B1 Kinin Receptor Does Not Contribute to Vascular Tone or Tissue Plasminogen Activator Release in the Peripheral Circulation of Patients With Heart Failure

Nicholas L.M. Cruden, George H. Tse, Keith A.A. Fox, Christopher A. Ludlam, Ian Megson, David E. Newby

Objective—Vascular expression of the B₁ kinin receptor is markedly upregulated with left ventricular dysfunction and angiotensin-converting enzyme (ACE) inhibition, but its function remains unclear. Inhibitors of ACE potentiate bradykinin-mediated B₂ receptor-dependent vasodilatation and tissue plasminogen activator (t-PA) release. We investigated the contribution of the B₁ receptor to the maintenance of vascular tone and t-PA release in patients with heart failure.

Methods and Results—Eleven patients were treated with enalapril (10 mg twice daily) or losartan (50 mg twice daily) in a randomized double-blind crossover trial. During week 6 of each treatment, patients received an intrabrachial infusion of Lys-des-Arg⁹-bradykinin (B₁ agonist; 1 to 10 nmol/min), bradykinin (30 to 300 pmol/min), Lys-[Leu⁸]-des-Arg⁹-bradykinin (B₁ antagonist; 1 to 10 nmol/min), and norepinephrine (60 to 540 pmol/min). Blood flow and t-PA release were measured using venous occlusion plethysmography and blood sampling. Bradykinin (P < 0.001 for all), but not Lys-des-Arg⁹-bradykinin, caused vasodilatation and t-PA antigen and activity release. Norepinephrine (P < 0.001), but not Lys-[Leu⁸]-des-Arg⁹-bradykinin, caused vasoconstriction. Compared with losartan, enalapril augmented bradykinin-mediated vasodilatation (P < 0.05) and t-PA release (P < 0.01 for all) but had no effect on B₁ receptor-mediated responses.

Conclusions—The B₁ kinin receptor does not have a major vasomotor or fibrinolytic role in patients with heart failure. Augmentation of kinin-mediated vasodilatation and t-PA release by ACE inhibition is restricted to the B₂ receptor.

Key Words: ACE inhibitors ■ bradykinin ■ heart failure ■ plasminogen activators ■ receptors

Bradykinin is the major effector for the kinin family of peptides in humans. It is released at sites of inflammation and coagulation and contributes to the systemic hemodynamic and anti-ischemic effects of ACE inhibitor therapy. Besides vasodilatation, bradykinin stimulates endothelial release of the pro-lytic factor, tissue plasminogen activator (t-PA), and these effects are mediated by the constitutively expressed B₂ kinin receptor.

Des-Arg⁹-bradykinin is the principal ligand for the B₁ kinin receptor in human plasma and is generated by carboxypeptidases after removal of the C-terminal arginine from bradykinin. The vascular B₁ receptor is normally expressed very weakly but is markedly upregulated in the presence of inflammation, ischemic left ventricular dysfunction, cardiovascular disease, and ACE inhibition. In animal studies, stimulation of the B₁ receptor produces vasodilatation and a reduction in blood pressure. Intense endothelial B₁ receptor expression has been demonstrated in atheromatous human blood vessels, and B₁ receptor stimulation induces dose-dependent vasodilatation in human coronary arteries in vitro. Whether the B₁ receptor contributes to the vascular effects of kinins in vivo in humans remains unknown.

ACE is the principal enzyme responsible for the rapid breakdown of bradykinin (plasma half life ≈15 seconds) to its inactive metabolites. In addition to increasing plasma bradykinin concentrations, ACE inhibition will favor bradykinin breakdown by alternative metabolic pathways including plasma carboxypeptidases, augmenting the generation of des-Arg⁹-bradykinin, and thereby potentiating both B₁ and B₂ receptor-mediated effects. B₂ receptor antagonism attenuates the vasodepressor effect of a single oral dose of captopril in healthy volunteers and subjects with hypertension. In patients with heart failure, the combined B₁ and B₂ kinin receptor antagonist, B9340, causes vasoconstriction in the forearm circulation in the presence, but not absence, of ACE inhibition, and when administered systemically, it attenuates the hemodynamic effects of chronic ACE inhibition.
However, the role of the B1 receptor in patients with heart failure and those treated with ACE inhibitor therapy remains to be established.

Although des-Arg9-bradykinin is the principal B2 agonist present in plasma, it has only modest affinity for the human B1 receptor and retains some activity at the human B2 receptor. Indeed, des-Arg9-bradykinin is only 100-fold more selective for the human B2 receptor. In contrast, Lys-des-Arg9-bradykinin, an endogenous tissue-based metabolite of kallidin, has ≈1000-fold greater affinity for the B1 receptor than des-Arg9-bradykinin and is inactive at the B2 receptor in humans. Substitution of the Phe8 residue in Lys-des-Arg9-bradykinin with Leu results in the formation of Lys-[Leu8]-des-Arg9-bradykinin, a synthetic peptide with potent selective inhibitory activity at the human B1 receptor.

Using custom-manufactured, clinical-grade preparations of Lys-des-Arg9-bradykinin and Lys-[Leu8]-des-Arg9-bradykinin, the aims of this study were to investigate whether the B2 receptor contributes to the vascular actions of kinins in the patients with heart failure treated with ACE inhibition.

**Methods**

This study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

**Drugs**

Pharmaceutical grade bradykinin (Clinalfa AG, Läufelfingen, Switzerland), Lys-des-Arg9-bradykinin (Clinalfa), Lys-[Leu8]-des-Arg9-bradykinin (Clinalfa), HOE-140 (Clinalfa), B9340 (Clinalfa), and norp Ephrine (Abbott Laboratories Ltd, Maidenhead, UK) were dissolved in physiological saline on the day of study. The doses of bradykinin and norp Ephrine were chosen based on the results of previous studies. The doses of Lys-des-Arg9-bradykinin and Lys-[Leu8]-des-Arg9-bradykinin were chosen based on the EC50 (B1 receptor, 0.2 nmol/L; B2 receptor, >30 000 nmol/L) and IC50 (B1, 1.3 nmol/L; B2, >30 000 nmol/L), respectively, for human kinin receptors.

**Human Umbilical Vein Studies**

To confirm the efficacy of the B1 receptor agonist, Lys-des-Arg9-bradykinin, and the B2 receptor antagonist, Lys-[Leu8]-des-Arg9-bradykinin, human umbilical cord was obtained from women aged 16 to 40 years undergoing routine cesarean section after uncomplicated pregnancy. Immediately after delivery, 10 cm umbilical cord was excised midway between placenta and child and placed in Krebs buffer solution (NaCl 6.954 mmol/L, KCl 4.7 mmol/L, CaCl2 2.5 mmol/L, MgSO4 1.17 mmol/L, NaHCO3 2 mmol/L, KH2PO4 1.18 mmol/L, EDTA 0.027 mmol/L, glucose 5.5 mmol/L, Fisher Scientific UK Ltd, Loughborough UK). Human umbilical vein was dissected into 3-mm rings as previously described. Three hours after delivery, umbilical vein rings were mounted on wire myographs, suspended in organ baths containing 10 mL Krebs solution, and stretched with an initial tension of 2 g (Multimyograph System 700MO; JP Trading, Denmark). Krebs solution was maintained at 37°C and continually bubbled with 95% O2/5% CO2. Changes in tension were measured using an isometric transducer (Mac Laboratory 8; Analog Digital Instruments Pty Ltd, Australia).

**Myography**

After 60-minute equilibration, during which the tension was readjusted at 15-minute intervals, maximal contraction to KCl (60 mmol/L) was determined on 3 occasions, interspersed by 15-minute washout periods. Tissue rings were incubated with captopril (1 μmol/L; Sigma Pharmaceuticals, St Louis, Mo) 30 minutes before performing cumulative dose responses to bradykinin (B1 receptor agonist; 10−11 to 10−6 mol/L) and Lys-des-Arg9-bradykinin (B1 receptor agonist; 10−11 to 10−6 mol/L) in the presence or absence of Lys-[Leu8]-des-Arg9-bradykinin (B2 receptor antagonist; 1 μmol/L), HOE-140 (B2 receptor antagonist; 1 μmol/L), or B9340 (combined B1/B2 receptor antagonist; 1 μmol/L). Peptide antagonists were applied 10 minutes before agonists to ensure that equilibrium was obtained. Maximal contraction to 60 mmol/L KCl was determined on a final occasion at the end of each experiment. A single concentration curve for bradykinin and Lys-des-Arg9-bradykinin was obtained for each ring, and experiments were performed in parallel with rings from the same tissue.

**Immunohistochemistry**

After contractile studies, human umbilical vein rings were fixed in formalin and embedded in paraffin wax. A hematoxylin and eosin stain was performed for each tissue section. B1 and B2 receptors were immunodetected using diaminobenzidine immunoprecipitation. Briefly, tissue was boiled in 0.01 mol/L sodium citrate (pH 6.0) for 8 minutes to facilitate antigen retrieval. Nonspecific binding sites were blocked with 1:100 donkey serum followed by 3% H2O2. Tissue sections were then incubated with the primary affinity purified goat polyclonal antibody specific for human the B1, or B2, kinin receptor (Santa Cruz Biotechnology, Inc) followed by the secondary antibody (donkey anti-goat IgG; Santa Cruz Biotechnology Inc) or with the secondary antibody alone (control). Finally, tissues were treated with a peroxidase–antiperoxidase strepavidin–biotin conjugating system (Dako K0690) and visualized by conventional light microscopy using liquid diaminobenzidine precipitant (Dako K3465).

**Clinical Study**

Eleven patients with symptomatic heart failure (New York Heart Association class II or III) and evidence of left ventricular systolic dysfunction (ejection fraction <40%, shortening fraction <20%, or left ventricular end diastolic dimension >55 mm) were recruited. Patients were maintained on maximally tolerated ACE inhibitor therapy for at least 6 months before enrollment. On study days, patients who attended fasted for 4 hours and diuretics were withheld for patient comfort.

When symptoms would allow, ACE inhibitor therapy was withdrawn for a period of 2 weeks (n = 7). After this and in place of their usual ACE inhibitor, patients were randomized to receive 6 weeks of treatment with enalapril 10 mg twice daily or losartan 50 mg twice daily in a double-blind, crossover trial. One patient withdrew because of worsening symptoms and was replaced. During week 2 of ACE inhibitor withdrawal and weeks 6 and 12 of the crossover trial, patients attended on 2 occasions at least 3 days apart and underwent an agonist study on one occasion and an antagonist study on the other. The study order was randomized between patients but remained constant for each patient.

**Intra-Arterial Drug Administration**

All studies were performed with patients lying supine in a quiet, temperature-controlled (22°C to 25°C) room. Under local anesthesia, a 27-gauge needle (Cooper Needle Works Ltd) was inserted into the brachial artery of the nondominant arm. The rate of intra-arterial drug infusion remained constant throughout at 1 mL/min.

**Forearm Blood Flow and Blood Pressure**

Forearm blood flow was measured at 10-minute intervals in both arms using venous occlusion strain gauge plethysmography as previously described. Heart rate and blood pressure were recorded in the noninfused arm at intervals throughout the study using a semi-automated noninvasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical Inc, Japan).

**Venous Sampling and Assays**

During agonist studies only, 17-gauge venous cannulae were inserted bilaterally into a large antecubital vein. Ten milliliters of blood were
withdrawn simultaneously from each arm at baseline and in the last minute of each drug infusion period and collected into acidified buffered citrate (Biopool Stabilyte, Umeå) for t-PA assays and citrate (Monovette, Sarstedt, Nuremberg) for plasminogen activator inhibitor (PAI) type-I (PAI-I) assays. Samples were kept on ice before being centrifuged at 2000g for 30 minutes at 4°C. Platelet-free supernatant was decanted and stored at −80°C before assay. Plasma t-PA and PAI-I antigen concentrations were determined using enzyme-linked immunoabsorbent assays and plasma t-PA activity by a photometric method.23

Protocol Design
After 30-minute equilibration with 0.9% saline, patients received an intra-brachial infusion of Lys-des-Arg9-bradykinin (1, 3, and 10 nmol/min) and bradykinin (30, 100, and 300 pmol/min) on one occasion (agonist protocol), and Lys-[Leu8]-des-Arg9-bradykinin (1, 3, and 10 nmol/min) and norepinephrine (60, 180, and 540 pmol/min) on the other (antagonist protocol). Study drugs were infused in random order for 10 minutes at each dose and separated by a 20-minute infusion of 0.9% saline.

Data Analysis and Statistics
Unless stated, all data are expressed as mean±SEM. Human umbilical vein responses are expressed as a percentage of the maximal contraction to 60 mmol/L KCl obtained at the end of each experiment. Plethysmographic data were extracted from chart data files and forearm blood flows were calculated as described previously.16 Estimated net release of t-PA antigen and activity were defined as the product of the infused forearm plasma flow (based on the mean hematocrit and forearm blood flow) and the concentration difference between the infused and noninfused arms.3,23 Statistical analyses were performed using analysis of variance (ANOVA) and statistical significance was taken at the 5% level.

Results
Human Umbilical Vein Studies
Immunohistochemistry confirmed intense immunolabeling of both B1 and B2 receptors on human umbilical vein (Figure I, available online at http://atvb.ahajournals.org). Consistent with previous work,18,20 bradykinin and Lys-des-Arg9-bradykinin caused dose-dependent constriction of human umbilical vein rings (Figure 1; P<0.001 for both). Lys-[Leu8]-des-Arg9-bradykinin and B9340 caused ~10-fold rightward shift and HOE-140 caused a modest leftward shift in the dose response curve for Lys-des-Arg9-bradykinin (Figure 1a; P<0.001, P<0.001, and P<0.05, respectively; ANOVA). In contrast, HOE-140 and B9340, but not Lys-[Leu8]-des-Arg9-bradykinin, caused a rightward shift in the dose response curve for bradykinin (Figure 1b; P<0.001, P<0.001, and P=not significant, respectively).

Clinical Study
Patients were predominantly male with mild to moderate congestive heart failure caused by ischemic heart disease (Table). There were no significant differences in heart rate, blood pressure, or baseline forearm blood flow during or between study days (Table I, available online at http://atvb.ahajournals.org). Compared with losartan, plasma ACE activity (42.2±11 versus 10.5±6.1 U/L, respectively; P<0.05) and angiotensin II concentrations (24.4±6.3 versus 7.8±1.6 pg/mL, respectively; P<0.05) were lower during treatment with enalapril.

Forearm Blood Flow
Bradykinin (P<0.0001 for all), but not Lys-des-Arg9-bradykinin (P=not significant for all), caused dose-dependent vasodilatation in all studies (Figure 2). Bradykinin-mediated vasodilatation was augmented in

Patient Characteristics (n=11)

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Data are expressed as number of patients or mean±SEM unless indicated. ACE indicates angiotensin-converting enzyme; DCM, idiopathic dilated cardiomyopathy; IHD, ischemic heart disease; LVEDD, left ventricular end diastolic diameter; NYHA, New York Heart Association.
patients treated with enalapril compared with losartan ($P<0.005$; Figure 3) or ACE inhibitor withdrawal ($P<0.05$; Figure 2).

Norepinephrine ($P<0.0005$ for all), but not Lys-[Leu$^8$]-des-Arg$^9$-bradykinin ($P$=not significant for all), caused dose-dependent vasoconstriction in all studies (Figure 2).

**Fibrinolytic Factors**

**Release of t-PA**

Bradykinin ($P<0.001$ for all), but not Lys-des-Arg$^9$-bradykinin ($P$=not significant for all), caused dose-dependent increases in plasma concentrations of t-PA antigen and activity in the infused arm and net release of t-PA antigen and activity in all studies (Figures 3 and 4). The bradykinin-mediated increase in plasma t-PA antigen and activity in the infused arm and net release of t-PA antigen and activity were augmented in patients treated with enalapril compared with losartan ($P=0.0005$ for all) or ACE inhibitor withdrawal ($P<0.005$ for all; Figures 3 and 4).

Consistent with systemic overspill,$^3$ bradykinin caused dose-dependent increases in plasma t-PA antigen and activity concentrations in the non-infused arm with enalapril therapy, and plasma t-PA antigen alone after ACE inhibitor withdrawal ($P=0.0001$ for all; Figure 3). Enalapril augmented the increase in t-PA compared with losartan therapy or ACE inhibitor withdrawal ($P<0.01$ for both; Figure 3).

**Plasma PAI-1 Antigen**

There were no significant differences in basal PAI-1 antigen concentrations between study days (data on file). Consistent with an increase in PAI-1-mediated clearance after marked t-PA release,$^24$ and a potential time effect,$^25$ plasma PAI-1 antigen concentrations declined during infusion of bradykinin in both the infused (47.6±7.9 ng/mL at baseline versus 43.8±7 ng/mL during bradykinin 300 pmol/min; $P<0.05$) and noninfused arms (51±8.2 ng/mL at baseline versus 44.5±7 ng/mL during bradykinin 300 pmol/min; $P<0.05$) in patients treated with enalapril but not losartan or during ACE inhibitor withdrawal.

**Discussion**

This is the first study to characterize the potential vasomotor and fibrinolytic role of the vascular B$_1$ kinin receptor in vivo. We have demonstrated that the peptidic B$_1$ receptor agonist, Lys-des-Arg$^9$-bradykinin, and antagonist, Lys-[Leu$^8$]-des-Arg$^9$-bradykinin, have no effect on vascular tone or endothelial t-PA release in the presence or absence of ACE inhibition. In contrast, and consistent with our previous unblinded and nonrandomized data,$^3$ ACE inhibition markedly augmented the vascular actions of bradykinin mediated via the B$_2$ receptor. We conclude that the B$_1$ receptor does not appear to have a major vasomotor or fibrinolytic role in the forearm circulation of patients with heart failure treated with chronic ACE inhibition.

Our findings are in contrast to previous in vitro and animal work demonstrating vasodilatation after B$_1$ receptor stimulation.$^6$–$^12$ Before concluding that the B$_1$ kinin receptor does not mediate vasodilatation or endothelial t-PA release in the forearm circulation of patients with heart failure, we must first consider the following possibilities: the doses of the Lys-des-Arg$^9$-bradykinin and Lys-[Leu$^8$]-des-Arg$^9$-bradykinin used in this study were inadequate; the custom-made peptides lacked biological activity; or the extent of ACE inhibition was insufficient to upregulate B$_1$ receptor expression.

We infused Lys-des-Arg$^9$-bradykinin at a dose that was at least 20-fold greater than that previously shown to produce
50% of the maximal hypotensive response in both primate (EC50 = 0.1 pmol/kg) and rodent studies (EC50 = 0.3 pmol/kg). Similarly, Lys-[Leu6]-des-Arg9-bradykinin was infused at a dose 20-fold greater than that previously shown to abolish B1 receptor-mediated vasomotor responses in animal models in vivo. To address the issue of biological activity, we examined vasomotor responses to the custom-made B1 agonist and antagonist in isolated human umbilical vein. The concentration-response curves obtained for Lys-des-Arg9-bradykinin and Lys-[Leu6]-des-Arg9-bradykinin were comparable with data from previous studies and confirm efficacy at concentrations predicted to be achieved in the infused human forearm circulation.

Previous rodent studies have demonstrated that besides cardiovascular inflammation, chronic ACE inhibition upregulates functional vascular B1 receptor expression. We have examined B1 receptor function in patients with heart failure treated with an effective evidence based dose of enalapril. Moreover, plasma concentrations of angiotensin II and ACE activity confirmed significant inhibition of the renin-angiotensin system with enalapril at this dose. From our findings, therefore, we can conclude that the B1 kinin receptor does not mediate vasodilatation or endothelial t-PA release in patients with mild to moderate heart failure treated with long-term ACE inhibitor therapy.

We have previously demonstrated that combined B1 and B2 receptor blockade, but not B2 receptor blockade, causes peripheral vasoconstriction in patients with heart failure treated with ACE inhibition. In our current study, however, selective B1 receptor antagonism had no effect on peripheral vascular tone. One potential explanation for this discrepancy is that the B1 receptor may only mediate the vasomotor effects of kinins in the absence of B2 receptor-mediated signaling. In support of this hypothesis, the B1 and B2 kinin receptors are coupled to similar G-protein subtypes and share the same intracellular signaling pathways. In transgenic mice lacking the B2 kinin receptor, the B1 receptor is upregulated and assumes vascular functions normally associated with the B2 receptor. Moreover, consistent with these data, we have demonstrated that the B2 receptor antagonist, HOE-140, augments the vasomotor responses to the B1 agonist, Lys-des-Arg9-bradykinin, in human umbilical vein in vitro. Future studies examining the effects of B1 receptor agonism and antagonism during concomitant administration of HOE-140 may help clarify the issue of kinin receptor cross-talk in the peripheral circulation of patients with heart failure.

Study Limitations

It has been suggested that the extent of the inflammatory response in patients with congestive cardiac failure correlates with the severity of underlying heart failure. We have examined B1 receptor function in patients with mild to moderate (New York Heart Association class II or III) heart failure. We cannot exclude the possibility that vascular B1 receptor expression may be restricted to patients with severe end-stage heart failure. In addition, the B1 kinin receptor has been implicated in a number of alternative biological processes, including leukocyte trafficking and ischemia-induced angiogenesis. It remains possible that B1 receptors, mediating processes other than vasodilatation or endogenous fibrinolysis, may be present in the human forearm vasculature. Finally, we have examined B1-mediated responses in the forearm circulation of patients with heart failure. Specific vascular beds may differ in their response to B1 agonists and the current findings cannot be extrapolated to the entire vasculature.

In conclusion, contrary to data from animal studies, we have demonstrated for the first time to our knowledge that the B1 kinin receptor does not mediate vasodilatation or endothelial t-PA release in the peripheral circulation of patients with heart failure treated with long-term ACE inhibition. Our findings suggest that the beneficial vascular effects of ACE inhibitor therapy attributed to kinins are restricted to those mediated by the B2 receptor and do not support a major role for the B1 kinin receptor as a potential therapeutic target in patients with heart failure.

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References


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