Nitric Oxide Modulates Tissue Plasminogen Activator Release in Normotensive Subjects and Hypertensive Patients

Chiara Giannarelli, Ferdinando De Negri, Agostino Virdis, Lorenzo Ghiadoni, Alessandro Cipriano, Armando Magagna, Stefano Taddei, Antonio Salvetti

Abstract—We evaluated the possible role of NO in modulating tissue plasminogen activator (t-PA) release in the forearm microcirculation of normotensive subjects and hypertensive patients. Essential hypertensive patients are characterized by endothelial dysfunction because of a reduced NO availability and also show an impaired t-PA release. In healthy volunteers and essential hypertensive patients, we studied local t-PA release and forearm blood flow changes (strain-gauge plethysmography) induced by intrabrachial administration of acetylcholine (0.45 and 1.5 μg/100 mL/min) and of sodium nitroprusside (0.5 and 1.0 μg/100 mL/min), an endothelium-dependent and -independent agonist, respectively. Acetylcholine was also repeated in the presence of intra-arterial infusion of the NO synthase inhibitor N\textsuperscript{G}-monomethyl-L-arginine (100 μg/100 mL/min). In normotensive subjects, vasodilation to acetylcholine was blunted by N\textsuperscript{G}-monomethyl-L-arginine. In these subjects, acetylcholine infusion induced a significant, dose-dependent increase in net forearm t-PA release. N\textsuperscript{G}-monomethyl-L-arginine significantly reduced basal t-PA release, as well as acetylcholine-induced t-PA release. In essential hypertensive patients, vasodilation to acetylcholine was reduced as compared with controls and resistant to N\textsuperscript{G}-monomethyl-L-arginine. In contrast to what was observed in healthy control subjects, in hypertensive patients, acetylcholine had no effect on t-PA release. Similarly, N\textsuperscript{G}-monomethyl-L-arginine failed to modify either the tonic or the agonist-induced t-PA release. Both tonic and agonist-induced release of NO are directly involved in t-PA release by endothelial cells. Essential hypertension, characterized by a reduction in tonic and stimulated NO availability, is also associated with impaired capacity of t-PA release, suggesting a major role of impaired NO availability in worsening both vasodilation and t-PA release. (Hypertension. 2007;49:1-7.)

Key Words: endothelium ■ tissue plasminogen activator ■ nitric oxide ■ acetylcholine ■ microcirculation ■ hypertension ■ essential

Vascular endothelium plays a primary role in the modulation of several important functions, including the regulation of vascular tone and fibrinolysis.1 In particular, mechanisms whereby endothelium modulates fibrinolysis include the production of tissue plasminogen activator (t-PA) and its main inhibitor, the plasminogen activator inhibitor type 1 (PAI-1).1,2 In healthy vessels, the activation of platelets or coagulation system induces a rapid release of t-PA from endothelial cells, which is a counterregulatory mechanism to clear intravascular fibrin, thereby preventing thrombus extension.3 It follows that, if acute t-PA release is defective, fibrinolytic activity is impaired, and this alteration may contribute to atherothrombotic events.4 Patients with essential hypertension show an increased risk of atherosclerosis and thrombotic-related complications, and a dysfunctioning endothelium represents an early major mechanism promoting the atherosclerotic process.5,6 In hypertensive patients, endothelial dysfunction is mainly caused by an increased production of oxidative stress, leading to impaired NO availability.7 In addition to its well-documented vasoactive properties, NO also participates in maintaining the antithrombotic properties of endothelial surface by different mechanisms, including the inhibition of platelet adhesion and aggregation.1,8 The role of NO pathway in the regulation of vascular tone is well documented.6 In contrast, its effects in promoting endogenous fibrinolysis by the regulation of endothelial t-PA release is less clear and only scanty investigated, with inconclusive results.8–10 Indeed, at the present time, whether NO promotes endothelial t-PA release is still not clarified. Therefore, the first aim of the present study was to assess whether NO is directly involved in local t-PA release in the forearm microcirculation of normotensive subjects. The second aim was to evaluate whether NO availability might be involved in impaired t-PA release in essential hypertension, a clinical condition characterized by reduced NO availability.7 We believe the latter as a crucial aim with clinical impact, because the restoration of NO availability might improve fibrinolysis in essential hypertension.
Methods

Subjects
The study population included 34 men with essential hypertension and 30 age-matched healthy male volunteers. Patients were recruited among newly diagnosed cases in our outpatient clinic. Inclusion criteria were age between 30 and 65 years, sitting clinical blood pressure (after 10 minutes of rest) values between 140 to 90 and 160 to 99 mm Hg, confirmed in 2 separate occasions within 1 month, and absence of target organ damage according to European guidelines. Each patient underwent a history, physical examination, ECG, and routine laboratory analysis. None had evidence of hyperlipidemia, diabetes, body mass index >30 kg/m², impaired renal function, or cardiovascular disease other than essential hypertension. Secondary forms of hypertension were excluded by routine diagnostic procedures. According to inclusion criteria, all were nonsmokers. Patients did not take any medications for ≥1 month before the study. The study protocol was approved by the local ethics committee and performed in accordance with guidelines of our institution. All of the patients were aware of the nature, purpose, and potential risks of the study and gave written consent to it. After the preliminary analysis of results obtained in 20 patients and 16 healthy subjects, power calculation of the study indicated the need to reach a sample size of 30 patients and 30 healthy subjects.

Experimental Procedures
The perfused-forearm model, which has been described previously in detail, was used. Studies were performed in the morning after an overnight fast, in a temperature-controlled room.

Intravenous catheters were placed in deep antecubital veins in both arms (experimental and contralateral forearm). After subdermal administration of 1% mevparcaine, a 20-gauge polyurethane catheter (BD Angiocath, Becton Dickinson Infusion Therapy Systems Inc) was inserted into the brachial artery of the nondominant arm for intra-arterial administration of drugs at systematically ineffective rates and intra-arterial blood pressure and heart rate monitoring using a digital monitor (Entour, Mennem Medical Ltd). After the placement of intravenous and intra-arterial catheters, subjects were allowed to rest 30 minutes before baseline measurement were made.

Forearm blood flow (FBF) was measured in both forearms by strain gauge plethysmography; the strain gauge was connected to plethysmography (EC-6, D.E. Hokanson Inc), and for each measurement, a cuff placed around the upper arm was inflated to 40 mm Hg with a rapid cuff inflator (model E-10, Hokanson Inc) to occlude venous outflow from the extremity. Circulation to the hand was excluded 1 minute before FBF measurement by inflation of a pediatric cuff around the wrist at suprasystolic blood pressure. Forearm volume was measured according to the water displacement method.

Experimental Design

Protocol 1: Effect of Acetylcholine on FBF and Local t-PA and PAI-1 Release
In 12 healthy subjects and 16 hypertensive patients, endothelium-dependent vasodilation was estimated by performing a dose-response curve to intra-arterial acetylcholine (0.45 and 1.5 μg/100 mL/min). A dose-response curve to intra-arterial sodium nitroprusside (0.5 and 1.0 μg/100 mL/min), a direct smooth muscle cell relaxant compound, was performed to assess endothelium-independent vasodilation. These rates were selected to induce a comparable vasodilation to that obtained with acetylcholine. Acetylcholine and sodium nitroprusside were infused in random order, and each dose was administered for 15 minutes. A free interval of 30 minutes was allowed between the different substances.

Protocol 2: Effect of NO Synthase Inhibition on Stimulated t-PA Release
To evaluate the effect of NO on vasodilation and endothelial release of t-PA, in 12 healthy subject and 12 hypertensive patients, acetylcholine (1.5 μg/100 mL/min) was infused in the absence and in the presence of intra-arterial infusion of L-monomethyl-L-arginine (L-NMMA), an NO synthase (NOS) inhibitor (Clinalfa AG; 100 μg/100 mL/min). Because L-NMMA modifies blood flow, the effects of acetylcholine were evaluated in the presence of NO clamp, which allows assessment of endothelial agonists in the presence of NOS blockade without changes in basal blood flow, thus avoiding any perturbation possibly altering the net balance of t-PA. Briefly, after 10 minutes of L-NMMA infusion, sodium nitroprusside was reinfused at adjusted dose (0.3 and 0.4 μg/100 mL/min) to neutralize the L-NMMA–induced vasoconstriction and to restore baseline FBF, as described previously in detail.

Protocol 3: Sham Study
As a control study, in an adjunctive group of 6 healthy subjects and 6 essential hypertensive patients, intra-arterial saline and glucose solution 5% were injected at the same infusion rates than those used for acetylcholine and sodium nitroprusside, respectively.

Blood Sampling and Biochemical Measurements
At baseline, venous blood samples were collected in lithium–heparin or EDTA tubes and immediately placed on ice. Plasma was immediately centrifuged and stored at ~70°C until assayed. Total serum cholesterol, triglycerides, high-density lipoprotein cholesterol, and plasma glucose were assessed by enzymatic methods (Roche, Diagnostic). Low-density lipoprotein cholesterol was calculated with Friedewald’s equation.

Before measurements of FBF, simultaneous arterial and venous samples were obtained from the infused arm before and after each dose of study drugs or vehicles. Infusions were interrupted during arterial sampling. All of the samples were obtained after the first 4 mL of blood were discarded. Blood samples were collected in tubes containing 1/9 vol 0.45 mol/L sodium citrate buffer (Vacutainer), kept on ice, and centrifuged immediately at 4°C and 3000g for 15 minutes; plasma was stored at ~70°C until the time of assay. Plasma concentrations of t-PA and PAI-1 antibodies were determined by ELISA (TecnocLine GmbH). All of the samples were assayed in duplicate on the same test plate. Intra-assay and interassay variation coefficients were <10%.

Data Analysis
Forearm plasma flow was determined by FBF and hematocrit. Net release or uptake rates for t-PA were calculated by the following formula: net release = (Cv−Ca)/[FBF×(101−hematocrit/100)], where Cv and Ca are the venous and arterial concentrations, respectively. The total amount of t-PA released was calculated using the area under the curve formula for repeated measures. Study population characteristics; basal venous, arterial, and venous–arterial concentrations; and t-PA balance at baseline were compared using the Wilcoxon test. Responses to acetylcholine and sodium nitroprusside were analyzed by 1-way repeated measures and 2-way (group and infusion) ANOVA for repeated measures. Results were expressed as mean ± SEM, except for those shown in Table 1 (mean ± SD). Findings were considered significant at P<0.05. Computations for the power calculation and for the statistical methods were performed with the use of the SAS System.

Drugs
Acetylcholine HCl (Farmigea SpA), L-NMMA (Clinalfa AG), and sodium nitroprusside (Malesci SpA) were obtained from commercially available sources and diluted to the desired concentration by the addition of normal saline. Sodium nitroprusside was dissolved in glucose solution 5% and protected from light by aluminum foil.

Results
Clinical hemodynamic and humoral characteristics of the study population are shown in Table 1.

Protocol 1: Effect of Acetylcholine on FBF and Local t-PA and PAI-1 Release
FBF Vasodilation to acetylcholine was significantly (P<0.001) reduced in hypertensive patients, as compared with control
subjects (Figure 1). The vascular response to sodium nitroprusside was found to be similar between the 2 groups (Figure 1). In both normotensive subjects and essential hypertensive patients, contralateral FBF did not change throughout the study (data not shown).

Fibrinolytic Components Release
At baseline, arterial and venous concentrations of t-PA antigen were lower in hypertensive patients as compared with normotensive subjects (Table 2; \( P<0.01 \)), with a lower venous–arterial difference among hypertensive patients as compared with control subjects (Table 2; \( P<0.05 \)). As a consequence, the basal release of t-PA across the forearm was significantly reduced in hypertensive patients when compared with normotensive subjects (Figure 1; \( P<0.01 \)). Venous concentration of t-PA antigen significantly progressively increased during the infusion of acetylcholine in normotensive subjects, an effect not observed in hypertensive patients (Table 2).

Because the arterial t-PA antigen concentrations in both normotensive and hypertensive groups did not alter significantly under agonist administration, during acetylcholine, infusion the venous–arterial concentration gradient of t-PA antigen significantly increased in normotensive subjects but not in hypertensive patients (Table 2). No increase of both venous and venous-arterial concentrations of t-PA was observed during sodium nitroprusside infusion in both groups (Table 2).

Concerning t-PA balance, acetylcholine infusion induced a significant, dose-dependent increase in net t-PA release across the forearm in normotensive subjects but not in hypertensive patients (Figure 1). The cumulative amount of t-PA antigen secreted during acetylcholine infusion was also significantly reduced in the hypertensive as compared with the normotensive group (Figure 2). During sodium nitroprusside infusion, the arterial t-PA antigen concentrations in both normotensive and hypertensive groups did not alter significantly under agonist administration, and the venous–arterial concentration gradient of t-PA antigen remained relatively stable across the infusion periods (Table 2).
TABLE 2. Arterial, Venous, and Venous–Arterial Concentrations Gradient of t-PA and PAI-1 at Baseline and After the Infusion of Acetylcholine and Sodium Nitroprusside

<table>
<thead>
<tr>
<th>Drug Doses (µg/100 mL tissue/min)</th>
<th>Normotensive Subjects (n=12)</th>
<th>Hypertensive Patients (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t-PA, A</td>
<td>t-PA, V</td>
</tr>
<tr>
<td>Baseline</td>
<td>6.7±0.5</td>
<td>7.2±0.6</td>
</tr>
<tr>
<td>ACH (0.45)</td>
<td>6.8±0.6</td>
<td>7.3±0.5</td>
</tr>
<tr>
<td>ACH (1.5)</td>
<td>6.8±0.6</td>
<td>8.0±0.3†</td>
</tr>
<tr>
<td>Baseline</td>
<td>7.1±0.4</td>
<td>7.6±0.5</td>
</tr>
<tr>
<td>SNP (0.5)</td>
<td>7.2±0.5</td>
<td>7.5±0.6</td>
</tr>
<tr>
<td>SNP (1.0)</td>
<td>7.2±0.6</td>
<td>7.5±0.5</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SEM. Ach indicates acetylcholine; SNP, sodium nitroprusside; A, arterial; V, venous; V-A, venous–arterial.

*P<0.05 vs normotensive subjects; †P<0.05 vs basal levels; ‡P<0.01 vs normotensive subjects.

side infusion, no significant increase in t-PA release in both normotensive and hypertensive group was seen (Figure 1). Arterial and venous concentrations of PAI-1 antigen at baseline were similar in both normotensive and hypertensive subjects, and no significant difference in arterial, venous, and venous–arterial concentration gradient of PAI-1 was observed after the infusion of acetycholine and sodium nitroprusside in both groups (Table 2). No significant difference in PAI-1 antigen balance resulted after the infusion of acetycholine and sodium nitroprusside in both normotensive and hypertensive groups (Figure 3). Contralateral venous–arterial concentrations of fibrinolytic components did not change throughout the study in both normotensive subjects and essential hypertensive patients (data not shown).

Protocol 2: Effect of NOS Inhibition on Stimulated t-PA Release

**FBF**

In normotensive subjects, L-NMMA infusion, which reduced basal FBF, significantly blunted the vasodilation to acetylcholine (Figure 4). In contrast, in hypertensive patients, the NOS inhibitor caused a smaller decrease in FBF and failed to affect the response to acetylcholine (Figure 4). In both normotensive subjects and essential hypertensive patients, contralateral FBF did not change throughout the study (data not shown).

**Fibrinolytic Component Release**

In normotensive subjects, t-NOXEA infusion decreased basal t-PA release (Figure 5). Moreover, in the presence of t-NOXEA, acetylcholine-induced t-PA release was impaired (Figure 5). In contrast, in hypertensive patients, no significant change on t-PA release was observed at the end of t-NOXEA–alone infusion (Figure 5) or during t-NOXEA plus acetylcholine (Figure 5). In both normotensive subjects and essential hypertensive patients, contralateral venous–arterial concentrations of t-PA antigen did not change throughout the study (data not shown).

**Protocol 3: Sham Study**

FBF did not change significantly during the infusions of saline and glucose solution 5% in both normotensive subjects, glucose: from 3.3±0.4 to 3.4±0.5 mL/100 mL/min; glucose: from 3.2±0.5 to 3.4±0.5 mL/100 mL/min) and hypertensive patients (FBF, saline: from 3.3±0.3 to 3.4±0.3 mL/100 mL/min; glucose: from 3.4±0.5 to 3.5±0.6 mL/100 mL/min).

No significant difference in t-PA balance was observed after the infusion of saline and glucose solution at 5% in both normotensive subjects (saline: from 1.6±0.1 to 1.7±0.2 mL/100 mL/min; glucose: from 1.5±0.2 to 1.6±0.3 mL/100 mL/min) and hypertensive patients (saline: from 0.4±0.1 to 0.5±0.3 mL/100 mL/min; glucose: from 0.5±0.2 to 0.6±0.1 mL/100 mL/min). Similarly, no effect on PAI-1 balance was observed during the infusions in both groups (data not shown).

**Discussion**

The present study shows that local intra-arterial infusion of acetylcholine, an endothelium-dependent agonist, induces a rapid release of t-PA across the forearm in normotensive subjects. Because sodium nitroprusside administration, despite a similar blood flow increment, had no such effect, these results confirm that the t-PA release is an endothelial property not dependent on flow increase.15–17 Worth noting, sodium nitroprusside is an exogenous NO donor, acting directly on vascular smooth muscle cells.18 Our finding that this com-
pound failed to stimulate t-PA release reinforces the concept that the relationship between NO and fibrinolysis mainly occurs within endothelial cells. Although evidence is concordant on the possibility to release t-PA by receptor-operated agonists, including methacholine\textsuperscript{15,17} and bradykinin,\textsuperscript{16,19} results with acetylcholine are discordant.\textsuperscript{16,19} However, a likely explanation is the short duration of acetylcholine infusion in negative articles (5 minutes per dose)\textsuperscript{16} as compared with our experimental conditions (15 minutes per dose). On the other hand, both methacholine (a stable derivative of acetylcholine) and acetylcholine stimulate the same muscarinic receptors,\textsuperscript{20} and, therefore, any possible difference in results should be related to methodologic problems. In the present study, we also observed that, in our hypertensive patients, characterized by a reduced endothelium-dependent vasodilation, acetylcholine administration failed to increase t-PA release, thus indicating an impairment of t-PA release in essential hypertension.

The concept of impaired t-PA release in response to endothelial stimuli in essential hypertension was assessed previously in a small population demonstrating an impaired endothelial t-PA release after desmopressin infusion.\textsuperscript{21} In addition, Ridderstrale et al\textsuperscript{22} showed an impaired release of t-PA in response to substance P in essential hypertension patients.

The demonstration that different endothelial agonists revealed an impaired endothelial t-PA release suggests that this fibrinolytic alteration is not related to a specific defect of the muscarinic receptor for acetylcholine or to an abnormality of a single intracellular signal-transduction pathway but to a more generalized abnormality of endothelial fibrinolytic properties in essential hypertension. However, our data are partially in contrast with results obtained from Hrafnkelsdottir et al,\textsuperscript{21} who observed no difference in terms of t-PA release between normotensive control subjects and hypertensive patients when methacholine was used. A likely explanation for this discrepancy could be that a smaller group of hypertensive patients (n=7) was evaluated in the report by Hrafnkelsdottir et al,\textsuperscript{21} by using a weaker stimulus.

The major novel finding of the present study concerns the demonstration in humans that endothelial NO is involved in t-PA release modulation. To test NO availability, we infused L-NMMA to block NO synthase. In normotensive subjects, as demonstrated previously,\textsuperscript{7} vasodilation to acetylcholine is blunted by L-NMMA, confirming the preservation of NO availability in healthy conditions. It is worth noting that, in these subjects, when L-NMMA was infused, we also observed a decrease in the venous t-PA concentration. Because FBF did not change because of the application of the NO-clamp technique, these results indicate a reduced basal t-PA release across the forearm vascular bed. In addition, L-NMMA infusion, which blocked agonist-induced NO release, blunted stimulated t-PA release in the forearm vascular bed. Taken together, these findings indicate a positive modulating effect of NO on tonic and stimulated endothelial t-PA release in healthy subjects.
In contrast, in essential hypertensive patients, vasoconstriction to L-NMMA was significantly reduced as compared with control subjects, confirming the presence of impaired basal NO release.\(^2^3\) Interestingly, in hypertensive patients, basal t-PA release was almost abolished, and, therefore, it was not possible to detect any eventual further reduction induced by L-NMMA administration. In addition, in essential hypertensive patients, the reduced vascular response to acetylcholine was totally resistant to L-NMMA, thus confirming the evidence of impaired NO availability in essential hypertension.\(^7\) Moreover, no modification on acetylcholine-induced t-PA release was observed under the simultaneous L-NMMA infusion. These data confirm and strengthen the concept of a strong relationship between NO and t-PA release. Indeed, in a clinical condition characterized by a reduced NO availability, such as essential hypertension,\(^7\) a blunted profibrinolytic property of endothelial cells occurs. Our results are in line with those obtained in previous animal models,\(^2^4,2^5\) where a strict relationship between endothelial t-PA release and NO pathway was documented. This hypothesis was already assessed in humans, with conflicting results. According to our results, Newby et al\(^8\) reported a blunted t-PA release secondary to substance P administration in the presence of L-NMMA in the forearm microcirculation of healthy subjects. In contrast, an increased t-PA release induced by bradykinin in the presence of L-NMMA was reported.\(^9\) These conflicting results may be related to the higher dose of L-NMMA used in the latter study (5 mg/min) as compared with that used in our report and that of Newby et al\(^8\) (4 μmol/min, which corresponds to 1 mg/min). Because the calculation of net t-PA balance is a function of plasma flow, it is conceivable that the greater perturbation in blood flow secondary to a high L-NMMA dose might account for the increase of t-PA balance in these patients.\(^9\) It is worth noting that, in our experimental condition, the use of NO clamp, which restored basal FBF after L-NMMA–induced vasoconstriction, overwhelmed this methodologic limit. Finally, Brown et al\(^1^0\) observed a lacking effect of L-NMMA infusion on bradykinin-stimulated t-PA release in healthy subjects, arguing against a modulatory influence of NO on endothelial fibrinolytic capacity. However, in this study, L-NMMA infusion induced only a slight reduction of vascular response to bradykinin,\(^1^0\) thus suggesting an incomplete endothelial NOS inhibition, a finding that does not provide conclusive results.

**Perspectives**

The results of the present study demonstrate that both tonic and agonist-induced release of NO are directly involved in t-PA release by endothelial cells. Essential hypertension, characterized by a reduction in tonic and stimulated NO availability, is also associated with impaired capacity of t-PA release, suggesting a major role of impaired NO availability in worsening both vasodilation and endothelial t-PA release. When considering that NO plays a prominent role in the modulation of vascular tone and of endothelial fibrinolytic properties as well, it is conceivable that both of these functions may contribute to the risk of atherothrombotic events in essential hypertensive patients.\(^2,2^6\) The definition of additional aspects characterizing endothelial dysfunction may increase the knowledge of the pathophysiology of atherothrombosis and provide further information for the development of specific strategies of treatment in essential hypertension.

**Disclosures**

None.

**References**

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