Age-Related Reduction of NO Availability and Oxidative Stress in Humans
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Abstract—Age-related endothelial dysfunction could be caused by an alteration in the L-arginine–NO system and the production of oxidative stress in both normotensive and hypertensive individuals. In 47 normotensive subjects and 49 patients with essential hypertension, we evaluated forearm blood flow (by strain-gauge plethysmography) modifications induced by intrabrachial sodium nitroprusside (1, 2, and 4 μg/100 mL per minute) and acetylcholine (0.15, 0.45, 1.5, 4.5, and 15 μg/100 mL per minute), an endothelium-independent vasodilator and an endothelium-dependent vasodilator, respectively. Acetylcholine was repeated in the presence of the NO synthase inhibitor N\(^\text{G}\)-monomethyl-L-arginine (L-NMMA, 100 μg/100 mL per minute), the antioxidant vitamin C (8 mg/100 mL per minute), or both. Vasodilation to acetylcholine, but not to sodium nitroprusside, was lower (\(P<0.01\)) in hypertensive patients compared with control subjects. Moreover, in both groups, endothelium-dependent vasodilation declined with aging. In normotensive subjects, the inhibiting effect of L-NMMA on response to acetylcholine decreased in parallel with advancing age, whereas vitamin C increased vasodilation to acetylcholine in only the oldest group (age \(>60\) years). In young hypertensive patients (age \(<30\) years), vasodilation to acetylcholine was sensitive to L-NMMA, whereas in hypertensive patients age \(>30\) years, vitamin C enhanced endothelium-dependent vasodilation and restored the inhibiting effect of L-NMMA on response to acetylcholine. In normotensive individuals, an earlier primary dysfunction of the NO system and a later production of oxidative stress cause age-related reduction in endothelium-dependent vasodilation. These alterations are similar but anticipated in hypertensive patients compared with normotensive subjects. (Hypertension. 2001;38:274-279.)

Key Words: endothelium ■ age ■ nitric oxide ■ oxidative stress ■ hypertension, essential

Aging and hypertension are well-documented cardiovascular risk factors.\(^1\)\(^2\) Most of the functional and structural vascular alterations that lead to cardiovascular complications are similar in aging and hypertension.\(^3\)\(^4\) Moreover, these vascular changes associated with essential hypertension are generally considered to be an accelerated form of the changes seen with aging.\(^5\)

Endothelial cells play an important local regulatory role by secreting substances that control both vascular tone and structure,\(^3\) including NO, which is derived from the metabolism of L-arginine by NO synthase,\(^6\) a constitutive enzyme that is present in endothelial cells. NO is produced and released under the influence of endothelial agonists—including acetylcholine, bradykinin, and others—acting on specific receptors, and by mechanical forces, such as shear stress.\(^3\) Experimental evidence indicates that almost the totality of cardiovascular risk factors, such as aging and hypertension, are characterized by the presence of endothelial dysfunction, which is mainly induced by the production and release of oxygen-derived free radicals,\(^7\) which cause NO breakdown.\(^8\)

In humans, the association of impaired endothelium-dependent vasodilation with essential hypertension and aging has been well documented in different vascular beds.\(^9\)\(^-\)\(^17\) In patients with essential hypertension, one of the main mechanisms leading to impaired endothelium-dependent vasodilation is the production of oxidative stress, which reduces NO availability.\(^18\) In these patients, when oxidative stress is removed by a scavenger such as vitamin C,\(^19\) NO availability is restored. In contrast, no data are available concerning NO availability and the role of oxidative stress in endothelial dysfunction associated with aging.

Therefore, the aim of the present study was to investigate whether a reduction in NO availability caused by oxidative stress participates in age-related endothelial dysfunction in humans and to assess whether the alterations documented in essential hypertension are an accelerated form of changes that occur with aging.

Methods
Patients
Forty-seven healthy subjects and 49 matched patients with essential hypertension (mean age, 44.5±14.9 and 47.6±11.3 years, respec-
Study Design
Endothelium-dependent vasodilation was estimated by a dose-response curve to intra-arterial acetylcholine (0.15, 0.45, 1.5, 4.5, and 15 μg/100 mL forearm tissue per minute for 5 minutes at each dose; Farmigea S.p.A.). Endothelium-independent vasodilation was assessed with a dose-response curve to intra-arterial sodium nitroprusside (Malesci, a direct smooth muscle cell relaxant compound (1, 2, and 4 μg/100 mL forearm tissue per minute for 5 minutes at each dose). Moreover, to evaluate a possible alteration in NO availability, acetylcholine was repeated in the presence of intra-arterial L-NMMA (100 μg/100 mL forearm tissue per minute; Bracco). To assess oxidative stress, acetylcholine was again repeated in the presence of intra-arterial vitamin C (8 mg/100 mL forearm tissue per minute; Bracco). Finally, to evaluate whether oxidative stress could determine a decrease in NO availability, a fourth dose-response curve to acetylcholine was repeated in the presence of simultaneous L-NMMA and vitamin C. Both L-NMMA and vitamin C were started 10 minutes before acetylcholine and continued throughout. After a 30-minute washout was allowed between each dose-response curve, and a 60-minute period was allowed when L-NMMA was infused.

Data Analysis
To compare vascular responses in subjects at different ages, the study population was divided into 4 subgroups (those age <30 years, 31 to 45 years, 46 to 60 years, and >60 years). Differences between 2 means were compared by the paired or unpaired Student t test, as appropriate. Because L-NMMA changed basal flow and because agonist-mediated vasodilation is dependent on vessel resting tone, responses to acetylcholine and sodium nitroprusside were analyzed by ANCOVA for repeated measures, which normalizes for differences in baseline FBF. The Scheffé test was applied for multiple comparison testing. Interaction between age and forearm vasodilation to acetylcholine and sodium nitroprusside was calculated by a multivariate analysis, with use of a multiple stepwise regression, to exclude the effects of blood pressure and plasma cholesterol. Results were expressed as mean±SEM.

Results
Apart from blood pressure values, clinical characteristics were not significantly different between the different age groups either among normotensive subjects or among patients with essential hypertension (Table).
whereas the response to sodium nitroprusside was similar (in normotensive subjects, FBF from 3.4±0.5 to 17.3±4.0 mL/100 mL per minute, +409%; in hypertensive patients, FBF from 3.3±0.5 to 15.0±2.8 mL/100 mL per minute, +354%; P=NS).

In normotensive subjects, the response to acetylcholine declined with age. Vasodilation to acetylcholine was reduced in the group age 30 to 45 years (FBF from 3.4±0.4 to 1.9±0.7 mL/100 mL per minute, +512%; P<0.01 versus the group age 31 to 45 years) and in the oldest group (age >60 years, FBF from 3.2±0.5 to 14.1±4.2 mL/100 mL per minute, +341%; P<0.01 versus the group age 46 to 60 years). Vasodilation to sodium nitroprusside was not different among the age subgroups. In patients with essential hypertension, the response to acetylcholine progressively declined from the <30-year group (FBF from 2.9±0.4 to 20.9±2.3 mL/100 mL per minute, +628%) to the 31- to 45-year group (FBF from 3.1±0.5 to 17.4±3.2 mL/100 mL per minute, +461%; P<0.01 versus <30-year group), 45- to 60-year (FBF from 3.3±0.7 to 14.8±3.7 mL/100 mL per minute, +349%; P<0.01 versus 31- to 45-year group), and >60-year (FBF from 3.2±0.5 to 12.8±4.0 mL/100 mL per minute, +299%) groups. Vasodilation to sodium nitroprusside was not different between the age subgroups. Moreover, age was inversely correlated with vasodilation to acetylcholine in normotensive subjects (r = −0.65, P<0.0001) and in patients with essential hypertension (r = −0.72, P<0.0001). No significant relationship was found between age and response to sodium nitroprusside (Figure 1).

**Effect of Aging on Response to L-NMMA**

In normotensive subjects, L-NMMA reduced FBF (from 3.3±0.5 to 2.0±0.5 mL/100 mL per minute, −39%). This effect was blunted by aging (in the <30-year group, from 3.0±0.5 to 1.7±0.4 mL/100 mL per minute, −43%; in the 31- to 45-year group, from 3.2±0.5 to 1.9±0.4 mL/100 mL per minute, −40%; in the 46- to 60-year group, from 3.5±0.5 to 2.2±0.5 mL/100 mL per minute, −37%; and in the >60-year group, from 3.2±0.4 to 2.2±0.4 mL/100 mL per minute, −31%).

In the presence of L-NMMA, response to acetylcholine was significantly reduced in the <30-year group (FBF from 1.7±0.4 to 5.8±2.3 mL/100 mL per minute, +554%; P<0.001 versus acetylcholine alone), 31- to 45-year (FBF from 1.90±0.4 to 6.9±2.7 mL/100 mL per minute, +278%; P<0.001 versus acetylcholine alone), and 46- to 60-year group (FBF from 2.2±0.5 to 7.5±2.2±0.4 mL/100 mL per minute, +241%; P<0.001 versus acetylcholine alone) subgroups (Figure 2). The inhibiting effect of L-NMMA on response to acetylcholine was progressively lower with advancing age (<30-year group, 68%; 31- to 45-year group, 57%; and 46- to 60-year group, 52%). However, in the oldest subgroup (age >60 years), the endothelium-dependent response was virtually unaffected by L-NMMA (FBF from 2.2±0.4 to 7.8±1.2 mL/100 mL per minute, +278%; P=NS versus acetylcholine alone; Figure 2).

L-NMMA-induced reduction in basal FBF was significantly (P<0.01) reduced in hypertensive patients compared with normotensive subjects (FBF from 3.1±0.5 to 2.1±0.5 mL/100 mL per minute, −33%) but was not influenced by aging (percent FBF reduction above baseline was −31% for the <30-year group, −33% for the 31- to 45-year group; −29% for the 46- to 60-year group; and −32% for the >60-year group).

In young (age <30 years) hypertensive patients, L-NMMA reduced vasodilation to acetylcholine (FBF from 2.3±0.5 to 12.8±2.2 mL/100 mL per minute, +496%; Figure 2). However, the degree of inhibition exerted by the NO synthase inhibitor was significantly lower in young hypertensive patients compared with the corresponding normotensive subgroup (27% versus 68%, respectively; P<0.001). In contrast, response to acetylcholine was resistant to L-NMMA in the subgroups of patients age 31 to 45 years (FBF from 2.2±0.4 to 10.9±2.4 mL/100 mL per minute, +445%), 46 to 60 years (FBF from 2.2±0.4 to 10.7±3.6 mL/100 mL per minute, +362%), and >60 years (FBF from 2.1±0.3 to 8.6±2.3 mL/100 mL per minute, +324%; Figure 2).
Effect of Aging on Response to Vitamin C

In normotensive subjects, vitamin C did not modify basal FBF or the vasoconstrictor effect of L-NMMA. Moreover, the response to acetylcholine was not modified by vitamin C in the 30-year (FBF from 2.9 ± 0.4 to 24.5 ± 4.2 mL/100 mL per minute, +739%), 31- to 45-year (FBF from 3.2 ± 0.5 to 22.7 ± 4.8 mL/100 mL per minute, +618%), and 46- to 60-year (FBF from 3.2 ± 0.5 to 19.4 ± 4.5 mL/100 mL per minute, +501%) subgroups (Figure 2). In the oldest (>60-year) subgroup, vitamin C significantly (P<0.01) enhanced vasodilation to acetylcholine (FBF from 3.1 ± 0.5 to 17.2 ± 3.5 mL/100 mL per minute, +463%) and restored the inhibiting effect of L-NMMA on response to the agonist (FBF from 2.2 ± 0.5 to 8.2 ± 1.8 mL/100 mL per minute, +272%; Figure 3).

In hypertensive patients, vitamin C did not change the response to acetylcholine in the youngest subgroup (FBF from 3.0 ± 0.4 to 21.1 ± 5.8 mL/100 mL per minute, +556%; P=NS versus acetylcholine alone), but it significantly (P≤0.01) enhanced the endothelium-dependent response in the older subgroups (31- to 45-year group, FBF from 3.1 ± 0.5 to 21.6 ± 3.2 mL/100 mL per minute, +598%; 46- to 60-year group, FBF from 3.1 ± 0.5 to 22.1 ± 6.5 mL/100 mL per minute, +581%; and >60-year group, FBF from 3.1 ± 0.5 to 21.5 ± 7.3 mL/100 mL per minute, +575%; Figure 2). Moreover, in these subgroups, vitamin C restored the inhibiting effect of L-NMMA on vasodilation to acetylcholine (31- to 45-year group, FBF from 2.1 ± 0.5 to 10.4 ± 3.2 mL/100 mL per minute, +395%; 46- to 60-year group, FBF from 2.3 ± 0.6 to 9.4 ± 3.1 mL/100 mL per minute, +352%; and >60-year group, FBF from 2.0 ± 0.3 to 8.1 ± 2.2 mL/100 mL per minute, +305%; Figure 3). Contralateral FBF did not change during the studies (data not shown).

Discussion

The present study was designed to assess the mechanisms responsible for age-related impairment of endothelium-
dependent vasodilation and the possible worsening influence of essential hypertension. In agreement with previous evidence, the present results indicated a blunted response to acetylcholine, but not to sodium nitroprusside, in hypertensive patients compared with control subjects, confirming the presence of endothelial dysfunction in essential hypertension. Moreover, in healthy subjects and in hypertensive patients, increasing age was associated with progressive and specific decrease in vasodilation to acetylcholine. Because the response to sodium nitroprusside was not strictly related to aging, these results reinforce the concept that advancing age is an independent factor leading to the progressive impairment of endothelium-dependent vasodilation in humans.

A novel finding of the present study is that in normotensive subjects, the reduction in endothelial function associated with aging seems to be mediated by a progressive reduction of NO availability, inasmuch as the inhibiting effect of L-NMMA on acetylcholine-induced vasodilation was progressively impaired by advancing age. It is worth noting that after the age of 60 years, the inhibiting effect of L-NMMA on response to acetylcholine was very weak, suggesting that in aged individuals NO availability is almost totally compromised. To assess the possible role exerted by oxidative stress, we tested the antioxidant vitamin C. Up to the age of 60 years, despite the evident decline in endothelium-dependent vasodilation, vitamin C did not modify the response to acetylcholine. In contrast, in the oldest individuals (age > 60 years) characterized by a profound alteration in NO availability, vitamin C not only enhanced the response to the endothelial agonist but also restored the inhibiting effect of L-NMMA on vasodilation to acetylcholine. Thus, in the present study, the use of L-NMMA and vitamin C, never tested before in investigating the mechanisms responsible for the previously demonstrated age-related endothelial dysfunction in humans, seems to indicate that the progressive impairment in endothelium-dependent vasodilation is caused by a progressive alteration of the L-arginine–NO pathway. Only in old age (after ~ 60 years) does the production of oxidative stress appear, leading to the complete compromise of NO availability.

In young patients with essential hypertension (age < 30 years), although the response to acetylcholine was still sensitive to L-NMMA, the degree of inhibition exerted by the NO synthase inhibitor on vasodilation to acetylcholine was significantly reduced compared with that in age-matched control subjects. However, in the oldest hypertensive subgroups, the response to acetylcholine was completely resistant to L-NMMA, indicating a pronounced alteration in NO availability. On the other hand, vitamin C, although ineffective in the youngest hypertensive subgroup, enhanced the vasodilating effect of acetylcholine in patients age > 30 years, and the extent of enhancement increased progressively with advancing age. Moreover, the antioxidant restored the inhibiting effect of L-NMMA on endothelium-dependent response. Taken together, these findings give new information on the mechanisms responsible for age-related endothelial dysfunction in hypertension. Thus, it is conceivable that in essential hypertension, oxidative stress is the main alteration leading to impaired endothelium-dependent vasodilation, possibly by a reduction in NO availability. Only in young patients with essential hypertension is NO availability at least partially preserved, and the impaired endothelial function in this group seems to be dependent on an alteration in the L-arginine–NO pathway.

All these results suggest that age-related impairment in endothelium-dependent vasodilation could be dependent on different mechanisms. In normotensive subjects up to the age of 60 years, a primary alteration in the L-arginine–pathway appears to be responsible for endothelial dysfunction, with oxidative stress playing a crucial role in old age only. In patients with essential hypertension, an impairment in the NO system is already present in young (age < 30 years) hypertensive patients, and oxidative stress production appears decades earlier in hypertensive individuals than in normotensive individuals (~ 30 and ~ 60 years, respectively). Thus, in patients with essential hypertension, the mechanisms involved in determining impaired endothelium-dependent vasodilation are similar to those observed in normotensive individuals. The main difference seems to be that essential hypertension anticipates the alterations that are characteristic of aging. Whether this alteration is a mere additive effect of 2 independent risk factors determining impairment in endothelium-dependent vasodilation or whether the endothelial dysfunction that occurs in hypertension is an accelerated form of dysfunction that occurs in aging remains to be established.

The alteration in the L-arginine–NO pathway could be related to reduced substrate availability or to the presence of an endogenous NO synthase inhibitor, such as asymmetric dimethyl-L-arginine. Both hypotheses are in agreement with available evidence demonstrating that L-arginine administration restored vasodilation to acetylcholine in normotensive and hypertensive study populations that was comparable to vasodilation in the present study population, possibly by correcting substrate deficiency or displacing asymmetric dimethyl-L-arginine. Various explanations may be considered regarding the possible sources of oxidative stress in aging and hypertension. Experimental evidence indicates that several systems could be responsible for the increased production of oxygen reactive species, including NADPH:NADP⁺, cyclooxygenase, tetrahydrobiopterin, and others.

Finally, another interesting finding of the present study is the effect of aging on basal release of NO, which was indirectly assessed as the degree of basal FBF reduction induced by L-NMMA. As previously demonstrated, vasoconstriction to L-NMMA is decreased in patients with essential hypertension compared with normotensive control subjects, confirming the presence of reduced basal NO release in essential hypertension. But although the vasoconstrictor effect of L-NMMA is progressively reduced by aging in normotensive subjects, this alteration is not evident in hypertensive patients. It is conceivable that in essential hypertension the effect of aging is masked by the presence of high blood pressure values, which, per se, represent a primary mechanism leading to reduction of basal NO release. In agreement with this hypothesis, the results of the present study indicate that the average vasoconstrictor effect of L-NMMA in the hypertensive study population (~ 30%) is
similar to that exerted by the NO synthase inhibitor in the oldest (>60-year) group of normotensive subjects.

In conclusion, the present study confirms that aging is an important factor altering endothelial function in humans. Moreover, the present study gives further insight into the mechanisms involved in age-related reduction in endothelium-dependent vasodilation, demonstrating that such mechanisms center, above all, on a primary alteration in the l-arginine–NO pathway; thus, oxidative stress appears to play a primary role, leading to compromised NO availability in aged individuals only. It is worth noting that essential hypertension is also characterized by an age-related reduction in endothelium-dependent vasodilation by mechanisms apparently similar to those observed in normotensive individuals. The striking difference is that the onset of these alterations is anticipated in hypertensive compared with normotensive individuals. Taken together, these results indicate that aging and hypertension have an additive effect on endothelial dysfunction. Whether impaired endothelium-dependent vasodilation in essential hypertension represents a mere acceleration of the changes seen with aging is an attractive hypothesis that needs to be further substantiated.

References

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