Inhibition of Endopeptidase-24.15 Decreases Blood Pressure in Normotensive Rats

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The potent vasodilatory peptide bradykinin is cleaved at the Phe\(^5\)-Ser\(^6\) bond in vitro by the metalloenzyme endopeptidase-24.15 (EC.4.24.15). We now report that intravenous infusion of N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate, a specific active site-directed inhibitor of endopeptidase-24.15, produces an immediate drop in mean arterial pressure of as much as 50 mm Hg in pentobarbital-anesthetized, normotensive rats. Arterial pressure recovers within 5 minutes. The B\(_2\) bradykinin antagonist \([\text{Arg}^6, \text{Hyp}^7, \text{Thi}^8, \text{D-Phe}^9]\)-bradykinin attenuates the decrease in mean arterial pressure resulting from treatment with the inhibitor. The endopeptidase-24.15 inhibitor potentiates the hypotensive effect of intravenous bradykinin infusion, increasing the maximal effect of the peptide by 47% and increasing the potency by almost 10-fold, while the response to intra-arterial bradykinin is less affected by the inhibitor. These results support a role for endopeptidase-24.15 in the inactivation of endogenous and exogenous bradykinin and suggest a direct involvement of the enzyme in the control of blood pressure.

The activation of the kallikrein-kinin system results in the formation of several potent vasoactive peptides including bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and Lys-bradykinin (kallidin). Although a clear role for kinins in cardiovascular homeostasis has not been established, these peptides have been implicated in regulation of blood pressure, regional blood flow, and the transvascular movement of water.\(^{1,2}\) Bolus intra-arterial infusion of bradykinin results in an acute reduction in mean arterial pressure, whereas intravenous infusion produces similar effects at 10-100-fold higher doses.\(^{3,4}\) The reduced response to intravenous administration appears to be due to a considerable "first-pass effect" due to enzymatic degradation in the pulmonary vascular bed.\(^{4,5}\) Bradykinin has a half-life after intravenous injection that is shorter than 30 seconds.\(^6\)

The enzymes that cleave kinins are often referred to as "kininas," although these enzymes hydrolyze a variety of other peptides both in vitro and in vivo. Bradykinin is widely considered to be cleaved in vivo primarily by two enzymes known as carboxypeptidase N\(^7\) (CpN; kininase I) and angiotensin converting enzyme\(^8\) (ACE; kininase II). However, several other enzymes have been implicated in kinin degradation as well, including aminopeptidases, endopeptidase-24.11\(^9,11\) (EP-24.11; "enkephalinase"), endopeptidase-24.15\(^12\) (EP-24.15), and endo-oligopeptidase A.\(^{13,14}\) Endo-oligopeptidase A cleaves bradykinin at the same site as EP-24.15, and may be identical to EP-24.15, despite one report to the contrary.\(^{15}\) Inhibitors of ACE and CpN reduce the bradykinin-degrading activity of rat plasma by 46% and 11%, respectively.\(^{16}\) In addition, enalaprilat, an inhibitor of ACE, slows the degradation of intravenously administered bradykinin, thereby potentiating its hypotensive effects, but has no depressor effect in normotensive rats.\(^{17}\) Although bradykinin has been shown to be an excellent substrate for CpN and EP-24.11 in vitro,\(^{10,11}\) inhibitors of these enzymes fail to increase plasma bradykinin, they do not potentiate the depressor effects of exogenously administered peptide, and they have little or no effect on blood pressure in normotensive animals.\(^{16}\)

EP-24.15 is a Zn\(^{2+}\)-containing neutral metalloendopeptidase found in a wide variety of tissues throughout the body. Purified EP-24.15 rapidly cleaves bradykinin as well as angiotensin I and II, neurotensin, luteinizing hormone releasing hormone, and several enkephalin-containing peptides.\(^{12,18}\) The enzyme prefers substrates with hydrophobic amino acids in the P\(_1\) and P\(_2\) positions, a proline in the P\(_3\) position and, in particular, a hydrophobic amino acid in the P\(_4\) position. Thus, bradykinin is readily cleaved at the
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Phe\textsuperscript{6} Ser\textsuperscript{6} bond by the purified enzyme.\textsuperscript{12} The present study examines the potential for the involvement of EP-24.15 in the degradation of endogenous bradykinin and the control of blood pressure.

**Methods**

Male Sprague-Dawley rats (150–250 g) fed ad libitum and maintained in a 12-hour light/dark cycle room were anesthetized with pentobarbital (55 mg/kg) for all studies. A polyethylene cannula (PE-50) filled with heparinized saline (200 units/ml) was introduced into the right femoral artery for continuous recording of blood pressure using a transducer (Statham Division, Gould Inc., Oxnard, Calif.) coupled to a strip chart recorder (Gould Co., Cleveland, Ohio). A second PE-50 cannula was placed in the femoral vein, whereby injections of inhibitor were made. The EP-24.15 inhibitor \textit{N}-(1-(R,S)-carboxy-3-phenylpropyl)-Ala-Ala-Phe-p-aminobenzoate (cFP-AAF-pAB) was given by bolus injection in 300 μl (approximately 5-second duration).

An additional intravenous cannula was introduced into the left femoral vein of some animals for infusion of the bradykinin antagonist [Arg\textsuperscript{2},Hyp\textsuperscript{3},Thi\textsuperscript{6},D-Phe\textsuperscript{7}]-bradykinin (Sigma Chemical Co., St. Louis, Mo.), which was administered at a rate of 50 μg/min (50 μl/min) for the period indicated.

For intravenous and intra-arterial infusions of bradykinin, rats were fitted with intra-atrial (via the jugular vein) or intracarotid cannulae, respectively. Arterial pressure was recorded in the femoral artery as described above while 100 μl doses of bradykinin (Bachem, Torrance, Calif.) were administered in ascending order from 0.03 to 30 μg/kg i.v. and from 0.003 to 10 μg/kg i.a. to establish a complete dose-response curve in each animal. Approximately 1 minute was allowed between infusions to establish baseline blood pressure. Hypotensive responses to repeated doses varied less than 5%, and administration of smaller doses subsequent to larger ones yielded identical responses, confirming the lack of tachyphylaxis to bradykinin. cFP-AAF-pAB (3.0 μmol/300 μl) was administered via a cannula in the femoral vein, and the dose–response curve was established after recovery from the hypotensive effects of the inhibitor (approximately 10–15 minutes was allowed). Curves were fitted to the data by an iterative technique using the general logistic equation\textsuperscript{19}:

\[
y = \frac{E_{\text{max}}}{\left(\frac{E_{50}}{x}\right)^m} + 1
\]

where \(y\) is maximum decrease in blood pressure produced by a given dose of bradykinin, \(x\) is dose of bradykinin, \(E_{\text{max}}\) is maximum depressor response to bradykinin, \(E_{50}\) is dose that produces a half-maximal decrease in blood pressure, and \(m\) is slope factor.

The EP-24.15 inhibitor cFP-AAF-pAB was synthesized as described previously.\textsuperscript{20} Briefly, Ala-Ala-Phe-pAB was made by stepwise elongation of pAB (Sigma) using hydroxysuccinimide esters of t-butyloxycarbonyl (BOC)–protected amino acids (Bachem). t-Butyloxycarbonyl–protected peptides were deblocked with trifluoroacetic acid (Sigma) after each step. In the final step, the inhibitor was produced by reductive amination of sodium α-ketophenylbutyrate (Chemical Dynamics, S. Plainfield, N.J.) by Ala-Ala-Phe-pAB using sodium cyanoborohydride (Sigma). This inhibitor has a \(K_i\) of 27 nM against EP-24.15 and inhibits EP-24.11 only at a very high concentration (\(K_i=17 \mu M\)).\textsuperscript{20}

**Results**

Intravenous infusion of the active site-directed EP-24.15 inhibitor cFP-AAF-pAB produced an immediate depressor response lasting 1–5 minutes, with a magnitude and duration that increased with dose (Figure 1). Infusion of 3 μmol cFP-AAF-pAB produced a maximum depressor response of 52 mm Hg. When the inhibitor was administered a second time
The reduction in mean arterial pressure was greatly diminished or absent, suggesting that compensatory responses to the initial dose remained in effect for a prolonged period. Infusion of as much as 15 μmol of the inhibitor produced little additional drop in mean arterial pressure (data not shown). However, infusion of 3 μmol cFP-AAF-pAB occasionally produced a larger decrease in blood pressure, resulting in irreversible shock. From previous studies, it can be estimated that the plasma concentration of cFP-AAF-pAB remains above the Kᵢ for at least 30 minutes after infusion of the 3 μmol dose. A relatively small increase in heart rate (less than 10%) was seen with the largest dose of EP-24.15 inhibitor (3 μmol). The effects of cFP-AAF-pAB on blood pressure were presumed to result from the blockade of degradation of some endogenous vasodilatory peptide substrates of EP-24.15, of which the kinins must be considered the most likely candidates.

We have used a B₂-bradykinin antagonist [Arg⁶,Hyp³, Thi¹⁴,D-Phe⁷]-bradykinin [Hyp, hydroxyproline; Thi, β-(2-thienyl)-alanine] to examine the involvement of kinins in the depressor response to the inhibitor of EP-24.15. Intravenous infusion of the B₂-bradykinin antagonist alone produced no direct change in arterial pressure, as shown in previous studies (Figure 2). The reduction in mean arterial pressure produced by bolus intravenous administration of 3.0 μmol cFP-AAF-pAB was greatly diminished in magnitude when the bradykinin antagonist was delivered by constant infusion. The maximum decrease in mean arterial pressure produced by the EP-24.15 inhibitor in this study was reduced from 37 mm Hg to 14 mm Hg in the presence of the bradykinin antagonist (p<0.001, t test).

Bolus intravenous infusion of bradykinin produces a transient, dose-related decrease in mean arterial pressure that exhibits no tachyphylaxis on repeated administration. To examine indirectly the in vivo cleavage of bradykinin by EP-24.15, the hypotensive effect of bradykinin was measured in the presence and absence of cFP-AAF-pAB (Figure 3). The inhibitor potentiated the depressor response to intravenous bradykinin administration, producing a decrease in the ED₅₀ of approximately 10-fold, from 1.89±0.16 to 0.244±0.51 μg/kg (p<0.0001). In addition, a 47% increase in the maximum depressor response to bradykinin (Eₘ₉ₐₓ) from 26.9±0.76 to 39.5±4.1 mm Hg is produced by prior administration of the EP-24.15 inhibitor (p<0.01; Figure 3). Infusion of bradykinin by the intra-arterial route produces a similar hypotensive response at much lower doses, presumably because the peptide is subject to degradation in the pulmonary vasculature. In our studies, infusion of bradykinin into the carotid artery increased the potency of the response to bradykinin by fivefold (E₅₀, 0.423±0.21 μg/kg) and increased the magnitude of the response by more than 75% (Eₘ₉ₐₓ, 47.5±5.6). When the inhibitor of EP-24.15 was infused intravenously before bradykinin administration (intracarotid), a small leftward shift in the dose–response curve was observed that was not statistically significant (ED₅₀, 0.140±0.03 μg/kg; p=0.11) and had a similar maximal response (Eₘ₉ₐₓ, 45.6±2.0; p=0.38).

Discussion

An extensive literature relating to the cardiovascular effects of kinins indicates that endogenous kinins could potentially alter local blood flow by an eicosanoid-mediated reduction in vascular resistance in specific vascular beds. However, it has not been established that endogenous kinins are generated in sufficient quantities to produce systemic effects. Kinins may instead serve as autocrine or paracrine factors that have little influence at distant sites but are in part responsible for maintenance of local blood flow. The results described above indicate that EP-24.15 rapidly degrades endogenous kinins as well as intravenously administered bradykinin and lend strong support to the hypothesis that the enzyme is involved in the regulation of kinin levels in vivo.
may be deduced, furthermore, that kinins are produced in sufficient quantities locally (or even systemically) in pentobarbital-anesthetized animals to produce a depressor response but that enzymatic degradation by EP-24.15 limits their effectiveness in producing this response. The relative contributions of other enzymes to the degradation of endogenous kinins in normotensive animals is not known. As mentioned above, specific active site-directed inhibitors of ACE, EP-24.11, and CpN have little effect on blood pressure in normotensive animals.

The short duration of the depressor response to the EP-24.15 inhibitor is remarkable in light of the persistence of the inhibitor in the circulation for at least 30 minutes. Interestingly, the depressor response to infusion of the EP-24.15 inhibitor is similar to the response seen during continuous infusion of bradykinin. In the latter case, a sharp drop in mean arterial pressure is seen that returns to baseline within minutes despite continued infusion. Compensatory mechanisms responsible for recovery of baseline blood pressure during constant infusion of bradykinin may include 1) increased sympathetic autonomic activity (baroreceptor reflex), 2) decrease in cardiac parasympathetic activity (increased heart rate), 3) increased rate of degradation of bradykinin, 4) exhaustion of the supplies of plasma or tissue kininogen, 5) exhaustion of mediators of kinin action or desensitization of their receptors, or 6) stimulation of the renin-angiotensin system. Mechanisms that are active in reducing the acute depressor effect of exogenously administered kinins would also be expected to attenuate the effects of endogenous kinins in the present study.

EP-24.15 is found in high specific activity in rat testis and brain, as well as in spleen, lung, kidney,
Liver, heart, skeletal muscle, and in serum itself.\textsuperscript{12,20} We have shown that EP-24.15 has access to circulating peptides; the half-life of intravenously administered luteinizing hormone releasing hormone is increased more than twofold by prior administration of cFP-AAF-pAB.\textsuperscript{21} Recently, we have developed a histochemical method to visualize EP-24.15. The enzyme is present in a subset of large and small blood vessels in brain and lung (C.J. Molineaux and J.M. Ayala, unpublished observations). Ishida et al\textsuperscript{17} showed that inhibitors of ACE and CpN prevented the bradykinin-degrading activity of rat plasma by 46\% and 11\%, respectively. EP-24.15, which was not examined in their studies, may account for the remainder of bradykinin hydrolysis by plasma.

Exogenously administered bradykinin also appears to be rapidly degraded by EP-24.15. Coadministration of the inhibitor of EP-24.15 increased the potency and magnitude of the depressor effects of intravenous bradykinin, making the response similar to that of intra-arterially administered peptide. In contrast, the inhibitor produced a relatively small effect on the intra-arterial dose-response curve. These results suggest that degradation of exogenously administered bradykinin by EP-24.15 occurs primarily within the pulmonary vasculature. A simple protection of bradykinin from degradation by EP-24.15 would be expected to yield a leftward shift in the dose–response curve with no increase in the magnitude of the response. The increase in $E_{\text{max}}$ of intravenous bradykinin produced by the EP-24.15 inhibitor may reflect an increase in the access of bradykinin to pulmonary or extrapulmonary vascular beds. A prolonged vasodilatory effect produced by the inhibitor may enhance access of bradykinin to a larger number of receptors. Alternatively, protection of some other (vasodilatory) peptides may contribute to this response in a similar manner.

Although the data discussed above lend support to the hypothesis that exogenous bradykinin is degraded by EP-24.15 within the pulmonary vasculature, these results must be interpreted with caution in regard to endogenous kinins. The hypotensive effect of the EP-24.15 inhibitor given alone and its reversal by a B2-bradykinin antagonist suggest that EP-24.15 is involved in the rapid degradation of endogenous kinins. This effect of the inhibitor might occur within specific capillary beds or within all capillary beds, dependent on the presence of enzyme, rate of formation of kinins from kininogens, and the proximity of receptors. Exogenous bradykinin administered by the intra-arterial route would be distributed throughout the capillary networks at distant sites, such that the dynamics of kinin delivery and receptor occupancy would be entirely different from those that occur after inhibitor treatment. Thus, the failure of inhibitor to dramatically influence the dose–response curve to intra-arterial bradykinin does not rule out the possibility that local changes in blood flow occur within specific vascular beds. Although kinins produced locally may affect arterial pressure acutely after administration of the EP-24.15 inhibitor, the physiological role of these peptides may lie in the control of local blood flow, as suggested by several authors.\textsuperscript{1,2} The contribution of EP-24.15 to control of local blood flow has yet to be determined.

Although several endopeptidases and exopeptidases have been shown to degrade bradykinin in vitro, evidence for the involvement of most of these enzymes in the regulation of kinin levels as part of normal homeostasis in vivo is less convincing. To our knowledge, no other specific peptidease inhibitors have been shown to reduce blood pressure in normotensive rats, although inhibitors of ACE and EP-24.11 have been shown to reduce blood pressure in hypertensive rats.\textsuperscript{23,26} While ACE inhibitors are thought to act primarily through blockade of conversion of angiotensin I to angiotensin II,\textsuperscript{27,28} work in several laboratories suggests that local protection of kinins is also involved in the antihypertensive action of ACE inhibitors: 1) the initial drop in arterial pressure that occurs after administration of ACE inhibitors in hypertensive animals is attenuated by bradykinin antisera and by a B2-bradykinin antagonist;\textsuperscript{29–31} 2) the increase in coronary blood flow observed after infusion of an ACE inhibitor in the isolated ischemic rat heart is reversed by a B2-bradykinin antagonist;\textsuperscript{32} and 3) the angiotensin II receptor antagonist saralasin is ineffective in certain forms of renin-independent hypertension, whereas ACE inhibitors are very effective.\textsuperscript{33} Inhibitors of EP-24.11 have antihypertensive activity in the deoxycorticosterone acetate–salt hypertensive rat,\textsuperscript{26} in the spontaneously hypertensive rat,\textsuperscript{34} and in humans.\textsuperscript{35} These inhibitors potentiate the renal (natriuretic) effects of atrial natriuretic factor as well as its depressor effects.\textsuperscript{26,34} However, the potentiation of the natriuretic effect (but not the depressor effect) of atrial natriuretic factor by the EP-24.11 inhibitor is fully reversed by a B2-bradykinin antagonist,\textsuperscript{36} indicating that protection of endogenous kinins, rather than atrial natriuretic factor, is responsible for the renal effects. While ACE is clearly involved in degradation of exogenously added kinins, the fact that in normotensive animals neither arterial plasma kinins nor arterial pressure are affected acutely in response to infusion of inhibitors of ACE, EP-24.11, CpN, or aminopeptidase\textsuperscript{15} suggests that these enzymes may not be involved in the control of local or systemic plasma kinins under normal conditions. Investigations are ongoing to determine whether plasma kinin levels are influenced by the administration of the EP-24.15 inhibitor, the extent to which the inhibitor affects blood pressure in hypertensive animals, and whether the depressor response to the inhibitor results from changes in blood flow to specific vascular beds. The inhibitor of EP-24.15 is expected to be a powerful tool in further studies of the mechanism of kinin action in vivo.

References


KEY WORDS: bradykinin • kallidin • angiotensin • peptide peptidohydrolases • enkephalinase • angiotensin converting enzyme
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