Research Paper

Journal of Vascular Research

J Vasc Res 2014;51:200–208
DOI: 10.1159/000362666

Received: January 13, 2014
Accepted after revision: March 28, 2014
Published online: June 4, 2014

Endothelium-Derived Hyperpolarizing Factor Mediates Bradykinin-Stimulated Tissue Plasminogen Activator Release in Humans

Ayaz M. Rahman[a] Jonathan R. Murrow[a] Muhiddin A. Ozkor[a]
Nino Kavtaradze[a] Ji Lin[b] Christine De Staercke[c] W. Craig Hooper[c]
Amita Manatunga[b] Salim Hayek[a] Arshed A. Quyyumi[a]

[a]Division of Cardiology, Department of Medicine, Emory University School of Medicine, [b]Rollins School of Public Health, Emory University, and [c]Division of Blood Disorders, National Center on Birth Defects and Developmental Disabilities, Centers for Disease Control and Prevention, Atlanta, Ga., USA

Key Words
Bradykinin · Endothelium · Endothelium-derived hyperpolarizing factors · Fibrinolysis · Tissue plasminogen activator

Abstract
Aims: Bradykinin (BK) stimulates tissue plasminogen activator (t-PA) release from human endothelium. Although BK stimulates both nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) release, the role of EDHF in t-PA release remains unexplored. This study sought to determine the mechanisms of BK-stimulated t-PA release in the forearm vasculature of healthy human subjects. ***Methods:*** In 33 healthy subjects (age 40.3 ± 1.9 years), forearm blood flow (FBF) and t-PA release were measured at rest and after intra-arterial infusions of BK (400 ng/min) and sodium nitroprusside (3.2 mg/min). Measurements were repeated after intra-arterial infusion of tetraethylammonium chloride (TEA; 1 μmol/min), fluconazole (0.4 μmol·min^{-1}·l^{-1}), and N^{G}-monomethyl-L-arginine (L-NMMA, 8 μmol/min) to block nitric oxide, and their combination in separate studies. ***Results:*** BK significantly increased net t-PA release across the forearm ($p < 0.0001$). Fluconazole attenuated both BK-mediated vasodilation (–23.3 ± 2.7% FBF, $p < 0.0001$) and t-PA release (from 50.9 ± 9.0 to 21.3 ± 8.9 ng/min/100 ml, $p = 0.02$). TEA attenuated FBF (–14.7 ± 3.2%, $p = 0.002$) and abolished BK-stimulated t-PA release (from 22.9 ± 5.7 to –0.8 ± 3.6 ng/min/100 ml, $p = 0.0002$). L-NMMA attenuated FBF ($p < 0.0001$), but did not inhibit BK-induced t-PA release (nonsignificant). ***Conclusion:*** BK-stimulated t-PA release is partly due to cytochrome P_{450}-derived epoxides and is inhibited by K^{+}_{Ca} channel blockade. Thus, BK stimulates both EDHF-dependent vasodilation and t-PA release.

Introduction

The endogenous fibrinolytic system is a crucial component of blood flow regulation and protects against intravascular thrombosis. Impairment in endogenous fibrinolysis characterized by inhibition of tissue plasminogen activator (t-PA) release is thought to be an underlying factor in endothelial dysfunction and atherothrombosis [1, 2].

Clinical Trial Registration: http://clinicaltrials.gov/ct2/show/NCT00166166, ClinicalTrials.gov identifier: NCT00166166.

KARGER

E-Mail karger@karger.com
www.karger.com/jvr

1018–1172/14/0513–0200$39.50/0

Prof. Arshed A. Quyyumi
Emory University School of Medicine
1462 Clifton Road NE, Suite F506
Atlanta, GA 30322 (USA)
E-Mail aquyyum@emory.edu

The endothelium regulates vasodilator tone and fibrinolytic capacity by releasing vasoactive substances, including nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) [3, 4]. Bradykinin (BK) is an endogenous cardioprotective vasoactive polypeptide that stimulates the release of these molecules and t-PA from the endothelium [5–7]. t-PA is synthesized in endothelial cells, stored in small dense granules and released in a dose-dependent manner in response to BK [6, 8]. t-PA converts plasminogen to a proteolytic enzyme, plasmin, which digests fibrin-dependent thrombi [9]. Although it is known that BK causes vasodilation via activation of the B_2 receptor and subsequent release of NO, prostacyclin, EDHF, and t-PA, the downstream mechanisms governing t-PA release have not been fully elucidated [5, 10, 11].

There is mounting preclinical evidence to suggest that t-PA release is mediated by EDHF [12, 13]. Persistent vasodilation after inhibition of NO and cyclooxygenase can be largely attributed to hyperpolarization by EDHF [3, 14]. The hallmark of hyperpolarization is activation of endothelial and/or smooth muscle cell calcium-activated potassium (K^+_{Ca}) channels and their inhibition by apamin and charybdotoxin in preclinical studies [3, 15]. In human studies, tetraethylammonium chloride (TEA) antagonizes K^+_{Ca} channels, thus facilitating characterization of EDHF-dependent responses [16, 17]. Endothelium-dependent hyperpolarization may also be partly due to the release of epoxyeicosatrienoic acids (EETs) from cytochrome P_{450}-dependent metabolism of arachidonic acid and subsequent stimulation of K^+_{Ca} channels on endothelial cells [18]. The role of EETs as potential EDHFs has been investigated in humans using azole compounds, such as fluconazole, which selectively inhibit epoxidation (EET generation) of arachidonic acid [14, 18, 19].

Since BK-mediated t-PA release may be crucial in maintaining the endogenous thrombolytic potential, it is important to determine its underlying mechanisms. Our overall goal was to investigate whether EDHF release stimulates t-PA, with the hypothesis that BK stimulates EDHF-mediated t-PA release in the human circulation. We investigated whether activation of the K^+_{Ca} channels and/or cytochrome P_{450}-derived EETs mediates t-PA release.

Methods

Subjects

Thirty-three healthy subjects aged 21–60 years and free of smoking, hypercholesterolemia, hypertension, diabetes, cardiovascular disease, medication use, or any other systemic disorder participated in the study (ClincalTrials.gov Identifier: NCT00166166; table 1). Written informed consent was obtained and the study was approved by the Emory University Institutional Review Board and the Food and Drug Administration.

Table 1. Subject characteristics

Male, n	21 (63.6%)
Age, years	40.3±1.9
Ethnicity, n	
White	8 (24.2%)
Black	21 (64.3%)
Hispanic	4 (12.1%)
Blood pressure, mm Hg	
Systolic	121.6±2
Diastolic	73±1.4
Heart rate, b.p.m.	68±1.5
Body mass index	28±1
Fasting blood glucose, mg/dl	86.4±1.4
Triglycerides, mg/dl	102.4±9.5
Total cholesterol, mg/dl	170±6.4
High-density lipoprotein cholesterol, mg/dl	54.3±2.3
Low-density lipoprotein, mg/dl	95.3±5.7
Tobacco smoking, %	0
Hematocrit, %	39.4±0.6

Experimental Protocols

Subjects were enrolled into 3 separate protocols (described below) performed sequentially. Measurements were performed after an overnight fast in a quiet, temperature-controlled (22–24°C) room. Subjects received 975 mg of oral aspirin 90 min prior to initiation of the study to inhibit prostanoid release [20, 21]. An intravenous catheter was placed into a deep antecubital vein for venous sampling and an arterial cannula into the brachial artery for arterial pressure monitoring, drug delivery, and blood sampling.

FBF Measurements

Simultaneous FBF measurements were obtained in both arms with the use of a dual-channel venous occlusion strain gauge plethysmograph (model EC6; DE Hokanson, Bellevue, Wash., USA) as previously described [14]. The mercury-filled silastic strain gauge was placed around the forearm and connected to a plethysmograph calibrated to measure the percent change in volume [14]. An upper arm cuff was inflated to 40 mm Hg to occlude venous outflow and FBF measurements were recorded every 15 s up to eight times, and a mean FBF value (in $ml \cdot min^{-1} \cdot 100\ ml^{-1}$) was computed. Forearm vascular resistance was calculated as the mean arterial pressure divided by FBF (expressed as $mm\ Hg/ml \cdot min^{-1} \cdot 100\ ml^{-1}$).

Protocol 1: Effect of K^+_{Ca} Channel Activation on BK-Stimulated t-PA Release

FBF was measured after a 30-min rest during saline infusion (total infusion rate $2.5\ ml \cdot min^{-1}$) and after intra-arterial infusions of 100, 200, and 400 ng/min of BK (Clinalfa, Laufelfingen, Switzerland) given for 8 min each (n = 18). After 30 min, intra-arterial TEA (Sigma-Aldrich, Allentown, Pa., USA, sterilized and tested for pyrogenicity by the Emory Investigational Drug Pharmacy) was infused at 1 μmol/min for 8 min. When given at 0.25–1

mg/min, TEA selectively inhibits K^+_{Ca} channels, but reduces FBF with BK only at 1 mg/min (<0.6 μmol/min) [16, 22–24]. This dose and timing was effective in attenuating FBF and agonist-stimulated vasodilation in previous studies [4, 16, 25]. While continuing the infusion of TEA, BK infusions were re-administered. Arterial blood pressure and FBF measurements were repeated in the last 2 min of each infusion. Arterial and venous blood was sampled at baseline prior to the infusion of drug and at peak doses of BK.

Protocol 2: Effect of Cytochrome P_{450} Metabolites to BK-Stimulated t-PA Release

FBF was measured at rest during saline infusion and after intra-arterial infusion of escalating doses of BK (n = 11). Thirty minutes later, fluconazole (Pfizer, New York, N.Y., USA) was infused intra-arterially at 0.4 $\mu mol \cdot min^{-1} \cdot l^{-1}$ for 5 min. This dose has been effective in attenuating vasodilation during sustained flow conditions [4, 17]. While continuing the infusion of fluconazole, escalating doses of BK were administered. Finally, combined intra-arterial infusions of fluconazole and TEA were given for 8 min and BK infusions were repeated. This protocol allowed to assess the contribution of cytochrome P_{450} metabolites alone and in combination with K^+_{Ca} channel activation to BK-stimulated t-PA release.

Protocol 3: Effect of NO on BK-Stimulated t-PA release

The contribution of NO to BK-stimulated t-PA release was also determined in the presence of K^+_{Ca} channel activation (n = 13). FBF was measured at rest and after intra-arterial infusion of BK. Thirty minutes later, TEA (1 μmol/min) was infused for 8 min and followed by repeat infusions of BK. After 30 min, TEA and N^G-monomethyl-L-arginine (L-NMMA; Clinalfa) at 8 μmol/min were infused together for 8 min to inhibit EDHF and NO synthesis, followed by repeat administration of BK in escalating doses while continuing infusions of the two antagonists. This dose and timing of L-NMMA were effective in attenuating FBF and agonist-stimulated vasodilation in previous studies [16, 25].

Effect of Sodium Nitroprusside on t-PA Release

Finally, in 30 subjects, the comparative contribution of the endothelium-independent vasodilator sodium nitroprusside (SNP) and BK was investigated. FBF was measured during rest and after intra-arterial infusion of escalating doses of BK. Thirty minutes later, SNP (Hospira, Lakeforest, Ill., USA) was infused intra-arterially at 1.6 and 3.2 μg/min.

Blood Sampling and Biochemical Assays

Simultaneous arterial and venous blood samples were obtained from the infused arm before and after peak doses of vasodilator drugs, cooled, centrifuged, and stored in tubes containing 0.5 M citrate buffer (Diapharma, West Chester, Ohio, USA) at –70°C. t-PA antigen levels were determined in duplicate using a two-site enzyme-linked immunosorbent assay (Diapharma) as described previously [26]. Net release or uptake rates at each time point were calculated: net release = $(C_V - C_A) \times \{FBF \times [(101 - \text{hematocrit}) / 100]\}$, where C_V and C_A represent the concentrations of t-PA in the brachial vein and artery, respectively.

Statistical Analysis

To determine the effects of the interventions on FBF and forearm vascular resistance, changes in the flow and resistance responses between the control, single blockade, and combined blockade were compared using repeated-measures ANOVA. To determine the effects of treatments on net t-PA release, changes in net t-PA release of control, single blockade, and combined blockade were compared using one-way ANOVA with the Tukey post hoc test. Changes in the response were converted to percentage changes of least-square means when presented in the text. Data are presented as means ± SEM in the text. The least-square means and standard errors are presented in the figures. A value of $p < 0.05$ was considered statistically significant.

Results

Baseline Subject Characteristics

Thirty-three healthy (age 40.3 ± 1.9 years, 64% male) normotensive, nondiabetic, nonobese, nonsmoking subjects with normal serum cholesterol levels were studied (table 1). During intrabrachial drug infusions, no changes in blood pressure or heart rate were observed.

Effect of K^+_{Ca} Channel Activation on FBF and BK-Stimulated t-PA Release

BK produced a dose-dependent increase in FBF (p = 0.002) and a decrease in vascular resistance (p = 0.03; fig. 1a, b). BK significantly increased the arteriovenous t-PA concentration gradient and net t-PA release across the forearm (from –0.3 ± 0.5 to 36.9 ± 5.5 ng/min/100 ml, p = 0.002; fig. 1c; table 2). There was no association between net t-PA release and age, sex, body mass index, total cholesterol, and LDL levels.

Infusion of TEA decreased resting FBF by 14% (p = 0.0001) and increased vascular resistance by 18% (p = 0.03). Co-administration of TEA with BK attenuated the overall response with a 15% (p = 0.002) lower FBF and a 21% (p = 0.03) higher vascular resistance (fig. 1a, b). TEA infusion effectively abolished t-PA release with BK (from 22.9 ± 5.7 to –0.8 ± 3.6 ng/min/100 ml, p = 0.0002) and the arteriovenous t-PA concentration gradient (table 2), indicating that the effect of BK on endothelial t-PA release is mediated entirely through K^+_{Ca} channel activation (fig. 1c).

Effect of Cytochrome P_{450} Metabolites and K^+_{Ca} Channel Activation on FBF and BK-Stimulated t-PA Release

Infusion of fluconazole decreased resting FBF by 19% (p = 0.001) and increased vascular resistance by 27% (p = 0.001). Fluconazole also blunted the overall vasodilator response to BK with a 23% (p < 0.0001) reduction in FBF and a 35% (p < 0.0001) higher vascular resistance (fig. 2a, b). Fluconazole infusion attenuated the t-PA release with

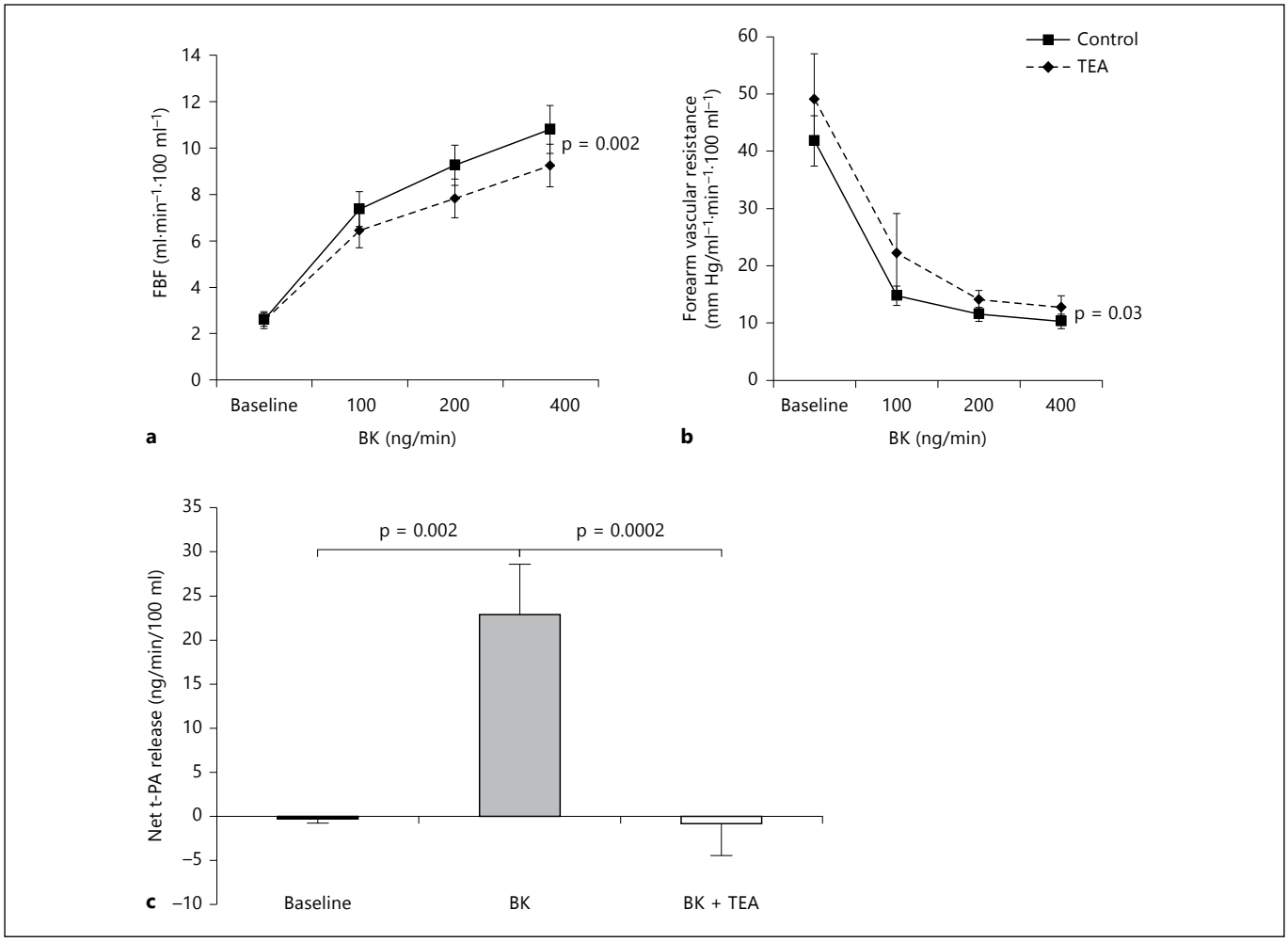

Fig. 1. Contribution of K^+_{Ca} channel activation to BK-stimulated vasodilation, and t-PA release. FBF (**a**) and forearm vascular resistance (**b**) changes in response to BK (100, 200, and 400 ng/min) alone, and after infusion of TEA (n = 18). Net t-PA release (**c**) in response to the peak dose of BK (400 ng/min) and after infusion of TEA. Means ± SEM.

Table 2. Effect of interventions on the arteriovenous gradient

	Venous-arterial t-PA difference, ng/ml				
	control (n = 33)	+ TEA (n = 18)	+ fluconazole (n = 11)	+ fluconazole + TEA (n = 10)	+ TEA + L-NMMA (n = 13)
Baseline	–0.2±0.3	–0.3±0.3	–0.2±0.8	–0.2±0.8	–0.2±0.4
BK (400 ng/min)	5.6±0.8*	0.03±0.7**	4.4±1.4**	1.6±0.4	2.6±0.9

* $p < 0.05$ vs. baseline, ** $p < 0.01$ vs. control.

BK (from 50.9 ± 9 to 21.3 ± 8.9 ng/min/100 ml, p = 0.02) and the arteriovenous t-PA concentration gradient (table 2), indicating significant contribution of cytochrome P_{450} metabolites to BK-stimulated t-PA release (fig. 2c). Infusion of TEA after fluconazole reduced FBF by an additional 20% ($p < 0.0001$, n = 10) and increased vascular resistance by a further 24% ($p < 0.0001$; fig. 2a, b). In the presence of fluconazole, infusion of TEA tended to attenuate the arteriovenous t-PA gradient (table 2), but the further reduction in net t-PA release did not reach statis-

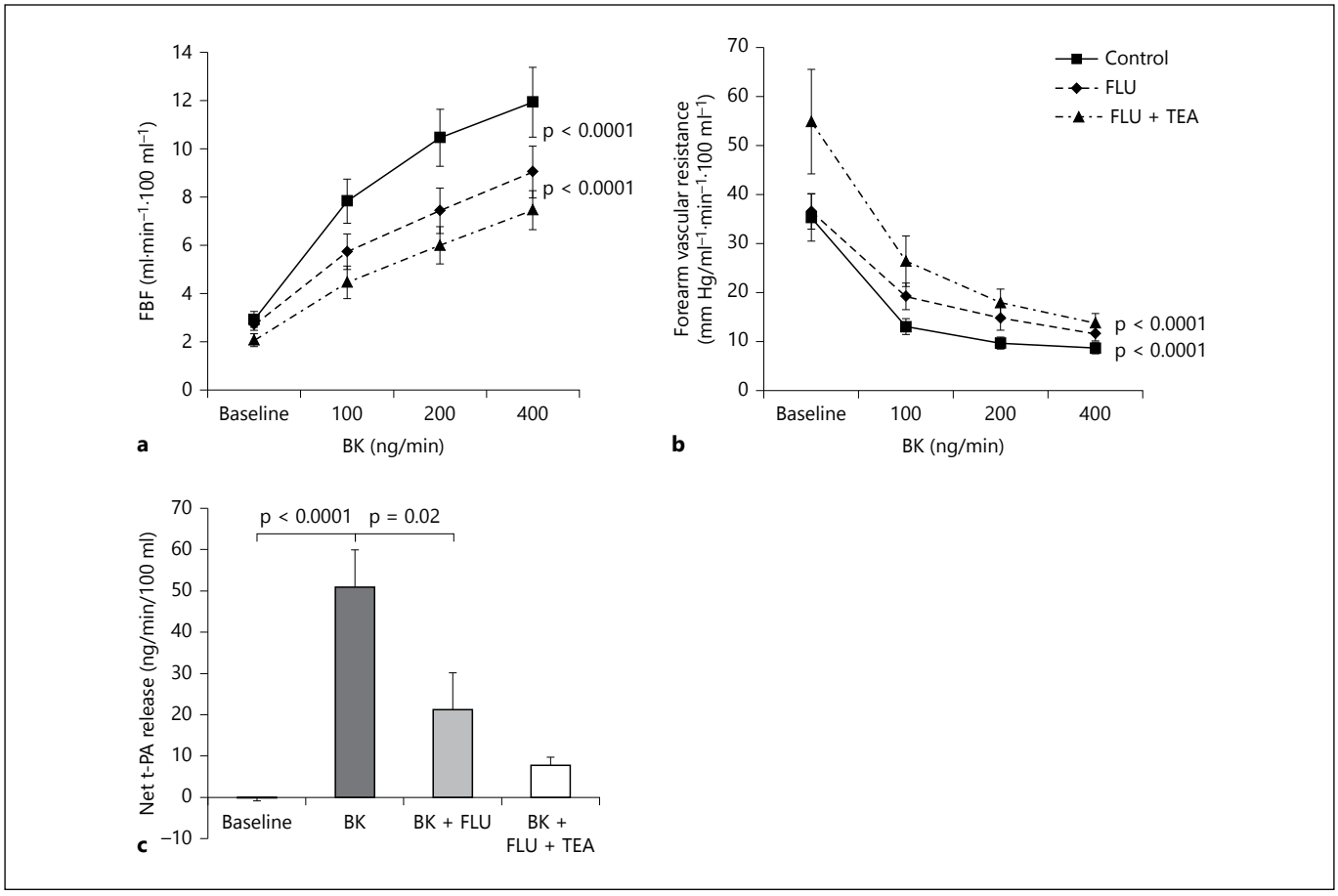

Fig. 2. Contribution of cytochrome P_{450} metabolites and K^+_{Ca} channel activation to BK-stimulated vasodilation and t-PA release. FBF (**a**) and forearm vascular resistance (**b**) changes in response to BK (100, 200, and 400 ng/min) alone, after infusion of fluconazole (FLU), and combined infusions of FLU and TEA (n = 11). Net t-PA release (**c**) in response to the peak dose of BK (400 ng/min) and after initial infusion of FLU, followed by combined FLU and TEA infusions. Means ± SEM.

tical significance (fig. 2c). However, TEA infusion attenuated the t-PA release with BK to a greater extent than fluconazole (85 ± 4 vs. 61 ± 11% reduction, respectively, p = 0.05), indicating that of the potential hyperpolarization mechanisms, K^+_{Ca} channel activation is the predominant EDHF signaling pathway mediating BK-stimulated t-PA release.

Effect of NO and K^+_{Ca} Channel Activation on FBF and BK-Stimulated t-PA Release

To assess the comparative contribution of K^+_{Ca} channel activation and NO to BK-stimulated t-PA release, individual and combined blockade with TEA and L-NMMA were studied. As seen previously, infusion of TEA attenuated the vasodilator response to BK with a 17% (p < 0.0001) reduction in FBF (fig. 3a). Similarly, t-PA release with BK was reduced from 20.4 ± 6 to 3.5 ± 3 ng/min/100 ml (p = 0.02), indicating a significant contribution of K^+_{Ca} channel activation to BK-stimulated t-PA release (fig. 3c). Infusion of L-NMMA after TEA reduced FBF by an additional 21% (p < 0.0001) and increased vascular resistance by a further 42% (p = 0.04; fig. 3a, b). In the presence of TEA, infusion of L-NMMA did not further affect net t-PA release (p = 0.25; fig. 3c; table 2), indicating that BK-stimulated t-PA release is through a K^+_{Ca} channel-dependent and NO-synthase-independent pathway.

Effect of SNP on t-PA Release

Infusions of BK and SNP resulted in similar increases in FBF (p = 0.7) and decreases in FVR (fig. 4a). However, in contrast to BK, the t-PA response (–1.8 ± 2 vs. –0.6 ± 0.4 ng/min/100 ml, nonsignificant) and the arteriovenous t-PA concentration gradient (–0.4 ± 0.3 vs. –0.2 ±

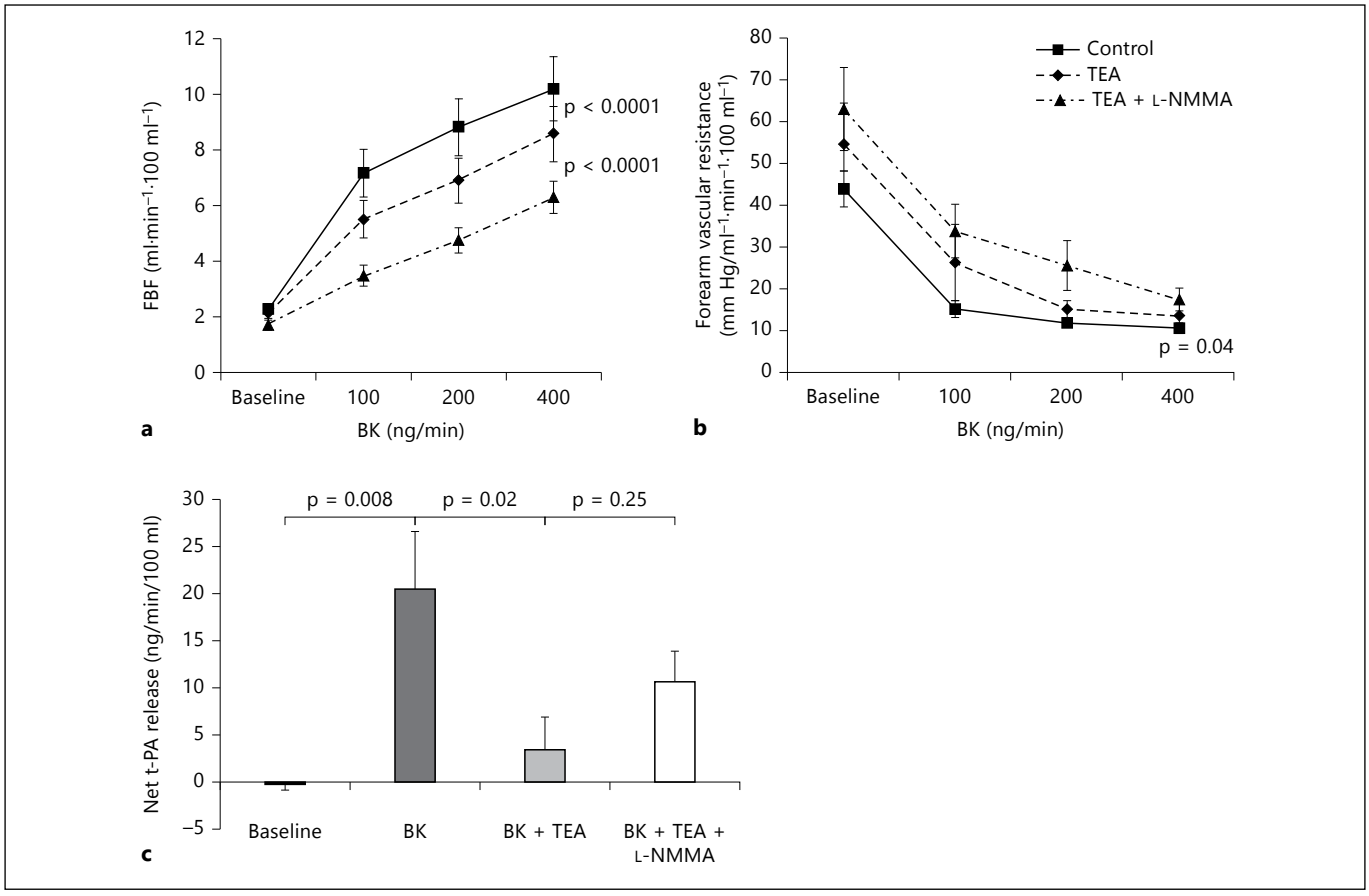

Fig. 3. Contribution of NO and K^+_{Ca} channel activation to BK-stimulated vasodilation and t-PA release. FBF (**a**) and forearm vascular resistance (**b**) in response to BK (100, 200, and 400 ng/min) alone, after initial infusion of TEA, and combined infusion of TEA and L-NMMA in healthy (n = 13) subjects. Means ± SEM. Box plot of net t-PA release (**c**) in response to the peak dose of BK (400 ng/min) and after infusion of TEA, followed by combined TEA and L-NMMA infusions. Minimum and maximum values are depicted by whiskers and the mean by the symbol marker.

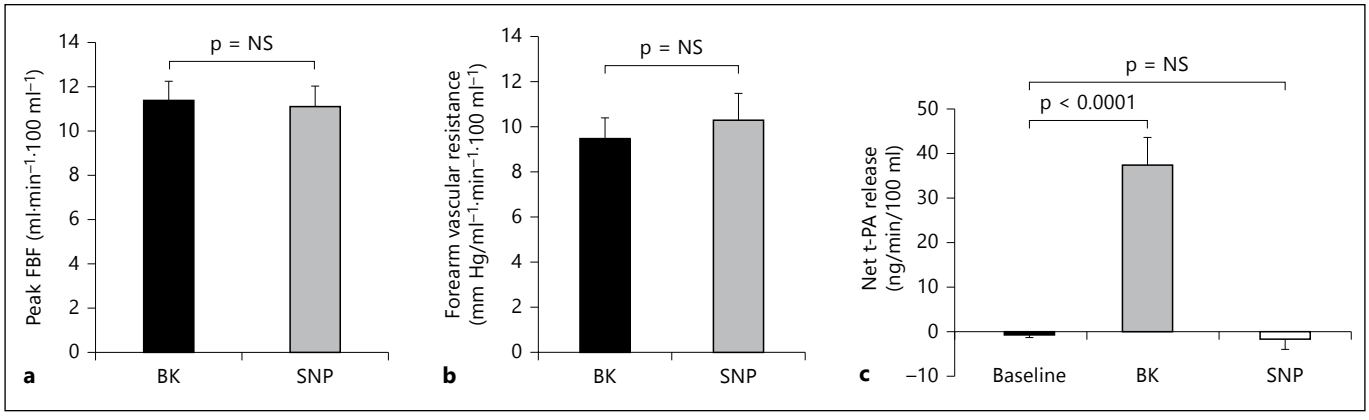

Fig. 4. Contribution of BK and SNP to vasodilation and t-PA release. FBF (**a**), forearm vascular resistance (**b**), and net t-PA release (**c**) in response to the peak doses of BK (400 ng/min) and SNP (3.2 μg/min). Means ± SEM. NS = Not statistically significant.

0.3, p = 0.5) remained unchanged with SNP, indicating that SNP does not stimulate endothelial t-PA release (fig. 4b).

Discussion

Herein, we demonstrate that although both NO and EDHF contribute to BK-induced vasodilation, t-PA release is solely mediated by EDHF signaling pathways. We confirmed that cytochrome P_{450}-derived EETs partly contribute to BK-stimulated t-PA release and now show that it is completely abolished by inhibition of K^+_{Ca} channels, the final site of action for several putative EDHFs [27]. Finally, we demonstrate a lack of contribution of NO to BK-stimulated t-PA release [11].

Endothelial fibrinolytic activity is an endogenous protective mechanism against the development and propagation of arterial thrombi [28]. In the fibrinolytic system, plasminogen is activated to plasmin that degrades fibrin into soluble fibrin degradation products [29]. The endothelium releases t-PA through a calcium- and G-protein-dependent pathway in response to a variety of endogenous mediators, including catecholamines, thrombin, and BK [30, 31]. Endogenous BK is produced by activation of the plasma kallikrein-kinin system on endothelial cells and BK B_2 receptor blockade inhibits t-PA release [10, 11].

In the intact human vasculature, endothelium-dependent hyperpolarization can be mediated by agonists such as BK and by physical stimuli including increases in shear stress that all increase intracellular calcium, causing opening of endothelial K^+_{Ca} channels and initiating downstream processes that explain the EDHF phenomena [3, 4]. Candidate EDHFs include EETs synthesized from the cytochrome P_{450}-dependent metabolism of arachidonic acid and hydrogen peroxide generated from the degradation of superoxides through various endothelial oxidases such as NADPH oxidase or through superoxide-dismutase-dependent dismutation [32, 33]. The availability of TEA, a specific K^+_{Ca} channel antagonist, for use in humans has enabled investigations of EDHF activity in the forearm microcirculation [16, 17, 22]. We now demonstrate that in addition to stimulating vasodilation, BK stimulates t-PA release that is completely abolished by K^+_{Ca} channel inhibition [6, 34].

Endothelium-derived cytochrome P_{450} metabolites of arachidonic acid can hyperpolarize membranes primarily by activating the K^+_{Ca} channels, although the identity of the specific EETs remains controversial [35–37]. Herein, we demonstrate that fluconazole, an inhibitor of cytochrome 2C9, attenuated BK-mediated t-PA release, suggesting that cytochrome P_{450} metabolites contribute significantly to t-PA release [19], observations that have been confirmed in both preclinical [14, 38] and clinical studies in both healthy and hypertensive subjects [27].

Because inhibition of EETs had a lesser effect than inhibition of K^+_{Ca} channels on BK-stimulated t-PA release, it appears that other factors that stimulate K^+_{Ca} channels also contribute to EDHF activity, which in preclinical studies has been attributed to hydrogen peroxide, gap junctions, or elevations in K^+ release from endothelial cells [3, 18, 39–41]. Hydrogen peroxide predominantly contributes to BK-induced human arteriolar vasodilation [42] and to t-PA release from rat hearts [43], but its role in the human circulation in vivo remains to be established. Thus, although hydrogen peroxide activates K^+_{Ca} channels and can function as an EDHF, its contribution to vascular homeostasis is complex [44]. It is not only a hyperpolarizing factor, but, depending on the species, blood vessel, and concentration, hydrogen peroxide can also be a signaling molecule and may also cause direct smooth muscle relaxation or depolarization of smooth muscles and vasoconstriction [3].

It may be argued that the BK-mediated t-PA release is at least partly flow mediated. However, with similar increases in blood flow with SNP, we and previous investigators found no increase in t-PA [6, 11, 27, 45, 46]. Moreover, infusion of L-NMMA selectively attenuated FBF without affecting the t-PA response to BK.

Limitations

L-NMMA, fluconazole, and TEA are competitive inhibitors and may not completely inhibit NO, cytochrome P_{450} pathways, and K^+_{Ca} channels, respectively, and thus likely underestimate the physiologic importance of these mediators in vivo [47, 48]. TEA also acts as a competitive inhibitor of the nicotinic receptor and reduces water permeability of human AQP1 channels; however, at the doses administered, it has been shown to selectively inhibit K^+_{Ca} channels [49, 50]. It is assumed that TEA effects are reflecting endothelium-dependent hyperpolarization as we cannot measure membrane potentials in this in vivo study. Fluconazole is not a specific inhibitor of cytochrome 2C9; however, we have shown significant inhibition of resting and BK-mediated vasodilation with this inhibitor [4]. Previous studies with a specific 2C9 inhibitor, sulfaphenazole, or with miconazole, have yielded contradictory data [19, 51–53]. We did not find any ethnic differences in BK-mediated t-PA release even though

ethnicity affects the vasodilator response to BK; however, our study was not powered to explore this issue [54]. Our experiments were conducted in the setting of cyclooxygenase inhibition and therefore are unable to explore possible interactions with prostaglandins. Nevertheless, previous studies demonstrated no alteration in BK-mediated t-PA release with cyclooxygenase inhibition [11, 55, 56].

Conclusions

We demonstrate that BK-stimulated t-PA release is via endothelium-dependent hyperpolarization, partly through cytochrome P_{450}-derived epoxides and ultimately via activation of K^+_{Ca} channels. Although BK stimulates both NO and EDHF-dependent vasodilation, BK-mediated t-PA release is purely EDHF dependent. Both endothelium-dependent vasodilation and endothelial fibrinolytic capacity, measured as t-PA release, appear to predict the risk of future cardiovascular events [57, 58]. Since abnormalities in the EDHF signaling pathway contribute to both abnormal vasodilation and increased risk of thrombosis, agonists that enhance EDHF activity, such as epoxide hydrolase inhibitors, need to be investigated for their thrombolytic actions.

Acknowledgment

The study was supported by National Institutes of Health Research Grant RO1 HL79115, and in part by PHS Grant UL1 RR025008 from the Clinical and Translational Science Award Program, and PHS Grant M01 RR00039 from the General Clinical Research Center program, National Institutes of Health, National Center for Research Resources, the British Cardiovascular Society Research Fellowship, National Blood Foundation, NIH NRSA T32 Training Grant, American College of Cardiology Foundation Keating Fellowship, and the American Heart Association Beginning Grant-in-Aid.

Disclosure Statement

The authors have no conflicts of interest to disclose.

References

1 Newby DE, Wright RA, Labinjoh C, Ludlam CA, Fox KA, Boon NA, Webb DJ: Endothelial dysfunction, impaired endogenous fibrinolysis, and cigarette smoking: a mechanism for arterial thrombosis and myocardial infarction. Circulation 1999;99:1411–1415.

2 Newby DE, McLeod AL, Uren NG, Flint L, Ludlam CA, Webb DJ, Fox KA, Boon NA: Impaired coronary tissue plasminogen activator release is associated with coronary atherosclerosis and cigarette smoking: direct link between endothelial dysfunction and atherothrombosis. Circulation 2001;103:1936–1941.

3 Feletou M, Vanhoutte PM: Endothelium-dependent hyperpolarizations: past beliefs and present facts. Ann Med 2007;39:495–516.

4 Ozkor MA, Murrow JR, Rahman AM, Kavtaradze N, Lin J, Manatunga A, Quyyumi AA: Endothelium-derived hyperpolarizing factor determines resting and stimulated forearm vasodilator tone in health and in disease. Circulation 2011;123:2244–2253.

5 Vanhoutte PM: Endothelium and control of vascular function. State of the art lecture. Hypertension 1989;13:658–667.

6 Brown NJ, Gainer JV, Stein CM, Vaughan DE: Bradykinin stimulates tissue plasminogen activator release in human vasculature. Hypertension 1999;33:1431–1435.

7 Linz W, Wiemer G, Scholkens BA: Beneficial effects of bradykinin on myocardial energy metabolism and infarct size. Am J Cardiol 1997;80:118A–123A.

8 Emeis JJ, van den Eijnden-Schrauwen Y, van den Hoogen CM, de Priester W, Westmuckett A, Lupu F: An endothelial storage granule for tissue-type plasminogen activator. J Cell Biol 1997;139:245–256.

9 Fox KA, Robison AK, Knabb RM, Rosamond TL, Sobel BE, Bergmann SR: Prevention of coronary thrombosis with subthrombolytic doses of tissue-type plasminogen activator. Circulation 1985;72:1346–1354.

10 Schmaier AH: The plasma kallikrein-kinin system counterbalances the renin-angiotensin system. J Clin Invest 2002;109:1007–1009.

11 Brown NJ, Gainer JV, Murphey LJ, Vaughan DE: Bradykinin stimulates tissue plasminogen activator release from human forearm vasculature through B_2 receptor-dependent, NO synthase-independent, and cyclooxygenase-independent pathway. Circulation 2000;102:2190–2196.

12 Node K, Ruan XL, Dai J, Yang SX, Graham L, Zeldin DC, Liao JK: Activation of Galpha s mediates induction of tissue-type plasminogen activator gene transcription by epoxyeicosatrienoic acids. J Biol Chem 2001;276:15983–15989.

13 Muldowney JA 3rd, Painter CA, Sanders-Bush E, Brown NJ, Vaughan DE: Acute tissue-type plasminogen activator release in human microvascular endothelial cells: the roles of Galphaq, PLC-beta, ip3 and 5,6-epoxyeicosatrienoic acid. Thromb Haemost 2007;97:263–271.

14 Halcox JP, Narayanan S, Cramer-Joyce L, Mincemoyer R, Quyyumi AA: Characterization of endothelium-derived hyperpolarizing factor in the human forearm microcirculation. Am J Physiol Heart Circ Physiol 2001;280:H2470–H2477.

15 Scotland RS, Madhani M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ, Ahluwalia A: Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. Circulation 2005;111:796–803.

16 Inokuchi K, Hirooka Y, Shimokawa H, Sakai K, Kishi T, Ito K, Kimura Y, Takeshita A: Role of endothelium-derived hyperpolarizing factor in human forearm circulation. Hypertension 2003;42:919–924.

17 Bellien J, Iacob M, Gutierrez L, Isabelle M, Lahary A, Thuillez C, Joannides R: Crucial role of NO and endothelium-derived hyperpolarizing factor in human sustained conduit artery flow-mediated dilatation. Hypertension 2006;48:1088–1094.

18 Miura H, Wachtel RE, Liu Y, Loberiza FR Jr, Saito T, Miura M, Gutterman DD: Flow-induced dilation of human coronary arterioles: important role of Ca(2+)-activated K(+) channels. Circulation 2001;103:1992–1998.

19 Bellien J, Joannides R, Iacob M, Arnaud P, Thuillez C: Evidence for a basal release of a cytochrome-related endothelium-derived hyperpolarizing factor in the radial artery in humans. Am J Physiol Heart Circ Physiol 2006; 290:H1347–H1352.
20 Hong SL: Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. Thromb Res 1980;18:787–795.
21 Duffy SJ, Tran BT, New G, Tudball RN, Esler MD, Harper RW, Meredith IT: Continuous release of vasodilator prostanoids contributes to regulation of resting forearm blood flow in humans. Am J Physiol 1998;274:H1174–H1183.
22 Honing ML, Smits P, Morrison PJ, Rabelink TJ: Bradykinin-induced vasodilation of human forearm resistance vessels is primarily mediated by endothelium-dependent hyperpolarization. Hypertension 2000;35:1314–1318.
23 Stanfield PR: Tetraethylammonium ions and the potassium permeability of excitable cells. Rev Physiol Biochem Pharmacol 1983;97:1–67.
24 Langton PD, Nelson MT, Huang Y, Standen NB: Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. Am J Physiol 1991;260:H927–H934.
25 Gilligan DM, Guetta V, Panza JA, Garcia CE, Quyyumi AA, Cannon RO 3rd: Selective loss of microvascular endothelial function in human hypercholesterolemia. Circulation 1994; 90:35–41.
26 Ridker PM, Vaughan DE, Stampfer MJ, Manson JE, Shen C, Newcomer LM, Goldhaber SZ, Hennekens CH: Baseline fibrinolytic state and the risk of future venous thrombosis. A prospective study of endogenous tissue-type plasminogen activator and plasminogen activator inhibitor. Circulation 1992;85:1822–1827.
27 Giannarelli C, Virdis A, De Negri F, Magagna A, Duranti E, Salvetti A, Taddei S: Effect of sulfaphenazole on tissue plasminogen activator release in normotensive subjects and hypertensive patients. Circulation 2009;119: 1625–1633.
28 Gorog DA: Prognostic value of plasma fibrinolysis activation markers in cardiovascular disease. J Am Coll Cardiol 2010;55:2701–2709.
29 Rijken DC, Lijnen HR: New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost 2009;7:4–13.
30 Stein CM, Brown N, Vaughan DE, Lang CC, Wood AJ: Regulation of local tissue-type plasminogen activator release by endothelium-dependent and endothelium-independent agonists in human vasculature. J Am Coll Cardiol 1998;32:117–122.
31 van den Eijnden-Schrauwen Y, Atsma DE, Lupu F, de Vries RE, Kooistra T, Emeis JJ: Involvement of calcium and G proteins in the acute release of tissue-type plasminogen activator and von Willebrand factor from cultured human endothelial cells. Arterioscler Thromb Vasc Biol 1997;17:2177–2187.
32 Fleming I, Michaelis UR, Bredenkotter D, Fisslthaler B, Dehghani F, Brandes RP, Busse R: Endothelium-derived hyperpolarizing factor synthase (cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. Circ Res 2001;88: 44–51.
33 Lambeth JD: Nox enzymes, ROS, and chronic disease: an example of antagonistic pleiotropy. Free Radic Biol Med 2007;43:332–347.
34 Witherow FN, Dawson P, Ludlam CA, Webb DJ, Fox KA, Newby DE: Bradykinin receptor antagonism and endothelial tissue plasminogen activator release in humans. Arterioscler Thromb Vasc Biol 2003;23:1667–1670.
35 Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, Busse R: Cytochrome P450 2C is an EDHF synthase in coronary arteries. Nature 1999;401:493–497.
36 Campbell WB, Gebremedhin D, Pratt PF, Harder DR: Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. Circ Res 1996;78:415–423.
37 Weston AH, Feletou M, Vanhoutte PM, Falck JR, Campbell WB, Edwards G: Bradykinin-induced, endothelium-dependent responses in porcine coronary arteries: involvement of potassium channel activation and epoxyeicosatrienoic acids. Br J Pharmacol 2005;145: 775–784.
38 Gauthier KM, Edwards EM, Falck JR, Reddy DS, Campbell WB: 14,15-Epoxyeicosatrienoic acid represents a transferable endothelium-dependent relaxing factor in bovine coronary arteries. Hypertension 2005;45:666–671.
39 Fleming I: Cytochrome P450 and vascular homeostasis. Circ Res 2001;89:753–762.
40 Fleming I, Busse R: Endothelium-derived epoxyeicosatrienoic acids and vascular function. Hypertension 2006;47:629–633.
41 Feletou M, Vanhoutte PM: Endothelium-derived hyperpolarizing factor: where are we now? Arterioscler Thromb Vasc Biol 2006;26: 1215–1225.
42 Larsen BT, Gutterman DD, Sato A, Toyama K, Campbell WB, Zeldin DC, Manthati VL, Falck JR, Miura H: Hydrogen peroxide inhibits cytochrome P450 epoxygenases: interaction between two endothelium-derived hyperpolarizing factors. Circ Res 2008;102:59–67.
43 Winnerkvist A, Wiman B, Valen G, Vaage J: Oxidative stress and release of tissue plasminogen activator in isolated rat hearts. Thromb Res 1997;85:245–257.
44 Matoba T, Shimokawa H, Kubota H, Morikawa K, Fujiki T, Kunihiro I, Mukai Y, Hirakawa Y, Takeshita A: Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. Biochem Biophys Res Commun 2002;290:909–913.
45 Newby DE, Wright RA, Ludlam CA, Fox KAA, Boon NA, Webb DJ: An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. Thromb Haemost 1997;78:1242–1248.
46 Pretorius M, Brown NJ: Endogenous nitric oxide contributes to bradykinin-stimulated glucose uptake but attenuates vascular tissue-type plasminogen activator release. J Pharmacol Exp Ther 2010;332:291–297.
47 Rees DD, Palmer RM, Hodson HF, Moncada S: A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. Br J Pharmacol 1989; 96:418–424.
48 Coleman HA, Tare M, Parkington HC: Endothelial potassium channels, endothelium-dependent hyperpolarization and the regulation of vascular tone in health and disease. Clin Exp Pharmacol Physiol 2004;31:641–649.
49 Akk G, Steinbach JH: Activation and block of mouse muscle-type nicotinic receptors by tetraethylammonium. J Physiol 2003;551:155–168.
50 Brooks HL, Regan JW, Yool AJ: Inhibition of aquaporin-1 water permeability by tetraethylammonium: involvement of the loop E pore region. Mol Pharmacol 2000;57:1021–1026.
51 Halcox JPJ, Narayanan S, Cramer-Joyce L, Mincemoyer R, Quyyumi AA: Characterization of endothelium-derived hyperpolarizing factor in the human forearm microcirculation. Am J Physiol Heart Circ Physiol 2001; 280:H2470–H2477.
52 Fichtlscherer S, Dimmeler S, Breuer S, Busse R, Zeiher AM, Fleming I: Inhibition of cytochrome P450 2C9 improves endothelium-dependent, nitric oxide-mediated vasodilatation in patients with coronary artery disease. Circulation 2004;109:178–183.
53 Passauer J, Bussemaker E, Lassig G, Pistrosch F, Fauler J, Gross P, Fleming I: Baseline blood flow and bradykinin-induced vasodilator responses in the human forearm are insensitive to the cytochrome P450 2C9 (CYP2C9) inhibitor sulphaphenazole. Clin Sci (Lond) 2003;105:513–518.
54 Rosenbaum DA, Pretorius M, Gainer JV, Byrne D, Murphey LJ, Painter CA, Vaughan DE, Brown NJ: Ethnicity affects vasodilation, but not endothelial tissue plasminogen activator release, in response to bradykinin. Arterioscler Thromb Vasc Biol 2002;22:1023–1028.
55 Ohlmann P, Martinez MC, Schneider F, Stoclet JC, Andriantsitohaina R: Characterization of endothelium-derived relaxing factors released by bradykinin in human resistance arteries. Br J Pharmacol 1997;121:657–664.
56 Nakashima M, Mombouli JV, Taylor AA, Vanhoutte PM: Endothelium-dependent hyperpolarization caused by bradykinin in human coronary arteries. J Clin Invest 1993;92: 2867–2871.
57 Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA: Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002;106:653–658.
58 Ridker PM, Vaughan DE, Stampfer MJ, Manson JE, Hennekens CH: Endogenous tissue-type plasminogen activator and risk of myocardial infarction. Lancet 1993;341:1165–1168.