Restoration of Nitric Oxide Availability After Calcium Antagonist Treatment in Essential Hypertension

Stefano Taddei, Agostino Virdis, Lorenzo Ghiadoni, Armando Magagna, Stefania Favilla, Alfonso Pompella, Antonio Salvetti

Abstract—Essential hypertension is associated with impaired endothelium-dependent vasodilation caused by oxygen free radical–induced nitric oxide (NO) breakdown. Because calcium antagonists can improve endothelial function in patients with essential hypertension, in this study we tested the hypothesis that this beneficial effect could be related to restoration of NO availability by antioxidant properties. In 15 healthy subjects and 15 hypertensive patients, we studied forearm blood flow (strain-gauge plethysmography) modifications induced by intrabrachial acetylcholine (ACh; 0.15, 0.45, 1.5, 4.5, and 15 μg/100 mL per minute), an endothelium-dependent vasodilator in basal conditions, during infusion of N\textsuperscript{G-}monomethyl-L-arginine (L-NMMA, 100 μg/100 mL forearm tissue per minute), an NO-synthase inhibitor, vitamin C (8 mg/100 mL forearm tissue per minute), and finally, simultaneous infusion of L-NMMA and vitamin C. The response to sodium nitroprusside (SNP; 1, 2, and 4 μg/100 mL forearm tissue per minute) was also evaluated. In control subjects, vasodilation to ACh was inhibited by L-NMMA and not changed by vitamin C. In hypertensive patients, vasodilation to ACh was blunted as compared with control subjects and resistant to L-NMMA. Vitamin C, which decreased plasma isoprostanes and increased plasma antioxidant capacity, increased the response to ACh and restored the inhibiting effect of L-NMMA. In hypertensive patients, the study was repeated after 3-month treatment with nifedipine gastrointestinal therapeutic system (30 to 60 mg/daily). Nifedipine treatment decreased circulating plasma lipoperoxides and isoprostanes and increased plasma antioxidant capacity. Moreover, nifedipine increased the vasodilation to ACh but not to SNP and restored the inhibiting effect of L-NMMA on ACh-induced vasodilation, whereas vitamin C no longer exerted its facilitating activity. These results indicate that nifedipine increases endothelium-dependent vasodilation by restoring NO availability, an effect probably determined by antioxidant activity. (Hypertension. 2001;37:943-948.)

Key Words: hypertension, essential ● endothelium ● nitric oxide ● endothelium-derived factors ● free radicals ● antioxidants ● calcium antagonists

Endothelium plays a primary role in the modulation of vascular tone.\textsuperscript{1} The major endothelium-derived relaxing factor is nitric oxide (NO), a labile substance derived from L-arginine by the activity of the enzyme NO-synthase.\textsuperscript{2} Moreover, endothelium can also produce contracting factors, including prostanoids (thromboxane A\textsubscript{2} and prostaglandin H\textsubscript{2})\textsuperscript{3} or superoxide anions.\textsuperscript{4}

Essential hypertension is characterized by impaired endothelium-dependent vasodilation\textsuperscript{5-10} as the result of a reduction in NO oxide availability\textsuperscript{8-10} caused by production of oxidative stress.\textsuperscript{10,11} It has been suggested that in patients with essential hypertension, endothelial dysfunction could act as a promoter of atherosclerosis,\textsuperscript{12} which is one of the most important complications associated with essential hypertension. Thus, it is conceivable that an adjunctive target for antihypertensive treatment could be represented by restoration of NO availability.

In different vascular districts, calcium antagonists can improve endothelium-dependent vasodilation. One of the mechanisms proposed to explain the beneficial effect of this class of drugs on endothelial function is antioxidant activity.\textsuperscript{13,14} Thus, the aim of this study was to evaluate whether treatment with the dihydropyridine calcium antagonist nifedipine may increase endothelium-dependent vasodilation by improving NO availability and whether the mechanism involved could be related to antioxidant activity.

Methods

Patients
The study population included 15 normotensive control subjects and 15 matched patients with essential hypertension. Hypertensive patients were recruited from among the newly diagnosed cases in our outpatient clinic and enrolled if never treated (n=11) or reporting a history of discontinued or ineffective pharmacological antihyperten-
sive treatment (n=4). Among the latter subgroup, no patient had previously been prescribed a calcium antagonist. Secondary forms of hypertension were excluded by routine diagnostic procedures. Individuals smoking >5 cigarettes per day and/or consuming >60 g of ethanol (corresponding to half a liter of wine) per day were likewise excluded from the study. Moreover, to avoid possible dropouts resulting from lack of blood pressure normalization by nifedipine treatment, hypertensive patients were tested for response to the compound 4 weeks before enrollment in the study. With this procedure, we screened 26 hypertensive patients to select 15 responders to nifedipine treatment. The clinical characteristics of the two study populations are shown in the Table. Approval for the protocol was obtained from the Ethics Committee of the University of Pisa, and all patients gave their written consent.

### Experimental Procedure

Vascular reactivity was assessed by the perfused forearm technique. Briefly, the brachial artery was cannulated for drug infusion at systemically ineffective rates, intra-arterial blood pressure, and heart rate monitoring. Another cannula was advanced into an ipsilateral deep forearm vein to perform deep venous blood samples. Forearm blood flow (FFB) was measured in both forearms (experimental and contralateral forearm) by strain-gauge venous plethysmography. Circulation to the hand was excluded 1 minute before FFB measurement by inflating a pediatric cuff around the wrist at suprasystolic blood pressure. Forearm volume was measured according to the water displacement method. Details concerning the method as performed in our laboratory, including sensitivity and reproducibility, have been published.

### Experimental Design

To assess whether oxygen free radicals can impair NO-mediated endothelium-dependent vasodilation in 15 normotensive subjects and 15 patients with essential hypertension, the dose-response curve to acetylcholine (ACh; cumulative increase of infusion rates: 0.15, 0.45, 1.5, 4.5, and 15 μg/100 mL forearm tissue per minute for 5 minutes at each dose) was performed during saline infusion (0.2 mL/min), in the presence of intra-arterial Nω-monomethyl-L-arginine (L-NMMA; 100 μg/100 mL forearm tissue per minute), in the presence of intra-arterial vitamin C (8 mg/100 mL forearm tissue per minute), and finally in the presence of simultaneous infusion of L-NMMA and vitamin C. In addition, endothelium-independent vasodilation was assessed with a dose-response curve to intra-arterial sodium nitroprusside (SNP), a direct smooth muscle cell relaxant compound (cumulative increase by 1, 2, and 4 μg/100 mL forearm tissue per minute for 5 minutes at each dose). Both L-NMMA and vitamin C were started 10 minutes before ACh and continued throughout. A 30-minute washout was allowed between each dose-response curve, whereas a 60-minute period was allowed after L-NMMA.

In the patients with essential hypertension, the above-described experimental design was performed at baseline and after 3-month nifedipine gastrointestinal therapeutic system (GITS) treatment at 30 mg once daily. After 4 weeks of treatment, patients whose blood pressure values were found to be >140 to 90 mm Hg (4 of 15) were given nifedipine GITS at 60 mg once daily. Additional clinic visits were scheduled every 4 weeks for the total duration of the study. Blood pressure was measured in our outpatient unit by a standard mercury sphygmomanometer and determined as the mean of 3 measurements made at 2-minute intervals after the patients had been seated for 10 minutes.

### Assessment of Effect of Vitamin C and Nifedipine on Oxidative Stress

The present control series was designed to evaluate the direct effect of vitamin C and nifedipine on oxidative stress. Moreover, because L-NMMA modifies basal flow, the effect of ACh in the presence of the NO clamp (which allows assessment of endothelial agonists in the presence of NO-synthase blockade without changes in basal flow) was also evaluated. Thus, in 6 adjunctive patients with essential hypertension, the previously described experimental design was repeated with the following modifications. After 10 minutes of L-NMMA infusion, SNP was coinfused (0.3 μg/100 mL tissue per minute for 5 minutes) to neutralize the L-NMMA–induced vasoconstriction and restore baseline FBF. SNP and L-NMMA were then continued throughout the ACh infusion (NO clamp system). By this technique, it is possible to compare the vasodilating effect of ACh under control conditions and NO-synthase blockade without the confounding effect of different baselines.

Ipsilateral venous samples for determination of circulating oxidant stress were collected at baseline and after vitamin C infusion. Finally, to investigate the effect of nifedipine on oxidative stress and antioxidant capacity, venous samples were also performed at baseline and after 3-month nifedipine GITS treatment.

Oxidative stress was evaluated in plasma through measurement of lipoperoxides (colorimetric assay) and isoprostanes (immunoenzymatic method)
**Figure 1.** FBF increase above basal level induced by intra-arterial ACh (left) and SNP (right) in normotensive patients and in patients with essential hypertension at baseline and after 12-week nifedipine GITS treatment (30 to 60 mg daily). Data are shown as mean±SD and expressed as percent increase above basal. *Significant difference between response to ACh before and after nifedipine treatment (P<0.05).*

**Data Analysis**

Data were analyzed in terms of changes in FBF and forearm vascular resistance (FVR) (ratio between intra-arterial mean pressure and FBF and expressed as SU). Differences between two means were compared by means of the paired Student’s t test. Responses to ACh and SNP were analyzed by ANOVA for repeated measures. Because basal FBF and FVR proved to be different in the various experimental steps, data were also analyzed as percent increase or decrease from baseline. In this case, Wilcoxon’s test was used to check the statistical significance. Results were expressed as mean±SD. Differences were considered statistically significant at a value of P<0.05. Computations for the statistical method described were performed with the SAS System.

**Results**

In normotensive subjects and patients with essential hypertension, age, gender, plasma cholesterol, glycemia, and smoking history were similar and within a normal range between the two study groups, who differed in blood pressure values (Table). In hypertensive patients, nifedipine significantly decreased blood pressure after 12 weeks of treatment, whereas heart rate, body weight, lipid profile, and glucose plasma levels were unchanged throughout the treatment period (Table).

**Endothelium-Dependent Vasodilation, NO Availability, and Oxidative Stress at Baseline**

The dose-dependent FBF increase induced by ACh was significantly (P<0.01) blunted in hypertensive patients (FBF: from 3.3±0.5 to a maximum of 17.9±3.4 mL/100 mL forearm tissue per minute) compared with normotensive control subjects (FBF: from 3.4±0.6 to a maximum of 25.8±3.9 mL/100 mL forearm tissue per minute) (Figure 1). In contrast, vasodilation to SNP was similar in normotensive subjects (FBF: from 3.3±0.6 to a maximum of 16.4±1.9 mL/100 mL forearm tissue per minute) and hypertensive patients (FBF: from 3.4±0.5 to a maximum of 17.9±3.4 mL/100 mL forearm tissue per minute). Analogously, ACh caused a decrease in FVR that was found to be significantly (P<0.01) reduced in hypertensive patients (FVR: from 36.7±7.7 to a minimum of 7.1±1.6 SU; −80.6% decrease) as compared with control subjects (FVR: from 27.1±5.8 to a minimum of 3.5±0.9 SU; −87.1% decrease), whereas the effect of SNP was similar in the two study populations (hypertensives: FVR from 36.7±7.7 to 7.3±1.0 SU; −80.1% decrease; normotensives: FVR from 27.8±6.1 to a minimum of 5.5±1.2 SU; −80.2% decrease).

In normotensive subjects, L-NMMA infusion, which decreased basal FBF (from 3.6±0.4 to 2.0±0.2 mL/100 mL forearm tissue per minute; P<0.01), significantly blunted vasodilation to ACh (FBF from 3.0±0.2 to 8.9±1.8 mL/100 mL forearm tissue per minute; P<0.01 versus ACh alone) (Figure 2) (FVR from 45.9±7.9 to 10.7±2.6 SU; −76.7% decrease; P<0.01 versus ACh alone). Vitamin C did not change either the response to ACh (FBF from 3.5±0.5 to 26.1±4.2 mL/100 mL forearm tissue per minute) or the inhibiting effect of L-NMMA on vasodilation to ACh (FBF from 2.1±1.0 to 9.3±2.1 mL/100 mL forearm tissue per minute) (Figure 2). Analogously, vitamin C did not change the FVR modifications induced by ACh in the absence (FVR: from 26.1±5.3 to a minimum of 3.5±0.7 SU; −86.6% decrease) or presence of L-NMMA (FVR: from 46.1±8.1 to a minimum of 9.8±2.7 SU; −78.7% decrease).

Results appeared to be different in hypertensive patients. L-NMMA infusion, which caused a lesser decrease in basal FBF (from 3.4±0.5 to 2.3±0.5 mL/100 mL forearm tissue per minute, P<0.01) as compared with normotensive control subjects (percent FBF decrease: 44% in normotensives versus 33% in hypertensives; P<0.01), did not change the ACh-induced FBF increase (from 2.3±0.5 to 11.8±3.7 mL/100 mL forearm tissue per minute, NS versus saline) (Figure 2) and FVR decrease (from 53.1±11.5 to 10.7±0.7 SU; −80.3% decrease; NS versus saline). Vitamin C infusion increased the response to ACh (FBF from 3.2±0.4 to 24.6±4.9 mL/100 mL forearm tissue per minute; P<0.01 versus ACh during saline) (Figure 2) (FVR from 38.5±8.1 to 5.0±1.1 SU; −86.6% decrease; P<0.02 versus ACh during saline). When the effect of L-NMMA was retested in the presence of vitamin C, the NO-synthase inhibitor blunted the vasodilating effect of ACh (FBF from 2.3±0.6 to 10.1±2.9 mL/100 mL forearm tissue per minute; P<0.01 versus ACh in the presence of vitamin C alone) (Figure 1) (FBF from 53.8±11.6 to 12.5±3.1 SU; −76.6% decrease; P<0.02 versus ACh in the presence of vitamin C alone).

**Effect of Nifedipine GITS on NO-Dependent Vasodilation in Patients With Essential Hypertension**

Nifedipine treatment for 12 weeks, though not affecting basal FBF, normalized basal FVR (from 36.7±7.7 to 30.2±6.0 SU) because of the significant reduction in blood pressure values (Table). Treatment with this calcium antagonist significantly (P<0.01) increased vasodilation to ACh (FBF: from 3.4±0.5 to a maximum of 23.6±3.6 mL/100 mL forearm tissue per minute) (Figure 1) (FBF from 29.7±4.5 to a minimum of 4.3±0.5 SU; −85.5% decrease) as compared with baseline.
In contrast, the response to SNP (FBF: from 3.4±0.6 to 15.9±3.6 mL/100 mL forearm tissue per minute) (Figure 1) (FVR from 30.2±6.0 to a minimum of 6.4±0.8 SU; −78.8% decrease) was not changed by treatment. Under nifedipine, L-NMMA, which caused a greater reduction in basal FBF over baseline (FBF from 3.6±0.3 to 2.2±0.4 mL/100 mL forearm tissue per minute; baseline, −33% versus nifedipine, −39%; P<0.01), significantly (P<0.001) blunted the response to ACh (FBF from 2.2±0.4 to 7.3±1.3 mL/100 mL forearm tissue per minute) (Figure 2) (FVR from 45.5±8.3 to 13.7±2.4 SU; −69.9% decrease). Moreover, vitamin C no longer increased the vasodilation to ACh (FBF from 3.1±0.4 to 23.1±3.9 mL/100 mL forearm tissue per minute). In both normotensive subjects and hypertensive patients, contralateral FBF underwent no significant change (data not shown).

Assessment of Effect of Vitamin C and Nifedipine on Oxidative Stress

In this adjunctive group of patients with essential hypertension, ACh exerted a dose-dependent increase in FBF (from 3.2±0.5 to 16.7±3.4 mL/100 mL forearm tissue per minute). L-NMMA infusion significantly decreased FBF (from 3.2±0.5 to 2.4±0.4 mL/100 mL forearm tissue per minute). Coinfusion of SNP successfully restored baseline FBF (from 2.4±0.4 to 3.1±0.5 mL/100 mL forearm tissue per minute). In the presence of SNP coinfusion, L-NMMA failed to modify the response to ACh (from 3.1±0.5 to 16.5±3.7 mL/100 mL forearm tissue per minute). Vitamin C infusion significantly (P<0.01) increased and decreased plasma venous FRAP (from 301±95 to 3189±1686 mmol/L) and isoprostanes (from 37.8±29.1 to 12.1±12.5 pg/mL), respectively, without changing lipoperoxides (data not shown). Moreover, the antioxidant increased the response to ACh (FBF from 3.2±0.4 to 24.1±4.4 mL/100 mL forearm tissue per minute; P<0.01 versus ACh during saline) and restored the inhibiting effect of L-NMMA on ACh (L-NMMA: FBF from 3.2±0.5 to 2.3±0.3; SNP: FBF from 2.3±0.3 to 3.2±0.4; ACh: FBF from 3.2±0.4 to 12.9±2.8 mL/100 mL forearm tissue per minute; P<0.01 versus ACh in the presence of vitamin C alone). Nifedipine treatment significantly (P<0.05) lowered plasma lipoperoxides (from 3.05±1.63 to 1.71±1.41 μmol/L) and isoprostanes (from 35.0±21.2 to 22.0±12.8 pg/mL) and increased FRAP (from 428±100 to 629±108 mmol/L). Moreover, the calcium antagonist significantly (P<0.01) increased the vasodilation to ACh (FBF: from 3.1±0.5 to 23.9±3.6 mL/100 mL forearm tissue per minute) as compared with baseline. The response to SNP was not changed by treatment (data not shown). L-NMMA caused a greater reduction in basal FBF (from 3.1±0.3 to 1.9±0.2 mL/100 mL forearm tissue per minute) as compared with baseline and, in the presence of NO clamp, significantly blunted the vascular response to ACh (SNP: FBF from 1.9±0.2 to 3.0±0.4; ACh: FBF from 3.0±0.4 to 10.3±3.1 mL/100 mL forearm tissue per minute). Vitamin C no longer increased the vasodilation to ACh (FBF from 3.1±0.4 to 23.1±3.9 mL/100 mL forearm tissue per minute). The response to NO clamp was not changed by treatment (data not shown).

**Figure 2.** ACh-induced increase in FBF in 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 presence of L-NMMA (100 mg/100 mL forearm tissue per minute) (○) in normotensive subjects (n=15) (top) and patients with essential hypertension (n=15) at baseline (medium) and after 12-week treatment with nifedipine GITS (30 to 60 mg daily) (bottom). Data are shown as mean±SD and, because L-NMMA modifies basal FBF, expressed as percent increase above basal. *Significant difference between infusion with and without L-NMMA (≤P<0.05).
Discussion

This investigation indicates that 3-month treatment with the dihydropyridine calcium antagonist nifedipine increases endothelium-dependent vasodilation in patients with essential hypertension by restoring NO availability. Moreover, because nifedipine decreases circulating parameters of oxidative stress and prevents the effect of the antioxidant vitamin C, these results suggest that the beneficial effect exerted by the calcium antagonist on endothelial function could be related to antioxidant activity.

In agreement with previous observations, the response to ACh but not to SNP was found to be reduced in patients with essential hypertension as compared with normotensive control subjects. Moreover, whereas L-NMMA inhibited the vasodilator response to ACh in normotensive subjects, it was ineffective in patients with essential hypertension. 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 ACh. These results confirm the presence of endothelial dysfunction in essential hypertension characterized by impaired NO availability caused by the production of oxidative stress.

Three-month treatment with nifedipine increased the vasodilation to ACh but not to SNP in patients with essential hypertension. Such a finding is in agreement with experimental data, indicating that calcium entry blockers increase endothelial function in various animal model vessels and hypertensive patients. In addition, nifedipine treatment can increase the L-NMMA–induced vasoconstrictor effect. This is in agreement with previous evidence that blood pressure normalization can increase tonic NO release.

However, the original findings of the present study are that under nifedipine treatment, first, L-NMMA clearly inhibited the vasodilation to ACh, and second, vitamin C did not alter either the vasodilating response to the muscarinic agonist or the inhibitory activity exerted by L-NMMA. The discovery that the presence of the calcium antagonist L-NMMA can inhibit vasodilation to ACh, an effect not exerted under baseline conditions, implies that NO availability is restored after treatment. This is a crucial issue because the main characteristic of endothelial dysfunction in essential hypertension is impaired NO availability. Note that the mere observation of an increased response to ACh is not equivalent to the demonstration of increased NO availability. This is because mediators different from NO (such as hyperpolarizing factors) can be responsible for agonist-evoked endothelium-dependent vasodilatation, at least in certain experimental conditions. It is relevant that the nifedipine effect is also observed in the presence of NO clamp, an experimental approach that allows comparison of the response to ACh in the absence and presence of NO-synthase blockade by L-NMMA without the possible confounding effect of changes in baseline flow. As regards the mechanism responsible for nifedipine-induced improvement in NO availability, mere blood pressure reduction is an unlikely explanation. Previous extensive evidence has demonstrated that blood pressure normalization per se is not a sufficient maneuver to improve endothelium-dependent vasodilation. Moreover, during the 3-month treatment period, the other cardiovascular risk factors that can impair endothelial function, including lipid or glicidic profile, showed no change. Therefore, the nifedipine effect is probably specific. The classic effect of calcium antagonists is inhibition of voltage-gated L-type calcium channels, which are represented on smooth muscle but not on endothelial cells. Through this mechanism, calcium antagonists could facilitate the relaxing response to endothelium-derived relaxing substances. However, this was not the case in our experimental conditions because nifedipine treatment did not change the response to SNP, ruling out any effect on endothelial responses mediated by drug activity on smooth muscle cells. On the other hand, it is well documented in several experimental models that calcium antagonists show antioxidant properties.

In this study, nifedipine treatment decreased plasma values of lipoperoxides and isoprostanes, whereas it increased plasma antioxidant capacity. Moreover, the calcium antagonist prevented the facilitating effect of the antioxidant vitamin C on vasodilation to ACh. Taken together, these findings support the possibility that the beneficial activity of nifedipine treatment on endothelial function is related to antioxidant activity. This hypothesis is further supported by the evidence that chronic treatment with nifedipine can improve vasodilation to ACh in the forearm circulation of normotensive patients with hypercholesterolemia, a positive effect obtained without modifications in blood pressure values or lipid profile. Considering that endothelial function in hypercholesterolemia is caused by oxidative stress-induced impairment in NO availability, it is conceivable that in these experimental conditions, the beneficial effect of calcium antagonists could likewise be mediated by antioxidant properties. This observation is also important in the context of the clinical characteristics of our study populations. Patients with essential hypertension show an increase, although statistically nonsignificant, in plasma cholesterol values as compared with normotensive control subjects. Thus, it cannot be excluded that the different lipid profile may play a role in the observed endothelial dysfunction of patients with essential hypertension. However, this eventualy reinforces the significance of the positive results shown by nifedipine because they were obtained in a population characterized by combined risk factors, with a possible additive effect on endothelial dysfunction.

It is important to observe that in our experimental conditions, vitamin C infusion confirmed its antioxidant ability by increasing plasma antioxidant capacity and decreasing isoprostanes without affecting lipoperoxides. It is very likely that the short duration of infusion rate (10 minutes) was not sufficient to reduce the systemic parameters of oxidative stress.

The results of this study can have important clinical implications. First, it has been documented that prolonged vitamin C treatment can lower blood pressure in patients with essential hypertension. Thus, the antioxidant properties of nifedipine could contribute to blood pressure–lowering properties of the compound. Moreover, it is well documented that NO has important antiatherogenic properties, whereas endo-
thelial dysfunction and the consequent NO deficiency, which is a characteristic not only of hypertension or hypercholesterolemia but also of the most important cardiovascular risk factors, can be a promoter of atherosclerosis. It is therefore conceivable that calcium entry blockers could have the additional effect of restoring NO availability, an effect that would help to prevent the development of atherosclerosis. In line with this possibility, both the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT) and Montreal Heart Study demonstrated a reduction in the number of new coronary lesions on angiography under calcium antagonist treatment. Moreover, future trials, such as Evaluation of Nifedipine and Cervastatin on Recovery of Endothelial Function (ENCORE) I and II, will investigate the effect of nifedipine treatment, alone or in combination with the statin, in patients with hypertension treated with a-blocker or a calcium channel antagonist. J Hypertens. 1996:14:1247–1255.


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