Potentiation of Kinin Analogues by Ramiprilat Is Exclusively Related to Their Degradation

Andreas Dendorfer, Siegmund Reißmann, Sebastian Wolfrum, Walter Raasch, Peter Dominiak

Abstract—The potentiation of kinin actions represents a cardioprotective property of ACE inhibitors. Although a clear contribution to this effect is related to the inhibition of bradykinin (BK) breakdown, the high efficacy of potentiation and the ability of ACE inhibitors to provoke a B2-receptor–mediated response even after receptor desensitization has also triggered hypotheses concerning additional mechanisms of kinin potentiation. The application of kinin analogues with enhanced metabolic stability for the demonstration of degradation-independent mechanisms of potentiation, however, has yielded inconsistent results. Therefore, the relation between the susceptibility of B2-agonists to ACE and the potentiation of their actions by ACE inhibitors was investigated with the use of minimally modified kinin derivatives that varied in their degree of ACE resistance. The B2-agonists BK, d-Arg-[Hyp3]-BK, [Hyp, Tyr(Me)3]-BK, [D-Phe3]-BK, [D-NMF7]-BK, and [Phe8(CH2-NH)Arg9]-BK were tested for degradation by purified rabbit ACE and for their potency in contracting the endothelium-denuded rabbit jugular vein in the absence and presence of ramiprilat. Purified ACE degraded d-Arg-[Hyp3]-BK and [Hyp, Tyr(Me)3]-BK at 81% and 71% of BK degradation activity, respectively, whereas other peptides were highly ([D-Phe3]-BK) or completely ([D-NMF7]-BK, [Phe8(CH2-NH)Arg9]-BK) resistant. The EC50 of BK-induced vasoconstriction (1.15±0.2 nmol/L) was reduced by a factor of 5.7 in the presence of ramiprilat. Likewise, d-Arg-[Hyp3]-BK and [Hyp, Tyr(Me)3]-BK were both significantly potentiated by a factor of 4.4, whereas the activities of the other agonists were not affected. Ramiprilat exerted no influence on the maximum contraction induced by any of the agonists. It is concluded that the potentiation of kinin analogues during ACE inhibition correlates quantitatively with the susceptibility of each substance to degradation by ACE. As such, no evidence of degradation-independent potentiating actions of ACE inhibitors could be obtained. (Hypertension. 2001;38:142-146.)

Key Words: bradykinin ■ angiotensin-converting enzyme ■ receptors, bradykinin ■ kinins ■ rabbits

Potentiation of kinin actions is a well-known property of angiotensin I–converting enzyme inhibitors, an effect attributed to a protection of kinins against ACE enzymatic degradation. This mechanism contributes to the therapeutic spectrum of ACE inhibitors in vivo. A variety of experimental studies have demonstrated that enhancement of kinin effectiveness is responsible for beneficial influences of ACE inhibitors on the cardiovascular system, such as reduction of infarct size, inhibition of myocardial hypertrophy and fibrosis, and protection against the development of hypertension (reviewed by Linz et al1). The contribution of endogenous kinins to the vasodilatory effect of an ACE inhibitor has recently been demonstrated in humans as well.2 Such kinin-mediated effects can arise because of the high extent to which ACE inhibitors potentiate the effectiveness of kinins. In volunteers, ACE inhibitor treatment sensitized the blood pressure response to injected bradykinin (BK) by a factor of α=40.3 However, attempts to correlate this intriguing effect with BK accumulation have failed because (1) plasma kinin levels increase only modestly after ACE inhibition,4 and (2) kinin potentiation by ACE inhibitors is also observed in experimental models in which kinin degradation should be negligible (eg, in superfused vessels5).

This fact, and the observation that after desensitization by prolonged kinin exposure an ACE inhibitor can reestablish a B2-receptor–mediated response in the continued presence of the desensitizing kinin concentration (a phenomenon addressed as “receptor resensitization”),6,7 has triggered hypotheses of degradation-independent mechanisms by which ACE inhibitors might potentiate the actions of BK. Such proposed mechanisms include stabilization of B2 receptors in a high-affinity state,8 attenuation of receptor sequestration and internalization,8,9 and enhancement of the spontaneous activity of unoccupied B2-receptors.5

The key approach for demonstrating such degradation-independent mechanisms of kinin potentiation and for excluding the consequences of reduced kinin cleavage is to use degradation-resistant kinin analogues. In fact, both potentia-
Degradation of BK and Kinin Analogues by ACE and Potentiation of Action by Ramiprilat

<table>
<thead>
<tr>
<th>B2-Agonist</th>
<th>Degradation by ACE (0.1 IU), pmol/min</th>
<th>EC50 of Venoconstriction, mmol/L</th>
<th>Maximum Venoconstriction, mN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without RAM</td>
<td>With RAM</td>
<td>Without RAM</td>
</tr>
<tr>
<td>BK</td>
<td>80.8±5.7</td>
<td>1.15±0.20</td>
<td>0.20±0.06*</td>
</tr>
<tr>
<td>d-Arg-[Hyp3]-BK</td>
<td>65.9±3.8</td>
<td>1.97±0.39</td>
<td>0.45±0.08*</td>
</tr>
<tr>
<td>[Hyp3, Tyr(Me)5]-BK</td>
<td>57.3±2.2</td>
<td>0.91±0.21</td>
<td>0.21±0.04*</td>
</tr>
<tr>
<td>[NPh5]-BK</td>
<td>7.0±0.7</td>
<td>1.43±0.27</td>
<td>1.26±0.30</td>
</tr>
<tr>
<td>[o-NM5]-BK</td>
<td>0</td>
<td>1.95±0.43</td>
<td>2.07±0.52</td>
</tr>
<tr>
<td><a href="CH2-NH">Phe5</a>Arg9]-BK</td>
<td>0</td>
<td>1.68±0.12</td>
<td>1.64±0.23</td>
</tr>
</tbody>
</table>

RAM indicates ramiprilat.

The activities of purified rabbit ACE (0.1 IU) in vitro and the potencies (EC50) and maximum efficacies of vasoconstriction in the absence and presence of ramiprilat (250 nmol/L) is depicted for BK and slightly modified kinin analogues. Data are presented as mean±SEM of 3 degradation experiments and of 6 to 7 studies on functional potentiation performed with each agonist. (*P<0.05 compared with the corresponding condition devoid of ramiprilat.)

Materials and Methods

Organ Bath Experiments

Male New Zealand White rabbits (weight, 3 to 3.5 kg; Charles River, Sulzfeld, Germany) were anesthetized with ketamine (15 mg/kg) and xylazine (6 mg/kg). Both jugular veins were removed and denuded of endothelium by means of a coarse steel rod. The organ bath incubation conditions were adopted from previous studies on kinin potentiation by ACE inhibitors. In brief, vessel rings were incubated in Krebs-Henseleit solution containing diconfocac (1 mmol/L) at a resting tension of 3 mN. Experiments with and without ACE inhibitor treatment (250 nmol/L ramiprilat, 30 minutes of preincubation) were performed independently. Venoconstriction was induced by cumulative 3-fold increases in peptide concentrations starting at 1 nmol/L and applied at 4-minute intervals. The experiments were performed in accordance with rules set by the state of Schleswig-Holstein.

ACE Activity of Rabbit Jugular Vein

ACE activity was determined in 8 endothelium-intact and endothelium-denuded segments of the jugular vein obtained from 2 rabbits, following an established protocol. In brief, vessel rings were homogenized in 40 parts (vol/wt) buffer containing 0.01 (vol/vol) Triton X-100, aprotinin (43 nmol/L), and soybean trypsin inhibitor (2.5 nmol/L). The extract was incubated at 37°C for 60 minutes with Abz-Gly-ω-nitro-Phe-Pro (83 mmol/L, pH 7.4) in the absence or presence of ramiprilat (250 nmol/L). The reaction was stopped with perchloric acid (0.3 mol/L final concentration), and the product (ω-aminobenzoylglycyl) was quantified by high-performance liquid chromatography and fluorimetric detection (320/412 nm). The ramiprilat-sensitive reaction was considered to represent the ACE activity. Protein content of the samples was determined by the method of Lowry et al.

Kinin Degradation by Purified ACE

BK and kinin analogues (1 μmol/L) were incubated at 37°C with purified rabbit ACE (0.1 IU, as determined with 83 mmol/L Abz-Gly-ω-nitro-Phe-Pro) in 1 mL HEPES-buffered Earle’s salt solution (pH 7.4). Aliquots were drawn at 0, 5, 10, 20, and 40 minutes and stimulated by addition of trifluoroacetic acid (120 mmol/L, final concentration). Intact peptides were determined by high-performance liquid chromatography as described earlier. Specificity of the assay was confirmed by inclusion of ramiprilat (250 nmol/L) in separate control experiments.

Substances

Bradykinin, purified rabbit ACE, and HEPES were purchased from Sigma-Aldrich. Abz-Gly-ω-nitro-Phe-Pro was obtained from Bachem. [Hyp5, Tyr(Me)5]-BK and [Phe5][Hyp3, Tyr(Me)8]-BK were bought from Calbiochem. The BK analogues t-Arg-[Hyp5]-BK, t-[D-NM5]-BK, t-[Ph3]-BK, and t-[Phe5]-BK were synthesized by a solid-phase method by means of the Boc-strategy. The specific activation of B2-receptors by these kinin analogues has been reported. Ramiprilat was kindly donated by Hoechst Marion Roussel. All other chemicals, of the highest analytical grade available, were obtained from Merck or Sigma-Aldrich.

Calculations and Statistics

Dose-response curves were constructed from the increases in isometric tension induced by each of the B2-agonists. Maximum tension and EC50 values were derived by nonlinear regression (Prism, GraphPad Software). For calculating degradation rates, the complete kinetics were fitted by means of a monoeponential function (BK(t) = BK0 e−kt, k = degradation rate). All quantitative data are given as mean±SEM. For each peptide, the parameters of vasoconstriction were compared between groups with and without ramiprilat treatment with the Student’s t test. Differences were considered statistically significant at an error level of P<0.05.

Results

Bradykinin and all investigated analogues induced a dose-dependent constriction of the jugular vein, the quantitative parameters of which are summarized in the Table. As exemplified in Figure 1, the potency of BK was increased by a factor of 5.7 after pretreatment with ramiprilat. A significant shift in potency by a factor of 4.4 during ACE inhibition

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was also observed for D-Arg-[Hyp\(_3\)]-BK and [Hyp,\(_3\)Tyr(Me)\(_8\)]-BK. On the other hand, the activities of the kinin analogues [D-Phe\(_5\)]-BK, [D-NMF\(_7\)]-BK, and [Phe\(_8\)c(CH\(_2\)-NH)Arg\(_9\)]-BK were not affected, a fact that is exemplarily depicted for [D-NMF\(_7\)]-BK in Figure 1. Ramiprilat exerted no influence on the maximum efficacy of any of the B\(_2\)-agonists (Table).

The intact rabbit jugular vein contained ACE at an activity of 8.4 \(\pm\) 1.4 nmol/min per gram of protein. The majority of this enzyme activity (7.7 \(\pm\) 1.9 nmol/min per gram of protein, corresponding to 92% of the intact vein) was still present in the endothelium-denuded preparation.

During in vitro incubation with purified rabbit ACE, BK was rapidly degraded, as were the peptides D-Arg-[Hyp\(_3\)]-BK and [Hyp,\(_3\)Tyr(Me)\(_3\)]-BK. On the other hand, the activities of the kinin analogues [D-Phe\(_5\)]-BK, [D-NMF\(_7\)]-BK, and [Phe\(_8\)c(CH\(_2\)-NH)Arg\(_9\)]-BK were not affected, a fact that is exemplarily depicted for [D-NMF\(_7\)]-BK in Figure 1. Ramiprilat exerted no influence on the maximum efficacy of any of the B\(_2\)-agonists (Table).

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During in vitro incubation with purified rabbit ACE, BK was rapidly degraded, as were the peptides D-Arg-[Hyp\(_3\)]-BK and [Hyp,\(_3\)Tyr(Me)\(_3\)]-BK (Figure 2). A low rate of degradation was also observed for [D-Phe\(_5\)]-BK, whereas [D-NMF\(_7\)]-BK and [Phe\(_8\)c(CH\(_2\)-NH)Arg\(_9\)]-BK were completely resistant to ACE (Table). Degradation was completely blocked by inclusion of ramiprilat in the incubation assay (data not shown).

**Discussion**

This study has shown that the ACE inhibitor–induced potentiation of kinin effects in the rabbit jugular vein is quantitatively correlated to the susceptibility of the individual agonist to degradation by ACE; it is completely abolished in the cases of 3 substances that are virtually or completely resistant to ACE. The rabbit jugular vein was proved to be a suitable model for the investigation of kinin potentiation by ACE inhibitors, as demonstrated in a previous study that confirmed that ramiprilat was a highly effective substance in this respect.\(^5\) In this model and in the rabbit isolated heart, a potentiation by ramiprilat not only of BK but of the B\(_2\)-agonist D-Arg-[Hyp\(_3\)]-BK had been observed earlier.\(^5,6\) Because of the rapid degradation of D-Arg-[Hyp\(_3\)]-BK by rabbit ACE, however, this effect may not be considered as a degradation-independent action of the ACE inhibitor. Rather, a quantitative relation exists for D-Arg-[Hyp\(_3\)]-BK and BK between their ACE-mediated degradation and their functional potentiation by the ACE inhibitor, which means that both phenomena are equally attenuated (by 19% and 22%, respectively) when D-Arg-[Hyp\(_3\)]-BK is administered instead of BK (Figure 3). A similar relation was also demonstrated for [Hyp,\(_3\)Tyr(Me)\(_3\)]-BK, indicating that the ability of a modified B\(_2\)-agonist to become potentiated by an ACE inhibitor does not represent a structure-related property but is causally linked to its degradation by ACE.

These results might also influence current hypotheses concerning the potentiation of kinins by ACE inhibitors. The majority of studies that have addressed the mechanisms of degradation-independent kinin potentiation with modified...
B₂-agonists have been performed with the substances D-Arg-
[Hyp⁹]-BK or [Hyp⁹] Tyr(Me)⁹]-BK⁵−⁸,18−20 which are not
resistant to rabbit ACE. Because the degradation stabilities of
these substances and of [Phe⁶ psi(CH₂-NH)Arg⁹]-BK in a recent
study²¹ have not been confirmed under the particular condi-
tions of the respective assays, the observed effects of poten-
tiation or resensitization by ACE inhibitors cannot be re-
garded as definitive proof for the involvement of degradation-
independent mechanisms.

By investigating the influence of ramiprilat on ACE-resis-
tant B₂-agonists, the present study has exploited only one
approach for demonstrating a degradation-independent poten-
tiation of kinin actions. Previous investigations have identi-
ied an increased efficacy of B₂ responses,⁵−⁷ a stabilization of
B₂-receptors in a high-affinity state,⁶ and a decreased internal-
ization of B₂-receptors⁶−⁹ as possible mechanisms by which
ACE inhibitors in combination with ACE may enhance
B₂-receptor signaling. Those findings were not obtained with
the use of stabilized kinin derivatives, and their interpretation
is not affected by the present results. The failure of ramiprilat
to potentiate stable kinins in the rabbit jugular vein only
indicates that those postulated mechanisms may not have been
present, properly activated, or sufficiently effective to
provoke functional potentiation. Naturally, this conclusion can
only be drawn for the specific kinin derivatives, ACE
inhibitor, and experimental model used in our study.

Because of the lack of degradation-independent kinin poten-
tiation in the rabbit jugular vein, the observed potentiation
by ACE inhibitors can only be explained by a reduction of
kinin degradation. When this line of reasoning is followed,
a 5.7-fold increase in the potency of BK during ACE
inhibition would reflect an equally effective increase in
functional kinin concentrations at the B₂-receptors. An alter-
tation to that extent can only occur if the normal activity of
ACE will reduce the availability of BK at the B₂-receptors to
<15% of the concentration administered. Clearly, the kinin
concentration in the large volume of the organ bath cannot be
lowered to this level by the ACE activity contained in the
vessel preparation, as has already been confirmed from BK
measurements in a similar setup.¹⁶ Therefore, it must be hypothesized that the functional B₂-receptors are not in direct
contact with the incubation medium but must be located
behind a metabolic barrier, a situation that can also be
described as a localization of B₂-receptors in a compartment
endowed with a highly active kinin metabolism. The actual
existence of a distribution compartment featuring such prop-
erties has already been identified in rat myocardium by tracer
transit studies that demonstrated a 3.1-fold increase of local
BK concentrations after ACE inhibition.²² These significant
local changes were not accompanied by major alterations of
BK concentrations in the perfusion medium, a situation
consistent with the constant overall peptide levels in the
organ bath. In addition, our study has shown that the rabbit
jugular vein, even in the absence of endothelium, contains
sufficient ACE activity to allow substantial alterations of
kinin concentrations to occur within the tissue.

Regarding an arrangement that would enable ACE to
determine kinin availability at the B₂-receptor site, a colocal-
ization of both proteins may in fact exist in membrane
compartments of individual cells. Properties shared by B₂-
receptors and ACE include a similar susceptibility of both
proteins to solubilization²³ and their presence within caveo-
ae.⁹,²⁴ A recent investigation has illustrated the functional
significance of a colocalization of B₂-receptors and ACE
within the membrane.²¹ In that study, the association of ACE
and B₂-receptors was disrupted by directing ACE to chole-
sterol-rich domains through genetic modification of its mem-
brane anchor; an alteration that abolished ramiprilat-induced
B₂-receptor resensitization. It may be hypothesized that such
close proximity of ACE and B₂-receptors to membrane
domains, or even a direct arrangement of the proteins,²¹
constitutes a minute compartment that enables an efficient
kinin metabolism and allows a profound kinin potentiation to
occur, although it might not be accessible for analysis of local
kinin levels.

The finding that functional potentiation of B₂-receptor–medi-
atated venoconstriction is absent after stimulation with ACE-re-
sistant kinin analogues and that it occurs as a graded pheno-
menon if stimulation is induced by partially stabilized peptides
indicates that ramipril exerts no degradation-independent poten-
tiation of kinin effects in the rabbit jugular vein.

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