

The Effect of Acute Angiotensin-Converting Enzyme and Neutral Endopeptidase 24.11 Inhibition on Plasma Extravasation in the Rat

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ABSTRACT

The effect of angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) inhibition on microvascular plasma leakage (extravasation) was evaluated in a rat model. Progressive inhibition of ACE using captopril caused increased extravasation when lung ACE was inhibited by >55%. In contrast, the selective inhibition of renal NEP by >90% using ecadotril did not increase extravasation. In NEP-inhibited rats, extravasation produced by the ACE inhibitors captopril and lisinopril was markedly enhanced. The dual ACE and NEP inhibitor omapatrilat, at oral doses of 0.03, 0.1, and 0.3 mg/kg, selectively inhibited lung ACE by 19, 61, and 76%, respectively, and did not cause significant extravasation. Doses of 1 and 10 mg/kg omapatrilat, which produced >90% inhibition of ACE and also inhibited renal NEP by 54 and 78%, respectively, significantly increased extravasation. In this model, bradykinin

and substance P produced extravasation that could be abolished by the bradykinin 2 (B2) receptor antagonist Hoe 140 (icatibant) or the neurokinin1 (NK1) antagonist CP99994 [(+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine], respectively. Bradykinin induced extravasation was also partially (~40%) inhibited by CP99994, indicating that a portion of the response involves B2 receptor-mediated release of substance P. In conclusion, this study is the first to relate the degree of ACE and/or NEP inhibition to extravasation liability in the rat model. Our data clearly demonstrate that ACE inhibitor-induced plasma extravasation is enhanced by concomitant inhibition of NEP. In addition, this study provides further evidence for the role for B2 and NK1 receptors in mediating plasma extravasation in the rat.

Since their introduction nearly three decades ago, the angiotensin I-converting enzyme inhibitors (ACEIs) have become one of the more effective and highly used treatments for hypertension and heart failure. The therapeutic efficacy of these agents is derived in large part from their ability to inhibit the conversion of angiotensin I to angiotensin II, a vasoactive peptide whose direct vasoconstrictor and aldosterone-releasing actions promote increased blood pressure. There are some data to suggest that part of the therapeutic effect of these agents may be due to decreased breakdown of bradykinin (BK), which is also a substrate for ACE (Bao et al., 1992; Linz and Schölkens, 1992; Linz et al., 1995). In an attempt to provide even greater antihypertensive efficacy, efforts have been directed toward developing vaso-peptidase inhibitors (VPI) that block not only ACE but also neutral endopeptidase (NEP). Inhibition of NEP should confer addi-

tional cardiovascular benefit by virtue of enhancing the hemodynamically favorable actions of the atrial natriuretic peptides, which are substrates for NEP (Robl et al., 1997; Burnett, 1999; Weber, 2001). Data from animal studies have indicated that the VPI omapatrilat does indeed confer greater cardiovascular benefit than ACE inhibitors alone (Trippodo et al., 1998; Bäcklund et al., 2001; Pu et al., 2002), and there is some data to suggest that this can also be seen in patients (Campese et al., 2001; Azizi et al., 2002; Ferrario et al., 2002). The development of omapatrilat, however, has been hampered by a 3-fold higher incidence of angioedema than observed with an ACEI (Coates, 2002; Campbell, 2003). Because it is possible that the greater degree of VPI may be also be responsible for the increased incidence of angioedema, we have used a rat model of plasma extravasation (Saria and Lundberg, 1983; Brokaw and White, 1994), an important component of angioedema, to evaluate the roles of ACE and NEP in this phenomenon. Plasma extravasation is an important component in angioedema and seems to be a reasonable way of studying angioedema in animals. Thus, C1

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ABBREVIATIONS: ACEI, angiotensin I-converting enzyme inhibitor; BK, bradykinin; VPI, vaso-peptidase inhibitor; NEP, neutral endopeptidase; ACE, angiotensin-converting enzyme; NK, neurokinin; TCA, trichloroacetic acid; B1, bradykinin1; B2, bradykinin2; Hoe 140, icatibant; CP99994, (+)-(2S,3S)-3-(2-methoxybenzylamino)-2-phenylpiperidine.

esterase inhibitor deficiency in humans leads to angioedema (Markovic et al., 2000); however, angioedema is not observed in C1 esterase inhibitor-deficient mice. Interestingly, these mice do show acute ACE inhibitor-induced worsening of plasma extravasation (Han et al., 2002).

Finally, we also evaluated the effects of BK, des-Arg9-BK, and substance P in the rat model because these peptides have either been implicated in angioedema in humans (Cugno et al., 2003) or have been shown to cause plasma extravasation in rats, mice, and guinea pigs (Saria et al., 1983; Abelli et al., 1991; Emanuelli et al., 1998).

Materials and Methods

Experimental Preparation(s). Male Sprague-Dawley strain rats weighing between 250 and 350 g were used in these studies. The procedures involving the use of rats in these experiments were reviewed and approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines (NIH publication 85-23). The animals were housed two per cage with free access to food and water and a 12-h light/dark cycle.

To determine the role of ACE and/or NEP inhibition on plasma extravasation, groups of rats received drug treatment orally using an 18-gauge dosing needle and 5-ml syringe. Approximately 50 to 55 min after dosing, the rats were anesthetized via intraperitoneal injection of 70 mg/kg sodium pentobarbital. When anesthesia was achieved (typically less than 10 min), Evans blue dye (30 mg/kg) in heparinized saline (30 U/ml) was administered intravenously at a dose volume of 0.2 ml/100 g b.wt. via the tail vein using a 26-gauge, 1.5-inch-long needle. Five minutes post-Evans blue injection, the thoracic and peritoneal cavities were opened via a single midline incision. A 0.8 to 1.0 cc blood sample was obtained by cardiac puncture using a heparinized 1-ml syringe and 23-gauge needle and placed on ice. The tip of the right atrium was then cut and a steel cannula, attached by latex tubing to a peristaltic pump (Harvard Apparatus Inc., Holliston, MA), was inserted into the heart at the bottom of the left ventricle and was slid up through ventricle until the tip of the cannula was visible in the aortic arch. The cannula was manually held in place using forceps clamped across the heart. The pump was then started and the vascular system was perfused with 120 ml of saline delivered at a rate of 40 ml/min, which results in a perfusion pressure pulse of 80 to 100 mm Hg. This procedure is similar to that described by others (Brokaw and White, 1994; Klitzman et al., 1995). The success of the perfusion was evaluated by visually monitoring the liver for the progressive removal of the dark bluish color caused by the presence of Evans blue in the liver tissue. Complete blanching of the liver was achieved in all cases. After perfusion, a section of the left lung, the entire left kidney, and a 15- to 20-mm segment of the trachea were removed. The lung and kidney were cut into four sections, placed into Falcon tubes, and flash frozen in liquid nitrogen. The blood, lung, and kidney samples were submitted for determination of ACE (plasma and lung) and NEP (kidney) activity. The trachea was cleared of any adhering fat and muscle, a cut was made along the entire length to expose the inner surface and the tissue was blotted dry using a paper hand towel. The trachea was weighed and placed into a 1.5-ml Eppendorf tube containing 0.5 ml of formamide. The tissue remained in the formamide for 24 to 48 h at room temperature to extract Evans blue from the tissue. Duplicate 200- μ l aliquots of each extract were then pipetted into wells of a 96-well clear plastic plate. The concentration of dye in the extracts was determined spectrophotometrically by measuring absorbance at 620 nm. The concentration was interpolated from an Evans blue standard curve run simultaneously and was expressed as nanograms of Evans blue per milligram of trachea (Brokaw and White, 1994; Klitzman et al., 1996).

For determination of extravasation liability of proinflammatory peptides, rats were anesthetized by intraperitoneal injection of 70

mg/kg pentobarbital sodium. The rats then received an intravenous dose of 30 mg/kg Evans blue dye in saline containing 30 U/ml heparin administered at a dose volume of 200 μ l/100 g b.wt. via tail vein injection. The Evans blue injection was followed immediately by intravenous injection of bradykinin, des-Arg9-bradykinin, or substance P. In some studies, the effect of selective B2 receptor blockade with Hoe 140 (10 μ g/kg i.v.) or selective NK1 receptor blockade with CP99994 (3 mg/kg i.v.) on bradykinin and substance P-mediated extravasation was also determined. In those studies, the selective antagonists were administered 3 to 5 min before Evans Blue injection. Five minutes after bradykinin, des-Arg9-bradykinin, or substance P injection, the thoracic and peritoneal cavities were opened via a single midline incision, and the rat was perfused as described above.

For evaluation of plasma ex vivo ACE activity, the blood collected by cardiac puncture was spun for 2 min at maximum speed in a Microfuge. Plasma was collected from the top. Thirty-five microliters of plasma was added to a conical-bottomed 96-well plate with 5 μ l of 1 M KCl, 0.5 M sodium borate, pH 8.3, and 3 μ M zinc sulfate. Ten microliters of 12.5 mM hippuryl-his-leu substrate (Cushman and Chung, 1971) dissolved in the same buffer was added to each well. The ACE activity was measured by the production of his-leu after 15 min at 37°C. The assay was terminated by the addition of 100 μ l of 10% trichloroacetic acid (TCA) followed by centrifugation of the plate to pellet precipitated proteins. Fifty microliters of the supernatant was added in duplicate to a black fluorometric plate containing 100 μ l of 2 mg/ml *o*-phthalaldehyde, 10% ethanol, and 50 μ l of 1 N NaOH. After 60 min, the plate was read in a fluorometer at 390-nm excitation and 460-nm emission. Standard curves were generated using his-leu.

For determination of lung ACE activity and kidney NEP activity, approximately 250 mg of tissue was homogenized in 6 volumes of 0.1 M KH_2PO_4 , pH 8.3, 0.3 M NaCl, and 1 μ M ZnSO_4 using a Teflon-glass motor-driven pestle. For lung ACE activity, 40 μ l of homogenate was added to conical-bottomed 96-well plates and warmed to 37°C for 5 min. Ten microliters of 7.5 mM hippuryl-his-leu (1.5 mM final) was added to each sample and incubated for 10 min at 37°C. One hundred microliters of 10% TCA was added to each well, and the plates were centrifuged to pellet precipitated proteins. Fifty microliters of supernatant was added to 100 μ l of 2 mg/ml *o*-phthalaldehyde in 10% ethanol and 50 μ l of 1 N NaOH in a black fluorometric plate. After 60 min, the plate was read in a fluorometer at 390-nm excitation and 460-nm emission. Standard curves were generated using his-leu.

Kidney NEP activity was measured by adding 35 μ l of homogenate to wells containing 5 μ l of buffer or 10 μ M phosphoramidon. Plates were warmed to 37°C for 5 min. Ten microliters of 2.5 mM *N*-dansyl-D-ala-gly-p-nitrophenyl-gly substrate (Florentin et al., 1984) was added to each sample to yield a 0.5 mM final concentration and incubated for 4 min at 37°C. One hundred microliters of 10% TCA was added, and plates were centrifuged to pellet precipitated proteins. Fifty microliters of supernatant was added to 100 μ l of 100% ethanol and 50 μ l of 1 N NaOH in a black fluorometric plate. After 10 min, plates were read at 590-nm emission, 320-nm excitation in a fluorometer. Standard curves were generated using *N*-dansyl-D-ala-gly in buffer. For both the ACE and NEP assays, homogenate protein was measured with a protein assay kit (Bio-Rad, Hercules, CA), and product production was corrected for protein.

Drugs. The selective ACE inhibitors captopril and lisinopril were dissolved in water and administered orally at doses of 0.3, 1, 3, 10, and 30 mg/kg (captopril) or 1 and 3 mg/kg (lisinopril) at a dose volume of 1.0 ml/100 g b.wt. The dual ACE/NEP inhibitor omapatrilat was dissolved in a 30% polyethylene glycol200/70% of 25% cyclodextran vehicle and was administered orally as described above at doses of 0.03, 0.1, 0.3, 1, or 10 mg/kg. The selective NEP inhibitor ecaodril was dissolved in the polyethylene glycol200/cyclodextrin vehicle and was administered orally at doses of 3, 10, neutral endopeptidase or 30 mg/kg.

In some studies, inhibition of both ACE and NEP was produced by the oral administration of various doses of the selective ACE inhibitor captopril or lisinopril in rats pretreated orally with the selective NEP inhibitor ecadotril.

Statistics. All data are expressed as the mean \pm S.E.M. The analysis of the differences in the extravasation measurement values between levels of treatments regimes used two-sample Wilcoxon tests. Based on the assumption of increasing extravasation with larger drug doses, one-sided tests were appropriate for the identification of the lowest dosage level with a significant increase in the extravasation values. The identification of these lowest dosages applied a fixed sequence test strategy (Westfall et al., 1999) for the comparisons of the treatment levels (bradykinin, captopril, des-Arg9-bradykinin, ecadotril, omapatrilat, and substance P) versus the common vehicle measurements. Acceptance of a significant difference at each sequential step was at the 0.05 *p* value level.

All other comparisons between dosage levels also had predetermined directions of changes in the extravasation values and used single-sided values from two-sample Wilcoxon tests. The reported *p* values for these comparisons were all less than 0.05 (Bonferroni adjusted *p* values were reported for the four comparisons of the captopril and ecadotril combinations and the two comparisons of the bradykinin and CP99994 combinations). The trend comparison for the bradykinin and Hoe combinations used the single-sided Jonckheere-Terpstra trend test. All statistical tests used SAS System Release 8.01 as the analysis software (proc npar1way for the exact Wilcoxon tests and proc freq for the Jonckheere-Terpstra test).

An unpaired Student's *t* test was used to test the effect of drug treatment on enzyme activity. Absolute enzyme rates of drug-treated rats were compared with enzyme rates of the vehicle-treated (control) rats. Acceptance of a significant difference between the groups was at the 0.05 *p* value level.

Results

Basal extravasation of Evans blue into the trachea of 29 vehicle-treated rats accumulated over the course of these studies was 8.3 ± 0.43 ng/mg tissue. Baseline plasma ACE, lung ACE, and renal NEP enzyme activity in vehicle-treated rats was 32.7 ± 1.7 nmol/ml/min and 6.5 ± 1.8 and 2.6 ± 0.2 nmol/mg protein/min, respectively.

Treatment with increasing oral doses of captopril produced dose-related inhibition of plasma and lung ACE activity and was without effect on renal NEP. The reductions in ACE activity were associated with increased extravasation as measured by tracheal Evans blue concentration (Fig. 1A). The lowest dose of captopril, 1 mg/kg, produced significant inhibition of plasma ACE (-47%) and lung ACE (-36%) and did not increase tracheal Evans blue concentration. At the 3 mg/kg dose, plasma and lung ACE were inhibited by 48 and 55%, respectively, and tracheal Evans blue was significantly increased to 13.4 ± 3 ng/mg. The progressively greater inhibition of ACE produced by the 10 and 30 mg/kg dose levels resulted in significant increases in plasma extravasation. Inhibition of plasma and lung ACE at the 30 mg/kg dose was essentially complete (91 and 99%, respectively) and was associated with a tracheal Evans blue content of 35 ± 9 ng/mg.

Oral doses of 0.03, 0.1, and 0.3 mg/kg of the dual ACE/NEP inhibitor omapatrilat produced dramatic, dose-related inhibition of both plasma and lung ACE with no inhibition of renal NEP (Fig. 1B). At these doses plasma ACE was more susceptible to inhibition by omapatrilat compared with lung ACE. As the dose was increased to 1 and 10 mg/kg, both plasma and lung ACE activity were reduced by greater than 90%, and inhibition of renal NEP now was apparent. First

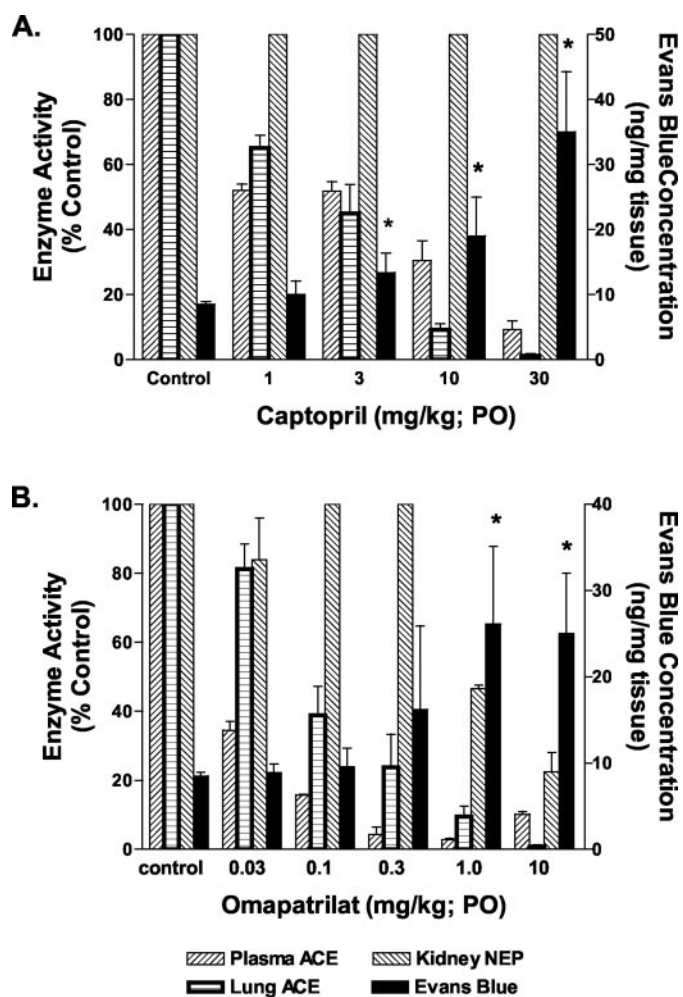


Fig. 1. A and B, effect of increasing doses of the selective ACE inhibitor captopril (A) or the dual ACE/NEP inhibitor omapatrilat (B) on plasma and lung ACE activity, renal NEP activity, and tracheal plasma extravasation. * denotes significant increase in Evans blue concentration at $p \leq 0.05$. All ACE values are significantly less than control. NEP was not significantly affected by captopril; NEP was significantly inhibited by omapatrilat at the 1 and 10 mg/kg doses only. $n = 4$ to 6/group.

evidence of an effect on plasma extravasation occurred at the 0.3 mg/kg dose level, a dose that markedly inhibited plasma ACE (-96%) and lung ACE (76%) but was devoid of NEP inhibition. Tracheal Evans blue content was 16.0 ± 7 ng/mg in this group. Although this value did not reach statistical significance, it is important to note that one of the four rats in this group exhibited a markedly increased tracheal Evans blue concentration of 35 ng/mg. When the dose was increased to 1 and 10 mg/kg, the inhibition of plasma ACE and lung ACE was virtually complete ($>90\%$), and renal NEP was inhibited by 53 and 78%, respectively. This profound effect on ACE and NEP was accompanied by a further significant increase in tracheal Evans blue content and the concentration seemed to plateau at ~ 25 ng/mg for both dose levels.

Effect of Increasing Lung ACE Inhibition in NEP-Inhibited Rats. The data with omapatrilat did not clearly define the role that NEP inhibition played in the extravasation of Evans blue into the trachea because the degree of both ACE and NEP inhibition varied with dose. To define the role of NEP in extravasation further, rats were treated with 3.0 mg/kg ecadotril, which resulted in a relatively consistent

background inhibition of ~74% in renal NEP (Fig. 2). These rats also received low doses of captopril (0.3 and 1 mg/kg) to produce graded inhibition of lung ACE. These low doses of captopril did not in themselves increase plasma extravasation. Under conditions of NEP inhibition, progressive, but relatively minor (<30%) inhibition of lung ACE evoked plasma extravasation (Fig. 2). The small inhibition of lung ACE (~12%) produced by the 0.3 mg/kg dose of captopril did not significantly increase the extravasation of Evans blue under conditions of simultaneous NEP inhibition. However, increasing the lung ACE inhibition to ~30% with a dose of 1 mg/kg captopril resulted in a significant increase in tracheal Evans blue concentration (22 ± 6 ng/mg). In rats treated with a dose of ecadotril (10 mg/kg) that produced >85% of renal NEP, the 1-mg/kg dose of captopril now produced ~50% inhibition of lung ACE and markedly increased tracheal Evans blue extravasation to 42 ± 13 ng/mg tissue. The reason for the greater reduction in lung ACE produced by the 1 mg/kg dose of captopril in the rats treated with 10 mg/kg ecadotril is unclear. One possible explanation is that the higher dose of ecadotril may have altered the pharmacokinetics (e.g., metabolism and protein binding) of captopril. In any event, it is evident that a 50% inhibition of lung ACE in animals having virtually no NEP activity dramatically increases plasma extravasation. It is important to note that the profound inhibition of renal NEP by the 3 and 10 mg/kg doses of ecadotril did not in itself increase Evans blue extravasation.

The selective ACE inhibitor lisinopril was also tested alone and in combination with ecadotril. Doses of 1 and 3 mg/kg lisinopril alone inhibited lung ACE by 63 and 83%, respectively, and did not increase tracheal Evans blue extravasation (Fig. 3). When the 1 mg/kg dose of lisinopril was tested in combination with a dose of ecadotril (10 mg/kg) that produced ~80% inhibition of kidney NEP activity, no increase in Evans blue extravasation occurred (7.7 ± 1.8 versus 11.1 ± 2.9 ng/mg tissue). A marked increase in extravasation did

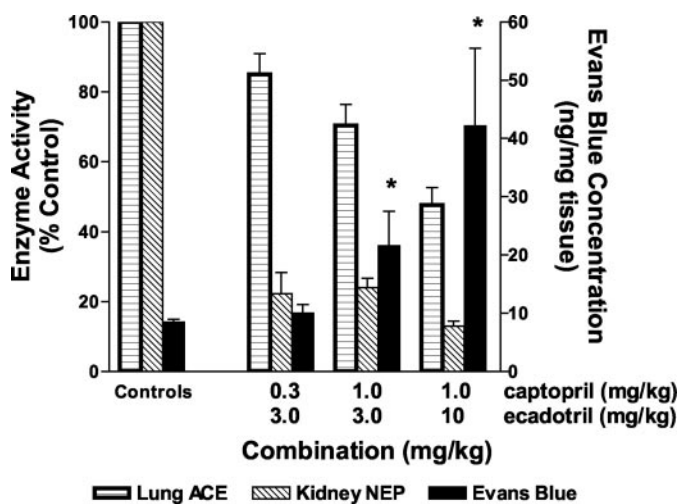


Fig. 2. Effect of increasing degrees of lung ACE inhibition (using captopril) imposed upon a relatively consistent degree of ecadotril-induced kidney NEP inhibition on tracheal plasma extravasation. * denotes significant increase in Evans blue concentration at $p \leq 0.05$. All NEP values are significantly lower than control at $p \leq 0.05$. The 0.3 mg/kg captopril dose did not significantly affect lung ACE; the 1 and 3 mg/kg captopril doses significantly inhibited lung ACE at $p \leq 0.05$. Captopril 0.3 mg/kg ($n = 3$); all other groups ($n = 7-8$).

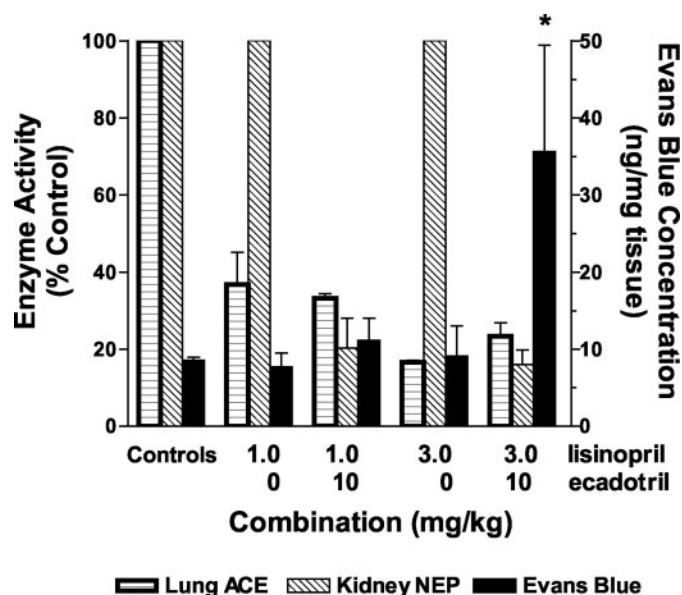


Fig. 3. Effect of increasing degrees of lung ACE inhibition (with lisinopril) imposed upon a relatively consistent degree of ecadotril-induced kidney NEP inhibition on tracheal plasma extravasation. * denotes significant increase in Evans blue concentration at $p \leq 0.05$. All NEP values in the ecadotril-treated rats are significantly lower than control at $p \leq 0.05$. The 1 and 3 mg/kg doses of lisinopril significantly inhibited lung ACE at $p \leq 0.05$. $n = 3$ to 4/group.

occur when a 3 mg/kg dose of lisinopril was administered in combination with ecadotril (9.1 ± 3.9 versus 35.6 ± 13.8 ng/mg tissue). For both combination groups, the degree of lung ACE inhibition was virtually identical to that produced by the 1 and 3 mg/kg doses of lisinopril when administered alone.

Dose-Response Relationships for des-Arg9-Bradykinin, Bradykinin, and Substance P in Producing Extravasation and the Role of Bradykinin1 (B1), Bradykinin2 (B2), and NK1 Receptors. The selective B1 receptor agonist des-Arg9-bradykinin did not affect plasma extravasation after intravenous doses of 10, 100, 300, and 1000 $\mu\text{g}/\text{kg}$ (Fig. 4). Intravenous challenges of 10, 30, 100, and 300 $\mu\text{g}/\text{kg}$ bradykinin produced dose-related increases in plasma extravasation as indicated by increases in tracheal Evans blue content (Fig. 3). Significant extravasation occurred at the 100 $\mu\text{g}/\text{kg}$ dose (Evans blue concentration 17 ng/mg tissue) and the 300 $\mu\text{g}/\text{kg}$ dose increased Evans blue concentration to 38 ng/mg tissue. Substance P was found to be 300- to 1000-fold more potent than bradykinin in causing plasma extravasation, producing a significant increase at an intravenous dose of 0.1 $\mu\text{g}/\text{kg}$. The difference in potency between bradykinin and substance P is likely explained by the extensive pulmonary metabolism of bradykinin that occurs after intravenous administration in the rat. However, substance P seemed to be somewhat less efficacious than bradykinin, producing a maximum Evans blue extravasation of 25 ng/mg tissue at both the 0.3 and 1 $\mu\text{g}/\text{kg}$ dose levels.

The robust increase in extravasation produced by 300 $\mu\text{g}/\text{kg}$ bradykinin was completely blocked by the B2 receptor antagonist Hoe 140 (Fig. 5A) and was partially (~40%) blocked by the selective NK1 receptor antagonist CP 99994 (Fig. 5B). The increase in plasma extravasation produced by substance P at 0.3 $\mu\text{g}/\text{kg}$ was unaffected by pretreatment

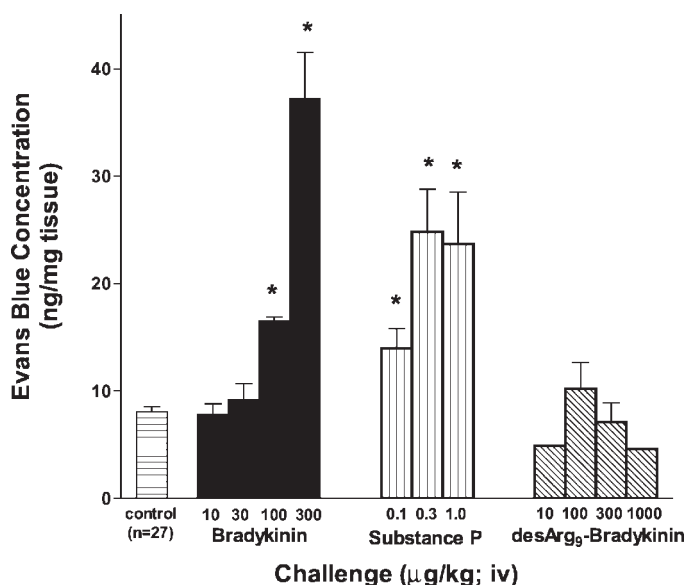


Fig. 4. Effect of kinins on extravasation of Evans blue into the rat trachea. Bradykinin, substance P, or des-Arg⁹-bradykinin was administered intravenously immediately after Evans blue injection, and the trachea was excised 5 min later. * denotes significant increase in Evans blue concentration at $p \leq 0.05$. $n = 3$ to 9/group for substance P; $n = 4$ to 10/group for bradykinin; and $n = 2$ to 4/group for des-Arg⁹-bradykinin.

with Hoe 140 (Fig. 4A) and was abolished by CP99994 (Fig. 5B).

Effect of Selective NEP Inhibition with Ecadotril on Plasma Extravasation. Oral doses of 10 and 30 mg/kg of the selective NEP inhibitor ecadotril reduced renal NEP activity by 88 and 99%, respectively. The 10-mg/kg dose was highly selective for NEP, producing no inhibition of lung ACE; however, a small (23%) but significant inhibition of lung ACE occurred at the 30 mg/kg dose. In spite of the marked inhibition of NEP produced by ecadotril, the drug did not increase tracheal Evans blue content (Fig. 6).

Discussion

The metabolism of the proinflammatory peptide bradykinin is accomplished by a number of endogenous enzymes, including ACE, carboxypeptidase N, aminopeptidase P, and NEP (Skidgel, 1992), and it has been shown that ACE is the key enzyme, being responsible for >70% of bradykinin hydrolysis in plasma and tissues (Prechel et al., 1995; Kuoppala et al., 2000; Dendorfer et al., 2001; Taylor-McCabe et al., 2001). Indeed, bradykinin is a highly preferred substrate for ACE, possessing a ~90-fold lower K_m and 24-fold higher K_{cat}/K_m than does angiotensin I (Jaspard et al., 1993). The contribution of the other enzymes to bradykinin metabolism seems to be minor under physiological conditions, but their roles may assume greater importance when ACE is inhibited. In the present study, we have demonstrated that selective inhibition of lung ACE by >55% with captopril caused plasma extravasation. However, the activity captopril may not be representative of all ACEIs. Specifically, the ACE inhibitor lisinopril did not cause extravasation at doses that produced as much as 83% inhibition of lung ACE. In addition, our experience suggests that inhibition of lung ACE by >90% is typically required to increase tracheal extravasation. For reasons that remain unclear, captopril possessed somewhat

greater liability to increase tracheal Evans blue extravasation than did lisinopril.

Extravasation after ACE inhibition seems to be due to bradykinin accumulation because other investigators have shown that extravasation produced by captopril in mice can be prevented by selective B2 receptor blockade (Emanuelli et al., 1998; Baluk et al., 1999) and that captopril-induced extravasation is not observed in B2 receptor knockout mice (Emanuelli et al., 1998). These data confirm the dominant role of ACE in bradykinin metabolism and clearly implicate bradykinin in ACE inhibitor-induced plasma extravasation in animal models. Whereas ACE inhibition alone caused plasma extravasation, we have also shown that a profound, selective inhibition of NEP was without effect, suggesting that NEP activity is of little consequence in the hydrolysis of bradykinin when ACE is fully active.

As further confirmation of the involvement of bradykinin and the B2 receptor in plasma extravasation in our model, we have demonstrated that bradykinin induced significant extravasation, a response that was completely blocked by the B2 receptor antagonist Hoe 140. In contrast, the selective B1 receptor agonist des-Arg⁹-bradykinin did not increase plasma extravasation. We also observed that bradykinin-induced extravasation was markedly worsened by relatively low doses of captopril, which produced only ~20% inhibition of ACE, but was unaffected by the selective NEP inhibitor ecadotril at doses that produced >80% inhibition of renal NEP activity (our unpublished data). These data are consistent with those reported by Klitzman et al. (1995) who demonstrated that bradykinin-induced plasma extravasation was not affected by the NEP inhibitor phosphoramidon but was potentiated by the ACE inhibitor enalaprilat.

Despite the apparent minor role for NEP in bradykinin metabolism under physiological conditions, we have found that in the presence of NEP inhibition a smaller degree of ACE inhibition was required to observe plasma extravasation. The reason for this is not clear; however, there are two possible explanations for this observation. NEP may assume a progressively greater contribution to bradykinin metabolism as ACE is inhibited. Thus, under conditions of ACE inhibition, the inhibition of additional bradykinin-metabolizing enzymes, including NEP, may cause further increases in bradykinin levels. Alternatively, inhibition of NEP may not affect bradykinin metabolism, but rather it may preserve or increase levels of substance P, which is known to be released via a bradykinin-mediated activation of sensory neurons (Geppetti, 1993; Kopp et al., 1997). The latter of these explanations, which provides for substance P component, seems to best explain our data. In these studies, we have shown that substance P is a potent inducer of plasma extravasation in the rat, an effect that can be totally blocked by the selective NK1 receptor antagonist CP99994 but is unaffected by the selective B2 receptor blocker Hoe 140. Furthermore, we have shown that although bradykinin-induced plasma extravasation can be totally blocked by Hoe 140, a substantial portion (~40%) of the bradykinin effect is also sensitive to CP99994. Clearly, these findings are consistent with the concept that bradykinin-induced plasma extravasation has two pharmacologically identifiable components, one being mediated directly by a bradykinin activation of B2 receptors and a second involving B2 receptor-mediated release of substance P. Although the metabolism of substance P has not been clearly

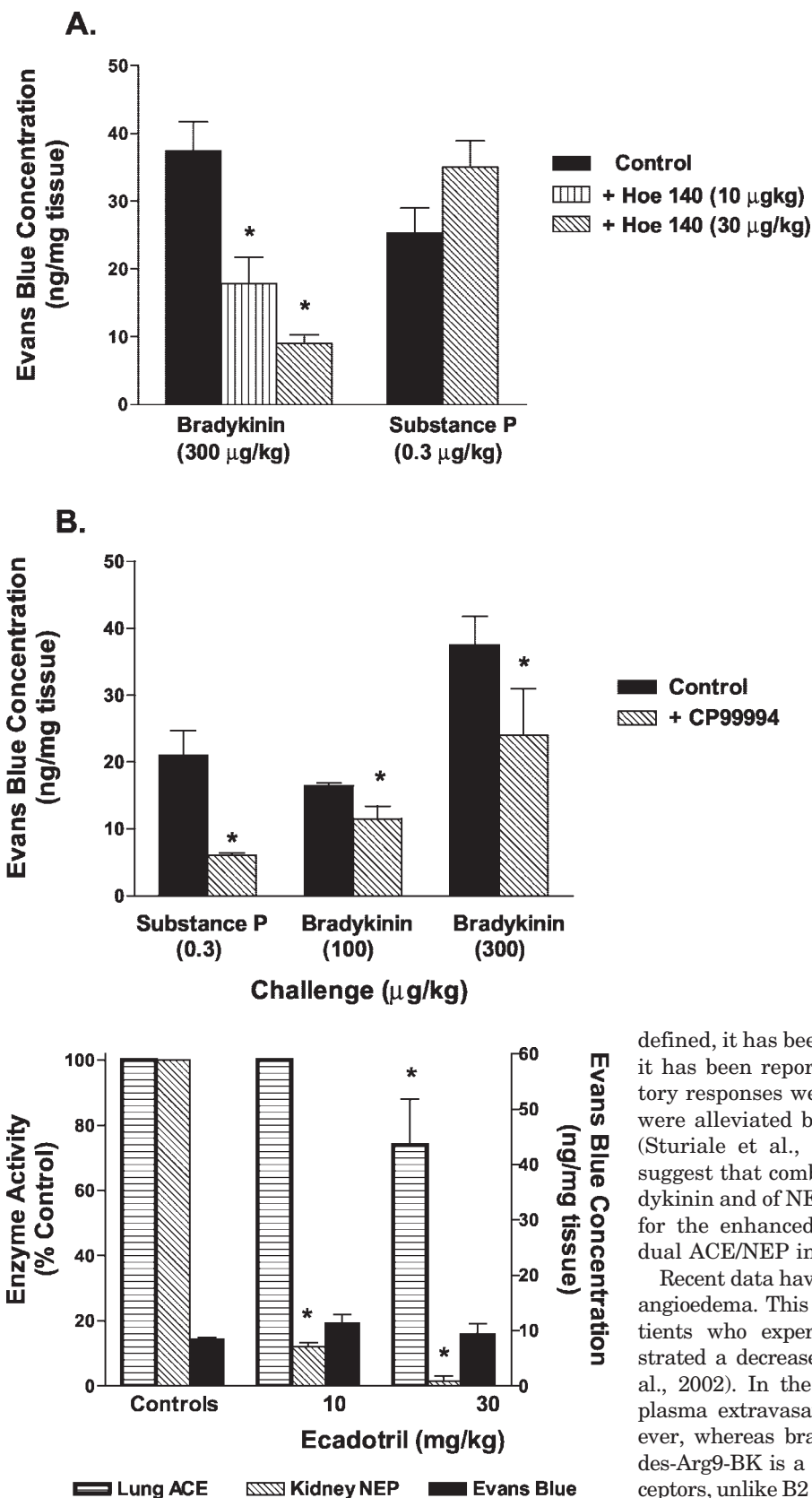


Fig. 5. A, effect of the B2 receptor antagonist Hoe 140 on bradykinin and substance P-evoked plasma extravasation. Hoe 140 was administered intravenously at 10 or 30 µg/kg. Bradykinin (300 µg/kg) or substance P (0.3 µg/kg) was injected intravenously 5 min after Hoe 140 administration. * denotes significant blockade of bradykinin-induced increase in tracheal Evans blue concentration at $p \leq 0.05$; Hoe 140 at 30 µg/kg did not affect substance P response. $n = 10$ for bradykinin control; $n = 4$ /group for Hoe 140 + bradykinin; $n = 7$ for substance P control; and $n = 3$ for Hoe 140 + substance P. B, effect selective NK1 receptor blockade with CP99994, 3 mg/kg i.v., on substance P and BK-stimulated plasma extravasation in rat trachea. The dose of substance P (SP) was 0.3 µg/kg. Bradykinin was tested at 100 and 300 µg/kg. * denotes significant inhibition of substance P or bradykinin response by CP99994 (CP) at $p \leq 0.05$. $n = 7$ for SP control; $n = 5$ for BK 100 µg/kg control; $n = 10$ for BK 300 µg/kg control; $n = 3$ for SP + CP; and $n = 4$ /group for BK + CP.

Fig. 6. Effect of selective NEP inhibition with ecadotril on the extravasation of Evans blue in rat trachea. Ecadotril was administered orally, and trachea was taken 60 min later. * denotes significant inhibition of lung ACE or renal NEP activity at $p \leq 0.05$. Profound inhibition of renal NEP was not associated with an increase Evans blue concentration. $n = 4$ /group.

defined, it has been shown that NEP plays a key role because it has been reported that substance P mediated inflammatory responses were potentiated in NEP knockout mice and were alleviated by the administration of recombinant NEP (Sturiale et al., 1999; Scholzen et al., 2001). These data suggest that combined inhibition of ACE metabolism of bradykinin and of NEP metabolism of substance P is responsible for the enhanced plasma extravasation we observed with dual ACE/NEP inhibition.

Recent data have implicated des-Arg9-BK rather than BK in angioedema. This is based on the observation that 50% of patients who experienced ACEI-induced angioedema demonstrated a decreased metabolism of des-Arg9-BK (Molinari et al., 2002). In the present study, we were unable to induce plasma extravasation by treatment with des-Arg9-BK; however, whereas bradykinin is a selective B2 receptor agonist, des-Arg9-BK is a selective agonist for the B1 receptor. B1 receptors, unlike B2 receptors, are not normally expressed but are up-regulated under inflammatory conditions (Marceau et al., 1998). There is also evidence that B1 receptors can be up-regulated in rats and mice during chronic treatment with ACE inhibitors (Marin-Castaño et al., 2002). It is possible, therefore, that B1 receptors are up-regulated in patients with angioedema

associated with ACEI treatment; however, this has yet to be demonstrated.

In summary, we have demonstrated that although NEP inhibition alone does not cause plasma extravasation, NEP inhibition can enhance ACEI-induced plasma extravasation. The studies with captopril or lisinopril administered alone or in combination with the NEP inhibitor ecadotril most clearly support this conclusion. Our studies with the vasopeptidase inhibitor omapatrilat fail to distinguish the contributions that ACE and NEP inhibition play in the extravasation produced by this agent in the rat model. The interpretation of the omapatrilat data is complicated by the fact that this agent possessed ~10-fold in vivo selectivity for ACE over NEP. Thus, the increased extravasation that we observed in some rats at lower doses of omapatrilat was likely driven solely by ACE inhibition. Because a profound inhibition of ACE occurred at doses of omapatrilat required to also inhibit NEP, the contribution of the NEP inhibition is obscured. Studies using a vasopeptidase inhibitor that nonselectively inhibits ACE and NEP or an agent that possesses some degree of NEP versus ACE selectivity may serve to more clearly resolve the roles that ACE and NEP inhibition play in the extravasation produced by these agents.

Studies using BK and substance P along with B2 and NK1 receptor antagonists suggest that the mechanism for the enhanced plasma extravasation seen with combined ACE/NEP inhibition may involve reduced metabolism of both BK and substance P. Any relevance to the enhanced angioedema observed in patients treated with the vasopeptidase inhibitor omapatrilat, however, has yet to be determined.

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