

Vitamin C Improves Endothelium-Dependent Vasodilation by Restoring Nitric Oxide Activity in Essential Hypertension

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Background—Essential hypertension is associated with impaired endothelium-dependent vasodilation. Inactivation of endothelium-derived nitric oxide by oxygen free radicals participates in endothelial dysfunction in experimental hypertension. To test this hypothesis in humans, we evaluated the effect of antioxidant vitamin C on endothelium-dependent responses in essential hypertensive patients.

Methods and Results—In 14 healthy subjects (47.1±4.8 years; blood pressure, 120.6±4.5/80.9±3.5 mm Hg) and 14 essential hypertensive patients (47.3±5.1 years; blood pressure, 153.9±7.1/102.3±4.1 mm Hg), we studied forearm blood flow (strain-gauge plethysmography) modifications induced by intrabrachial acetylcholine (0.15, 0.45, 1.5, 4.5, and 15 $\mu\text{g} \cdot 100 \text{ mL}^{-1} \cdot \text{min}^{-1}$) or sodium nitroprusside (1, 2, and 4 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute), an endothelium-dependent and -independent vasodilator, respectively, in basal conditions and during infusion of intrabrachial vitamin C (2.4 mg/100 mL forearm tissue per minute). In hypertensive patients but not in control subjects, vitamin C increased ($P < 0.01$) the impaired vasodilation to acetylcholine, whereas the response to sodium nitroprusside was unaffected. Moreover, in another 14 hypertensive patients (47.1±5.2 years; blood pressure, 155.2±6.9/103.7±4.5 mm Hg), the facilitating effect of vitamin C on vasodilation to acetylcholine was reversed by N^G -monomethyl-L-arginine (100 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute), a nitric oxide synthase inhibitor, suggesting that in essential hypertension superoxide anions impair endothelium-dependent vasodilation by nitric oxide breakdown. Finally, because in adjunctive 7 hypertensive patients (47.8±6.1 years; blood pressure, 155.3±6.8/103.5±4.3 mm Hg), indomethacin (50 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute), a cyclooxygenase inhibitor, prevented the potentiating effect of vitamin C on vasodilation to acetylcholine, it is possible that in essential hypertension a main source of superoxide anions could be the cyclooxygenase pathway.

Conclusions—In essential hypertensive patients, impaired endothelial vasodilation can be improved by the antioxidant vitamin C, an effect that can be reversed by the nitric oxide synthase inhibitor N^G -monomethyl-L-arginine. These findings support the hypothesis that nitric oxide inactivation by oxygen free radicals contributes to endothelial dysfunction in essential hypertension. (*Circulation*. 1998;97:2222-2229.)

Key Words: hypertension ■ endothelium ■ nitric oxide ■ endothelium-derived factors ■ free radicals ■ antioxidants

Endothelium plays a primary role in the modulation of vascular tone.¹ The major endothelium-derived relaxing factor is NO,² a labile substance derived from L-arginine by the activity of the enzyme NO synthase,³ which can be specifically inhibited by L-arginine analogues such as L-NMMA.^{4,5} Moreover, endothelium can also produce EDCFs, which are mainly cyclooxygenase-dependent prostanoids (TXA₂ and PGH₂)⁶⁻⁸ or superoxide anions.⁹

Essential hypertension is characterized by impaired endothelium-dependent vasodilation to specific agonists¹⁰⁻¹⁸ because of the presence of an alteration in the L-arginine-NO pathway^{13,14,18,19} and production of cyclooxygenase-dependent EDCFs.^{12,19} It is worth noting that in essential hypertensive patients blockade of EDCF production by cyclooxygenase inhibition can restore the L-arginine-NO pathway,¹⁹ suggesting that the alteration in the NO system can at least partially

be caused by cyclooxygenase derivatives. However, the nature of these EDCFs still remains unidentified in human hypertension.

Superoxide anions are the predominant products of univalent reduction of oxygen and are mediators of vascular injury.²⁰ They can be produced from a variety of sources, including oxidative enzymes such as xanthine oxidase or cyclooxygenase.^{21,22} Under physiological conditions, these oxygen free radicals are potent chemical inactivators of NO.²³⁻²⁶ It has been proposed that endothelial dysfunction associated with hypertension could be determined by augmented production of superoxide anions, which could impair the ability of endothelium to induce NO-mediated relaxations of underlying smooth muscle cells.²⁷⁻²⁹ Moreover, oxygen free radicals produce contractions of rat aorta that are augmented in the spontaneously hypertensive rat and, inter-

Received November 24, 1997; revision received January 7, 1998; accepted January 30, 1998.

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Selected Abbreviations and Acronyms

EDCF = endothelium-derived contracting factor
FBF = forearm blood flow
L-NMMA = N ^G -monomethyl-L-arginine
NO = nitric oxide
PGH ₂ = prostaglandin H ₂
SOD = superoxide dismutase
TXA ₂ = thromboxane A ₂

estingly, are reduced in the presence of cyclooxygenase inhibitors and PGH₂/TXA₂ receptor antagonists but not in the presence of TXA₂ synthetase inhibitors.^{30,31} Therefore, the EDCFs do not necessarily have to be prostanoids, but superoxide anions can also be classified as EDCFs.³² Finally, treatment with the antioxidant SOD³³ or the inhibitor of xanthine oxidase oxypurinol lowers blood pressure in the spontaneously hypertensive rat but not in controls³⁴; moreover, acutely induced hypertension has been shown to induce superoxide generation, which can be reversed by SOD administration in cats.³⁵

Whether a similar alteration is operating in human essential hypertension is still to be determined. Therefore, the aim of the present study was to investigate the possibility that oxygen free radicals could be responsible for the impairment in the L-arginine–NO system of patients with essential hypertension. To address this issue, we used vitamin C, which is an important antioxidant capable of scavenging oxygen-derived free radicals³⁶ and sparing other endogenous antioxidants from consumption.³⁷ The possibility that oxygen free radicals could originate from cyclooxygenase activity was also evaluated.

Methods**Patients**

The study population included 35 normotensive control subjects and 47 matched essential hypertensive patients. Subjects with hypercholesterolemia (total cholesterol >240 mg/dL), diabetes mellitus, cardiac and/or cerebral ischemic vascular disease, impaired renal function, and other major pathologies were excluded from the study. Likewise, subjects or patients smoking more than five cigarettes per day and/or consuming >60 g of ethanol (corresponding to half a liter of wine) per day were excluded from the study. In accordance with institutional guidelines, all patients were aware of the investigational nature of the study and gave written consent. Any pharmacological treatment was discontinued for at least 2 weeks before the study was performed.

Subjects, defined as normal according to the absence of familial history of essential hypertension and blood pressure values <140 to 90 mm Hg, were characterized by a mean age of 46.4±5.1 years and blood pressure values of 120.8±4.3/80.7±3.2 mm Hg. Essential hypertensive patients were recruited from among the newly diagnosed patients in our outpatient clinic if they reported the presence of positive family history of essential hypertension and when supine arterial blood pressure (after 10 minutes of rest) measured by mercury sphygmomanometer three times at 1-week intervals for 1 month was consistently found to be >140/90 mm Hg. Secondary forms of hypertension were excluded by routine diagnostic procedures. Mean age was 47.1±5.4 years, and blood pressure values were 154.8±6.8/102.2±4.4 mm Hg. Because the patients were newly diagnosed, they had never been treated, and the known history of hypertension had lasted 2.4±0.6 years. The demographic and clinical characteristics of the two groups are shown in the Table.

Characteristics of Study Subjects

Parameter	Normotensive Subjects (n=35)	Essential Hypertensive Patients (n=47)
Age, y	46.4±5.1	47.1±5.4
Age range, y	38–60	39–63
Sex, M/F	22/13	26/16
Weight, kg	72.1±3.9	72.4±4.3
Systolic blood pressure, mm Hg	120.8±4.3	154.8±6.8
Diastolic blood pressure, mm Hg	80.7±3.2	102.2±4.4
Plasma glucose, mg/dL	80.9±5.1	83.1±4.9
Plasma total cholesterol, mg/dL	184.3±16.8	189.2±14.6
Plasma HDL cholesterol, mg/dL	42.8±3.3	38.4±3.1
Plasma LDL cholesterol, mg/dL	113.2±9.1	116.5±14.6
Body mass index, kg/m ²	21.2±0.5	21.5±0.5
FBF, mL · 100 mL ⁻¹ · min ⁻¹	3.6±0.5	3.6±0.5

Values are mean±SD.

Experimental Procedure

All studies were performed at 8:00 AM after an overnight fast with the subjects lying supine in a quiet, air-conditioned room (22°C to 24°C). A polyethylene cannula (21 gauge, Abbot) was inserted into the brachial artery under local anesthesia (2% lidocaine) and connected through stopcocks to a pressure transducer (model MS20, Electromedics) for systemic mean blood pressure (1/3 pulse pressure+diastolic pressure) and heart rate monitoring (model VSM1, Physiocontrol) and for intra-arterial infusions. FBF was measured in both forearms (experimental and contralateral forearms) by strain-gauge venous plethysmography (LOOSCO, GL LOOS).³⁸ Circulation to the hand was excluded 1 minute before each sampling or FBF measurement by inflation of a pediatric cuff around the wrist at suprasystolic blood pressure. Details concerning the sensitivity and reproducibility of the method as performed in our laboratory have already been published.³⁹

Forearm volume was measured according to the water displacement method. Drug infusion rates were normalized to 100 mL tissue by alteration of the drug concentration in the solvent while the pump speed of infusion was kept constant. Drugs were infused at systemically ineffective rates through separate ports via three-way stopcocks.

Experimental Design**Effect of Vitamin C on Endothelium-Dependent and Endothelium-Independent Vasodilation**

To evaluate whether oxygen free radicals can selectively impair endothelium-dependent vasodilation in human hypertension, in 14 essential hypertensive patients and 14 normotensive control subjects, endothelium-dependent vasodilation was estimated by performing a dose-response curve to intra-arterial acetylcholine (cumulative increase in infusion rates: 0.15, 0.45, 1.5, 4.5, 15 µg/100 mL forearm tissue per minute, 5 minutes at each dose) while endothelium-independent vasodilation was assessed with a dose-response curve to intra-arterial sodium nitroprusside, a direct smooth muscle cell relaxant compound⁴⁰ (cumulative increase: 1, 2, and 4 µg/100 mL forearm tissue per minute, 5 minutes at each dose). These rates were selected to induce vasodilation comparable to that obtained with acetylcholine. Both acetylcholine and sodium nitroprusside were infused under control conditions (saline infusion at 0.2 mL/min) and in the presence of intrabrachial vitamin C (2.4 mg/100 mL forearm tissue per minute), which was started 10 minutes before the agonists and continued throughout. Previous evidence has indicated that this vitamin C infusion rate is effective in determining a local forearm concentration shown in vitro to be capable of protecting human plasma from free radical-mediated lipid peroxidation.³⁶ A similar

infusion rate has been used by other authors to demonstrate that oxidative stress causes endothelial dysfunction in type II diabetic patients⁴¹ and chronic smokers.⁴² The acetylcholine or sodium nitroprusside infusion sequence was randomized. A 30-minute washout was allowed between each dose-response curve.

Effect of L-NMMA on Response to Acetylcholine in the Absence and Presence of Vitamin C

To assess whether superoxide anions can impair NO-mediated endothelium-dependent vasodilation, in 14 normotensive subjects and 14 essential hypertensive patients, the dose-response curve to acetylcholine was performed according to the following design: during saline (0.2 mL/min), in the presence of intra-arterial L-NMMA (100 μ g/100 mL forearm tissue per minute), in the presence of intra-arterial vitamin C (2.4 mg/100 mL forearm tissue per minute), and finally in the presence of simultaneous infusions of L-NMMA and vitamin C. Both L-NMMA and vitamin C were started 10 minutes before acetylcholine and continued throughout. A 30-minute washout was allowed between each dose-response curve, and a 60-minute period was allowed when L-NMMA was infused.

Effect of Cyclooxygenase Inhibition on Response to Acetylcholine in the Presence of Vitamin C

This series was designed to indirectly evaluate whether cyclooxygenase activity could be a source of superoxide anions in human essential hypertension. First, we performed a dose-ranging analysis for indomethacin and vitamin C to identify the rate at which each compound exerts its maximal effect on the response to acetylcholine. Thus, in two adjunctive groups of essential hypertensive patients ($n=6$ for each group), the dose-response curve to acetylcholine was performed during saline (0.2 mL/min) and repeated in the presence of increasing rates of indomethacin (5, 15, 50, and 100 μ g/100 mL forearm tissue per minute) or vitamin C (0.8, 2.4, 8, and 16 mg/100 mL forearm tissue per minute). After the titration study, in 7 normotensive subjects and 7 essential hypertensive patients, the dose-response curve to intra-arterial acetylcholine was performed during saline (0.2 mL/min), in the presence of cyclooxygenase inhibition by intra-arterial indomethacin (50 μ g/100 mL forearm tissue per minute started 10 minutes before acetylcholine and continued throughout), in the presence of intra-arterial vitamin C (2.4 mg/100 mL forearm tissue per minute started 10 minutes before acetylcholine and continued throughout), and finally in the presence of simultaneous infusions of indomethacin and vitamin C. Finally, in another 7 essential hypertension patients, the above-described experimental design (association of indomethacin with vitamin C) was repeated, testing the antioxidant at 8 mg/100 mL forearm tissue per minute. A 30-minute washout was allowed between each dose-response curve, and a 60-minute period was allowed when indomethacin was infused.

Drugs

Acetylcholine HCl (Farmigea SpA), indomethacin (Liometafen, Chiesi Farmaceutici SpA), L-NMMA (Clinalfa AG), vitamin C (Bracco), and sodium nitroprusside (Malesci) were obtained from commercially available sources and diluted freshly to the desired concentration by the addition of normal saline. Sodium nitroprusside was dissolved in glucose solution and protected from light by aluminum foil.

Data Analysis

Because arterial pressure did not change significantly during the study, all data were analyzed in terms of FBF. FBF increments were taken as evidence of local vasodilation. Clinical characteristics of study subjects shown in the Table were compared by unpaired Student's *t* test. Responses to acetylcholine and sodium nitroprusside were analyzed by ANOVA for repeated measures, and Scheffé's test was applied for multiple comparison testing. Results are expressed as mean \pm SD.

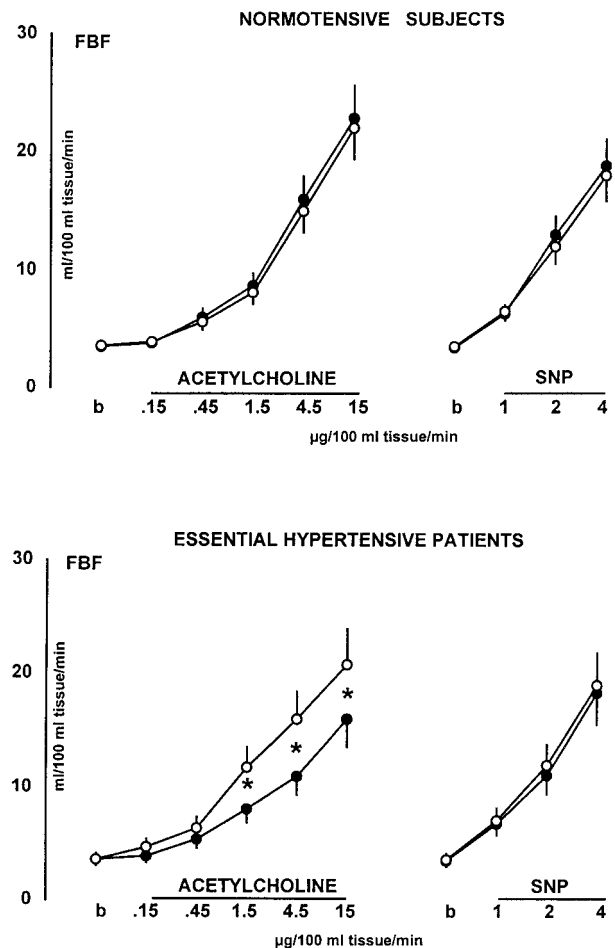


Figure 1. FBF increase above basal (b) induced by intra-arterial acetylcholine (left) and sodium nitroprusside (SNP; right) in the absence (saline at 0.2 mL/min) (●) and presence (○) of vitamin C (2.4 mg/100 mL forearm tissue per minute) in normotensive subjects ($n=14$; top) and essential hypertensive patients ($n=14$; bottom). Data are shown as mean \pm SD and expressed as absolute values. *Significant difference between the response to acetylcholine in the absence and presence of vitamin C ($P<0.05$).

Results

Effect of Vitamin C on Endothelium-Dependent and Endothelium-Independent Vasodilation

Vasodilation to acetylcholine was significantly ($P<0.01$) blunted in essential hypertensive patients (FBF rose from 3.6 ± 0.5 to a maximum of 16.5 ± 2.7 mL/100 mL forearm tissue per minute with the highest dose) compared with normotensive control subjects (FBF rose from 3.6 ± 0.6 to a maximum of 22.8 ± 3.3 mL/100 mL forearm tissue per minute with the highest dose) (Figure 1). In contrast, the vasodilating effect of sodium nitroprusside was similar in normotensive subjects and essential hypertensive patients (FBF rose from 3.5 ± 0.4 to a maximum of 18.9 ± 3.2 mL/100 mL forearm tissue per minute with the highest dose and from 3.4 ± 0.4 to a maximum of 18.0 ± 3.0 mL/100 mL forearm tissue per minute, respectively; $P=NS$) (Figure 1). Vitamin C administration did not change basal FBF in either normotensive or hypertensive subjects. However, whereas in healthy control subjects the free radical scavenger did not change the response to acetylcholine (FBF rose from 3.5 ± 0.6 to a maxi-

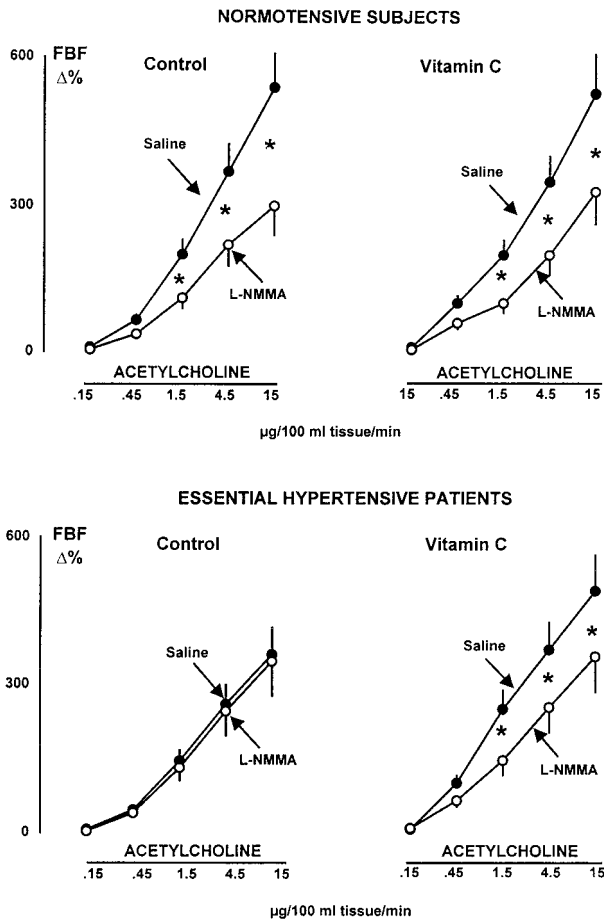


Figure 2. Acetylcholine-induced increase in FBF in the absence (left) and presence (right) of vitamin C (2.4 mg/100 mL forearm tissue per minute) under control conditions (saline at 0.2 mL/min) and in the presence of L-NMMA (100 μ g/100 mL forearm tissue per minute) in normotensive subjects (n=14; top) and essential hypertensive patients (n=14; bottom). Data are shown as mean \pm SD and, because L-NMMA modifies basal FBF, are expressed as percent increase above basal. *Significant difference between infusion with and without L-NMMA ($P < 0.05$).

of 22.0 \pm 3.1 mL/100 mL forearm tissue per minute with the highest dose) (Figure 1), in essential hypertensive patients vitamin C significantly increased the vasodilating effect of the muscarinic agonist (FBF rose from 3.5 \pm 0.6 to a maximum of 20.8 \pm 2.6 mL/100 mL forearm tissue per minute with the highest dose) (Figure 1). In contrast, the response to sodium nitroprusside was not affected by vitamin C in normotensive subjects and essential hypertensive patients (FBF rose from 3.5 \pm 0.6 to a maximum of 18.1 \pm 3.3 mL/100 mL forearm tissue per minute with the highest dose and from 3.6 \pm 0.5 to a maximum of 19.1 \pm 2.4 mL/100 mL forearm tissue per minute, respectively; $P = \text{NS}$) (Figure 1). In both normotensive subjects and essential hypertensive patients, contralateral FBF did not significantly change throughout the study (data not shown).

Effect of L-NMMA on Response to Acetylcholine in the Absence and Presence of Vitamin C

In normotensive subjects, L-NMMA infusion, which caused a decrease in basal FBF (from 3.6 \pm 0.4 to 2.2 \pm 0.2 mL/100 mL

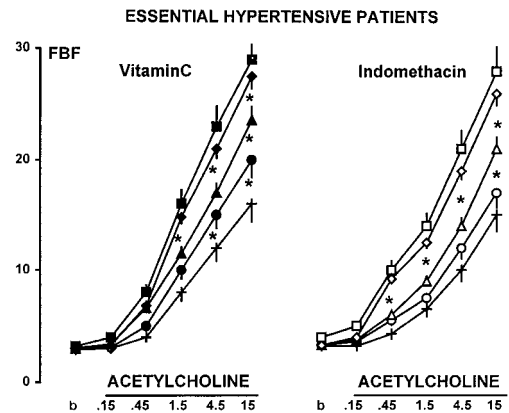


Figure 3. Acetylcholine-induced increase in FBF in the presence of saline (0.2 mL/min) (+) or vitamin C at 0.8 (●), 2.4 (▲), 8 (◆), and 16 (■) mg/100 mL forearm tissue per minute and in the presence of saline (+) or indomethacin at 5 (○), 15 (△), 50 (◇) and 100 (□) μ g/100 mL forearm tissue per minute in two distinct groups of essential hypertensive patients (n=6 each). Data are shown as mean \pm SD and expressed as absolute values. *Significant difference between infusion under control conditions and in the presence of different infusion rates of indomethacin or vitamin C ($P < 0.05$).

forearm tissue per minute; $P < 0.01$), significantly blunted the vasodilating effect of acetylcholine (saline, from 3.6 \pm 0.5 to 22.7 \pm 3.7 mL/100 mL forearm tissue per minute; L-NMMA, from 2.2 \pm 0.2 to 9.9 \pm 1.8 mL/100 mL forearm tissue per minute; $P < 0.01$ versus acetylcholine alone) (Figure 2). Vitamin C did not change either the response to acetylcholine (from 3.7 \pm 0.5 to 23.1 \pm 3.2 mL/100 mL forearm tissue per minute) or the inhibiting effect of L-NMMA on vasodilation to acetylcholine (from 2.2 \pm 10.0 to 10.9 \pm 2.1 mL/100 mL forearm tissue per minute) (Figure 2). In contrast, in essential hypertensive patients, L-NMMA infusion, which caused a smaller decrease in basal FBF (from 3.5 \pm 0.5 to 2.6 \pm 0.2 mL/100 mL forearm tissue per minute; $P < 0.01$) compared with normotensive control subjects (percent FBF decrease, 39% versus 25%, respectively; $P < 0.01$), did not change the response to acetylcholine (saline, from 3.6 \pm 0.5 to 16.7 \pm 2.3 mL/100 mL forearm tissue per minute; L-NMMA, from 2.6 \pm 0.2 to 9.9 \pm 1.9 mL/100 mL forearm tissue per minute; $P = \text{NS}$ versus saline) (Figure 2). Vitamin C infusion increased the response to acetylcholine (from 3.6 \pm 0.4 to 21.1 \pm 2.6 mL/100 mL forearm tissue per minute; $P < 0.01$ versus acetylcholine during saline) (Figure 2). When the effect of L-NMMA was retested in the presence of vitamin C, the NO-synthase inhibitor blunted the vasodilating effect of acetylcholine (from 2.6 \pm 0.2 to 11.4 \pm 1.8 mL/100 mL forearm tissue per minute; $P < 0.01$ versus acetylcholine in the presence of vitamin C alone) (Figure 2). In both normotensive subjects and essential hypertensive patients, contralateral FBF did not significantly change throughout the study (data not shown).

Effect of Cyclooxygenase Inhibition on Response to Acetylcholine in the Presence of Vitamin C

The preliminary titration study showed that acetylcholine-induced vasodilation (from 30.0 \pm 0.5 to 15.1 \pm 2.1 mL/100

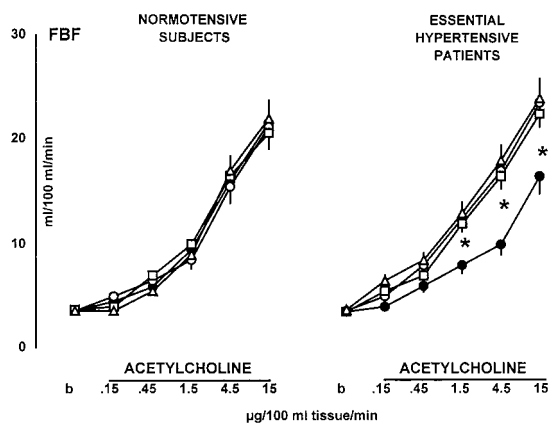


Figure 4. Acetylcholine-induced increase in FBF in the presence of saline (0.2 mL/min) (●); indomethacin (50 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute) (□); vitamin C (8 mg/100 mL forearm tissue per minute) (○); and simultaneous indomethacin and vitamin C (Δ) in normotensive subjects ($n=7$) and essential hypertensive patients ($n=7$). Data are shown as mean \pm SD and expressed as absolute values. *Significant difference between infusion under control conditions and in the presence of vitamin C, indomethacin, or vitamin C plus indomethacin ($P<0.05$).

mL forearm tissue per minute) was increased by indomethacin in a dose-dependent manner (Figure 3). The cyclooxygenase inhibitor reached its maximum effect at the infusion rate of 50 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute (from 3.3 ± 0.4 to $25.7 \pm 2.9 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.001$ versus saline), with no further increase at the greatest concentration of 100 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute (from 3.4 ± 0.4 to $26.9 \pm 2.8 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.001$ versus saline and $P=\text{NS}$ versus indomethacin at 50 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute) (Figure 3). Vitamin C also dose dependently increased the response to acetylcholine (from 30.0 ± 0.2 to $16.2 \pm 2.1 \text{ mL}/100 \text{ mL}$ forearm tissue per minute), with a maximum effect obtained with the infusion rate of 8 mg/100 mL forearm tissue per minute (from 3.1 ± 0.5 to $27.5 \pm 3.1 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.001$ versus saline) (Figure 3) and no further increase obtained with 16 mg/100 mL forearm tissue per minute (from 3.2 ± 0.5 to $28.7 \pm 3.6 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.001$ versus saline and $P=\text{NS}$ versus vitamin C at 8 mg/100 mL forearm tissue per minute) (Figure 3).

When the activities of indomethacin and vitamin C were compared in normotensive control subjects, acetylcholine-dependent vasodilation (from 3.6 ± 0.4 to $21.8 \pm 2.9 \text{ mL}/100 \text{ mL}$ forearm tissue per minute) was not significantly increased by intrabrachial infusion of indomethacin (from 3.5 ± 0.4 to $21.4 \pm 2.6 \text{ mL}/100 \text{ mL}$ forearm tissue per minute, $P=\text{NS}$ versus acetylcholine during saline) or vitamin C (from 3.6 ± 0.5 to $21.1 \pm 3.1 \text{ mL}/100 \text{ mL}$ forearm tissue per minute, $P=\text{NS}$ versus acetylcholine during saline) or the simultaneous administration of indomethacin and vitamin C (FBF, from 3.6 ± 0.4 to $21.9 \pm 2.6 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P=\text{NS}$ versus acetylcholine during saline) (Figure 4). In essential hypertensive patients, acetylcholine infusion caused a dose-dependent vasodilation (from 3.7 ± 0.5 to $17.9 \pm 2.3 \text{ mL}/100 \text{ mL}$ forearm tissue per minute) that was significantly increased both by indomethacin (50 $\mu\text{g}/100 \text{ mL}$

forearm tissue per minute) (from 3.5 ± 0.4 to $24.3 \pm 3.1 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.01$ versus acetylcholine alone) and vitamin C (2.4 mg/100 mL forearm tissue per minute) (from 3.6 ± 0.5 to $20.3 \pm 2.4 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.05$ versus acetylcholine alone). Indomethacin-induced increase in vasodilation to acetylcholine was greater than the potentiation exerted by vitamin C (percent increase above basal of maximum to acetylcholine: during saline, $392.6 \pm 45.3\%$; during indomethacin, $595.8 \pm 67.4\%$ [$P<0.001$ versus saline]; during vitamin C, $468.8 \pm 55.3\%$ [$P<0.01$ versus saline and $P<0.05$ versus indomethacin]). When indomethacin was coinfused with vitamin C, these compounds did not exert an additive effect compared with that exerted by indomethacin alone (from 3.7 ± 0.4 to $23.7 \pm 3.1 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.01$ versus acetylcholine during saline) (percent increase above basal of maximum to acetylcholine, 540.4 ± 67.2 ; $P=\text{NS}$ versus indomethacin). However, in the final group of hypertensive patients, vitamin C at the higher infusion rate of 8 mg/100 mL forearm tissue per minute significantly increased the response to acetylcholine (saline, from 3.5 ± 0.5 to $16.5 \pm 2.4 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; vitamin C, from 3.6 ± 0.6 to $23.6 \pm 3.2 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.01$ versus acetylcholine alone), and this effect was no longer different from that exerted by indomethacin (50 $\mu\text{g}/100 \text{ mL}$ forearm tissue per minute) (from 3.5 ± 0.5 to $22.5 \pm 3.0 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.01$ versus acetylcholine alone). Again, when indomethacin and vitamin C were coinfused, the potentiating effect of the combined compounds was similar to that observed with each one infused alone (from 3.7 ± 0.5 to $24.1 \pm 3.2 \text{ mL}/100 \text{ mL}$ forearm tissue per minute; $P<0.01$ versus acetylcholine during saline) (Figure 4). In both normotensive subjects and hypertensive patients, contralateral FBF did not change significantly (data not shown).

Discussion

In the present investigation, we tested the hypothesis that increased NO breakdown by oxygen free radicals accounts for the impaired endothelium-dependent vasodilation in patients with essential hypertension. In agreement with previous observations,¹⁰⁻¹⁸ the response to acetylcholine but not to sodium nitroprusside was found to be reduced in essential hypertensive patients compared with normotensive control subjects, confirming the presence of endothelial dysfunction in essential hypertension. However, the impaired endothelium-dependent vasodilation was markedly improved by vitamin C in essential hypertensive patients. This effect of the superoxide anion scavenger was specific, because it was observed neither in healthy control subjects nor on endothelium-independent vasodilation induced by sodium nitroprusside. Taken together, these findings indicate that antioxidant vitamin C improves endothelial function in essential hypertensive patients, probably by directly scavenging oxygen free radicals within the vasculature, and strongly suggest that superoxide anion production is a likely candidate to account for endothelial dysfunction in essential hypertension.

This possibility is reinforced by the results of the L-NMMA study. In agreement with previous evidence, al-

though intrabrachial administration of L-NMMA clearly inhibited the vasodilating effect of acetylcholine in normotensive subjects, it was found to be ineffective in essential hypertensive patients, indicating an alteration in the NO system. However, when L-NMMA was retested in the same patients simultaneously with vitamin C, we observed that the NO-synthase inhibitor clearly antagonized the response to acetylcholine, indicating that in the presence of superoxide anion scavenging, the activity of the L-arginine-NO pathway is restored. A likely explanation of these findings could be that in basal conditions the L-NMMA effect is masked by the fact that superoxide anions destroy NO produced by NO-synthase activity. However, when vitamin C scavenges oxygen free radicals and therefore increases the ability of acetylcholine to produce NO, it also unmasks the inhibitory activity of L-NMMA.

Therefore, taken together, these findings indicate that oxygen free radicals may be responsible for endothelial dysfunction in essential hypertension, probably through NO breakdown.

As regards the origin of oxidative stress in human hypertension, it is of interest that available data indicate the cyclooxygenase pathway to be the main source of oxygen free radicals. Thus, previous evidence has indicated that cyclooxygenase-dependent mechanisms induce endothelial dysfunction in essential hypertension,^{12,19} because indomethacin can increase the response to acetylcholine in hypertensive patients but not in healthy control subjects. Moreover, it has recently been demonstrated that cyclooxygenase-derived EDCFs at least partially impair endothelium-dependent vasodilation by inactivating NO formation,¹⁹ suggesting that these factors could include oxygen free radicals. This possibility seems to be confirmed by the present study. Thus, cyclooxygenase inhibition by indomethacin caused a potentiating effect on the response to acetylcholine of the same degree as that exerted by vitamin C. Moreover, when the two compounds were coinjected, no further potentiation of the vasodilating effect of acetylcholine was observed. Therefore, because the potentiation of indomethacin is similar to that exerted by vitamin C and no additive effect was observed from the association of these compounds, oxygen free radical production is probably the main cyclooxygenase-dependent mechanism responsible for endothelial dysfunction in essential hypertension. However, to rule out the possibility that prostanoids (TXA₂ and PGH₂)⁵⁻⁸ may also be produced in the same experimental conditions, studies with specific PGH₂/TXA₂ receptor antagonists or TXA₂ synthetase inhibitors are needed to elucidate this issue. Unfortunately, at the present time, such compounds are not available for human use.

To further address the mechanism through which superoxide anions can inactivate NO, it is worth considering the recent demonstration that intra-arterial CuZn SOD is devoid of any effect on vasodilation to acetylcholine in the forearm of essential hypertensive patients.⁴³ Because the main difference between vitamin C and CuZn SOD is the respective high and low ability of these scavengers to penetrate into endothelial cells,^{32,36} it is likely that the major part of NO destruction occurs within the endothelium. This possibility is in agreement with the present finding of a lack of vitamin

C-induced improvement in the vasodilating response to the NO donor sodium nitroprusside, whose effect should be impaired by extra-endothelial oxygen free radical production.

Finally, it is important to note that superoxide anions seem to be operating mainly when endothelial cells are stimulated by acetylcholine. Thus, intrabrachial vitamin C was ineffective in modifying basal flow, suggesting that superoxide anions are not tonically produced. This possibility is confirmed by the finding that vitamin C did not change the basal vasoconstrictor effect of L-NMMA. As previously demonstrated,^{12,33} L-NMMA-induced vasoconstriction is blunted in hypertensive patients compared with control subjects, indicating that NO basal release in human vasculature is defective in essential hypertensive patients.^{13,18,19,44} This finding is confirmed in the present study, because the response to intrabrachial L-NMMA was reduced in essential hypertensive patients compared with control subjects. However, vitamin C did not change the vasoconstrictor effect of L-NMMA, suggesting that oxygen free radical production is not the cause of impaired tonic NO-mediated regulation of basal flow.

Assessment of the clinical relevance of the present results should take into account the fact that superoxide anion production has been found to cause endothelial dysfunction in presence of several cardiovascular risk factors. Thus, in non-insulin-dependent diabetic patients⁴¹ or smokers,⁴² intrabrachial administration of vitamin C can increase the impaired endothelium-dependent vasodilation to methacholine and acetylcholine, respectively. Moreover, in patients with coronary artery disease and hypercholesterolemia, the association of antioxidant and cholesterol-lowering therapy by probucol and lovastatin, respectively, can induce greater improvement in endothelium-dependent coronary artery vasomotion to acetylcholine compared with cholesterol-lowering therapy alone.⁴⁵ This line of evidence clearly suggests that oxygen-derived free radical production could be a common mechanism accounting for impaired endothelium-dependent vasodilation associated with cardiovascular risk factors. Because there is increasing evidence that endothelial dysfunction contributes to the development of atherosclerotic disease,⁴⁶⁻⁴⁹ it can be hypothesized that oxidative stress could be one of the most important pathogenetic mechanisms. In line with this possibility, it has been demonstrated that in patients with atherosclerotic coronary artery disease, oral administration of vitamin C selectively reverses endothelial vasomotor dysfunction of the brachial artery.⁵⁰ Finally, epidemiological studies indicate an association between increased intake of antioxidant vitamins and reduced risk of coronary disease,⁵¹⁻⁵³ whereas the Cambridge Heart Antioxidant Study indicates that 1-year treatment with the antioxidant vitamin E reduces the rate of nonfatal myocardial infarction in patients with angiographically proven symptomatic coronary atherosclerosis.⁵⁴

In conclusion, the present results indicate that in essential hypertensive patients the antioxidant vitamin C improves endothelium-dependent vasodilation by at least partially restoring L-arginine-NO pathway activity. This finding supports the hypothesis that oxygen-derived free radicals, possibly cyclooxygenase-derived superoxide anions, could be responsible for endothelial dysfunction in essential hypertension. Because the same mechanism is operating in presence of several cardiovascular risk factors, reversing endothelial dysfunction by antioxidant therapy could be important in reduc-

ing the development of cardiovascular disease. Therefore, the results of ongoing studies such as the Heart Outcome Prevention Evaluation study⁵⁵ and the Oxford Heart Protection Study,⁵⁶ evaluating the long-term effect of oral antioxidant vitamin supplementation on cardiovascular morbidity and mortality, will be crucial to our understanding of the effective clinical relevance of oxidative stress in human cardiovascular disease.

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