Bradykinin and Its Metabolite Bradykinin 1–5 Inhibit Thrombin-Induced Platelet Aggregation in Humans

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ABSTRACT

Bradykinin 1–5 is a major stable metabolite of bradykinin, formed by the proteolytic action of angiotensin-converting enzyme. In vitro and animal studies suggest that bradykinin 1–5 possesses biological activity. This study tests the hypothesis that bradykinin 1–5 affects vasodilation, fibrinolysis, or platelet aggregation in humans. Graded doses of bradykinin (47–377 pmol/min) and bradykinin 1–5 (47–18,850 pmol/min) were infused in the brachial artery in random order in 36 healthy subjects. Forearm blood flow (FBF) was measured, and simultaneously obtained venous and arterial plasma samples were analyzed for tissue plasminogen activator (t-PA) antigen. In seven subjects each, α- and γ-thrombin-induced platelet aggregation was measured in platelet-rich plasma obtained from antecubital venous blood at baseline and during peptide infusions. Bradykinin caused dose-dependent increases in FBF and net t-PA release (P < 0.001 for both). Bradykinin 1–5 did not affect FBF (P = 0.13) or net t-PA release (P = 0.46) at concentrations >1500 times physiologic. In contrast, both bradykinin and bradykinin 1–5 inhibited α- and γ-thrombin-induced platelet aggregation (P < 0.01 versus baseline). Bradykinin 1–5 inhibited γ-thrombin-induced platelet aggregation 50% at a calculated dose of 183 ± 3 pmol/min. Neither bradykinin nor bradykinin 1–5 affected thrombin receptor-activating peptide-induced platelet aggregation, consistent with the hypothesis that bradykinin and bradykinin 1–5 inhibit thrombin-induced platelet aggregation by preventing cleavage of the thrombin receptor and liberation of thrombin receptor-activating peptide. This study is the first to demonstrate biological activity of bradykinin 1–5 following in vivo administration to humans. By inhibiting thrombin-induced platelet aggregation without causing vasodilation, bradykinin 1–5 may provide a model for small molecule substrate-selective thrombin inhibitors.

Bradykinin (BK) is a vasoactive peptide that exhibits cardioprotective effects. Bradykinin promotes vasodilation (Vanhoutte, 1989), exerts antiproliferative effects (Linz and Scholkens, 1992), inhibits thrombin-induced platelet activation (TRAP) in vitro (Hasan et al., 1996), stimulates endothelial tissue plasminogen activator (t-PA) release (Smith et al., 1985), and contributes to many of the effects of angiotensin-converting enzyme (ACE) inhibition (Gainer et al., 1998; Labinjoh et al., 2001; Minai et al., 2001). Bradykinin stimulates t-PA release from the human forearm vasculature in a dose-dependent manner through a bradykinin B2 receptor-dependent pathway (Brown et al., 2000). Systemic bradykinin is rapidly metabolized via ACE in humans to BK1–5 (Murphey et al., 2000a). The identification of BK1–5 as the major stable metabolite of systemic bradykinin in humans raises the question of whether this peptide itself exhibits biological activity, just as studies have demonstrated that degradation products of angiotensin II, once thought to be inactive, have biological activity (Kerins et al., 1995; Brosnihan et al., 1996; Freeman et al., 1996).

Studies in animal models suggest that BK1–5 possesses biological activities. Specifically, BK1–5 has been shown to prolong survival in lipopolysaccharide-treated rats, when given at concentrations of 1 nM (Morinelli et al., 2001), to inhibit thrombin-induced platelet activation and electrocyte-induced coronary thrombosis in dogs (Hasan et al., 1996, 1999) and to prevent ipsilateral thrombin-induced brain edema in rats (Jiang et al., 2002). In human studies, net t-PA release correlates with total kinin and BK1–5 concentrations during bradykinin infusion (Murphey et al., 2000a); however,
it is not known whether this is because the concentration of the stable BK1–5 reflects bradykinin dose or because BK1–5 itself stimulates t-PA release. Therefore, the purpose of this study was to compare the effects of bradykinin and its metabolite BK1–5 on vasodilatation, t-PA release, and on thrombin-induced platelet aggregation.

Materials and Methods

Subjects. Thirty-six healthy nonsmoking subjects were studied. 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, electrocardiogram, and routine laboratory analysis. No study subject had evidence of hypertension, hyperlipidemia, cardiovascular disease, or other systemic condition. Twenty-two men and 14 women were studied. Twenty-nine subjects were white, and seven were black. The mean age was 29.8 ± 1.1 years; mean body mass index was 26.0 ± 0.6 kg/m². Mean arterial pressure and heart rate were 83.8 ± 1.5 mm Hg and 66.0 ± 1.8 beats per min, respectively.

Experimental Protocol. Studies were performed in the morning in a temperature-controlled room as described in detail elsewhere (Brown et al., 1999). After placement of intravenous and intraarterial catheters, subjects were allowed to rest 30 min before baseline measurements were made. Forearm blood flow (FBF) was measured by mercury-in-silastic strain-gauge plethysmography (Hokanson et al., 1975). After measurement of FBF, simultaneous arterial and venous samples were obtained from the infused arm. Blood pressure was monitored in the contralateral arm with an automated line measurements were made. Forearm blood flow (FBF) was calculated from the FBF, t-PA activity in-creases with t-PA antigen in response to bradykinin (Brown et al., 1999), t-PA activity was not measured separately. Arteriovenous concentration gradients were calculated by subtracting the plasma concentrations measured in simultaneously collected venous and arterial blood. Forearm plasma flow was calculated from the FBF, and arterial hematocrit was corrected for 1% trapped plasma. Thus, individual net release or uptake rates at each time point were calculated by the formula:

\[
\text{Net release} = (C_V - C_A) \times \frac{\text{FBF} \times (101 - \text{hematocrit})}{100}
\]

where \(C_V\) and \(C_A\) represent the concentration of t-PA in the brachial vein and artery, respectively.

Platelet Aggregation Assay. Blood for measurement of platelet aggregation was collected from the forearm venous catheter during infusion of bradykinin and BK1–5. After the first 3 ml of blood were discarded, blood was drawn into an acid-citrate-dextrose solution [9:1 (v/v); composition, 25 g/l dextrose, 22 g/l sodium citrate, and 8 g/l citric acid, in water, pH 5.0] at room temperature. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200g for 20 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation of the remaining blood at 1000g for 20 min. Aggregation was assessed using the principle of light transmission (Platelet Aggregation Profiler, model PAP-4; Biodata, Hatboro, PA). Agonist (10 μl) was added to 490 μl of PRP, which was continuously stirred at 37°C. Platelet-poor plasma (500 μl) was used as the blank. The total extent of aggregation was used as the response parameter for agonist-stimulated platelet aggregation and was expressed as a percentage of light transmission. The amount of light transmission obtained with PPP was used as 100%. Initially, aggregation was induced with increasing concentrations of either α-thrombin (from 0.2 to 3 IU, specific activity 2997.00 IU/mg; Sigma, St. Louis, MO) in seven subjects or TRAP 1–6, SFLLRN (from 50 nM to 20 μM; Peninsula Laboratories, Merseyside, UK), in eight subjects during infusion of bradykinin (377 pmol/min) or BK1–5 (18,850 pmol/min). The concentration of α-thrombin required to induce at least 75% platelet aggregation was determined at baseline for each subject, and this concentration was used in subsequent studies. For TRAP 1–6, a concentration of 2 μM was used for aggregation studies.

To exclude artifactual α-thrombin-induced fibrin formation during the aggregation assays, further studies using γ-thrombin (which does not catalyze the cleavage of fibrinogen) in concentrations from 100 nM to 1 μM confirmed the antithrombin effect of both peptides. In seven subjects, the antithrombin dose-response curve of BK1–5 was defined using γ-thrombin at a concentration of 500 nM.

Statistics. Data are presented as mean ± S.E.M. The effects of increasing doses of bradykinin and BK1–5 on FBF, net t-PA release, and BK1–5 levels in venous blood were assessed by a general linear model-repeated measures ANOVA, in which the with-subject variables were drug and dose increments. Post hoc comparisons were made using the paired t test or Wilcoxon signed rank test, as appropriate. The effects of bradykinin and BK1–5 versus baseline on α-thrombin- or TRAP 1–6–induced platelet aggregation were also assessed by repeated measures ANOVA followed by Bonferroni’s testing for multiple comparisons. The ED50 for BK1–5 inhibition of γ-thrombin-induced platelet aggregation was calculated using the Prism graphing program (version 4.0; GraphPad Software, Inc., San Diego, CA). A two-tailed P value of less than 0.05 was considered statistically significant. All statistical analyses were performed with the statistical package SPSS for Windows (version 10.0; SPSS, Inc., Chicago, IL).

Results

BK1–5 Concentration. Figure 1 shows venous BK1–5 concentration during both intra-arterial infusion of bradykinin and BK1–5. The venous concentration of BK1–5 increased in a dose-dependent fashion during bradykinin (from...
41.6 ± 10.9 to 484.2 ± 106.4 fmol/ml; P < 0.005 for dose effect) and BK1–5 infusions (from 48.9 ± 13.0 to 2099.5 ± 707.6 fmol/ml; P < 0.01 for dose effect) in those subjects who received equimolar doses of the two agonists from 47 to 377 pmol/min. Venous concentrations of BK1–5 were significantly higher during intra-arterial administration of BK1–5 than during administration of bradykinin over this dose range (P < 0.001). Concurrent administration of enalaprilat prevented the increase in venous BK1–5 concentrations during infusion of bradykinin (from 49.7 ± 31.1 to 56.5 ± 11.0 fmol/ml). In subjects who received BK1–5 up to doses of 18,850 pmol/min, venous concentrations of BK1–5 increased in a dose-dependent manner from 44.7 ± 17.1 to 79,359 ± 12,824 fmol/ml (P < 0.001 for dose effect).

**Hemodynamics and FBF.** Local infusions of bradykinin and BK1–5 did not affect mean arterial pressure; therefore, data are presented as FBF. Figure 2A shows the effect of bradykinin and BK1–5 on FBF. Bradykinin significantly increased FBF in a dose-dependent fashion from 3.6 to 22 ml/min/100 ml at baseline to 71.9 to 169 ml/min/100 ml at the 377 pmol/min dose (P < 0.001 for dose effect). There was no effect of BK1–5 on FBF at doses up to 18,850 pmol/min (P = 0.13 for dose effect). Thus, FBF was significantly higher during intra-arterial administration of intact bradykinin than during intra-arterial administration of BK1–5 in those subjects who received equimolar doses of both agonists from 47 to 377 pmol/min (P < 0.01).

**Fibrinolytic Parameters.** Figure 2B shows net t-PA release across the forearm during bradykinin and BK1–5 infusions. Bradykinin caused a dose-dependent increase in net t-PA release from –1.9 ± 1.1 ng/min/100 ml at baseline to 71.9 ± 16.9 ng/min/100 ml at the 377 pmol/min dose (P < 0.001). However, there was no effect of BK1–5 on net t-PA release at doses up to 18,850 pmol/min (P = 0.92; P = 0.46). Thus, net t-PA release was significantly higher during intra-arterial administration of intact bradykinin than during intra-arterial administration of BK1–5 for drug doses ranging from 94 to 377 pmol/min (P < 0.05) in those subjects who received equimolar doses of both agonists. Net PAI-1 extraction increased significantly with increasing doses of bradyki-
TRAP 1–6-induced platelet aggregation (response to 2 μM TRAP 1–6 was 52.2 ± 9.9, 54.56 ± 8.7, and 56.13 ± 6.6% at baseline and during bradykinin and BK1–5 infusions, respectively; P = 0.95).

**Thrombin-Induced Platelet Aggregation.** Because α-thrombin can proteolyze fibrinogen, producing clot formation that can interfere with platelet aggregometry, studies were repeated with γ-thrombin at concentrations from 100 nM to 1 μM. Both intra-arterial bradykinin and BK1–5 inhibited γ-thrombin-induced platelet aggregation (data not shown). Further studies were undertaken to define the dose response for the antithrombin effect of BK1–5 with doses ranging from 37 to 754 pmol/min. As shown in Fig. 5, BK1–5 potently inhibited γ-thrombin (500 nM)-induced platelet aggregation with an ED₅₀ of 183 ± 2 pmol/min. Simultaneously obtained venous plasma samples were also analyzed for BK1–5 concentrations, and mean plasma concentrations of BK1–5 were 1635 ± 311 fmol/ml during the 188 pmol/min infusion. In contrast, when BK1–5 was added in vitro, a concentration of 7 mM was required to inhibit γ-thrombin-induced platelet aggregation (not shown). There was no effect of PPP from subjects infused with BK1–5 at 18,850 pmol/min on γ-thrombin-induced aggregation of platelets from unexposed individuals (70.0 ± 5.1 versus 69.3 ± 3.2% control; P = 0.864).

**Discussion**

Bradykinin is a short-lived vasoactive peptide, with a reported half-life in vivo of 17 s (Ferreira and Vane, 1967), that is rapidly metabolized in the circulation to BK1–5 (Murphey et al., 2000b). BK1–5, the product of two sequential cleavages of bradykinin by ACE at the Pro⁷-Phe⁸ and Phe⁸-Ser⁹ bonds (Murphey et al., 2000a), has been identified as the major stable metabolite of bradykinin in vivo in human subjects, with a terminal half-life of minutes (Murphey et al., 2000b). In studies in which increasing doses of bradykinin were infused intravenously, plasma BK1–5 concentrations increased in direct proportion to the dose of bradykinin administered (Murphey et al., 2000b). The conversion of bradykinin to...
BK1–5 is genetically determined by an insertion/deletion polymorphism in the ACE gene, such that individuals with the D/D genotype, which is associated with the highest level of plasma ACE activity, have the highest ratio of BK1–5 to bradykinin (Murphey et al., 2000a). The present study is the first to indicate that BK1–5 exhibits biological activity in humans and inhibits thrombin-induced platelet aggregation. In addition, bradykinin also inhibits thrombin-induced platelet aggregation, even when its degradation to BK1–5 is prevented by concurrent ACE inhibition.

Previous studies indicate that bradykinin and BK1–5 inhibit thrombin-induced platelet aggregation in vitro (Hasan et al., 1996, 1999). For example, in PRP, BK1–5 inhibits α-thrombin-induced platelet activation with an IC_{50} of 0.5 mM (Hasan et al., 1996). In vivo in rabbits, plasma BK1–5 concentrations of 8.7 μM caused measurable inhibition of γ-thrombin-induced platelet aggregation for up to 4 h (Hasan et al., 1999). Likewise, in dogs, local BK1–5 concentrations of 0.6 mM were effective in delaying coronary thrombosis induced by electrolytic injury (Hasan et al., 1999). In the present study in humans, supraphysiological concentrations of bradykinin and BK1–5 inhibited α- and γ-thrombin-induced but not TRAP 1–6-induced platelet aggregation. The lack of an effect of BK1–5 on TRAP 1–6-induced platelet aggregation is consistent with the data of Hasan et al. (2003), indicating that BK1–5 inhibits thrombin-induced platelet activation in vitro by preventing the cleavage of the protease-activated receptor (PAR-1) at its cleavage site, arginine 41, thereby preventing the exposure of the PAR-1 autoactivating tethered ligand. In addition, BK1–5 may inhibit thrombin by binding to the PAR-4 thrombin receptor (Nieman et al., 2005).

In the present study, we determined the ED_{50} for BK1–5 inhibition of γ-thrombin-induced platelet aggregation. The concentration of BK1–5 achieved during intra-arterial administration that resulted in maximal platelet inhibition was approximately 50- to 75-fold higher than physiological concentrations (Murphey et al., 2004; Pretorius et al., 2004) but 150,000-fold lower than the concentrations required to inhibit platelet aggregation or in vitro (Hasan et al., 1996, 1999) or in vivo in dogs. Species differences may account for the difference in the potency of BK1–5 in dogs and humans. However, as in our study, increased potency of BK1–5 for platelet aggregation in vivo compared with in vitro has been observed previously. Nieman et al. (2004) reported that BK1–5 inhibits thrombosis in mice at doses that produce physiologic concentrations, whereas micromolar concentrations of BK1–5 were required to inhibit platelet aggregation in vitro. It is possible that intravascular concentrations of BK1–5 exceed circulating concentrations, given expected local concentrations of high molecular weight kininogen (670 nM) (Colman and Schmaier, 1997), a known substrate for the generation of bradykinin and BK1–5.

Alternatively, increased potency following in vivo compared with ex vivo exposure of platelets to BK1–5 could result either if BK1–5 released an endogenous thrombin inhibitor in vivo or if BK1–5 altered platelet function in vivo via a mechanism that persisted during isolation of platelets. In favor of the latter mechanism, we found no effect of PPP from subjects who had been exposed to the highest concentration of BK1–5 on thrombin-induced aggregation of platelets from unexposed individuals. In addition, Schmaier and co-workers have reported difficulty competing BK1–5 binding to immobilized PAR-1 using soluble PAR-1 (Hasan et al., 2003). Taken together, these findings suggest stable binding of BK1–5 to PAR-1 following in vivo exposure.

In the present study, we found that bradykinin caused a significant dose-dependent increase in FBF and net t-PA release, as previously reported by our laboratory (Brown et al., 1999, 2000). These effects are known to be mediated by stimulation of the B2 receptor, as HOE 140 (icatibant, a specific B2 receptor antagonist) blocks the effect of bradykinin (Brown et al., 2000). In contrast, BK1–5 had no effect on FBF or t-PA release. This is consistent with data from Morinelli and collaborators who reported that, at concentrations of approximately 1.0 nM, BK1–5 did not significantly displace the binding of [³H]bradykinin from B2 receptors in cultured vascular smooth muscle cells, suggesting that BK1–5 does not have affinity for B2 receptors at those concentrations. These data suggest that BK1–5 exerts no B2 receptor-mediated effects in the human forearm vasculature, as measured by changes in FBF and t-PA release under the experimental conditions set out in this study.

An unexpected finding in the study was the observation that bradykinin caused a significant increase in net PAI-1 extraction across the forearm vasculature, an effect that was not observed with BK1–5. An effect of bradykinin on PAI-1 extraction has not been observed in prior studies (Brown et al., 1999, 2000). To exclude the possibility that circadian variation in PAI-1 could have influenced calculated PAI-1 extraction across the forearm, we determined whether treatment order influenced PAI-1 extraction during bradykinin infusion. In this analysis, there was no effect of the order of randomization on the degree of net PAI-1 extraction, suggesting that the effect was independent of the time of sample collection. Furthermore, simultaneous sampling of venous and arterial blood for measurement of PAI-1 antigen should have negated any effect of circadian variation in PAI-1 concentrations on calculated arteriovenous gradients. The mechanism for the apparent effect of bradykinin on vascular PAI-1 extraction is not clear. PAI-1 is cleared primarily by the liver (Pearson and Guthrie, 1982). Whereas bradykinin stimulates the release of nitric oxide and nitric oxide decreases PAI-1 expression (Bouchie et al., 1998), an effect of bradykinin on PAI-1 expression would not probably be observed over the time course of this study. Most likely, the apparent increase in PAI-1 extraction across the forearm reflects the dilutional effect of bradykinin-stimulated flow on venous PAI-1 concentrations, an effect that may have been magnified by the longer duration of bradykinin infusion in this study compared with our prior studies (Brown et al., 1999, 2000).

The findings of this study must be interpreted in the context of clinical studies. ACE inhibitors prevent the degradation of bradykinin to BK1–5 and decrease the incidence of thrombotic events (AIRE Study Investigators, 1993; Yusuf et al., 2000). Conversely, the ACE D/D genotype, associated with enhanced degradation of bradykinin to BK1–5 (Murphey et al., 2000a), has been associated with increased risk of thrombosis in some studies (Rigat et al., 1990). Taken together, these studies are consistent with the findings that both bradykinin and BK1–5 inhibit thrombin-induced platelet aggregation, whereas only bradykinin increases t-PA release. However, the administration of bradykinin at pharmacologic doses, even when given in the coronary artery, is
limited by B2 receptor-mediated hypotension, particularly in the setting of ACE inhibition (Minai et al., 2001). In contrast, the lack of effect of BK1–5 at the B2 receptor could make it feasible to administer this peptide or related small-molecule derivatives at pharmacological doses as an antplatelet agent.

In summary, this study demonstrates that bradykinin and BK1–5, the stable degradation product of bradykinin, inhibit thrombin-induced platelet aggregation in humans. Currently available antplatelet agents, including aspirin, glycoprotein IIb/IIIa inhibitors, and ADP-receptor antagonists, have improved outcomes in the acute coronary syndrome and following percutaneous coronary intervention but are associated with both resistance and complications (Topol, 2001; Stein-hubl et al., 2002; Stone et al., 2002). BK1–5 inhibits platelet aggregation in humans through a novel mechanism without causing vasodilation and thus may serve as a model for new pharmacological antplatelet strategies.

References


