A Novel Stable Inhibitor of Endopeptidases EC 3.4.24.15 and 3.4.24.16 Potentiates Bradykinin-Induced Hypotension

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Abstract—We have developed a novel inhibitor of the metalloendopeptidases EC 3.4.24.15 (EP24.15) and EC 3.4.24.16 (EP24.16), N-[1-(R, S)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-p-aminobenzoate (JA2), in which α-aminoisobutyric acid (Aib) is substituted for an alanine in a well-described but unstable inhibitor, cFP-AAY-pAB. This substitution increases the resistance of the inhibitor to degradation without altering potency. In the present study, we investigated the effects of JA2 (5 mg/kg) on the responses of mean arterial pressure to bradykinin, angiotensin I, and angiotensin II in conscious rabbits. The depressor responses to both low (10 ng/kg) and high (100 ng/kg) doses of bradykinin were increased 7.0±2.7-fold and 1.5±0.3-fold, respectively, during the 30 minutes after JA2 administration (mean±SEM, n=8). Bradykinin potentiation was undiminished 4 hours after JA2 injection. In contrast, the hypertensive effects of angiotensins I and II were unaltered, indicating that the bradykinin-potentiating effects were not due to angiotensin-converting enzyme inhibition. These data suggest that JA2 is not only a potent and specific inhibitor of EP24.15 and EP24.16 but is also stable in vivo. Furthermore, the potentiation of bradykinin-induced hypotension by JA2 suggests for the first time a role for one or both of these peptidases in the metabolism of bradykinin in the circulation. (Hypertension. 2000;35:626-630.)

Key Words: bradykinin ■ angiotensin ■ circulation ■ angiotensin-converting enzyme ■ endopeptidase inhibition

Vasoactive peptides such as bradykinin and angiotensin play a crucial role in the regulation of blood pressure. The level of these peptides within the vasculature depends on the relative rates of production and degradation by peptidases present either in the plasma or on the extracellular membranes of vascular cells. Several members of the thermolysin-like metalloendopeptidase family are known to participate in the metabolism of vasoactive peptides. These include angiotensin-converting enzyme (ACE; EC 3.4.15.1), which both generates angiotensin II and degrades bradykinin, neutral endopeptidase (NEP; EC 3.4.24.11), which can cleave a number of vasodilator peptides to inactive products, and endothelin-converting enzyme (ECE), which generates the vasoconstrictor endothelin and may also contribute to bradykinin metabolism. Inhibitors of ACE markedly reduce blood pressure, particularly in instances in which the renin-angiotensin system is activated. Recent work with specific angiotensin and bradykinin receptor antagonists indicate that a portion of the hypotensive response to ACE inhibitors is attributable to blockade of bradykinin degradation. Similarly, inhibition of NEP reduces blood pressure by interfering with the degradation of both bradykinin and atrial natriuretic peptide. Thus enhanced levels of vasodilatory peptides, as well as reduction of constrictor peptide levels, may have beneficial effects in hypertension.

In addition to ACE, NEP, and ECE, the thermolysin-like metalloendopeptidase family includes the closely related enzymes EC 3.4.24.15 (EP24.15) and EC 3.4.24.16 (EP24.16). These enzymes are known to specifically cleave several bioactive peptides including bradykinin in vitro, but their exact function in vivo remains unclear, largely because of the unavailability of potent, specific, and stable inhibitors of these enzymes. A role for these peptidases in bradykinin metabolism was first suggested by Genden and Molineaux, who reported the potentiation of bradykinin-induced hypotension in rats after infusion of an EP24.15/16 inhibitor, N-[1-(R, S)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate (cFP-AAF-pAB). Subsequent studies, however, demonstrated that the observed effect of this inhibitor, or the tyrosine analogue cFP-AAY-pAB, was due to inhibition of ACE by a metabolite of the inhibitor (generated at least in part by NEP) rather than through the inhibition of EP24.15 or EP24.16. Thus the role of these peptidases in bradykinin degradation in the circulation remained in doubt, for want of a stable and specific inhibitor.

We have recently developed a novel EP24.15/16 inhibitor, JA2, in which the second alanine residue of cFP-AAY-pAB has been replaced with an α-aminoisobutyric acid (Aib). This substitution renders the inhibitor resistant to cleavage by membrane peptidases including NEP but does not affect...
potency against EP24.15 or EP24.16. In the present study, we demonstrate in conscious rabbits that JA2 administration potentiates the hypotensive effect of bradykinin without affecting resting arterial pressure; responses to angiotensin I were unaffected, confirming that JA2 has no effect on ACE activity. These results suggest that EP24.15 and/or EP24.16 are accessible to the intravascular space and thus may participate in the metabolism of bradykinin, although their exact role in circulatory control remains unknown. Furthermore, these results indicate that the inhibitor JA2 is stable in vivo and may be used in future studies to examine other purported functions of EP24.15 and EP24.16.

Methods

Materials

N-[1-(R, S)-carboxy-3-phenylpropyl]-Ala-Alb-Tyr-p-aminobenzoate (JA2) was synthesized as described by Shrimpton et al. This compound was found to inhibit both recombinant rat EP24.15 (K = 23 nmol/L) and to a lesser extent, EP24.16 (K = 610 nmol/L) but not ACE, ECE, aminopeptidase P, or neutral endopeptidase at concentrations up to 100 µmol/L. JA2 was radiolabeled with 125I with iodogen and purified by extraction on a Sep-Pak C18 column (Waters). Other drugs used in this study include bradykinin, angiotensin I, and angiotensin II (Auspex) and 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals Inc).

In Vivo Studies

Eight rabbits of a cross-bred English strain and of either sex were used, weighing 2.2 to 2.8 kg (mean 2.5). The experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved in advance by the Monash University Department of Physiology/Central Animal Services Animal Ethics Committee.

Preparations for Experiments

Experimental procedures were essentially performed as previously described. On the morning of each study the rabbit was placed in a 15×40×18-cm box fitted with a wire mesh lid, and intravascular catheters were inserted under local analgesia (1% lignocaine; xylocaine, Astra Pharmaceuticals). A catheter (Insyte; Deseret Medical) was inserted into the central ear artery to measure arterial pressure and to take blood samples when required. A nylene catheter (ID 0.50 mm; OD 0.63 mm) with a dead space of 40 µL was inserted into the marginal ear vein and advanced 11 to 15 cm so that the tip was near the right atrium for bolus drug administration. Each rabbit was then allowed at least 30 minutes to recover before the experimental procedures began. At the completion of the study day, the catheters were removed.

Experimental Protocol

Each of the 8 rabbits was studied on 2 separate occasions, 7 days apart. On each day the rabbits received either JA2 or its vehicle; the order of these treatments was randomized. The effects on arterial pressure of right atrial administration of bradykinin (10 and 100 ng/kg), angiotensin I (10 and 100 ng/kg), and angiotensin II (10 and 100 ng/kg) were tested. The 6 vasoactive peptide treatments were administered in random order at 5-minute intervals (25 minutes total) in volumes of 0.1 mL/kg. The rabbits were then treated with either JA2 (5 mg/kg, a near-maximal dose of the parent compound, cFP-AA-pAB9) or its vehicle (10% wt/vol 2-hydroxypropyl-β-cyclodextrin in 154 mmol/L NaCl; 1 mL/kg), and the effects of the vasoactive peptides were retested 5 minutes and 210 minutes later. In 4 of the rabbits, the dose of JA2 was spiked with 1.4×10^6 cpm of the iodinated material. In these rabbits, arterial blood samples (1 mL) were collected immediately before, 10 and 30 seconds, and 1, 5, 30, 60, and 240 minutes after administration of JA2 or its vehicle. These blood samples were processed for determination of plasma levels of JA2 and its metabolites with the use of methods similar to those described by Lew et al (see below).

Recording of Hemodynamic Variables

Arterial pressure was measured by connecting the arterial catheter to a Statham P23Dc strain gauge, set to zero at the level of the animal’s heart. The signals were amplified and recorded on a Neotrace pen recorder (Neomedix Systems) and sent to an Olivetti M24 computer equipped with an A-D converter, which provided 2-second mean values of mean arterial pressure (MAP, mm Hg) and heart rate (HR, beats/min).

The effects of the vasoactive peptides were determined as the peak change in MAP compared with the mean for the 10-second period before peptide administration. Resting MAP and HR were defined as the mean levels of these variables across the six 10-second periods before the peptides were administered.

Statistical Analysis

Four-way ANOVA was used to test for effects of JA2 on responses to the vasoactive compounds. The factors were rabbit, dose (10 or 100 ng/kg of the vasoactive peptides), treatment (JA2 or vehicle), and time (before or 5 or 210 minutes after administration of JA2 or vehicle). The error mean square comprised all interaction terms that included the factor rabbit. The treatment by time interaction was tested whether the effects of JA2 on the magnitude of responses to the vasoactive peptides differed from that of vehicle. This analysis allowed us to test our hypothesis in a global, within-animal fashion, and the outcome was independent of any possible effects of time on responses to the vasoactive peptides. In addition, to specifically test for the effects of time in vehicle-treated rabbits, the data for the 2 study days were also analyzed separately to test the effects of time within each treatment (Ptime×treatment, df 2,35).

A similar analysis was used to test whether JA2 or its vehicle influenced resting levels of MAP and HR. The statistical computer software package SYSTAT was used for statistical analyses. A value of P≤0.05 was considered significant. All data are expressed as mean±1 SEM.

High-Performance Liquid Chromatography

Plasma (1 mL) was added to 5 mL of methanol to precipitate protein and centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was collected into 5-mL tubes and dried in a Speed-Vac concentrator (Savant). Samples were then reconstituted in 0.08% TFA, and the total radioactivity was determined before analysis by high-performance liquid chromatography (HPLC). Samples were injected onto a NovaPack C18 column (8.00 mm ID×100 mm) contained within a radial compression module (Waters Associates), and constituents were eluted from the column by a linear (30 minutes) gradient from 3% to 100% solvent B (70% CH3CN/0.08% TFA; solvent A = 0.08% TFA) at a flow rate of 1 mL/min. Chromatography was performed with a Waters HPLC system consisting of an automated gradient controller (model 680), 2 model 510 pumps, a U6K injector, and a model 441 detector (214 nm). The metabolism of 125I-JA2 was assessed by counting radioactivity (Packard RiaStar γ-counter) in 70 fractions (0.5 mL) collected from each HPLC run with a FRAC-100 fraction collector (Pharmacia). The plasma concentration of noniodinated JA2 was estimated by relating the area of the UV absorbance peak to concentration by use of a series of JA2 standards.

Results

Metabolism of 125I-JA2 In Vivo

In a previous study in which radioiodinated cFP-AA-pAB was used to follow its in vivo degradation, we observed significant and rapid metabolism of the labeled inhibitor, with the generation of several radioactive fragments including 125I-Tyr-pAB (Figure 1). The appearance of this fragment is indicative of cleavage at the Ala-Tyr bond, thus confirming generation of the ACE inhibitor cFP-AA. Five minutes after
injection, $^{125}$I-Tyr-pAB represented 15% of the total radioactivity in plasma; other metabolites retained on the reverse-phase column accounted for 20% of the radiolabel. In contrast, HPLC analysis in the present study revealed no radioactive metabolites of $^{125}$I-JA2 (other than free $^{125}$I, which was not retained on the column), and specifically there was no evidence of $^{125}$I-Tyr-pAB within the first 5 minutes after injection (Figure 1). These results suggest that the Aib-Tyr bond in JA2 is resistant to hydrolysis in vivo under the conditions of these studies. The plasma concentration of JA2 5 minutes after administration of 5 mg/kg was estimated to be 4.2 μmol/L (3.0 to 5.4, 95% confidence limits, n=4), somewhat higher than that observed at the same time point for the same dose of cFP-AAY-pAB (2.9 μmol/L (1.2 to 6.9); 10), which is consistent with a lower rate of degradation.

**Effects of JA2 on Resting MAP and HR**

Before treatment with JA2 or its vehicle, resting MAP and HR averaged 83±1 mm Hg and 214±6 beats/min, respectively. Neither JA2 nor its vehicle significantly affected the level of MAP (P<time*>time*<treatment*<0.58; P<time*treatment*<0.98), but HR tended to increase similarly after both JA2 treatment and vehicle treatment (P<time*<0.004; P<time*treatment*<0.18) (Table).

**Effects of JA2 on Responses to Bradykinin, Angiotensin I, and Angiotensin II**

Our analysis indicated a significant effect of JA2 treatment on responses to bradykinin (P<time zaman=0.02; Figure 2). Before treatment with JA2, right atrial administration of bradykinin (10 and 100 ng/kg) was followed by transient reductions in MAP of 2±1 and 13±1 mm Hg, respectively. These re-
responses were augmented by JA2, both 5 minutes (7±6 and 21±6 mm Hg, respectively) and 210 minutes (12±3 and 31±4 mm Hg respectively) after inhibitor administration. Thus the depressor response to 10 ng/kg bradykinin was increased 7.0±2.7-fold and 7.5±1.8-fold, respectively, during the periods 5 to 30 and 210 to 235 minutes after JA2 administration. At the same times, the depressor response to 100 ng/kg bradykinin was increased 1.5±0.3-fold and 2.5±0.5-fold, respectively (P_{time=treatment}=0.001). In contrast, responses to the 2 doses of bradykinin remained relatively stable after vehicle treatment (P_{time=treatment}=0.21).

Before treatment with JA2 or its vehicle, right atrial administration of angiotensin I (10 and 100 ng/kg) was followed by transient increases in MAP of 11±1 and 33±1 mm Hg, respectively, and right atrial administration of angiotensin II (10 and 100 ng/kg) was followed by transient increases in MAP of 19±1 and 41±2 mm Hg, respectively. These responses were not significantly affected by vehicle or JA2 treatment (Figure 2).

**Discussion**

The development of a novel inhibitor of the metalloendopeptidases EP24.15 and EP24.16, which is specific, potent and, most importantly, stable, has allowed us to investigate the role of these enzymes in bradykinin metabolism in the circulation of conscious rabbits. Administration of the inhibitor (JA2) significantly potentiated the hypotensive response to exogenous bradykinin, without affecting pressor responses to angiotensin I (Figure 2). This suggests that unlike the parent compound cFP-AAY-pAB, JA2 is not degraded to any significant extent to form an ACE inhibitor. Indeed, we have previously shown that JA2 is resistant to degradation by kidney membranes in vitro, whereas in the present study, analysis of radiolabeled JA2 in plasma samples also suggested a much greater stability of this compound in the circulation compared with cFP-AAY-pAB (Figure 1). Potentiation of bradykinin-induced hypotension by JA2 in the absence of ACE inhibition indicates that EP24.15 and/or EP24.16 may participate in the metabolism of this peptide in the bloodstream. Indeed, this potentiation was of a similar magnitude as that seen with the ACE inhibitor captopril in the same experimental model (≈7-fold and 2-fold for 10 ng/kg and 100 ng/kg bradykinin, respectively; Reference 9). Thus the present studies suggest that EP24.15 and/or EP24.16 may be included in the growing array of kininases, which includes ACE, NEP, ECE, carboxypeptidases, and aminopeptidases, present in the circulation. Inhibition of any of these peptidases, particularly ACE, increases the effects and/or plasma concentration of exogenous bradykinin severalfold (reviewed in References 5, 14, and 15); however, there are several reports suggesting the presence of other, uncharacterized kininases.5,15,16 This apparent redundancy in bradykinin-degrading peptidases reflects the necessity of rapid turnover of such a potent vasoactive and proinflammatory peptide. The contribution of EP24.15 and/or EP24.16 relative to other peptidases can now be assessed with JA2 in combination with other specific inhibitors, under both normal and pathophysiological conditions, as well as within individual vascular beds.

Although the present results suggest a role for EP24.15 and/or EP24.16 in the metabolism of bradykinin, the localization of these enzymes in the circulation is not yet known. Early reports indicated very low levels in serum and plasma,¹⁷ a finding replicated in our laboratory (Norman MU, Lew RA, and Smith AI, unpublished results, 1999), although EP24.15-like activity has been detected in rat hypophysial portal blood.¹⁸ Recent work in this laboratory has demonstrated the presence of both EP24.15 and EP24.16 in cultured aortic
endothelial cells, a proportion of which was located extracellularly (Lew RA, Gerreyn SB, Little PJ, and Smith AI, manuscript submitted, 1999). Thus, like ACE and NEP, EP24.15 and/or EP24.16 may degrade bradykinin at the endothelial cell monolayer lining the bloodstream. Further work is needed to determine the exact cellular and subcellular localization of these peptidases and their site of action in the metabolism of bradykinin as well as assessing their role in the degradation of other bioactive peptides.

Despite potentiating the effects of exogenous bradykinin, resting arterial pressure was unaffected by JA2 administration. Similar results were seen with cFP-AAY-pAB in this animal model, in which ACE was the predominant enzyme inhibited. However, this inhibitor did elicit a fall in resting blood pressure in anesthetized rats, probably caused by inhibition of ACE. Similarly, ACE inhibitors often have only modest effects on blood pressure in normotensive, salt-replete animal models, in contrast to the profound effects seen in a number of hypertensive models. Indeed, in the same experimental model used here (conscious rabbit), MAP was unchanged by captopril administration. Thus the changes in resting blood pressure observed after peptidase inhibitor administration are very much dependent on preexisting conditions.

In summary, we have developed and used a stable inhibitor of EP24.15 and EP24.16 to demonstrate, for the first time, that these peptidases participate in the degradation of bradykinin within the circulation. Further studies to define the exact intravascular distribution of these enzymes and their relative contribution to the metabolism of bradykinin and other vasoactive peptides, both in healthy and diseased states, can now be undertaken. In addition, it is our aim to refine further inhibitors such as JA2, which can better distinguish between the closely related EP24.15 and EP24.16, to determine the precise physiological roles of these 2 metalloendopeptidases.

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