Potentiation of the Vascular Response to Kinins by Inhibition of Myocardial Kininases

Andreas Dendorfer, Sebastian Wolfrum, Ulrich Schäfer, John M. Stewart, Noriaki Inamura, Peter Dominiak

Abstract—Inhibitors of angiotensin I–converting enzyme (ACE) are very efficacious in the potentiation of the actions of bradykinin (BK) and are able to provoke a B₂ receptor–mediated vasodilation even after desensitization of this receptor. Because this activity cannot be easily explained only by an inhibition of kinin degradation, direct interactions of ACE inhibitors with the B₂ receptor or its signal transduction have been hypothesized. To clarify the significance of degradation-independent potentiation, we studied the vasodilatory effects of BK and 2 degradation-resistant B₂ receptor agonists in the isolated rat heart, a model in which ACE and aminopeptidase P (APP) contribute equally to the degradation of BK. Coronary vasodilation to BK and to a peptidic (B6014) and a nonpeptidic (FR190997) degradation-resistant B₂ agonist was assessed in the presence or absence of the ACE inhibitor ramiprilat, the APP inhibitor mercaptoethanol, or both. Ramiprilat or mercaptoethanol induced leftward shifts in the BK dose-response curve (EC₅₀ = 3.4 nmol/L) by a factor of 4.6 or 4.9, respectively. Combined inhibition of ACE and APP reduced the EC₅₀ of BK to 0.18 nmol/L (ie, by a factor of 19) but potentiated the activity of B6014 (EC₅₀ = 0.9 nmol/L) only weakly without altering that of FR190997 (EC₅₀ = 0.5 nmol/L). Desensitization of B₂ receptors was induced by the administration of BK (0.2 µmol/L) or FR190997 (0.1 µmol/L) for 30 minutes; the vascular reactivity to ramiprilat or increasing doses of BK was tested thereafter. After desensitization with BK, but not FR190997, an additional application of ramiprilat provoked a B₂ receptor–mediated vasodilation. High BK concentrations were still effective at the desensitized receptor. The process of desensitization was not altered by ramiprilat. These results show that in this model, all potentiating actions of ACE inhibitors on kinin-induced vasodilation are exclusively related to the reduction in BK breakdown and are equivalently provoked by APP inhibition. The desensitization of B₂ receptors is overcome by increasing BK concentrations, either directly or through the inhibition of ACE. These observations do not suggest any direct interactions of ACE inhibitors with the B₂ receptor or its signal transduction but point to a very high activity of BK degradation in the vicinity of the B₂ receptor in combination with a stimulation-dependent reduction in receptor affinity. (Hypertension. 2000;35:32-37.)

Key Words: bradykinin • angiotensin-converting enzyme • receptor, bradykinin • rats • heart

Inhibitors of angiotensin I–converting enzyme (ACE) have been proved to be clinically effective against diseases such as hypertension, congestive heart failure, and myocardial infarction. Through their main mechanism of action (ie, inhibition of the peptidase ACE), ACE inhibitors not only prevent the activation of angiotensin I but also eliminate a degradation pathway for the vasodilatory peptide bradykinin (BK) and thus increase the availability and effectiveness of kinins. The results of experimental studies show that kinins can contribute to the hypotensive effect of ACE inhibition and that they may be of prime importance in its cardioprotective actions, including reduction in infarct size, improvement in performance and energetic state of ischemic myocardium, and prevention of ventricular hypertrophy and remodeling (reviewed by Linz et al¹). These effects arise because of the high extent to which ACE inhibitors can potentiate kinin effectiveness. In volunteers, ACE inhibitor treatment sensitized the blood pressure response to injected BK by a factor of 20 to 50.²

This outstanding effectiveness of ACE inhibitors in kinin potentiation was also observed in various experimental models, in which the BK doses required to produce in vitro vasodilation or blood pressure reduction were as much as 120-fold lower during ACE inhibition.³,⁴ Such an activity cannot be easily explained only by a reduced BK degradation because, in the circulation, ACE is only one of a number of kininases (eg, carboxypeptidase N in plasma, aminopeptidase P, carboxypeptidase M, and neutral endopeptidase on endo-
thelial cells), and plasma kinin levels increase only modestly after ACE inhibition. On the other hand, local ACE activities present in tissue or cellular compartments could effectively determine kinin concentrations at these sites. In the rat heart, the influence of kininase inhibitors on local kinin concentrations has been established in tracer transit studies that demonstrated a marked increase in kinin concentrations during ACE inhibition specifically in an extravascular distribution compartment.

Additional observations have been made suggesting that some of the kinin potentiation properties of ACE inhibitors might be related to mechanisms independent of the inhibition of kinin degradation; for example, (1) ACE inhibitors act as kinin mimetics under conditions in which kinin breakdown should be negligible, (2) ACE inhibitors can enhance the effects of degradation-resistant BK analogs, (3) ACE inhibitors show structure-related differences in their kinin potentiation activities, and (4) ACE inhibitors can provoke a kinin-mediated response even when B2 receptors have been desensitized through kinin pretreatment. The last phenomenon is addressed further here as “receptor resensitization.” A number of explanations for these activities have been hypothesized. In addition to inhibition of BK breakdown, ACE inhibitors could act directly on B2 receptors or their signal transduction by increasing receptor affinity, preventing receptor internalization, or enhancing second-messenger activation. However, none of these mechanisms have ever been demonstrated in a physiological model that can reveal the classic consequences of kinin potentiation, namely a leftward shift in the BK dose-response curve.

Therefore, the aim of the present study was to characterize kinin-mediated vasodilation with respect to the potencies of BK and degradation-stable B2 agonists and to the kinetics of receptor desensitization and resensitization. The influences of both the ACE inhibitor ramiprilat and the aminopeptidase P (APP) inhibitor mercaptoethanol on these parameters were investigated in the isolated rat heart; this represents the only model in which the influence of kininases on kinin concentrations in the perfusion medium, as well as in tissue compartments, has been thoroughly investigated. The fact that ACE and APP contribute about equally to kinin metabolism in the rat myocardium provides the additional opportunity to compare the consequences of reduced kinin breakdown provoked by ACE and APP inhibition. Furthermore, the use of 2 degradation-resistant B2 agonists, the peptidic kinin analog B6014 and the nonpeptidic B2 agonist FR190997, provided a control for the actions of kininase inhibitors that would not attribute to a reduction in kinin breakdown.

Methods

Preparation
The study was performed in accordance with the guidelines of the government of Schleswig-Holstein. Isolated perfused Langendorff hearts were prepared from anesthetized (50 mg/kg pentobarbital IP) male Wistar rats (250 to 300 g body wt; Charles River) as previously described. The hearts were perfused at constant flow (15 mL/min) with Krebs-Ringer solution consisting of (in mmol/L) 116 NaCl, 26.2 NaHCO3, 5.4 KCl, 1 NaH2PO4, 2.5 CaCl2, 0.81 MgSO4, 5.6 glucose, and 2 pyruvate (pH 7.4, 37°C) that had been equilibrated with 95% O2/5% CO2, and perfusion pressure was recorded. Drugs were applied as stock solutions (100-fold final concentrations) that were continuously added to the perfusion medium at a rate of 150 µL/min. Dose-response curves were obtained through the sequential applications of B2 agonists lasting either 1 minute (BK, B6014) or 2 minutes (FR190997). Increasing doses (0.01 to 100 nmol/L) were administered after perfusion pressures had returned to baseline levels (5- to 20-minute intervals). In some experiments, B2 responses were desensitized by the continuous infusion of BK (0.05 or 0.2 µmol/L) or FR190997 (0.1 µmol/L) for 30 minutes. Subsequent treatments with ramiprilat or increased BK concentrations were performed in the continued presence of the respective B2 agonists in their desensitizing concentrations.

Substances
Ramiprilat and HOE140 (icatibant) were kindly donated by Prof. G. Wiemer (Hoechst Marion Roussel, Frankfurt, Germany). Chemical and pharmacological properties of the peptidic B2 agonist B6014 (Arg-Pro-Hyp-Gly-Thi-Ser-r-Pro-Thi-Arg) and the nonpeptidic B2 agonist FR190997 have been described elsewhere. All other chemicals were obtained in the highest quality available from Merck or Sigma.

Calculations and Statistical Analysis
Dose-response curves were constructed from the acute reductions in perfusion pressures during BK applications. Maximum responses and EC50 values were calculated for each individual experiment through the use of nonlinear regression. All quantitative data are given as mean±SEM of 5 to 10 independent experiments. Parameters of vasodilation were compared among the treatment groups with the use of ANOVA with Tukey’s post hoc test. Differences were considered to be statistically significant at an error level of P<0.05.

Results

Vasodilatory Potency of BK
Basal perfusion pressures stabilized at an average level of 101±2.8 mm Hg, which did not differ among the treatment groups. During BK application, perfusion pressures dropped to a stable minimum within 1 minute. BK reduced perfusion pressures with an EC50 value of 3.4±0.4 nmol/L (n=10), reaching a maximum decrease of 44±4 mm Hg (Figure 1). The initiation of ramiprilat treatment (250 nmol/L) induced a slight and transient decrease in perfusion pressure in some preparations (average 2±1 mm Hg). During ACE inhibition, the BK dose-effect curve showed a leftward shift (EC50=0.7±0.2 nmol/L, P<0.05 versus control, n=9) without any changes in maximum efficacy (Figure 1). The alterations in BK responses induced by the APP inhibitor mercaptoethanol (1 mmol/L final concentration) were equivalent to those seen with ramiprilat. The application of mercaptoethanol caused an initial 12.6±4.6 mm Hg reduction in perfusion pressure. This was an acute and reversible reaction that did not influence perfusion pressures at the start of the experiments. The EC50 value of BK during APP inhibition was lowered to 0.69±0.16 nmol/L (P<0.05 versus control, n=6), without any change in the maximum response (Figure 1). The combined inhibition of ACE and APP led to a further increase in BK potency (EC50=0.18±0.05 nmol/L, P<0.05 versus all groups, n=8; Figure 1). The observed 19-fold increase in BK potency was nearly identical to the product of the potentiating factors of ramiprilat (factor 4.6) and mercaptoethanol (factor 4.9) alone. This indicates that each inhibitor of BK degradation acted independently and that their individual effects could be reproduced entirely.
when they were administered in combination. With combined inhibitor application, the maximum effect of BK was diminished to 25±2 mm Hg. This phenomenon may have been due to a long-lasting decrease in basal perfusion pressure occurring after administration of BK in high doses. This incomplete recovery resulted in an average perfusion pressure that was 23 mm Hg lower at the end of the experiments than in the control group. The absolute effectiveness of BK was not impaired under these conditions, because it produced the same minimum perfusion pressures as in the control group (60±6 versus 58±5 mm Hg).

**Effects of Stable B₂ Agonists**

Identical experiments performed with the stable B₂ agonist B6014 revealed that this BK derivative potently provoked vasodilation in basal pressures (see Results) (n=6 to 9).

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Influence of kininase inhibitors on dose-response curve of BK-induced vasodilation. Maximum decrease in coronary perfusion pressure during sequential bradykinin infusions was determined under control conditions and after inhibition of ACE, APP, or both with ramiprilat and mercaptoethanol, respectively. EC₅₀ values of individual dose-response curves are indicated with dashed lines. Inhibition of each kininase shifted dose-response curve of bradykinin to left by factors of 4.6 and 4.9, respectively. Combined inhibition of ACE and APP reduced EC₅₀ by a factor of 19. Only under this condition was the maximum effect of BK reduced EC₅₀ by a factor of 19. Only under this condition was the maximum decrease in perfusion pressure attenuated due to a difference in basal pressures (see Results) (n=6 to 9).

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Influence of kininase inhibitors on vasodilatory potentials of degradation-resistant B₂ agonists. Maximum decrease in coronary perfusion pressure (CPP) obtained during application of peptidic B₂ agonist B6014 and nonpeptidic B₂ agonist FR190997 was determined under control conditions and during combined inhibition of ACE and APP by ramiprilat and mercaptoethanol (2-ME). B6014 produced same maximum effect as bradykinin. Treatment with ramiprilat and mercaptoethanol reduced EC₅₀ of B6014 by a factor of 1.4. Maximum vasodilation induced by FR190997 was less pronounced than that provoked by BK (28±2 vs 44±4 mm Hg). Combined kininase inhibition failed to influence either potency or maximum effectiveness of FR190997 (n=8 to 10).

2), FR190997 had a slower onset of action than BK, so 2-minute applications were required to establish a stable effect.

**Receptor Resensitization and Desensitization**

The ability of ACE inhibitors to provoke an acute BK-mediated response subsequent to B₂ receptor desensitization by BK treatment (ie, to resensitize the receptor) has also been attributed to an activity unrelated to ACE inhibition. This effect was reproduced in the rat heart, in which the vasodilatory effect of BK (0.2 μmol/L) ceased during a 30-minute application (78±8% loss of vasodilation, half-maximum desensitization after 20±3 minutes, n=5) but was completely restored on subsequent treatment with ramiprilat (250 nmol/L; Figure 3). Consistent with previous studies, this resensitization was abolished by the B₂ antagonist icatibant (HOE140, 1 μmol/L) administered 2 minutes before ramiprilat (data not shown). The same experiment performed with the stable agonist FR190997 (0.1 μmol/L) induced an equivalent course of desensitization, but the subsequent application of ramiprilat was ineffective (Figure 3).

To test the hypothesis that kininase inhibition might overcome B₂ receptor desensitization by increasing BK concentrations at the receptor site, high doses of BK were applied to the desensitized receptor. As shown in Figure 4, the receptor desensitized with 0.2 μmol/L BK was still responsive even to slight increases in BK concentrations and could be stimulated in this way to maximum vasodilation. The half-maximum dilation occurred at a BK concentration of 0.4 μmol/L, which was equivalent to a 2-fold increase in the continuously applied desensitizing BK concentration.
Figure 3. Resensitization of vascular response to B<sub>2</sub> agonists. Decrease in coronary perfusion pressure (CPP) induced by BK (0.2 μmol/L) or stable B<sub>2</sub> agonist FR190997 (0.1 μmol/L) desensitized during 30-minute continuous application. After desensitization with bradykinin, addition of ramiprilat (0.25 μmol/L) provoked a nearly maximum vasodilation (ie, a resensitization of response) in continued presence of BK (0.2 μmol/L), whereas ramiprilat was ineffective during treatment with FR190997. Typical tracings from 3 independent experiments are depicted.

Figure 4. Maintained reactivity of vascular response to BK after receptor desensitization. Desensitization of BK-induced vasodilation was produced within 30 minutes in absence or presence of ramiprilat with equipotent concentrations of BK (0.2 and 0.05 μmol/L, respectively). Sequential increases in BK concentrations dose-dependently restored vasodilatory responses up to maximum effectiveness. Despite a ~100-fold reduction in BK potency compared with basal receptor state, 2-fold increases in continuously applied desensitizing BK concentrations (marked by dashed lines) induced a marked vasodilatory response of desensitized receptors. Pretreatment with ramiprilat (bottom) did not influence course or extent of receptor desensitization or maintained responsiveness to increased kinin concentrations (n=5).

Discussion

The present results show that the investigated aspects of kinin potentiation by ACE inhibitors in the rat heart depend in full on the degradability of the B<sub>2</sub> agonist, because neither the potency of vasodilation nor the activation of the desensitized receptor was influenced by ramiprilat when FR190997 was applied. This exclusion of an untypical activity of the ACE inhibitor was confirmed by the use of the second major kininase of rat heart, APP, as a positive control. APP has been demonstrated to be equal to ACE with respect to BK degradation in different compartments of the rat myocardium, and the inhibition of APP was as effective as the inhibition of ACE in shifting the BK dose-response curve to lower concentrations, an effect that was independent of and additive to that of ramiprilat.

The failure of ramiprilat to influence the potency of degradation-stable B<sub>2</sub> agonists suggests that the ACE inhibitor exerted no significant influence on the affinity of the B<sub>2</sub> receptors. This interpretation is in agreement with binding studies in which it was also found that there were no alterations in B<sub>2</sub> receptor affinity in the presence of ACE inhibitors. In only 1 model of transfected Chinese hamster ovary cells expressing the human B<sub>2</sub> receptor and ACE genes was an increase in the density and affinity of BK binding reported in the presence of enalaprilat. This effect was dependent on the presence of ACE, and it occurred only when enalaprilat was applied in concentrations substantially higher than those needed for the inhibition of the enzymatic activity of ACE, a condition that was not investigated in the present study.

As with the majority of previous investigations, ACE inhibition, and in this case, also inhibition of APP, was found to be highly effective in potentiation of the vascular effects of BK. In the rat heart, the leftward shift of the BK dose-response curve to 19-fold lower concentrations might be explained by extensive BK metabolism in specific tissue or cellular compartments. Tracer transit studies in this model have demonstrated highly active BK metabolism in an extravascular distribution compartment, where local BK concentrations were increased by factors of 3.1 or 8.7 through the inhibition of ACE or ACE plus APP, respectively. Because only 10% of the total amount of applied BK entered this compartment, even the very effective local kinin metabolism contributed to only a minor extent to the overall kinin degradation in rat heart, which cleaved 39% of the applied BK during 1 myocardial passage. As such, the significant increase in local BK concentrations occurring during kininase inhibition in this rat heart compartment is not reflected in the coronary perfusate, but it is nearly sufficient to account for the degrees to which kinins were potentiated if one assumes that similar conditions of kinin breakdown will also prevail in the functional compartments of the B<sub>2</sub> receptors.

Because the interstitial space of the rat myocardium has been described as a distribution compartment that can be reached by exogenous BK only after extensive degradation,
B₂ receptors might be localized within this space (i.e., at the basal side of the endothelium). However, alternative locations of the B₂ receptors should also be considered. Even within the cell surface, a colocalization of B₂ receptors and ACE has been suggested. Because stimulated B₂ receptors and internalized BK have been found to be associated with caveolae, these vesicles may also be involved in the formation of a compartment in which kinin levels are highly sensitive to kininase inhibition.

In the isolated perfused rat heart, ramiprilat conferred neither potentiation nor resensitization of B₂-mediated responses when degradation-resistant agonists were used. These findings do not reflect the potentiation properties of ACE inhibitors reported with stabilized kinin derivatives in earlier studies. However, complete degradation stability of the B₂ agonists d-Arg-[Hyp]²BK and [Hyp⁸,Tyr(Me)⁹]BK, which have so far been exclusively used for such investigations, has not yet been demonstrated. As such, the failure of ramiprilat and mercaptoethanol to potentiate the effects of B6014 and FR190997 in this study excludes these experimental imponderabilities and demonstrates the absence of degradation-independent actions of the ACE inhibitor. Likewise, this consideration excludes the possibility that endogenous, authentic BK is present in the isolated perfused heart in an amount sufficient to provoke kinin-like actions of ACE inhibitors or to influence the dose-response curve of B₂ agonists.

Another phenomenon that has been attributed to a direct interaction of ACE inhibitors with B₂ receptors was the ability of ACE inhibitors to provoke a BK-dependent reaction after B₂ receptors had been desensitized. However, this effect can be related to a direct resensitizing influence on receptor regulation only if the desensitized receptor will not respond to increasing BK doses, a prerequisite that has not been established in previous studies. In contrast, the present study demonstrated a maintained reactivity to high BK concentrations in the desensitized state that can be interpreted as a stimulation-dependent loss of BK potency. Although the basal dose-response curve of BK was shifted rightward by a factor of ~100 after desensitization, the BK concentrations that still provoked vasodilation under those conditions corresponded to just a 2-fold increase in the continuously applied BK concentration (Figure 4). If one assumes that ACE inhibitors potentiate kinin actions only by enhancing kinin availability at the B₂ receptors, it is obvious that such treatment would increase the intact fraction of continuously applied BK to the extent (i.e., by a factor of 4.6) that the desensitized B₂ receptors would again become activated. This idea is confirmed by the absence of a resensitizing effect of ramiprilat after desensitization with and in the presence of FR190997 (Figure 3), demonstrating that an increase in kinin concentrations is an indispensable mechanism for this action of the ACE inhibitor.

On the other hand, the affinity loss of the desensitized B₂ receptors might be a target for a direct influence of ACE inhibitors. After long-term activation, B₂ receptors undergo internalization and intracellular processing, whereby B₂ receptor affinity is not necessarily affected. However, a stimulation-induced shift of B₂ receptors to a low-affinity state has been demonstrated by binding studies in cultured fibroblasts. In Chinese hamster ovary cells expressing the human B₂ receptor and ACE, these mechanisms were attenuated by enalapril, which stabilized the receptor in a high-affinity conformation and inhibited its internalization. To investigate the influence of ACE inhibition on the desensitization of B₂ receptors and the loss of BK potency during continued stimulation in our model, the desensitization experiments were repeated in the presence of ramiprilat with the use of equipotent BK concentrations. Under these conditions, the time course and extent of desensitization and the subsequent shift in receptor affinity were reproduced exactly, thus indicating that the ACE inhibitor exerted no influence other than to increase the availability of BK at its receptors. The mechanisms responsible for the loss of B₂ receptor affinity can only be speculated but may involve phosphorylation of B₂ receptors, altered G protein availability or direct receptor-receptor interactions. None of these mechanisms have been identified as a target for the direct influences of ACE inhibitors.

A future perspective arises from the observation that APP was equal to ACE with respect to kinin potentiation. This agrees with earlier studies demonstrating the BK potentiation potential of APP inhibitors in whole animal models. In these studies, potentiation of BK-induced hypotension in rats was more effectively induced by ACE inhibition than by APP inhibition, which seemed to be related to the predominant role of ACE in BK metabolism in the lung. In the rat heart, however, nearly identical kinin-degrading activities and functional distributions of ACE and APP have been demonstrated. Because this equivalence coincides with a nearly identical significance of both enzymes for kinin potentiation as shown in this study, APP and ACE must have the same strategic locations in relation to the B₂ receptors; therefore, APP, which is known to reside as an extracellular enzyme on endothelial membranes, should be colocalized in membrane domains with ACE, and possibly also with B₂ receptors. If similar enzymatic conditions exist in human myocardium (mRNA for APP has already been detected in this tissue), then the inhibition of APP may even confer a more cardioselective potentiation of kinin effects.

The interpretation of the results of this study is restricted by the use of mercaptoethanol for APP inhibition, which may seem to be a nonspecific approach. Unfortunately, apstatin, the only alternative APP inhibitor suitable for use in intact tissues, was not available in amounts sufficient for this kind of study. On the other hand, mercaptoethanol has been described as an effective and selective inhibitor of APP that will not inhibit ACE at the concentrations that we used. This was confirmed for the rat heart in BK degradation studies that showed the ACE products 1-7-BK and 1-5-BK were still produced in the presence of mercaptoethanol (data not shown). There may be some concern that mercaptoethanol acts rather nonspecifically due to its activity as a sulphydryl agent; however, such effects did not influence the actions of the stable B₂ agonists used in this study, so the potentiation of BK by mercaptoethanol under the conditions described here can be regarded as a specific consequence of APP inhibition. A possible further limitation of the present
study is inevitably connected to the use of modified B2 agonists that might lack some property of the natural agonist that is essential for the ACE inhibitors to exert a direct potentiation activity. This seems rather unlikely, however, because the observed interdependence between the potency and the degradation of kinins was confirmed not only with the use of 2 structurally different B2 agonists but also with an unrelated inhibitor of an alternative kinin degradation pathway (ie, APP), whose BK potentiation actions were equal and even additive to those of the ACE inhibitor.

This study shows in the isolated perfused rat heart that the effective potentiation of kinins by ACE inhibitors does not involve direct influence on the B2 receptors or their signal transduction but is related to a highly active kinin degradation in the vicinity of the B2 receptors. A local increase in BK concentrations is also responsible for the reconstitution by ACE inhibitors of kinin effects after desensitization, which can occur due to the maintained reactivity to increased BK concentrations in this condition. Likewise, ramiprilat exerts no influence on the process of receptor desensitization itself. Because APP was found to be as significant as ACE with respect to the degradation of kinins, APP inhibitors can further enhance the effects of endogenous kinins beyond the potential of ACE inhibitors, a mechanism that may prove to be clinically useful.

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