. Interestingly, large artery stiffening shows a broadly similar behavior with aging, supporting the hypothesis of a cause–effect relationship between the 2 alterations.30
Among others, oxidative stress is a recognized dramatic inducer of redox-sensitive proinflammatory signaling pathways, contributing to inflammation and vascular growth. More recently, a causative role played by oxidative stress in promoting vascular collagen deposition, via immune activation, was observed. Such proposal fits with our findings, showing collagen deposition together with oxidant generation and some degree of vascular growth in the oldest subgroup. Further studies will clarify the presence of collagen deposition during the intermediate decades of life and whether mechanisms other than oxidative stress might trigger such signaling cascade.

The second major novel finding identifies a different impact exerted by the hypertensive disease on age-related vascular changes. M/L ratio, although similar in NT and HT within the youngest age, becomes higher among HT after 30 years of age. The direct relationship between age and M/L ratio was dramatically steeper among HT than among NT, indicating that hypertension influences structural changes only after 30 years of age, but it triggers a steeper progression rate over time. When globally considered, HT showed a predominant eutrophic vascular remodeling. However, starting from 46 to 60 years of age, some degree of hypertrophic remodeling occurred, as confirmed by increased GI. This is not in disagreement with previous reports, in which the vascular remodeling associated with essential hypertension is mainly eutrophic in middle-aged (around 50 years) patients. In addition, when hypertension is longstanding, smooth muscle cell growth may predominate over apoptosis, and remodeling may be hypertrophic. Accordingly, hypertension duration, although playing only a marginal role in causing alteration in M/L ratio, emerged as a determinant of MCSA, independently of age.

The higher impact of hypertensive disease on vascular structural changes is likely caused by intravascular ROS, which in turn promotes vascular fibrosis. As dihydroethidium revealed, ROS appeared decades earlier in HT compared with NT, and it represents a major contributor, together with other factors not investigated in the present study, such as the insulin resistance, the hemodynamic load, or the activated renin–angiotensin system, in promoting vascular cell growth. Accordingly, a direct correlation between intravascular superoxide and indexes of vascular remodeling emerged, specifically among HT. Our histochemical analysis strengthens this hypothesis, revealing the presence of an enhanced collagen deposition already detectable in youngest HT subgroup and dramatically augmented in advancing age.
Different is the impact exerted by hypertension on age-related endothelial dysfunction. Exposure to hypertensive disease, although inducing an early impairment of endothelial function, however, does not worsen the age-associated reduction of NO availability. Accordingly, with advancing age, the progressive NO impairment runs in 2 parallel lines in HT and NT. Moreover, no significant age–hypertension interaction emerged. Taken together, these data indicate that hypertension anticipates the age-related endothelial dysfunction, a phenomenon identified as an early vascular aging, but such deleterious
effect remains constant with advancing age. This is supported by the fact that hypertension duration does not affect the age-related decline in endothelial function: indeed, in a multiple regression model, only age, but not hypertension duration, is associated with inhibition by L-NAME on Ach.

Although the impaired NO availability represents the common final effect, different mechanisms seem to be adopted by aging and hypertensive disease. Indeed, in contrast to what is seen in NT, vessels from HT showed an early and progressive detection of superoxide generation. These findings agree with our functional experiments, showing a progressive potentiating effect of the antioxidant tempol on Ach-evoked relaxation in HT, whereas its effect among NT was detectable only in the oldest subgroup. Therefore, it is conceivable that in NT up to the age of 60 years, a primary alteration in the substrate of NO generation (ie, L-arginine) seems to be responsible for endothelial dysfunction, with superoxide playing a role in advanced age only. In HT, the reduced NO availability is largely caused by oxidative stress, which shows up decades earlier than in NT. These data are in line with previous data showing that L-arginine was able to potentiate the endothelial function in young NT, while in >60-year-old individuals, it was no longer effective. On the contrary, among HT, the beneficial effect of L-arginine disappeared earlier, and endothelial dysfunction was reversed only by an antioxidant compound.4,35

In conclusions, in small resistance arteries, physiological aging shows a progressive eutrophic vascular remodeling and a reduced NO availability. In advanced age, some degree of oxidative stress and fibrosis emerge. In hypertensive patients, NO availability is early reduced, but the progression rate with age seems to be similar. Conversely, structural alterations are mainly characterized by enhanced collagen deposition, likely driven by intravascular ROS, and the progression rate with age is steeper.

Perspectives

The reduced NO availability favors the atherosclerotic disease. As well, vascular structural change predicts cardiovascular events in high-risk populations. Although limited by the cross-sectional design, which does not allow us to fully elucidate the interaction between the hypertensive disease and aging, our findings may contribute to improve our knowledge of the vascular biology of aging.

Acknowledgments

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Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

- Age induces an eutrophic vascular remodeling, with a switch toward a hypertrophic remodeling in the advanced age. Collagen deposition is the major contributor.
- Hypertension amplifies the degree of hypertrophic remodeling, determining a steeper progression rate over time. Collagen deposition and oxidant excess contribute.
- Hypertension anticipates, without worsening, the progressive reduction of nitric oxide availability associated with aging.

**What Is Relevant?**

- Our findings contribute to improve our knowledge on age-related vascular changes at the level of peripheral microcirculation and the impact of hypertensive disease on such alterations.

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**Summary**

In small arteries, advancing age is associated with an eutrophic remodeling, with a slight switch toward a hypertrophic remodeling in the advancing age. Vascular collagen deposition is a contributor. The hypertensive disease anticipates and strongly amplifies the degree of hypertrophic remodeling, determining a steeper progression rate over time. Major contributors of such vascular changes are collagen and reactive oxygen species excess. Hypertension also anticipates the age-related reduction in nitric oxide availability, but the progression rate with age seems to be similar.
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http://hyper.ahajournals.org/content/suppl/2016/10/31/HYPERTENSIONAHA.116.08041.DC1

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Supplemental Methods

Study population.

Essential hypertensive patients and normotensive patients were recruited among consecutive individuals, referred to the Department of Clinical and Experimental Medicine or the Department of Surgery of the University of Pisa, who underwent laparoscopic surgery for cholecystectomy caused by gallbladder stones or adrenalectomy for a benign and non-functioning adrenal mass over 3.5 cm in size. Venous blood samples were taken with the participants in the supine position, for standard hematology and serum biochemistry tests. With respect to hypertensive patients, the major inclusion criterion was a clinic blood pressure value (after 10 minutes of rest) >140/90 mmHg, confirmed on 2 separate occasions within 1 month, according to current European Guidelines. Secondary forms of hypertension were excluded by routine diagnostic procedures, including morphological and hormonal investigations when adrenal mass was detected. Other exclusion criteria included clinical or biochemical evidence of thyroid dysfunction, ethanol consumption (more than 60 g per day), dyslipidemia, diabetes mellitus, smoking, body mass index >30 kg/m², renal or liver impairment, and established cardiovascular disease. Patients were never treated for hypertension or they had not received any medication for at least 15 days before enrollment in the study. Previous antihypertensive treatment is detailed in Table S2. Among women in postmenopausal status, no one was receiving hormone replacement therapy. Among those in fertile status, no one had received hormone treatment or had a pregnancy for > 6 months before the study.

Preparation, Mounting and Measurements in Small Arteries

Small arteries (150 to 300 µm) were isolated from subcutaneous tissue immediately after biopsy sample procurement and mounted on a pressurized myograph. Vessel segments (≈ 2 mm long) were mounted onto 2 glass cannulas, one of which was positioned until the vessel walls were parallel, and equilibrated in physiologic salt solution (mmol/L: NaCl 120, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 2.5, EDTA 0.026, and glucose 5.5) bubbled
continuously with 95% air and 5% CO₂ to achieve pH 7.4 at 37°C. Vessels were pressurized at 60 mm Hg. All functional experiments were performed by measuring dilatory responses to agonists in vessels precontracted with norepinephrine (NE, 10⁻⁶ mol/L). The dose of 1 µM NE was chosen after preliminary experiments of concentration-response curves to NE (from 1 nM to 100 µM) in vessels from normotensive subjects and hypertensive patients. Vessels from the two groups showed concentrations-dependent contractions to the first four concentrations of NE (from 1 nM to 1 µM), resulting in 50-60% of vessel contraction at the dose of 1 µM, similarly in healthy condition or hypertensive disease.

Media cross-sectional area (CSA) was obtained by subtraction of the internal CSA from the external CSA: CSA = (π/4) x (De² - Di²) where De and Di are external and lumen diameters, respectively.

The remodeling and growth indices were also calculated. The remodeling index quantifies how much of the vascular structural alteration may be explained by a rearrangement of the same material around a narrowed lumen, without cell growth. The growth index quantifies the relative component of vascular smooth muscle cell growth.

The remodeling index was calculated as 100x[(Di)n-(Di)remodel]/[(Di)n-(Di)h] where (Di) indicates internal diameter; n, normotensive control group (< 30 years); h, normotensive older groups or hypertensive vessels. (Di)remodel=[(De)h²-(4xCSAn/π)]⁰.⁵, where (De)h is the external diameter of normotensive older groups or hypertensive vessels and CSAₙ was the CSA of normotensive control vessels.

The growth index was calculated as (CSAh-CSAn)/CSAn where CSAn was media CSA of normotensive control group (< 30 years), and CSAₙ was media CSA of normotensive older groups or hypertensive vessels.

**Detection of vascular superoxide anion generation.**

The in situ production of superoxide anion was measured by means of the fluorescent dye dihydroethidium (DHE; Sigma). Three slides per segment were analyzed simultaneously after incubation with Krebs solution at 37°C for 30 min. Krebs-HEPES buffer containing 2 µM DHE was then applied to each section and evaluated under a Leica TCS SP8 confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) using a 561-nm excitation wavelength laser. All frames (2.048x2.048 pixels) were captured by means of microscope with 20 x
objective non oil lens, using a power scanning laser and gain level of which would avoid any possible saturation because all fluorescent pixels were detected by image analysis system. The percentage of arterial wall area stained with the red signal was estimated using an imaging software (McBiophotonics Image J; National Institutes of Health, Bethesda, MD).

**Histochemical staining**

For each patient, tissue samples were formalin-fixed and paraffin-embedded and then sectioned [3-µm thickness] just before use. In particular, they were incubated in 0.04% Fast Green for 15 min, washed with distilled water and then incubated in 0.1% Fast Green and 0.04% Sirius Red in saturated picric acid for 30 min. Then, they were dehydrated and mounted with DPX Mounting. Collagen fibers appeared red, while the non-collagen proteins were green. Quantitative estimations of histochemical stainings were carried out independently by two blind investigators (C.S. and C.I.). Each investigator analyzed all tissue specimens under study. The respective values were then averaged and plotted in graphs in accordance with previously described criteria.\(^1\) Briefly, for each vessel, 3 non-adjacent sections were captured by a Leica DMRB microscope equipped with the digital camera DFC480. All images, which were captured with 400x objective, were quantitatively estimated for Sirius Red-stained collagen fibers. To detect the specific threshold of pink/red for collagen fibers, a square was applied upon the color of interest and recorded by Image Analysis System ‘L.A.S. software v.4’. Positive tissue areas were automatically estimated on the basis of the total pixel number and intensity. The whole vessel wall areas were manually circumscribed and automatically calculated. Data were expressed as percentage of \(\Sigma\) of positive-stained area / \(\Sigma\) of tissue area examined of whole vessel wall in three sections for each vessel.

**References**

TABLE S1. Clinical and Plasma Parameters of NT and HT divided according to Age Profile.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>&lt;30 Years</th>
<th>31-45 Years</th>
<th>46-60 Years</th>
<th>&gt;60 Years</th>
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</tr>
<tr>
<td>NT (n=9)</td>
<td>26.3±2.4</td>
<td>38.8±1.4</td>
<td>53.7±3.7</td>
<td>63.0±2.0</td>
</tr>
<tr>
<td>HT (n=9)</td>
<td>27.4±1.7</td>
<td>39.2±3.6</td>
<td>52.6±4.9</td>
<td>64.0±1.9</td>
</tr>
<tr>
<td>Male/Female</td>
<td>4/5</td>
<td>5/7</td>
<td>6/6</td>
<td>5/3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.5±2.5</td>
<td>25.3±2.1</td>
<td>26.3±4.1</td>
<td>26.1±4.3</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>126.9±8.5</td>
<td>127.2±6.9</td>
<td>128.3±6.6</td>
<td>132.4±5.2</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>77.9±3.3</td>
<td>80.5±4.6</td>
<td>80.6±4.1</td>
<td>83.8±3.5</td>
</tr>
<tr>
<td>Glycaemia, mg/dL</td>
<td>82.1±3.5</td>
<td>79.5±8.2</td>
<td>85.9±9.2</td>
<td>83.8±8.6</td>
</tr>
<tr>
<td>Total chol, mg/dL</td>
<td>192.9±28.7</td>
<td>198.1±18.1</td>
<td>207.1±17.2</td>
<td>203.8±22.2</td>
</tr>
<tr>
<td>HDL chol, mg/dL</td>
<td>49.6±8.3</td>
<td>48.1±8.1</td>
<td>49.3±9.7</td>
<td>40.5±6.5</td>
</tr>
<tr>
<td>LDL chol, mg/dL</td>
<td>113.8±32.1</td>
<td>120.0±17.3</td>
<td>132.4±22.8</td>
<td>139.0±24.4</td>
</tr>
<tr>
<td>eGFR, mL/min</td>
<td>95.6±5.9</td>
<td>88.6±4.5</td>
<td>80.4±10.3</td>
<td>78.4±9.5</td>
</tr>
</tbody>
</table>

Values are mean ±SD. NT, normotensive subjects; HT, essential hypertensive patients; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; chol, cholesterol; eGFR, estimated glomerular filtration rate (x 1.73 m²). * P<0.05 vs NT counterpart
TABLE S2. Previous Antihypertensive Treatment and Vascular Parameters of NT and HT divided according to Age Profile.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>&lt;30 Years</th>
<th>31-45 Years</th>
<th>46-60 Years</th>
<th>&gt;60 Years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-antagonists (%)</td>
<td>-</td>
<td>22</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>ACE-inhibitors (%)</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>41</td>
</tr>
<tr>
<td>Ang II receptor antagonists (%)</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>Alpha1-blockers (%)</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Beta-blockers (%)</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Diuretics (%)</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Remodeling index (%)</td>
<td>-</td>
<td>101.9</td>
<td>104.1</td>
<td>91.8</td>
</tr>
<tr>
<td>Growth index (%)</td>
<td>-</td>
<td>10.7</td>
<td>12.3</td>
<td>17.7</td>
</tr>
</tbody>
</table>

NT, normotensive subjects; HT, essential hypertensive patients; Ang, angiotensin.
Supplemental Figures

**Figure S1.** Endothelium-dependent relaxation in small vessels from essential hypertensive patients and normotensive subject

Relaxations to acetylcholine without or with L-NAME in essential hypertensive patients (n=42, Panel A) and normotensive subjects (n=41, Panel B). Data presented as means ± SD. *P<0.05; †P<0.001.
Figure S2. Impact of tempol on endothelium-dependent relaxation in essential hypertensive patients and normotensive subjects.

Potentiating effect of tempol on maximal response to acetylcholine in essential hypertensive patients or normotensive subjects, divided according to the age ranges. Data presented as means ± SD.