Potentiation of Bradykinin Effect by Angiotensin-Converting Enzyme Inhibition Does Not Correlate With Angiotensin-Converting Enzyme Activity in the Rat Mesenteric Arteries

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Abstract—Angiotensin-converting enzyme (kininase II [ACE]) inhibitors are capable of potentiating bradykinin (BK) effects by enhancing the actions of bradykinin on B2 receptors independent of blocking its inactivation. To investigate further the importance of ACE kininase activity on BK-induced vasodilation, we investigated the effect of inhibiting ACE, as well as other kininases, on both BK metabolism and vasodilator effect in preparations that exhibit increased ACE activity. Mesenteric arterial beds obtained from 1-kidney, 1-clip hypertensive rats presented augmented ACE and angiotensin I converting activities compared with normotensive rats. The isolated and perfused mesenteric beds were exposed to BK for 15 minutes in the absence or in the presence of kininase inhibitors; then, the perfusate was collected for analysis of the products of BK metabolism by high-performance liquid chromatography. BK was metabolized to the fragments BK(1-8), BK(1-7), and BK(1-5), and the recovery of intact BK was reduced by 47% in the hypertensive group. Recovery of BK was increased in both groups in the presence of a kininase I inhibitor and in the hypertensive group by neutral endopeptidase 24.11 inhibitor; however, ACE inhibition did not affect BK metabolism in both groups. In contrast, only the ACE inhibitor potentiated the vasodilator effect of BK in a mesenteric bed preconstricted with phenylephrine; the increase in BK effect, nevertheless, was not greater in arteries from hypertensive rats that presented an increased ACE activity when compared with those in the normotensive group. These data demonstrated that ACE inhibitor–induced potentiation of BK vasodilator effects is not related to their actions on BK degradation. (Hypertension. 2007;50:110-115.)

Key Words: bradykinin ■ ACE activity ■ kininase I ■ endopeptidases ■ ACE inhibitor

The nonapeptide bradykinin (BK) has important pharmacological effects on blood vessels, heart, and kidney; among these, the most conspicuous is the transient hypotensive effect when BK is administered into the systemic circulation in all of the species studied.1 This effect results from resistance vessel dilation in several beds mediated by the release of endothelial relaxing factors after the activation of B2 receptors.2,3 The remarkably short half-life of BK in vivo has been attributed to the rapid enzymatic degradation by several peptidases (collectively known as “kininases”) present in plasma and tissues. The role that a particular kininase plays in the metabolism of BK depends on its localization and the presence of other peptidases in plasma or tissue. The predominant kininases that degrade BK in most tissues are the metallopeptidases kininase II (angiotensin-converting enzyme [ACE]), kininase I (carboxypeptidase M and N), and neutral endopeptidase 24.11 (NEP).4–7 ACE is primarily a surface enzyme that removes the C-terminal dipeptide from BK, which leads to its complete inactivation. ACE eventually cleaves further its primary metabolite, BK(1-7), into the shorter fragment, BK(1-5). NEP, another membrane-bound peptidase cleaves 2 bonds in BK (Gly4-Phe5 and Pro7-Phe8). Kininase I–type carboxypeptidases cleave the C-terminal basic amino acid Arg from BK generating BK(1-8), whereas carboxypeptidase N is present in the plasma and carboxypeptidase M is a widely distributed ectoenzyme anchored to plasma membranes. The membrane localization of ACE, NEP, and carboxypeptidase M makes them ideally suited to regulate BK activity when expressed in the kidney and vascular tissue.

The well-known potentiation of BK actions by ACE inhibitors has been attributed to a protection of BK against ACE enzymatic degradation, an effect that seems to contribute to the large therapeutic spectrum of ACE inhibitors.8 However, studies using cultured transfected or native cells propose that, other than the protection of BK from degrada-
tion, ACE inhibitors may also re sensitize or potentiate the effects of ligands on B<sub>2</sub> receptors by inducing protein–protein cross-talk between ACE and the receptor.9–12 In contrast, studies in isolated perfused rat heart<sup>13</sup> or porcine coronary artery<sup>14</sup> suggest that inhibition of ACE in the immediate vicinity of B<sub>2</sub> receptors is a more likely explanation for the potentiation of BK by ACE inhibitors than a direct interaction between ACE and B<sub>2</sub> receptors. The key approach for these controversial studies was to use BK analogs containing amino acid substitutions that confer a varied degree of resistance to breakdown by ACE and affinity to B<sub>2</sub> receptor. To avoid these inconveniences and to further investigate the possibility that the ACE inhibitor–induced potentiation of BK has a metabolic origin, we examined the effect of ACE inhibition on the metabolism and effect of BK in the isolated mesenteric arterial bed (MAB) of normotensive control and 1-kidney, 1-clip (1K1C) hypertensive rats that exhibit an increased vascular ACE activity.15–17 Thus, to investigate the importance of ACE, as well as NEP and kininase I–like activities, in modulating the effect of BK in vascular tissue, we evaluated in the rat MAB the effect of their inhibitors on the metabolism of exogenously administered BK by determining the recovery of intact BK and its degradation products and the main functional response, the BK B<sub>2</sub> receptor–mediated vasorelaxation.

**Methods**

**Animals**

The experiments were performed using male Wistar rats that were housed in a room at 20°C with a 12-hour light/dark cycle and allowed free access to tap water and standard rat chow. All of the surgical procedures were performed on ether-anesthetized rats, and experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the Animal Care and Use Committee of the Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo. To induce 1K1C hypertension, a silver clip (1K1C) was placed around the left renal artery, and right nephrectomy was performed in 180- to 200-g rats. Systolic blood pressure was measured weekly by the tail-cuff method, and only the animals that presented blood pressure >170 mm Hg were used 5 to 6 weeks after clipping. Age-matched intact normotensive rats were used as control.

**Blood Pressure Measurements**

On the day of the experiment, blood pressure of conscious rats was recorded (HP-7754A recorder and HP-1280C transducer, Hewlett Packard) through a catheter implanted into the carotid artery 24 hours earlier under ether anesthesia.

**Preparation of the Isolated MAB**

After blood pressure measurement, the MAB was isolated under anesthesia, as described previously.15,18 and perfused through a cannula placed into the superior mesenteric artery with a modified Krebs solution (in <mml:mi>mol</mml:mi>/L) NaCl 120.0, KCl 4.7, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.43, NaHCO<sub>3</sub> 25.0, KH<sub>2</sub>P<sub>4</sub> 1.17, glucose 11.0, and EDTA 0.03 maintained at 37°C (pH 7.4) and equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. MAB perfusate were altered to keep the perfusion solution recirculating through the MAB. In this way, 4-mL volumes of perfusing solution were recirculated for a 15-minute period. At the beginning of the recirculating period, BK(100 nmol) was injected into the solution perfusion in the absence or the presence of kininase inhibitors. The assay conditions were determined considering the sensitivity of our amino acid analyzer to determine the concentration of each BK peptide formed and that BK(100 nmol) was completely metabolized in a 30-minute period of recirculation across the MAB as determined in a pilot experiment. Then, the whole MAB perfusate (volume 4 mL) was collected in polypropylene tubes containing 150 µL of trifluoroacetic acid 5%. BK and its degradation products present in the perfusate were extracted by reverse-phase chromatography on Shimadzu SLC-6b (Japan) equipment fitted with a Sep-Pak C18 column, and the BK peptides were separated by high-performance liquid chromatography using a reversed-phase column (Shim Pack CLC-ODS, 4.6 mm×15 cm) at a flow rate of 1 mL/min and a 30-minute period gradient of 20% to 70% (vol/vol) acetonitrile in 0.01% (vol/vol) H<sub>2</sub>PO<sub>4</sub>. BK and its metabolite peptides were detected by absorbance at 214 nm and identified using peptide standards. The material corresponding with each peak was collected, dried under vacuum, and subjected to amino acid analysis after acid hydrolysis for determination of the peptide concentration. The kininase inhibitors added to the solution perfusion at the beginning of the equilibration period were (N = 5 each): ramiprilat (5 µmol/L) or captopril (5 µmol/L) to inhibit ACE; DL-2-mercaptoethyl-3-guanidinopentane (MGTA; 50 µmol/L), an inhibitor of basic carboxypeptidases as kininase I; phosphoramidon (10 µmol/L) to inhibit NEP; o-phenantrone (1 mmol/L), an inhibitor of metalloproteinases; or the combination of ramiprilat and MGTA or phosphoramidon.

**Evaluation of BK Vasodilator Effect**

The mesenteric arteries were perfused at a constant flow of 4 mL/min with Krebs solution containing indomethacin (5.5 µmol/L). Blood pressure was continuously monitored throughout the experiments. After a 30-minute stabilization period, phenylephrine (2.5 to 50 µmol/L) was added to the perfusion solution until a stable perfusion pressure of ~100 mm Hg was achieved. BK (2.5 to 160 ng) was administered by bolus injection of 5 to 50 µL of the peptide solution into the perfusion stream. For the assessment of the contribution of different kininase activities to the effect of BK, the following specific inhibitors were added to the solution perfusion at beginning of the infusion (N = 7 each): ramiprilat (5 µmol/L), MGTA (50 µmol/L), phosphoramidon (10 µmol/L), or the combination of ramiprilat and MGTA. The endothelium-dependent vasodilation responses elicited by acetylcholine (2.5 to 40 ng) were also assessed in phenylephrine-precontracted preparations in the absence or in the presence of the protease inhibitors (N = 7 each) to monitor alterations in relaxant capacity after different inhibitor treatments.

**ACE Activity**

ACE activity in MAB homogenates was determined as described previously.15,17 using Hip-His-Leu as substrate, and expressed in picomoles of His-Leu per minute per microgram of protein. Protein content in tissue homogenate was determined using Coomassie reagent. Angiotensin (Ang) I to Ang II converting activity was also estimated by the ratio of the doses of both peptides that elicited a 35-mm Hg increase in mesenteric perfusion pressure. These doses were determined from the dose–response curves obtained by bolus injection of Ang I (100 to 800 pmol) and Ang II (20 to 200 pmol) into the MAB maintained at the same conditions described for the study of the vasodilator effect of BK. In addition, to verify the role of the ACE-independent pathway in Ang I conversion,19,20 single responses to Ang II (200 pmol) and Ang I (800 pmol) were determined in the same preparation (N = 5) before and after the addition of captopril (10 µmol/L).

**Drugs**

Acetylcholine, BK, BK(1-8), BK(1-7), BK(1-5), indomethacin, o-phenantrone, captopril, and phenylephrine were purchased from...
Sigma Chemical Co. and MGTA and phosphoramidon from Calbiochem. Ramiprilat was provided by Hoescht-Marion-Roussel.

**Data Analysis**

Vasodilator responses were calculated as the percentage decrease in mesenteric perfusion pressure, and the differences between dose–response curves obtained in the absence or presence of kininase inhibitors were evaluated using multivariate ANOVA for repeated measures. For comparisons between BK peptide data without and with protease inhibitors, ANOVA with the Dunnett test for multiple comparisons was used. Finally, Student's *t* test was used when only 2 mean values were compared. Data are expressed as mean±SEM, and differences were considered significant at a value of *P*<0.05.

**Results**

**Arterial Pressure**

Mean arterial pressure of 1K1C rats was significantly higher than that of control rats (184±2 versus 104±1 mm Hg; *P*<0.0001).

**Effect of Kininase Inhibitors on the Metabolism of BK**

Figure 1A shows an original high-performance liquid chromatography tracing that represents the spectrum of BK metabolites obtained from the rat MAB perfusate. Under control conditions, BK was mostly metabolized to the fragments BK(1-8), BK(1-7), and BK(1-5) from the mesenteric bed perfusate isolated from normotensive and 1K1C hypertensive rats, and no significant amino-terminal degradation could be detected. The recovery of intact BK was greater (*P*<0.0001) in MAB perfusate of normotensive than 1K1C rats (Figure 1B). Although the production of the fragment BK(1-5) in samples from the 1K1C group was greater (*P*<0.003), no significant difference was found in BK(1-8) and BK(1-7) fragments between samples from the 2 groups of rats. In the presence of the metalloproteinase inhibitor o-phenantroline, ~80% of BK was recovered intact in both groups, and the recovery of BK(1-8), BK(1-7), and BK(1-5) was markedly depressed or abolished (Figure 1C).

The effect of kininase inhibitors on the recovery of intact BK and its metabolites from the perfusate of MAB from both normotensive and hypertensive rats is presented in Figure 2. Under control conditions, BK was mostly metabolized to the fragments BK(1-8), BK(1-7), and BK(1-5) in MAB from both normotensive and hypertensive rats, and no significant amino-terminal degradation could be detected. The recovery of intact BK was greater (*P*<0.0001) in MAB perfusate of normotensive than 1K1C rats (Figure 1B). Although the production of the fragment BK(1-5) in samples from the 1K1C group was greater (*P*<0.003), no significant difference was found in BK(1-8) and BK(1-7) fragments between samples from the 2 groups of rats. In the presence of the metalloproteinase inhibitor o-phenantroline, ~80% of BK was recovered intact in both groups, and the recovery of BK(1-8), BK(1-7), and BK(1-5) was markedly depressed or abolished (Figure 1C).

The effect of kininase inhibitors on the recovery of intact BK and its metabolites from the perfusate of MAB from both normotensive and hypertensive rats is presented in Figure 2. The ACE inhibitor ramipril did not alter the recovery of intact BK or its metabolites in both groups. Another ACE inhibitor, captopril, exhibited the same effect (38.6±6.9 versus 32.3±3.4 pmol of intact BK in control). MGTA prevented the formation of BK(1-8) and increased the recovery of intact BK in samples from normotensive (by 49%; *P*<0.03) and hypertensive (by 101%; *P*<0.001) rats, whereas the generation of BK(1-7) and BK(1-5) was not affected. The combination of ramiprilat and MGTA produced no further
effect on BK metabolism than that already observed with MGTA alone. Phosphoramidon significantly \( P < 0.05 \) increased the recovery of BK and reduced the recovery of BK(1-7) in samples from hypertensive rats \( P < 0.001 \) but not from normotensive and, as expected, did not significantly change the recovery of either BK(1-8) or BK(1-5) in both groups. In the presence of both ramiprilat and phosphoramidon, the recovery of BK peptides was similar to that observed in the presence of the NEP inhibitor alone, except that an additional decrease in the formation of BK(1-7) was observed in both groups.

**Effect of Kininase Inhibitors on the Vasodilator Effect of BK**

The addition of the different kininase inhibitors did not affect basal perfusion pressure of phenylephrine-constricted MAB, and their effect on BK-induced vasodilation is shown in Figure 3. In the absence of kininase inhibitors, BK elicited a dose-related fall in perfusion pressure that was significantly reduced \( P < 0.0001 \) in MAB of hypertensive compared with that of normotensive rats. Dose–response curves to BK were shifted to the left in the presence of the ACE inhibitor ramiprilat in preparations of normotensive \( P < 0.0001 \) and hypertensive \( P < 0.0007 \) groups. Although maximal vasodilator response was increased by ramiprilat in the normotensive group \( 74.7 \pm 3.3\% \) versus \( 88.3 \pm 3.0\% \; P < 0.05 \), it was not affected in the hypertensive group. MGTA or phosphoramidon added to the perfusion solution had no significant effect on BK-induced dose–response curves obtained in preparations from both groups. The potentiation of BK responses in the presence of both ramiprilat and MGTA was of the same extent of that observed with ramiprilat alone in the 2 groups.

**ACE Activity**

ACE activity determined in mesentery homogenate of hypertensive rats was significantly higher than that of normotensive rats \( 1.40 \pm 0.17 \) versus \( 0.59 \pm 0.09 \) pmol of His-Leu/min per microgram of protein; \( P < 0.007 \). The increases in perfusion pressure elicited by different doses of Ang II and Ang I in MAB of normotensive and hypertensive rats are shown in Figure 5A. Although the responses to Ang II were smaller

The dose–response curves elicited by acetylcholine, an endothelium-dependent vasodilator agent, in MAB of normotensive and 1K1C hypertensive rats were not affected by the kininases inhibitors (Figure 4). However, as observed for BK, the responses to acetylcholine were depressed in arteries of hypertensive rats \( P < 0.0001 \) compared with those in the normotensive group.
rats that presented an augmented ACE activity. In addition, preparations from normotensive rats, as well as from 1K1C ACE inhibition on the metabolism of BK was observed in combination with MGTA. Interestingly, the lack of effect of metabolism of BK in perfused MAB either alone or in contrast, ACE inhibition had no protective effect on the potency ratio of Ang I and Ang II to induce vasoconstriction on isolated MAB of normotensive and hypertensive rats. This study has become clear that ACE is not the only relevant kininase-like activity present in the rat mesenteric vasculature, though the vasodilator effect of BK was potentiated only in the presence of an ACE inhibitor. Moreover, the magnitude of ACE inhibitor–induced potentiation of BK vasodilator effects is not related to ACE activity.

In agreement with previous data, ACE activity was greatly increased in MAB of 1K1C hypertensive rats. Also, an increase in the extent of Ang I conversion to Ang II in isolated preparations from 1K1C was observed by comparing the potency ratio of Ang I and Ang II to induce vasoconstriction on isolated MAB of normotensive and hypertensive rats. A possible role for non-ACE pathways in the increased Ang I conversion to Ang II in MAB of 1K1C rats could not be demonstrated in our experiments, because the ACE inhibitor–resistant response to Ang I was of similar magnitude in both groups. These data are consistent with a functional increase of ACE activity in MAB of 1K1C hypertensive rats.

The fact that less intact BK was recovered from MAB perfusate of 1K1C rats than control rats is consistent with the hypothesis of an increased kininase activity in this model of hypertension. However, if we take the reduction of the nonapeptide BK degradation caused by the addition of each kininase inhibitor as a measure for the kininase activity of the respective enzyme, a basic carboxypeptidase-like activity is the most important identified kininase in perfused mesenteric arteries of both normotensive and 1K1C rats. In fact, an MGTA-sensitive carboxypeptidase activity is responsible for most of the identified metabolism of BK in the absence of peptidase inhibitors, and its inhibition, demonstrated by the complete disappearance of its direct product BK(1-8), significantly increases the recovery of intact BK in both groups. It is worth mentioning that a carboxypeptidase activity may be secreted by the perfused MAB into the perfusion solution, and this activity was described as the major degradative pathway of BK by enzymes found in the perfusate. In contrast, ACE inhibition had no protective effect on the metabolism of BK in perfused MAB either alone or in combination with MGTA. Interestingly, the lack of effect of ACE inhibition on the metabolism of BK was observed in preparations from normotensive rats, as well as from 1K1C rats that presented an augmented ACE activity. In addition, the failure of BK(1-7) levels, 1 of the products of BK hydrolysis by ACE, to decrease in the presence of an ACE inhibitor is indicative of the operative of alternative pathways, such as NEP, in the formation of this peptide from BK. In fact, when both ACE and NEP inhibitors were combined, the recovery of BK(1-7) was almost abolished in both groups. It is noteworthy that an NEP-like activity seems to play an important role in metabolizing BK in 1K1C arteries, because in the presence of phosphoramidon alone, the recovery of intact BK augmented 58% associated with a significant decrease in BK(1-7) formation in this preparation. NEP plays a major role in the metabolism of kinin peptides in urine, and increased urinary kinin levels may contribute to the natriuretic effects of NEP inhibition. Kinins may also mediate the cardiac effects of NEP inhibition.

An important kininase activity in perfused MAB could not be attributed to ACE, NEP, or carboxypeptidases by the kininase inhibitors used and was responsible for the release of the fragment BK(1-5). Interestingly, the generation of this product from BK was significantly augmented in arteries from hypertensive rats and might be responsible, together with NEP, for the increased metabolism of BK observed in these preparations. This activity could be ascribed to the metalloendopeptidases EC 3.4.24.15 (EP24.15) or EC 3.4.24.16 (EP24.16). This hypothesis is supported by the demonstration that the hypotensive responses to BK were potentiated by the administration of a stable inhibitor of these enzymes, N-(1-[R,S]-carboxy-3-phenylpropyl)-Ala-Aib-Tyr-p-amino-benzoate. In addition, the presence of active EP24.16 was demonstrated in cultured endothelial cells, supporting the assumption that this enzyme may be involved in the metabolism of BK in vascular tissue.

To establish the relative contribution of these kininases in modulating the effect of BK, we determined the effect of selective kininase inhibitors on the BK-induced vasodilation of the mesenteric arteries. Although MGTA and phosphoramidon-susceptible activities could be detected in the perfused MAB, their inhibitors did not affect the responses to BK in both groups. Interestingly, the potentiation of BK vasodilator effects by captopril in the hypertensive group was not greater than that found in the normotensive group, though the former presented increased vascular ACE activity. These findings indicate that an augmented metabolism of BK by ACE in 1K1C arteries has little contribution to the reduced vasodilation induced by BK. An endothelial dysfunction that is commonly associated with the hypertensive state could be responsible for the reduced responsiveness to BK in 1K1C arteries. This interpretation is supported by the fact that the endothelium-dependent vasodilator responses to acetylcholine were also reduced in MAB of hypertensive rats.

Altogether, our study shows that different carboxy- and endopeptidases with kininase activity are present in the MAB and could be detected when BK was allowed to recirculate for 15 minutes through the vascular bed. However, a role for these kininase activities in modulating the vasodilator effect of BK injected as a bolus in mesenteric arteries could not be demonstrated. The fact that potentiation of BK effects in the presence of an ACE inhibitor in arteries of 1K1C hypertensive rats that showed a functional increase in
ACE activity was not greater than that observed in arteries of normotensive rats implies that ACE inhibitors might potentiate BK effects in a manner not related to their actions on BK degradation.

Perspectives
Considering the very short half-life of BK, data obtained from tissue homogenates or a prolonged period of BK incubation with plasma or other organic fluids may disclose kininase activities that do not play any relevant role in metabolizing BK and, consequently, modulating the effect of BK in vivo. Furthermore, our findings in intact arterial bed isolated from rats show that ACE inhibitor–induced potentiation of BK vasodilation does not correlate with ACE activity and, in addition, strongly suggest that the ACE–B2 receptor interaction is not limited to transfected or isolated cells.

Source of Funding
This work was supported by a grant from Fundação de Amparo à Pesquisa do Estado de São Paulo.

Disclosures
None.

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Hypertension. 2007;50:110-115; originally published online April 30, 2007;
doi: 10.1161/HYPERTENSIONAHA.106.085761

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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