Bradykinin Stimulates Tissue Plasminogen Activator Release From Human Forearm Vasculature Through B₂ Receptor–Dependent, NO Synthase–Independent, and Cyclooxygenase-Independent Pathway

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Background—Bradykinin stimulates dose-dependent tissue plasminogen activator (tPA) release from human endothelium. Although bradykinin is known to cause vasodilation through B₂ receptor–dependent effects on NO, prostacyclin, and endothelium-derived hyperpolarizing factor production, the mechanism(s) underlying tPA release is unknown.

Methods and Results—We measured the effects of intra-arterial bradykinin (100, 200, and 400 ng/min), acetylcholine (15, 30, and 60 μg/min), and nitroprusside (0.8, 1.6, and 3.2 μg/min) on forearm vasodilation and tPA release in healthy volunteers in the presence and absence of (1) the B₂ receptor antagonist HOE 140 (100 μg/kg IV), (2) the NO synthase inhibitor L-N²-monomethyl-L-arginine (L-NMMA, 4 μmol/min intra-arterially), and (3) the cyclooxygenase inhibitor indomethacin (50 mg PO TID). B₂ receptor antagonism attenuated vasodilator (P=0.004) and tPA (P=0.043) responses to bradykinin, without attenuating the vasodilator response to nitroprusside (P=0.36). L-NMMA decreased basal forearm blood flow (from 2.35±0.31 to 1.73±0.22 mL/min per 100 mL, P=0.01) and blunted the vasodilator response to acetylcholine (P=0.013) and bradykinin (P=0.07, P=0.038 for forearm vascular resistance) but not that to nitroprusside (P=0.47). However, there was no effect of L-NMMA on basal (P=0.7) or bradykinin-stimulated tPA release (P=0.45). Indomethacin decreased urinary excretion of the prostacyclin metabolite 2,3-dinor-6-keto-prostaglandin F₁α (P=0.04). The vasodilator response to endothelium-dependent (P=0.019 for bradykinin) and endothelium-independent (P=0.019) vasodilators was enhanced during indomethacin administration. In contrast, there was no effect of indomethacin alone (P=0.99) or indomethacin plus L-NMMA (P=0.36) on bradykinin-stimulated tPA release.

Conclusions—These data indicate that bradykinin stimulates tPA release from human endothelium through a B₂ receptor–dependent, NO synthase–independent, and cyclooxygenase-independent pathway. Bradykinin-stimulated tPA release may represent a marker for the endothelial effects of endothelium-derived hyperpolarizing factor. (Circulation. 2000;102:2190-2196.)

Key Words: bradykinin ■ endothelium ■ endothelium-derived factors ■ nitric oxide synthase ■ plasminogen activators

Bradykinin (BK) is a vasoactive polypeptide that exhibits cardioprotective effects. BK promotes vasodilation, exerts antiproliferative effects, inhibits thrombin-induced platelet activation, and stimulates tissue plasminogen activator (tPA) release from endothelium, where it is stored in small dense granules. Recent data indicate that BK stimulates tPA release from the human forearm vasculature in a dose-dependent manner. The finding that neither acetylcholine (ACh) nor sodium nitroprusside (SNP) stimulated tPA release under the same experimental conditions suggests that this effect of BK is specific and flow independent. Although BK is known to cause vasodilation through the B₂ receptor and through its effects on NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF), the mechanism underlying stimulation of tPA release has not been studied.

The purpose of the present study was to elucidate the mechanism(s) of BK-stimulated endothelial release of tPA from human vasculature. To assess whether BK stimulates tPA release through its B₂ receptor, we measured the effect of intra-arterial BK on tPA release from the forearm vasculature of healthy volunteers in the presence and absence of the specific B₂ receptor antagonist HOE 140. To assess the contribution of NO to BK-stimulated tPA release, we measured the effect of BK in the presence and absence of the NO synthase (NOS) inhibitor L-N²-monomethyl-L-arginine (L-NMMA). Finally, to evaluate the contribution of prostacyclin to BK-stimulated tPA release, we compared the effect of...
intra-arterial BK in the presence and absence of the cyclooxygenase (COX) inhibitor indomethacin.

Methods

Subjects
Eighteen healthy subjects were studied; 8 subjects participated in more than one protocol (1 subject participated in protocols 1 and 3, and 7 subjects participated in protocols 2 and 3). Informed consent was obtained. The study was approved by the Vanderbilt University Institutional Review Board and conducted according to the Declaration of Helsinki. Each subject underwent a history, physical examination, ECG, and routine laboratory analysis. None had evidence of hypertension, hyperlipidemia, cardiovascular disease, or other systemic conditions. All were nonsmokers. Twelve men and 6 women were studied. Eleven subjects were white, and 7 were African American. We have observed previously that ethnicity does not affect the tPA response to BK (authors’ unpublished data, 2000). The mean age was 28.9±2.1 years; mean body mass index was 26.5±0.8 kg/m². Mean arterial pressure (MAP) and heart rate (HR) were 84.9±1.7 mm Hg and 60.4±2.2 bpm, respectively. All subjects were studied under salt-replete conditions.

Experimental Protocol
Studies were performed in the morning in a temperature-controlled room. Subjects were studied while supine and fasted. Intravenous catheters were placed in the antecubital veins in both arms. After subdural administration of 1% lidocaine, a 20-gauge polyurethane catheter (Cook Inc) was inserted into the brachial artery of the nondominant arm for intra-arterial administration of drugs. Before infusion of vasoactive drugs, arterial catheter patency was maintained by infusion of 5% dextrose in water at a rate of 1 mL/min. After the placement of intravenous and intra-arterial catheters, subjects were allowed to rest 30 minutes before baseline measurements were made. Blood pressure was monitored in the contralateral arm with an automated blood pressure cuff throughout the study. After measurement of basal forearm blood flow (FBF) and blood sampling, graded doses of SNP (Gensia Siccor Pharmaceuticals), ACh (Miochol-E, Ciba Vision), or BK (Calbiochem, sterilized and acidified sodium tetraborate pentahydrate) were infused in random order according to the protocols described below. SNP was infused at 0.8, 1.6, and 3.2 μg/min; ACh was infused at 15, 30, and 60 μg/min; and BK was infused at 100, 200, and 400 ng/min. During the highest dose of BK, forearm venous BK concentrations of ~500 fmol/mL are achieved. Each dose was infused for 5 minutes, and FBF was measured during the last 2 minutes. Drug concentrations in the infusate were adjusted to maintain infusion volumes at 1 mL/min. Before infusion of each drug, the 30-minute rest period and basal measurements were repeated.

Forearm Perfusion Measurements
FBF was measured by Silastic-in- mercury strain-gauge plethysmography. The wrist was supported in a sling to raise the forearm above the level of the atrium, and the strain gauge was placed at the widest part of the forearm. The strain gauge was connected to a chart recorder. 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) to occlude venous outflow from the extremity. The hand was excluded from the measurement of blood flow by inflation of a pediatric sphygmomanometer cuff around the wrist to 200 mm Hg before and during measurements of FBF. Flow measurements were recorded for ~7 of 15 seconds, and the slope was derived from the first 3 or 4 pulses; 5 to 7 such readings were obtained for each mean value. Forearm vascular resistance (FVR) was calculated as MAP/FFB.

Protocol 1: Effect of BK Receptor Antagonism
SNP and BK were administered intra-arterially in random order according to the general protocol (n=9). Thirty minutes after administration of the vasodilators, subjects were given 100 μg/Kg IV HOE 140 (gift from Hoechst, Frankfurt, Germany) over 1 hour in the contralateral arm. We and others have shown that this dose inhibits the forearm vasodilator response to intra-arterial BK without affecting systemic blood pressure or HR.9,10 Thirty minutes after completion of the HOE 140 infusion, intra-arterial infusions of SNP and BK were repeated. Pilot studies demonstrated that there was no tachyphylaxis to BK over this time interval.

Protocol 2: Effect of NOS Inhibition
ACh, SNP, and BK were administered intra-arterially in random order according to the general protocol (n=9). Thirty minutes after infusion of the last vasodilator, baseline measurements were repeated. L-NMMA (Clinalfa AG) was then infused continuously at a rate of 4 μmol/min. This dose has been shown to blunt the endothelium-dependent vasodilator response to ACh and BK in the human vasculature.11 After 25 minutes, measurement of baseline FBF and blood sampling was repeated, and ACh, SNP, and BK infusions were repeated in the presence of L-NMMA.

Protocol 3: Effect of COX Inhibition
Twelve subjects participated for 2 study days. Before each study day, subjects were given either placebo or indomethacin (50 mg PO TID) in identical-looking capsules for 3 days in random order. This dose of indomethacin inhibits the prostacyclin response to systemic BK administration in humans.12 Urine concentrations of the stable metabolite of prostacyclin, 2,3-dinor-6-keto-prostaglandin F1α, were measured during placebo and indomethacin administration to ensure adequate inhibition of COX. On each study day, ACh, SNP, and BK were administered intra-arterially in random order according to the general protocol. In 7 subjects, intra-arterial infusions were repeated during L-NMMA administration, as outlined above, during both placebo and indomethacin study days.

Blood Sampling and Biochemical Assays
After measurement of FBF, simultaneous arterial and venous samples were obtained from the infused arm before and after each dose of study drug. Infusions were interrupted during arterial sampling. All samples were obtained after the first 3 mL of blood was discarded. Blood samples were collected on ice and centrifuged immediately, and plasma was stored at ~70°C until the time of assay. Blood for measurement of plasminogen activator inhibitor-1 (PAI-1) and tPA was collected in tubes containing 0.105 mol/L acidified sodium citrate, and antigen levels were determined by using a 2-site ELISA (Biopool AB) as previously described.13 tPA activity was measured by using a commercially available assay (Biopool AB) during 6 BK infusions in 3 randomly selected subjects (2 infusions per subject). Because changes in tPA activity paralleled changes in tPA antigen, only tPA antigen was measured in the remainder of the subjects and is reported in the present study.

Arteriovenous concentration gradients were calculated by subtracting the plasma level measured in simultaneously collected venous and arterial blood. Forearm plasma flow was calculated from the FBF and arterial hematocrit corrected for 1% trapped plasma. Thus, individual net release or uptake rates at each time point were calculated by the following formula: net release = (Cv−Ca)*[ (FBF×(101−hematocrit/100)), where Ca and Cv represent the concentration of tPA in the brachial vein and artery, respectively.

Statistical Analysis
Data are presented as mean±SEM. Because there was no effect of HOE 140, L-NMMA, or indomethacin on systemic blood pressure, data are presented in terms of FBF. Where statistical significance is marginal, analysis using FVR is also provided. Comparisons between drugs were made by ANOVA with repeated measures in which the within-subject variables were drug and dose increment.
When appropriate, data were logarithmically transformed before analysis. Post hoc comparisons were made by the paired t test or Wilcoxon signed rank test, as appropriate. A 2-tailed value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Effect of BK Subtype B2 Receptor Blockade**

Intra-arterial infusion of both BK (\( P = 0.011 \)) and SNP (\( P = 0.003 \)) caused significant dose-dependent increases in FBF, and the vasodilator response to the 2 drugs was not significantly different (\( P = 0.11 \), Figure 1). As reported previously, intra-arterial administration of BK significantly increased net tPA release across the forearm (\( P = 0.004 \)), whereas there was no effect of SNP on tPA release (\( P = 0.7 \)). Neither agonist altered net PAI-1 extraction across the forearm (\(-1.0 \pm 0.3 \text{ ng/min per 100 mL at 400 ng/min BK versus } -0.1 \pm 1.4 \text{ ng/min per 100 mL at baseline}\)). There was no effect of the BK receptor antagonist HOE 140 on basal MAP (\( P = 0.66 \)), HR (\( P = 0.35 \)), FBF (\( P = 0.69 \)), or net tPA release (\( P = 0.08 \)). Coadministration of HOE 140 significantly attenuated the FBF response to BK (effect of HOE 140 \( P = 0.004 \)) but not that to SNP (\( P = 0.36 \)). The BK receptor antagonist HOE 140 effectively abolished the tPA response to BK (BK versus BK + HOE 140, \( P = 0.043 \)); thus, the tPA response to BK was no longer significant (\( P = 0.32 \)).

**Effect of NOS Inhibition**

Figure 2 shows the effect of L-NMMA on the FBF response to ACh, SNP, and BK. All 3 vasodilators caused a significant increase in FBF (\( P < 0.001 \) for each). As noted earlier, there was an increase in net tPA release across the forearm in response to BK (\( P < 0.001 \), Figure 3A), without a concomitant change in PAI-1 (net PAI-1 extraction \(-7.4 \pm 8.6 \text{ ng/min per 100 mL at the 400 ng/min dose versus } -0.8 \pm 0.8 \text{ ng/min per 100 mL at baseline}\)). There was no effect of intra-arterial infusion of L-NMMA on baseline MAP (\( P = 0.920 \)), HR (\( P = 0.2 \)), or net tPA release (\( P = 0.7 \)). However, treatment with L-NMMA significantly decreased basal FBF (1.73 \pm 0.22 versus 2.35 \pm 0.31 \text{ mL/min per 100 mL in the absence of L-NMMA, } P = 0.01 \). Treatment with L-NMMA blunted the vasodilator response to the endothelium-dependent vasodilators ACh (\( P = 0.013 \)) and BK (\( P = 0.07 \) for FBF, \( P = 0.038 \) for FVR) (Figure 2). There was no effect of L-NMMA on the vasodilator response to SNP (\( P = 0.47 \)). There was no effect of NOS inhibition on the tPA response to BK (effect of L-NMMA, \( P = 0.45 \); Figure 3A); thus, the effect of BK on net tPA release remained significant (\( P < 0.001 \)).

**Effect of COX Inhibition**

Treatment with indomethacin significantly decreased the urinary excretion of the stable metabolite of prostacyclin, 2,3-dinor-6-keto-prostaglandin F1\( \alpha \) (0.065 \pm 0.001 ng/mg creatinine versus 0.235 \pm 0.050 ng/mg creatinine during placebo, \( P = 0.04 \)). All 3 vasodilators significantly increased FBF (\( P < 0.001 \) for each). There was an increase in net tPA release increased net tPA release across the forearm (\( P = 0.004 \)}, whereas there was no effect of SNP on tPA release (\( P = 0.7 \)). Neither agonist altered net PAI-1 extraction across the forearm (\(-1.0 \pm 0.3 \text{ ng/min per 100 mL at 400 ng/min BK versus } -0.1 \pm 1.4 \text{ ng/min per 100 mL at baseline}\)). There was no effect of the BK receptor antagonist HOE 140 on basal MAP (\( P = 0.66 \)), HR (\( P = 0.35 \)), FBF (\( P = 0.69 \)), or net tPA release (\( P = 0.08 \)). Coadministration of HOE 140 significantly attenuated the FBF response to BK (effect of HOE 140 \( P = 0.004 \)) but not that to SNP (\( P = 0.36 \)). The BK receptor antagonist HOE 140 effectively abolished the tPA response to BK (BK versus BK + HOE 140, \( P = 0.043 \)); thus, the tPA response to BK was no longer significant (\( P = 0.32 \)).

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in response to BK \( (P<0.001, \text{Figure 3B}) \), without a change in PAI-1 (net PAI-1 extraction \( -9.9 \pm 7.7 \) ng/min per 100 mL during 400 ng/min BK versus \( -0.8 \pm 0.5 \) ng/min per 100 mL at baseline). There was no effect of indomethacin on baseline MAP \( (P=0.56) \), HR \( (P=0.16) \), FBF \( (P=0.08, P=0.12 \text{ for FVR}) \), or net tPA release \( (P=0.08) \). The vasodilator responses to both the endothelium-independent vasodilator SNP \( (P=0.019) \) and the endothelium-dependent vasodilator BK \( (P=0.019) \) were greater during indomethacin than during placebo (Figure 4). The effect of indomethacin on the vasodilator response to ACh \( (P=0.059 \text{ for FBF}, P=0.08 \text{ for FVR}) \) did not reach statistical significance. In contrast, there was no effect of treatment with indomethacin on the tPA response to BK \( (P=0.99, \text{Figure 3B}) \).

**Effect of Combined NOS and COX Inhibition**

As in the absence of indomethacin, coadministration of L-NMMA in the presence of indomethacin significantly decreased baseline FBF (from 2.85 \pm 0.35 to 1.91 \pm 0.23 mL/min per 100 mL, \( P=0.004 \)) but did not affect MAP \( (P=0.55) \), HR \( (P=0.19) \), or basal tPA release \( (P=0.36) \). The effect of L-NMMA on the vasodilator responses to ACh \( (effect \text{ of L-NMMA in the presence of indomethacin, } P=0.059) \) and BK \( (effect \text{ of L-NMMA in the presence of indomethacin, } P=0.038) \) was similar in the presence and absence of indomethacin. There was no effect of combined treatment with L-NMMA and indomethacin on the tPA response to BK \( (P=0.36, \text{Figure 3C}) \).

**Discussion**

The regulated release of tPA from the endothelium plays a major role in the defense against endogenous thrombosis, particularly in the coronary vasculature.\(^{14} \) tPA is synthesized by endothelial cells, stored in small dense granules, and released through a calcium-dependent and G protein–dependent pathway in response to thrombin and numerous agonists.\(^{5,15,16} \) Studies in animals suggest that BK is 1000-fold more potent than agonists such as histamine, norepinephrine, vasopressin, and ACh in stimulating the acute release of tPA from the vasculature.\(^{4} \) BK is produced locally through activation of the kallikrein-kinin system on the surface of endothelial cells.\(^{17} \) BK is known to cause vasodilation through the B\(_2\) receptor and subsequent effects on NO, prostacyclin, and EDHF production.\(^{1} \) Data from the present study indicate that the effect of BK on endothelial tPA release is also mediated through activation of the B\(_2\) subtype receptor but occurs through a NOS- and COX-independent pathway.

Over the last 8 years, the availability of a sensitive and specific BK B\(_2\) receptor antagonist, HOE 140,\(^{7} \) has permitted elucidation of the physiology of the endogenous kallikrein-kinin system and, in particular, of the contribution of BK to the cardioprotective effects of ACE inhibitors. Hence, it is now well established that BK causes vasodilation and natriuresis and exerts anti-ischemic, anti-atherosclerotic, and anti-inflammatory effects through the activation of the B\(_2\) receptor.\(^{18,19} \) Endogenous BK contributes to the favorable effects of ACE inhibitors on blood pressure, on ischemic preconditioning, and on myocardial remodeling after infarction.\(^{20} \) The present study extends these observations on the role of BK in vascular physiology. As reported previously in humans,\(^{8} \) we observed that B\(_2\) receptor antagonism specifically blocked BK-induced vasodilation; in addition, we observed that B\(_2\) receptor antagonism blocked the effect of BK on tPA release from the forearm vascular endothelium.

Because treatment with HOE 140 antagonized the vasodilator and the tPA response to BK simultaneously, we...
cannot exclude the possibility that the effect of HOE 140 on BK-induced tPA release was secondary to decreased flow. However, several lines of evidence suggest that the effect of BK on tPA release is not flow dependent. First, in this and previous studies, it has been consistently demonstrated that SNP does not induce tPA release at doses that increase FBF and shear stress.6,21,22 Second, treatment with L-NMMA and indomethacin selectively modulated vasodilation without altering the tPA response to BK. Regardless, the finding that B2 receptor antagonism blocks the effects of exogenous BK on tPA release from the human vasculature lays the groundwork for studies elucidating the contribution of endogenous BK to the effects of ACE inhibitors on endothelial tPA release in humans. Recently, ACE inhibition has been shown to potentiate the tPA response to exogenous BK but not substance P.23

In the present study, intra-arterial administration of the NOS inhibitor L-NMMA decreased basal FBF and blunted the vasodilator response to the endothelium-dependent vasodilators ACh and BK but not to the endothelium-independent vasodilator SNP. This is consistent with data from previous studies demonstrating that NOS inhibition decreases basal FBF and attenuates the FBF response to BK.11,24 In contrast, coadministration of L-NMMA had no effect on the tPA response to BK when administered either alone or in combination with indomethacin. This finding conflicts with the observations of Newby et al,25 who reported that coadministration of L-NMMA attenuated the tPA response to another agonist, substance P; however, Tranquille and Emeis26 observed no effect of N6-nitro-L-arginine on BK-stimulated tPA release from rat vasculature. To the extent that substance P and BK activate unique receptors and hyperpolarize endothelial cells through distinct intracellular pathways,27 it is possible that these 2 agonists stimulate tPA release through different mechanisms. On the other hand, an NO–independent effect of BK on tPA release is further supported by the finding in this and numerous other studies that SNP, an NO donor, does not stimulate endothelial tPA release.6,21,22

Studies in vitro and in vivo suggest that prostaglandins do not contribute significantly to the vasodilator response to BK in human vasculature. Thus, pretreatment with COX inhibitors such as aspirin or indomethacin does not alter vasodilation to BK in isolated coronary or resistance arteries.28–30 Similarly, previous studies have demonstrated that the forearm and systemic vasodilator responses to BK in humans are not mediated by prostaglandin release.12,31

The present study also does not support a significant contribution of prostacyclin to the vasodilator response to BK. To the contrary, pretreatment with indomethacin resulted in a significantly increased vasodilator response not only to endothelium-dependent vasodilator BK but also to SNP, suggesting a generalized effect on vascular smooth muscle relaxation. The effect of COX inhibition on agonist-induced vasodilation depends on the relative balance between vasodilating prostanoids such as prostacyclin and vasoconstricting eicosanoids such as prostaglandin F2α and thromboxane A2.32 In addition, COX-dependent endothelium-derived factors such as superoxide anion cause vasoconstriction by decreasing the half-life of NO.33 The finding that the FBF response to both endothelium-dependent and -independent vasodilators was increased during COX inhibition suggests that under the salt-replete conditions of the present study, COX-derived constricting factors predominated over vasodilating factors.

In contrast to the effect of indomethacin on agonist-induced vasodilation, there was no effect of COX inhibition on BK-stimulated tPA release. These data suggest that BK stimulates tPA release through a COX-independent pathway. This is consistent with the findings of van den Eijnden-Schrauwen et al,15 who have reported that thrombin-stimulated tPA release in human umbilical vein endothelial cells is not blocked by 5,8,11,14-eicosatetraenoic acid or indomethacin at doses that completely inhibited thrombin-induced prostacyclin synthesis. The lack of effect of indomethacin on tPA release despite an enhanced flow response to BK also provides further supportive evidence of a flow-independent effect of BK on tPA release.

The main finding of the present study, that combined treatment with L-NMMA and indomethacin did not alter the effect of BK on tPA release, suggests the hypothesis that BK stimulates tPA release through EDHF. EDHF causes endothelium-dependent NOS/COX inhibitor–insensitive hyperpolarization and relaxation of vascular smooth
muscle that is blocked by inhibitors of calcium-activated potassium channels such as charbydotoxin and apamin but not by inhibitors of ATP-sensitive potassium channels. The identity of EDHF may vary among vascular beds and species, mounting evidence suggests that EDHF is a cytochrome P450 epoxyenase-derived product of arachidonic acid. The contribution of EDHF to BK-stimulated vasodilation in human arteries in vitro has been demonstrated and recent work by Taddei et al indicates that prevention of hyperpolarization by ouabain attenuates the forearm vasodilator response to BK in hypertensive patients. However, ouabain acts by blocking Na+,K+-ATPase rather than by specifically inhibiting the effects of EDHF. Clearly, in vitro studies using calcium-dependent potassium-channel inhibitors are needed to explore the possibility that EDHF contributes to the NOS/COX-insensitive BK-stimulated release of tPA. Such studies could elucidate a new physiological role for EDHF and suggest a compensatory pathway whereby endothelial fibrinolytic function may be preserved in patients in whom NO availability is diminished.

Finally, pharmacological doses of BK were used in the present study to elucidate the mechanism of BK-induced tPA release. Venous concentrations of BK achieved during the highest dose are ~10-fold higher than those measured in patients taking ACE inhibitors and 2-fold higher than those measured in the coronary sinus of patients undergoing coronary angioplasty. In addition, although previous investigators have demonstrated that endogenous BK contributes to human coronary vasodilation, it is not possible to extrapolate findings in the forearm regarding the mechanism of BK-stimulated tPA release to the coronary vasculature. Additional studies are needed to assess the contribution of endogenous BK to tPA release in the coronary circulation.

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