Tissue-Type Plasminogen Activator Release in Healthy Subjects and Hypertensive Patients

Relationship With β-Adrenergic Receptors and the Nitric Oxide Pathway

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

Abstract—The relationship between adrenergic stimuli and NO in modulating tissue-type plasminogen activator (t-PA) release from endothelial cells was investigated in normotensive subjects and essential hypertensive patients. Sympathetic activation, a well-known stimulus for endogenous fibrinolysis, is also involved in the determination of cardiovascular risk in essential hypertension. However, the existence of cross-talk between adrenergic stimuli and NO availability in modulating t-PA release is not well established yet. We assessed the release of t-PA in the forearm microcirculation of 58 normotensive subjects (mean age: 47±9 years) and 44 essential hypertensive patients (mean age: 48±11 years) under specific intra-arterial adrenergic stimuli. Intrabrachial infusion of epinephrine (0.1 to 0.3 μg/100 mL per minute) induced greater t-PA release in normotensive subjects as compared with essential hypertensive patients (P<0.05). However, inhibition of NO synthase with Nω-monomethyl-L-arginine (100 μg/100 mL per minute) infusion blunted epinephrine-induced t-PA release in normotensive subjects (P<0.05) but not in essential hypertensive patients. In normotensive subjects, t-PA release by epinephrine was not affected by phentolamine (8 μg/100 mL per minute) coinfusion and was abolished in the presence of propanolol (10 μg/100 mL per minute). Intrabrachial isoproterenol (0.03 μg/100 mL per minute) induced a significant increase in t-PA release (P<0.01), an effect blunted by Nω-monomethyl-L-arginine (P<0.05). In essential hypertensive patients, the response to isoproterenol was impaired as compared with normotensive subjects and was unaffected by Nω-monomethyl-L-arginine coinfusion. In conclusion, the results of the present study demonstrate that adrenergic-induced t-PA release is mediated by β-adrenergoreceptors via a mechanism involving the NO pathway. Our results show an impaired adrenergic-stimulated t-PA release among essential hypertensive patients, probably mediated via a reduced NO availability. This impaired fibrinolytic activity might contribute to the increased cardiovascular risk associated with hypertension. (Hypertension. 2008;52:314-321.)

Key Words: t-PA β receptors NO endothelium hypertension essential

The endogenous fibrinolytic system contributes to the maintenance of vessel patency via the cleavage of insoluble fibrin by plasmin. The activation of plasmin by tissue-type plasminogen activator (t-PA) is a physiological process that, clearing inappropriate intravascular fibrin deposition, prevents vascular atherothrombotic events.1,2 The coagulation factors thrombin and activated factor X, acting as counterregulatory mechanisms during fibrin deposition, are considered the main stimuli for acute t-PA release from endothelial cells.2 Thus, the vascular endothelium plays a major regulatory role in endogenous fibrinolysis.3 One of the main mechanisms by which a healthy endothelium regulates acute release of t-PA involves the NO pathway.4 In certain pathological conditions characterized by reduced NO availability, such as essential hypertension, in which an impaired endothelium-dependent vasodilation occurs,5 a concurrent impairment in t-PA release is observed.4,6 Of note, t-PA release and endothelium-dependent vasodilation can predict the risk of cardiovascular events.7,8

Another important stimulus involved in modulating the fibrinolytic system is adrenergic activation.9 Because the sympathetic nervous system plays a major role in cardiovascular homeostasis and is involved in the determination of cardiovascular risk,10 the relationship between adrenergic activation and the fibrinolytic system could be relevant in elucidating the pathophysiology of atherothrombosis. Previous human studies have measured circulating t-PA concentrations after systemic stimuli, such as mental stress,11 exercise, or intravenous drug infusions,12,13 which do not necessarily reflect the local amount of t-PA released by endothelial cells.14 This is a crucial issue, because effective fibrinolysis must be sustained by local t-PA release, which cannot be measured by systemic assessment.15,16 The relationship between the adrenergic system and endothelial fibri-
nolysis is currently not fully characterized, and local t-PA release after adrenergic stimulation represents a major issue requiring further investigation.14

In the present study we hypothesized that the (adrenergic-mediated) t-PA release in response to the adrenergic stimulus is mediated by the NO pathway. Therefore, we have assessed t-PA release in the forearm microcirculation of normotensive subjects and in patients with essential hypertension under specific adrenergic stimuli. In particular, we investigated which adrenergic receptor subtype (ie, α- or β-adrenoceptor) is involved and the contribution of NO in modulating t-PA release in healthy conditions.

Materials and Methods

Subjects

The study population included 58 healthy male volunteers and 44 patients with essential hypertension. Patients were recruited among newly diagnosed case subjects in our outpatient clinic. Inclusion criteria were between 30 and 65 years and sitting blood pressure of 150/90 mm Hg and 160/90 mm Hg, confirmed on 2 separate occasions within a period of 1 month according to European guidelines.17 Exclusion criteria were age between 30 and 65 years and sitting blood pressure ≥ 160/90 mm Hg, renal or liver impairment, and established cardiovascular diseases other than essential hypertension. Secondary forms of hypertension were excluded by routine diagnostic procedures. Patients either were never treated for hypertension or they did not receive any medications for ≥1 month before enrollment in the study.

The study protocol was approved by the local ethics committee and performed according to the guidelines of our institution. All of the patients were aware of the nature, purpose, and potential risks of the study and gave their written consent.

Experimental Procedures

The perfused-forearm model used in this study has been described previously in detail. Briefly, intravenous catheters were placed into the deep antecubital vein of each arm (experimental and contralateral forearm as control) and the brachial artery of the nondominant arm cannulated for drug infusion at systemically ineffective rates, as well as for the intra-arterial blood pressure and heart rate monitoring. Forearm blood flow (FBF) was measured in both forearms by strain gauge venous plethysmography (EC-6, DE Hokanson, Inc). Before FBF measurement, simultaneous arterial and venous samples for t-PA and plasminogen activator inhibitor (PAI)-1 antigen concentrations were collected before and after each infusion of the study drug. Each dose was infused for 15 minutes. Infusions were interrupted during arterial sampling.

Plasma concentrations of t-PA and PAI-1 antigen were determined by ELISA (Tecnoclone GmbH). All of the samples were assayed in duplicate on the same test plate. Intra-assay and interassay coefficients of variation were <10%. Coefficient of variation for FBF repeated measurements in our laboratory is <10%.9

Experimental Design

Relationship Between Adrenergic Stimuli and Acute t-PA and PAI-1 Release in Normotensive Subjects and Essential Hypertensive Patients

t-PA and PAI-1 release in response to intra-arterial administration of 2 doses of epinephrine (0.1 and 0.3 μg/100 mL per minute) were measured in 18 normotensive and 16 essential hypertensive subjects. Intra-arterial ouabain (0.3 and 0.7 μg/100 mL per minute), a vasoconstrictor compound that acts via direct hyperpolarization of vascular smooth cells,15 was used as a control. Epinephrine and ouabain were infused in random order and each dose for a duration of 15 minutes.

NO Contribution to Adrenergic-Mediated t-PA Release in Normotensive Subjects and Essential Hypertensive Patients

To evaluate the contribution of NO to adrenergic-mediated t-PA release, another group of 12 normotensive subjects and 12 hypertensive patients was subjected to infusion of epinephrine (0.1 μg/100 mL per minute) both in the absence and presence of the NO synthase (NOS) inhibitor Nω-monomethyl-L-arginine (t-NMMA; 100 μg/100 mL per minute). Because t-NMMA reduces blood flow, the effect of epinephrine was evaluated in presence of the NO clamp, which allows assessment of endothelial agonists in the presence of NO synthase blockade without change in basal blood flow, thus avoiding any perturbation that could alter net-forearm t-PA release. Briefly, after 10 minutes of t-NMMA infusion, sodium nitroprusside was coinfused at an adjusted dose (0.3 and 0.4 μg/100 mL per minute) to restore the FBF reduced by t-NMMA infusion to baseline, as described previously in detail.18 Because sodium nitroprusside is an exogenous NO donor acting directly on vascular smooth muscle cells,20 it does not stimulate t-PA release from endothelial cells in vivo.21

Receptor Characterization of Adrenergic-Mediated t-PA Release in Normotensive Subjects

To characterize the receptor subtype involved in the adrenergic-induced t-PA release, in another group of 12 normotensive subjects, epinephrine (0.1 μg/100 mL per minute) was infused either in the presence of phentolamine (8 μg/100 mL per minute) or of propanolol (10 μg/100 mL per minute), an α- and β-receptor blocker, respectively. Each drug was preinfused for 10 minutes in random order. The effectiveness of these infusion rates was validated in previous studies.22,23

Effect of Selective β-Receptor Stimulation and NO Contribution on t-PA Release in Normotensive Subjects and Essential Hypertensive Patients

To confirm the role of β-receptors in endothelial t-PA release, in another group of 16 normotensive subjects and 16 hypertensive patients, isoproterenol (0.03 μg/100 mL per minute), a β-selective agonist, was infused in the absence and presence of t-NMMA, using the NO clamp technique. Isoproterenol was infused for 15 minutes. To exclude the possible confounding effect of flow increase, a dose-response curve to intra-arterial sodium nitroprusside (0.5 to 1.0 μg/100 mL per minute), a direct smooth muscle cell relaxant, was performed.

Data Analysis

Forearm plasma flow was determined by FBF and hematocrit. Net forearm release or uptake rates for t-PA and PAI-1 were calculated by the following formula: net release = (Cv–Ca)×(FBF×Cv–Ca)×100g/100 mL per minute), a direct smooth muscle cell relaxant, was performed.

Drugs

Epinephrine (Biologici Italia), isoproterenol (Abbott), t-NMMA (Clinalfa AG), sodium nitroprusside (Malesci SpA), ouabain
**Table. Clinical Characteristics of Study Group**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normotensive Subjects (n=58)</th>
<th>Hypertensive Patients (n=44)</th>
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<tr>
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<td>Plasma vitamin B₁₂, pg/mL</td>
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<td>299.1±60.4</td>
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<tr>
<td>CRP, mg/dL</td>
<td>3.1±0.9</td>
<td>2.8±0.8</td>
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</table>

Data are presented as means±SDs or number of subjects, unless otherwise specified. SBP indicates systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein.

*P<0.01 vs normotensive subjects.

(Clinalfa AG), propanolol (Astra Zeneca), and phentolamine (Novartis) were obtained from commercially available sources and diluted to the desired concentration by the addition of normal saline. Sodium nitroprusside was dissolved in 5% glucose solution and protected from light by aluminum foil.

**Results**

Clinical characteristics of the study population are shown in Table. Groups were similar in characteristics except for systolic and diastolic blood pressure values, which were significantly higher in the hypertensive patients. No changes in intra-arterial blood pressure or heart rate were observed during intrabrachial drug infusion (data not shown).

**Adrenergic Stimuli and Acute t-PA and PAI-1 Release in Normotensive Subjects and Essential Hypertensive Patients**

Normotensive subjects showed a dose-dependent vasoconstriction in response to epinephrine that was significantly lower than that observed in the hypertensive patients (Figure 1A). In contrast, vasoconstriction to ouabain was similar in the 2 groups (Figure 1B).

At baseline, arterial and venous concentrations of t-PA were significantly (P<0.05) lower in hypertensive patients than in the normotensive subjects (arterial: 0.5±0.1 versus 3.7±0.3 ng/mL; venous: 0.5±0.1 versus 3.8±0.2 ng/mL). In the normotensive group, net t-PA release significantly increased during epinephrine infusion, a maximal effect being observed with the lower dose of the agonist (Figure 1C). By contrast, in hypertensive patients, t-PA release was reduced, reaching a peak effect with the higher dose of epinephrine (Figure 1C). Accordingly, the total amount of t-PA released was significantly lower in hypertensive patients as compared with control subjects (1.2±0.4 versus 4.3±1.7 ng/100 mL of forearm tissue; P<0.01). No significant difference in net t-PA release was recorded in either group after ouabain infusion (Figure 1D). At baseline, arterial and venous concentrations of PAI-1 antigen were similar in normotensive subjects and hypertensive patients (arterial: 29.8±4.8 versus 27.6±5.2 ng/mL; venous: 30.0±5.0 versus 27.7±5.7 ng/mL). No significant difference in net PAI-1 release after epinephrine infusion was noted in either the normotensive subjects (from 0.5±0.2 to 0.7±0.3 ng/min per 100 mL of forearm tissue) or the hypertensive patients (from 0.7±0.3 to 0.6±0.2 ng/min per 100 mL of forearm tissue). Similarly, no significant changes in t-PA release were observed after ouabain infusions in both normotensive (from 0.8±0.3 to 0.9±0.4 ng/min per 100 mL of forearm tissue) and hypertensive (from 0.8±0.3 to 1.0±0.5 ng/min per 100 mL of forearm tissue) groups.

**NO Contribution to Adrenergic-Mediated t-PA Release in Normotensive Subjects and Essential Hypertensive Patients**

In normotensive subjects, a lower vasoconstriction to intrabrachial epinephrine as compared with hypertensive patients was observed (Figure 2A and 2B). Vasoconstriction in response to epinephrine in normotensive subjects was increased by the NOS blockade with L-NMMA, which significantly reduced basal FBF (Figure 2A). In hypertensive patients, L-NMMA infusion, which reduced basal FBF insignificantly, did not modify epinephrine-induced vasoconstriction (Figure 2B).

In normotensive subjects, the lower dose of epinephrine induced a significant increase in t-PA release (Figure 2C). Moreover, the NOS blockade with L-NMMA decreased basal t-PA release (from 0.2±0.1 to −0.1±0.0 ng/min per 100 mL of forearm tissue; P<0.05) and significantly blunted epinephrine-induced t-PA release (Figure 2C). In contrast, epinephrine-induced t-PA release in hypertensive patients was significantly lower as compared with normotensive subjects (P<0.01), and L-NMMA coinfusion failed to affect either constitutive (from 0.1±0.0 to 0.1±0.1 ng/min per 100 mL of forearm tissue) or stimulated t-PA release (Figure 2D).

**Receptor Characterization of Adrenergic-Mediated t-PA Release in Normotensive Subjects**

In the group of normotensive subjects, a nonsignificant vasoconstriction in response to the lower dose of epinephrine was observed (Figure 3A). Phentolamine preinfusion significantly increased FBF (Figure 3A). In the presence of α-blockade by phentolamine, epinephrine induced a significant vasodilation (Figure 3A). By contrast, in the presence of β-blockade with propanolol, which failed to significantly affect FBF when preinfused, epinephrine induced a significant vasoconstrictor effect (Figure 3B).

Intrabrachial infusion of the lower dose of epinephrine significantly increased net t-PA release, a response not affected by phentolamine coinfusion (Figure 3C). In contrast, epinephrine-induced t-PA release was abolished in the presence of propanolol (Figure 3D).
Effect of Selective β-Receptor Stimulation and NO Contribution on t-PA Release in Normotensive Subjects and Essential Hypertensive Patients

Normotensive subjects exhibited vasodilation to isoproterenol similar to that observed in hypertensive patients (Figure 4A and 4B). In healthy subjects, vasodilation to isoproterenol was significantly reduced by L-NMMA infusion (Figure 4A), whereas in hypertensive patients L-NMMA infusion only caused nonsignificant reduction in vasodilation to isoproterenol (Figure 4B). Vascular response to sodium nitroprusside was found to be similar in the 2 groups (normotensive subjects: FBF from 3.5±0.2 to 18.2±0.9 mL/min per 100 mL of forearm tissue; hypertensive patients: FBF from 3.5±0.3 to 18.4±0.6 mL/min per 100 mL of forearm tissue; P value not significant).

In normotensive subjects, isoproterenol induced a significant increase in net t-PA release versus baseline (Figure 4C). L-NMMA infusion, which reduced constitutive t-PA release (from 0.2±0.1 to −0.2±0.0 ng/min per 100 mL of forearm tissue; P<0.05), significantly blunted isoproterenol-induced t-PA release (Figure 4C). By contrast, in hypertensive patients, isoproterenol-induced t-PA release was significantly reduced as compared with normotensive subjects, and L-NMMA infusion failed to significantly affect both constitutive (0.2±0.0 to 0.1±0.0 ng/min per 100 mL of forearm tissue) and stimulated t-PA release (Figure 4D). No significant increase in t-PA release was observed during sodium nitroprusside infusion (from 0.2±0.1 to 0.3±0.1 ng/min per 100 mL of forearm tissue). In both groups, contralateral FBF and venous-arterial concentrations of t-PA remained unchanged throughout each protocol (data not shown).

Discussion

The main finding of the present study is that, in physiological conditions, adrenergic stimulation induces t-PA release via the activation of β-adrenergic–mediated NO pathway. Contrastingly, in hypertensive patients, β-adrenergic activation of the t-PA/NO pathway is impaired, an alteration potentially involved in the hypofibrinolytic state characterizing this clinical condition.4,6

In normotensive subjects, epinephrine infusion caused a dose-dependent vasoconstriction and a net release of t-PA but not of its inhibitor PAI-1. In the same experimental conditions, the infusion of ouabain, despite inducing a similar vasoconstriction, failed to increase the release of t-PA and PAI-1. Taken together, these results indicate that epinephrine- and possibly adrenergic-mediated t-PA release in healthy subjects is a specific and flow-independent endothelial property.

In normotensive subjects, vasoconstriction to epinephrine was significantly lower as compared with hypertensive patients, in line with the previous findings of an imbalance of adrenoreceptors in essential hypertension.25,26 Although epinephrine is a potent nonselective adrenergic agonist with a higher affinity for β-adrenoceptors, it induces vasoconstriction when infused locally.27 This effect, related to the higher
density of α-receptors in peripheral arterioles, is part of the hemostatic effect of epinephrine, which is also mediated via platelet aggregation and the increase of several clotting factors. The present results show that epinephrine also participates in the modulation of local fibrinolysis via the activation of t-PA release in the microcirculation of healthy subjects.

Epinephrine-induced t-PA release was blunted in hypertensive patients, a finding that strongly reinforces the concept of impaired t-PA release in essential hypertension, as reported previously with different stimuli, such as desmopressin, substance P, and acetylcholine. This is a crucial issue because only t-PA acutely released and incorporated into the growing thrombus effectively activates plasminogen to plasmin, thereby being protected from its main circulating inhibitor PAI-1.

The major novel finding of the present study is the demonstration that epinephrine-induced t-PA release is mediated by the activation of the NO pathway. The inhibition of NO with L-NMMA significantly reduced basal and epinephrine-induced t-PA release in normotensive subjects, thereby confirming a positive modulating effect of NO on both tonic and stimulated t-PA release in healthy conditions.

The NO pathway also plays a crucial role in modulating the vascular effects of epinephrine in healthy conditions. In normotensive subjects, L-NMMA, an NOS inhibitor, decreased basal flow and accentuated the vasoconstriction in response to epinephrine. The latter effect is probably related to the NO activation induced by the β-adrenergic component of the epinephrine response.

By contrast, no significant modification in either tonic or epinephrine-induced t-PA release was observed during L-NMMA coinfusion in hypertensive patients. Moreover, in hypertensive patients, basal vasoconstriction to L-NMMA was reduced, and the inhibition of NOS did not have any effect on the vascular response to epinephrine. Taken together, these findings confirm the presence of impaired NO availability in essential hypertension, which accounts for the reduced endothelial t-PA release capacity in this clinical condition, as already documented with different stimuli.

To elucidate the receptor subtype involved in the modulation of fibrinolysis by adrenergic stimuli, we assessed the effect of epinephrine on t-PA release in the presence of either phentolamine, an α-antagonist, or propanolol, a β-antagonist, in a group of normotensive subjects. The results show that α-blockade, which induced significant vasodilation to epinephrine, failed to affect t-PA release.

In contrast, the blockade of β-adrenoceptors with propanolol, which potentiated vasoconstriction to epinephrine, significantly blunted t-PA release after epinephrine infusion. These findings support the possibility that epinephrine-induced t-PA release across the forearm microcirculation is mediated by β-adrenoceptors. A final demonstration is provided by the study with the selective β-adrenergic agonist isoproterenol. In normotensive subjects isoproterenol caused vasodilation and a parallel t-PA release. Note that both effects were blunted by L-NMMA. In hypertensive patients, whereas vasodilation to isoproterenol was similar to that of controls, but unaffected by L-NMMA coinfusion, β-adrenoceptor–induced t-PA release was still present but significantly reduced.
The results of the present study are in contrast with those of a previous study in which a lacking effect of NOS inhibition on bradykinin-induced t-PA release was reported. Indeed, in this study, L-NMMA infusion failed to significantly affect vasodilation to bradykinin in healthy subjects. Therefore, an incomplete endothelial NOS inhibition, which may account for the lacking effect on t-PA release, cannot be excluded.

The discrepancy between the vascular and fibrinolytic effects of isoproterenol observed in hypertensive patients requires further explanation. It is conceivable that, whereas mechanisms compensating for the reduced NO availability, eg, hyperpolarization, may account for the similar degree of vasodilation to bradykinin in healthy subjects, Therefore, an incomplete endothelial NOS inhibition, which may account for the lacking effect on t-PA release, cannot be excluded.

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The major limitation of the present in vivo clinical study concerns the assessment of dynamic endothelial t-PA release in the forearm. Indeed, although this vascular district is less susceptible to atherothrombosis, it is less invasive and is considered a valid surrogate of the coronary circulation. Moreover, the dynamic assessment of t-PA release across the entire vascular forearm bed does not necessarily reflect the local capacity for t-PA release at the site of developing thrombus.

In the present study we did not measure t-PA and PAI-1 activity or fibrin degradation products to estimate the activity of the fibrinolytic system. However, several findings indicate that, in the presence of no demonstrable release of PAI-1 antigen across the forearm, as in our experimental conditions, t-PA antigen concentration increases in parallel with t-PA activity.

**Perspectives**

The results of the present study demonstrate that adrenergic-induced t-PA release is mediated by β-adrenoreceptors via a noncompensatory endothelial-derived hyperpolarizing factor pathway. According to our findings, 5,6-epoxyeicosatrienoic acid–mediated t-PA release was not affected by the ouabain-dependent part of the endothelial-derived hyperpolarizing factor pathway.

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mechanism that involves the NO pathway. In essential hypertension, the reduced NO availability impairs adrenergic-mediated t-PA release, possibly playing a crucial role in determining the hypofibrinolytic state characterizing this clinical condition. The reduced dynamic endothelial t-PA release could be part of a generalized endothelial dysfunction that characterizes essential hypertensive patients and contributes to the risk of atherothrombotic events.7,41 Thus, understanding the mechanisms underlying stimulated t-PA release could lead to specific therapeutic strategies to improve endothelial fibrinolytic function and possibly reduce cardiovascular risk in essential hypertension.

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Disclosures

None.

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