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The Effect of Acute Angiotensin Converting Enzyme (ACE) and Neutral Endopeptidase 24.11 (NEP) Inhibition on Plasma Extravasation in the Rat

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ABSTRACT

The effect of ACE and 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 of 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 which could be abolished by the bradykinin 2 (B2) receptor antagonist, Hoe 140, or the neurokinin1 (NK1) antagonist, CP99994, 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.

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Since their introduction nearly 3 decades ago, the angiotensin I converting enzyme (ACE) inhibitors have become one of the more effective and highly utilized 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 (AI) to angiotensin II (AII), 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 anti-hypertensive efficacy, efforts have been directed towards developing vasopeptidase inhibitors (VPI) that block not only ACE, but also neutral endopeptidase (NEP). Inhibition of NEP should confer additional cardiovascular benefit by virtue of enhancing the hemodynamically favorable actions of the atrial natriuretic peptides which are substrates for NEP (Robl, 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 three-fold higher incidence of angioedema than observed with an ACEI (Coates, 2002; Campbell, 2003). Since 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 appears to be a reasonable way of studying angioedema in animals. Thus, CI esterase inhibitor deficiency in man 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-Arg^9-BK and Substance P in the rat model since these peptides have either been implicated in angioedema in man (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; Emanueli et al., 1998).

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METHODS

Experimental Preparation(s):

Male, Sprague-Dawley strain rats weighing between 250-350 grams 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 NIH guidelines (NIH Publication 85-23). The animals were housed 2/cage with free access to food and water and a 12-hour 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 cc syringe. Approximately 50-55 minutes following dosing the rats were anesthetized via intraperitoneal injection of 70 mg/kg of sodium pentobarbital. When anesthesia was achieved (typically less than 10 minutes), Evans Blue dye (30 mg/kg) in heparinized saline (30 U/ml) was administered intravenously at a dose volume of 0.2 cc/100 grams of body weight via the tail vein using a 26 gauge, one and one-half inch long needle. Five minutes post Evans Blue injection, the thoracic and peritoneal cavities were opened via a single midline incision. A 0.8-1.0 cc blood sample was obtained by cardiac puncture using a heparinized 1 cc syringe and 23G 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), 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

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cc of saline delivered at a rate of 40 cc/minute which results in a perfusion pressure pulse of 80-100 mmHg. This procedure is similar to that described by others (Brokaw and White, 1994; Klitzman et al., 1996). 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. Following perfusion, a section of the left lung, the entire left kidney and a 15-20 mm segment of the trachea were removed. The lung and kidney were cut into 4 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 cc of formamide. The tissue remained in the formamide for 24-48 hours at room temperature to extract Evans Blue from the tissue. Duplicate 200 µl 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 ng of Evans Blue/mg 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 of pentobarbital sodium. The rats then received an intravenous dose of 30 mg/kg of Evans Blue dye in saline containing

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30 U/ml heparin administered at a dose volume of 200 μL/100 grams body weight 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 μg/kg; iv) or selective NK-1 receptor blockade with CP99994 (3 mg/kg; iv) on bradykinin and Substance P mediated extravasation was also determined. In those studies the selective antagonists were administered 3-5 minutes prior Evans Blue injection. Five minutes following 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 minutes at maximum speed in a microfuge. Plasma was collected from the top. Thirty-five μl of plasma was added to a conical bottom 96 well plate with 5 μl 1M KCl, 0.5M sodium borate pH 8.3 and 3 μM zinc sulfate. Ten μl 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 μl 10% trichloroacetic acid (TCA) followed by centrifugation of the plate to pellet precipitated proteins. Fifty μl of the supernatant were added in duplicate to a black fluorometric plate containing 100 μl of 2 mg/ml o-phthaldialdehyde 10% ethanol and 50 μl 1 N NaOH (Roth, 1971). After 60 minutes, the plate was read in a fluorometer at 390 nm excitation and 460 nm emission. Standard curves were generated using his-leu.

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For determination of lung ACE activity and kidney NEP activity approximately 250 mg of tissue were homogenized in 6 volumes of KH_2PO_4 0.1M pH 8.3, 0.3 M NaCl and 1 μM $ZnSO_4$ using a teflon-glass motor driven pestle. For lung ACE activity 40 μl of homogenate was added to conical bottomed 96 well plates and warmed to 37°C for 5 minutes. Ten μl of 7.5 mM hippuryl-his-leu (1.5 mM final) was added to each sample and incubated for 10 minutes at 37°C. One hundred μl of 10% TCA was added to each well and the plates were centrifuged to pellet precipitated proteins. Fifty μl of supernatant was added to 100 μl of 2 mg/ml o-phthaldialdehyde in 10% ethanol and 50 μl of 1 N NaOH in a black fluorometric plate. After 60 minutes, 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 μl of homogenate to wells containing 5 μl of buffer or 10 μM phosphoramidon. Plates were warmed to 37°C for 5 minutes. Ten μl of 2.5 mM N-dansyl-D-ala-gly-p-nitrophe-gly substrate (Florentin et al., 1984) was added to each sample to yield a 0.5 mM final concentration and incubated for 4 minutes at 37°C. One hundred μl of 10% TCA were added to and plates were centrifuged to pellet precipitated proteins. Fifty μl of supernatant were added to 100 μl of 100% ethanol and 50 ul 1 N NaOH in a black fluorometric plate. After 10 minutes, plates were read at 590 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

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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 grams of body weight. The dual ACE/NEP inhibitor omapatrilat was dissolved in a 30% PEG200/70% 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, ecadotril, was dissolved in the PEG200/cyclodextrin vehicle and was administered orally at doses of 3, 10 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 ± SEM.

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

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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 CP 99994 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 to enzyme rates of the vehicle treated (control) rats. Acceptance of a significant difference between the groups was at the 0.05 p-value level.

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RESULTS

Basal extravasation of Evan Blue into the trachea of 29 vehicle treated rats accumulated over the course of these studies was 8.3 ± 0.43 ng/mg of tissue.

Baseline plasma ACE, lung ACE and renal NEP enzyme activity in vehicle treated rats was 32.7 ± 1.7 nmoles/ml/min, 6.5 ± 1.8 and 2.6 ± 0.2 nmoles/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 (Figure 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 (Figure 1B). At these doses plasma ACE was more susceptible

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to inhibition by omapatrilat when compared to 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 evidence of an effect on plasma extravasation occurred at the 0.3 mg/kg dose level, a dose which markedly inhibited plasma ACE (-96%) and lung ACE (76%) but was devoid of NEP inhibition. Tracheal Evans Blue content was 16.0 $\pm$ 7 ng/mg in this group. Although this value did not reach statistical significance, it is important to note that 1 of the 4 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.

The effect of increasing lung ACE inhibition in NEP inhibited rats:

The data with ompatrilat did not clearly define the role that NEP inhibition played in the extravasation of Evans Blue into the trachea since 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 of ecadotril which resulted in a relatively consistent background inhibition of ~74% in renal NEP (Figure 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

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ACE evoked plasma extravasation (Figure 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 of 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 of 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 of ecadotril is unclear. One possible explanation is that the higher dose of ecadotril may have altered the phamacokinetics (metabolism, protein binding etc) 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 of lisinopril alone inhibited lung ACE by 63 and 83%, respectively, and did not increase tracheal Evans Blue extravasation (Figure 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 vs 11.1 ± 2.9 ng/mg tissue). A marked increase in extravasation did occur when a 3 mg/kg dose of lisinopril was administered in combination with ecadotril (9.1 ± 3.9 vs 35.6 ± 13.8 ng/mg tissue). For both

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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 B1, B2 and NK1 receptors:

The selective B1 receptor agonist, des-arg9-bradykinin, did not affect plasma extravasation following intravenous doses of 10, 100, 300 and 1000 μg/kg (Figure 4). Intravenous challenges of 10, 30, 100 and 300 μg/kg of bradykinin produced dose related increases in plasma extravasation as indicated by increases in tracheal Evans Blue content (Figure 3). Significant extravasation occurred at the 100 μg/kg dose (Evans Blue concentration= 17 ng/mg tissue) and the 300 μg/kg dose increased Evans Blue concentration to 38 ng/mg tissue. Substance P was found to be 300-1000-fold more potent than bradykinin in causing plasma extravasation, producing a significant increase at an intravenous dose of 0.1 μg/kg. The difference in potency between bradykinin and Substance P is likely explained by the extensive pulmonary metabolism of bradykinin that occurs following intravenous administration in the rat. However, Substance P appeared 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 μg/kg dose levels.

The robust increase in extravasation produced by 300 ug/kg of bradykinin was completely blocked by the B2 receptor antagonist, Hoe 140 (Figure 5A) and was partially (~40%) blocked by the selective NK1 receptor antagonist, CP 99994 (Figure 5B). The increase in plasma extravasation produced by Substance P at 0.3 μg/kg was

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unaffected by pretreatment with Hoe 140 (Figure 4A) and was totally abolished by CP 99994 (Figure 5B).

The 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 (Figure 6).

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DISCUSSION

The metabolism of the pro-inflammatory 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; Dendorfer et al., 2001, Taylor-McCabe et al., 2001). Indeed, bradykinin is a highly preferred substrate for ACE, possessing a ~90-fold lower Km and 24-fold higher K_{cat}/K_m than does angiotensin I (Jaspard, 1993). The contribution of the other enzymes to bradykinin metabolism appears to be minor under physiologic 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 ACE inhibitors. Specifically, the ACE inhibitor, lisinopril, did not cause extravasation at doses which 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 following ACE inhibition appears to be due to bradykinin accumulation since other investigators have shown that extravasation produced by captopril in mice can be prevented by selective bradykinin2 (B2) receptor blockade

(Emanueli et al., 1998; Baluk et al., 1999) and that captopril-induced extravasation is not observed in B2 receptor knockout mice (Emanueli 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 which was completely blocked by the B2 receptor antagonist, Hoe 140. In contrast, the selective bradykinin1 (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 (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 physiologic conditions, we have found that in the presence of NEP inhibition a smaller

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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, 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. Further, we have shown that while 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. While the metabolism of Substance P has not been clearly defined, it has been shown that NEP plays a key role since 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 has implicated des-Arg^{9}-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-Arg^{9}-BK (Molinaro et al., 2002). In the present study, we were unable to induce plasma extravasation by treatment with des-Arg^{9}-BK, however, whereas bradykinin is a selective B2 receptor agonist, des-Arg^{9}-BK is a selective agonist for the B1 receptor. B1 receptors, unlike B2 receptors, are not normally expressed but are upregulated under inflammatory conditions (Marceau et al., 1998). There is also evidence that B1 receptors can be upregulated in rats and mice during chronic treatment with ACE inhibitors (Marin-Castaño et al., 2002). It is possible, therefore, that B1 receptors are upregulated in patients with angioedema associated with ACEI treatment, however, this has yet to be demonstrated.

In summary, we have demonstrated that while NEP inhibition alone does not cause plasma extravasation, NEP inhibition can enhance ACE inhibition 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

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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. Since 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 NK-1 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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FIGURE LEGENDS

Figure 1A 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=4-6/group

Figure 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).

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Figure 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= 3-4/group.

Figure 4. Effect of kinins on extravasation of Evans Blue into the rat trachea. Bradykinin, Substance P or des-Arg_9-bradykinin was administered intravenously immediately following Evans Blue injection and the trachea was excised 5 minutes later. * denotes significant increase in Evans Blue concentration at $p \leq 0.05$. n=3-9/group for Substance P; n=4-10/group for bradykinin; n=2-4/group for des-Arg_9-bradykinin.

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Figure 5A. 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 ug/kg) or Substance P (0.3 ug/kg) were injected intravenously 5 minutes following 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; n=3 for Hoe 140 + Substance P.

Figure 5B. Effect selective NK1 receptor blockade with CP99994, 3 mg/kg; IV, on Substance P and bradykinin (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 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; n=4/group for BK + CP.

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Figure 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 minutes 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

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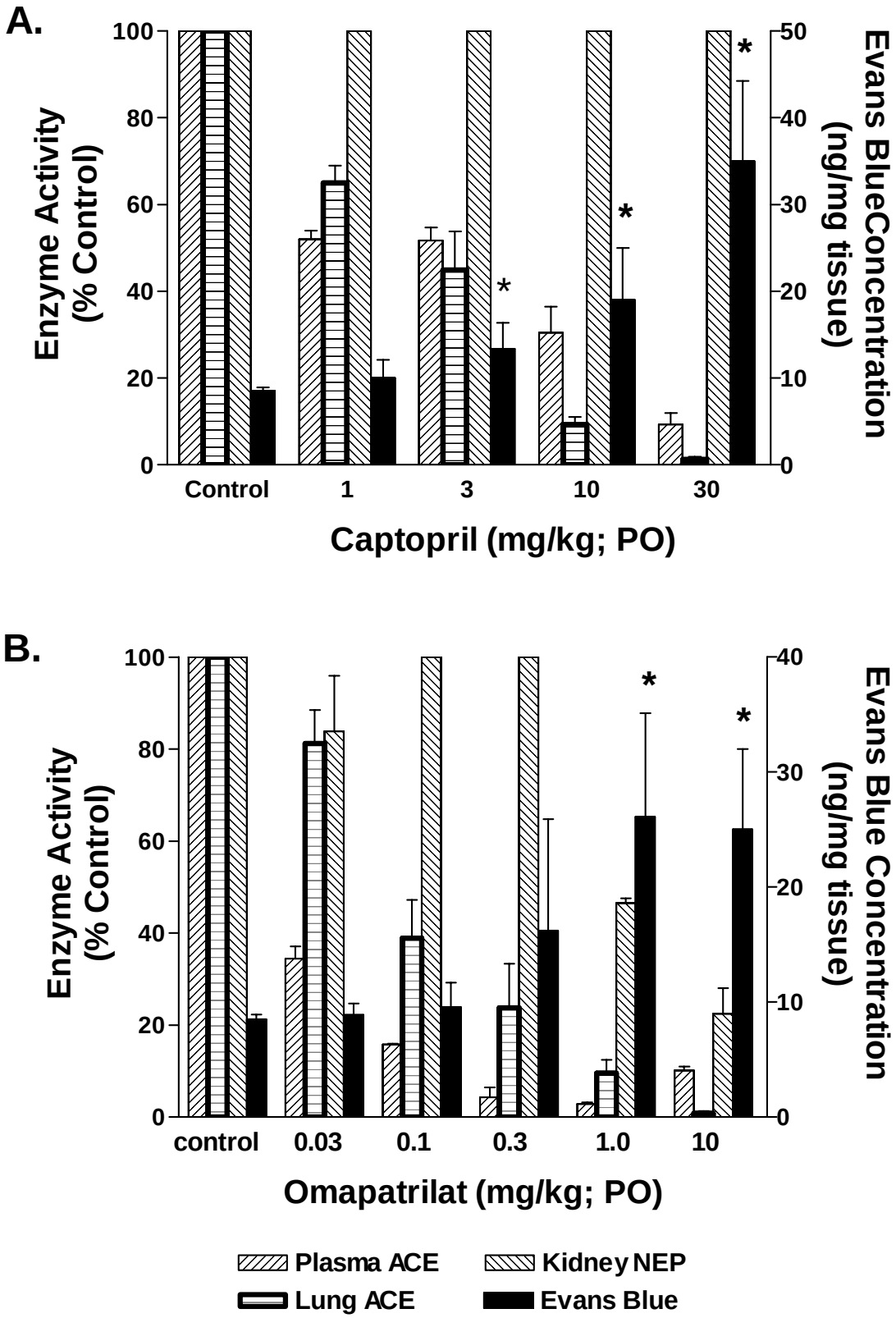

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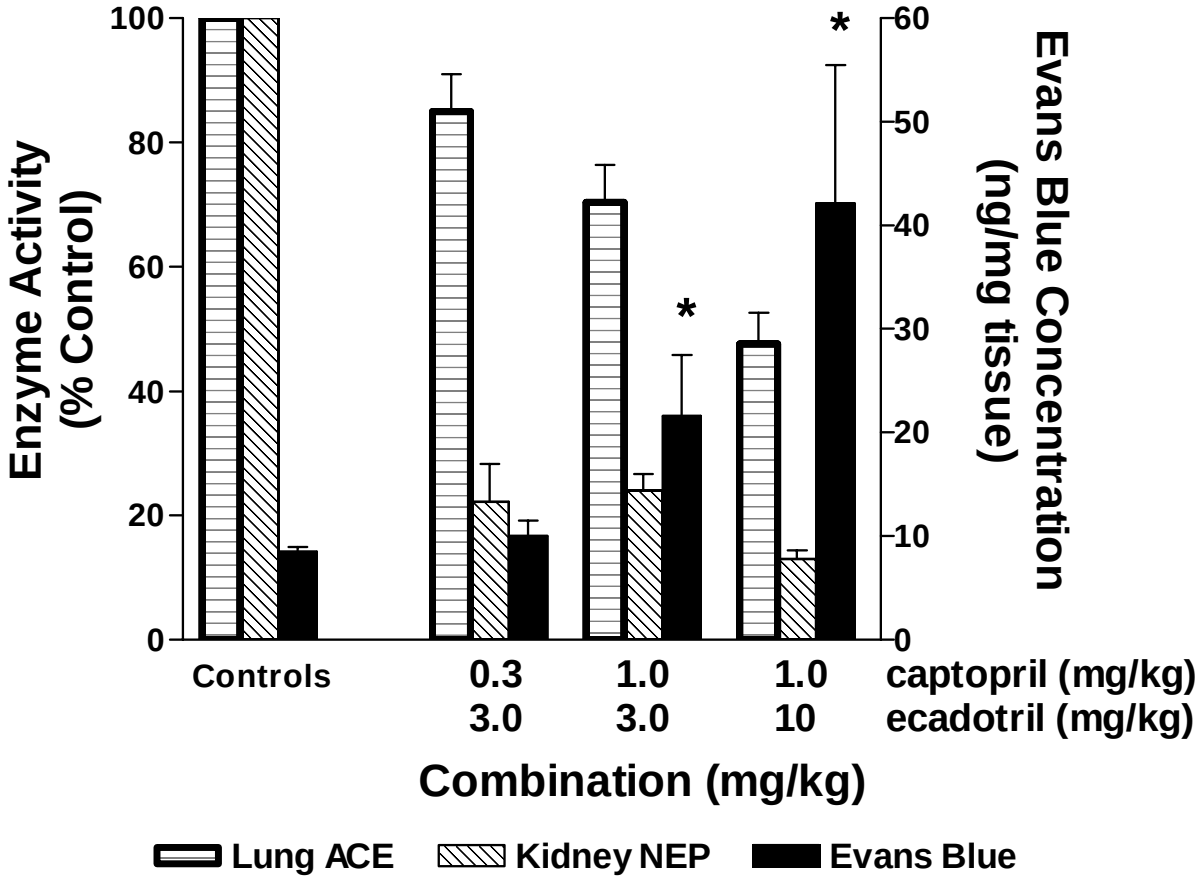

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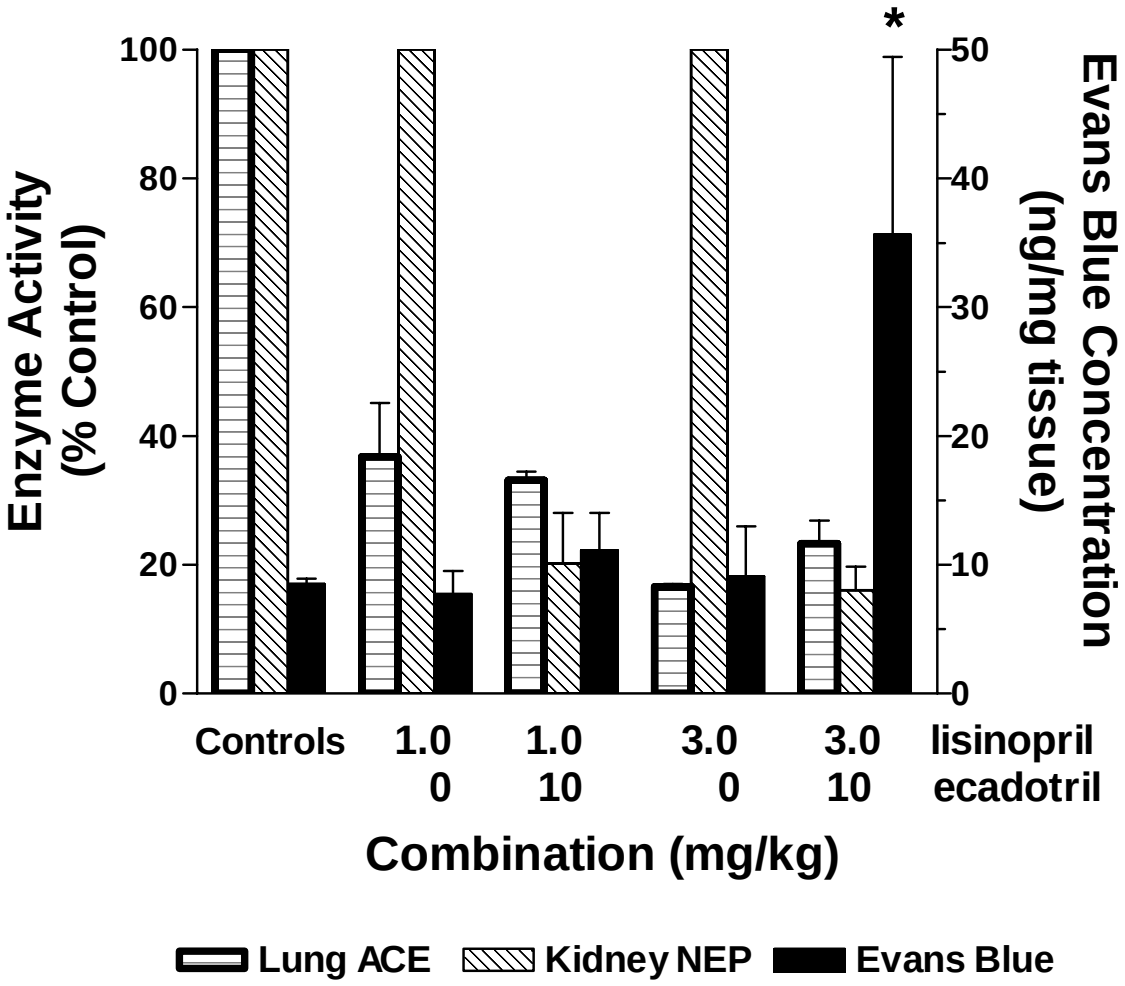

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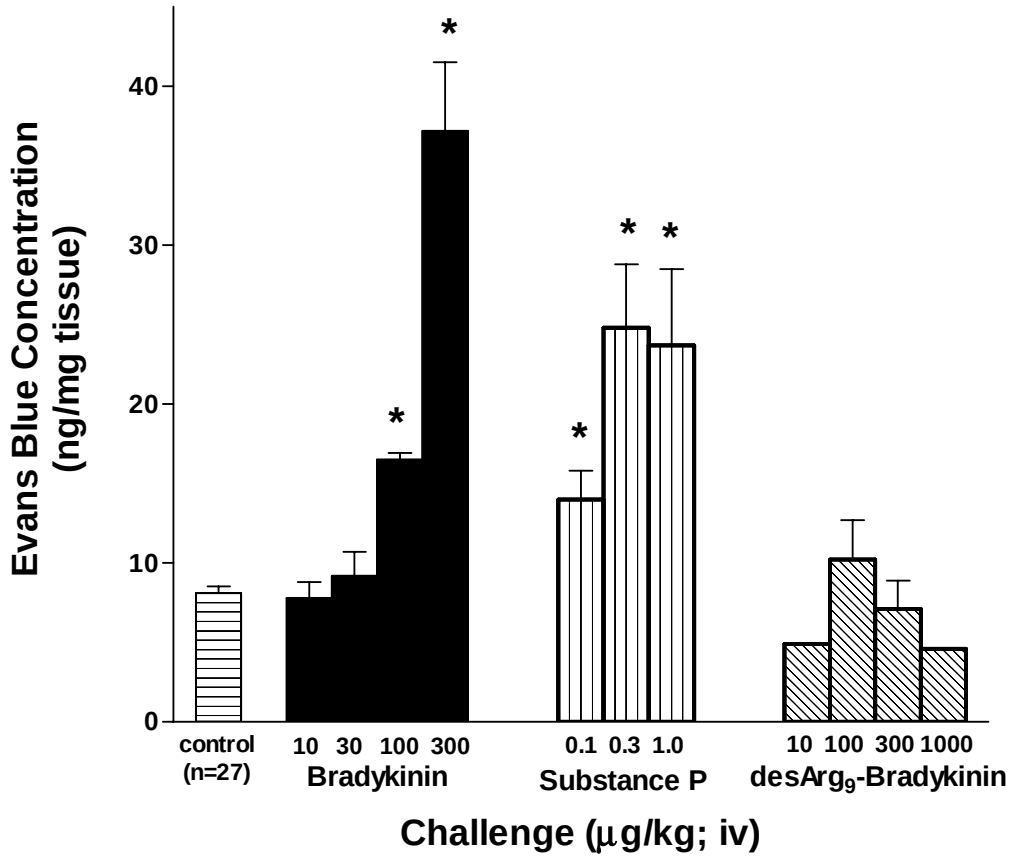

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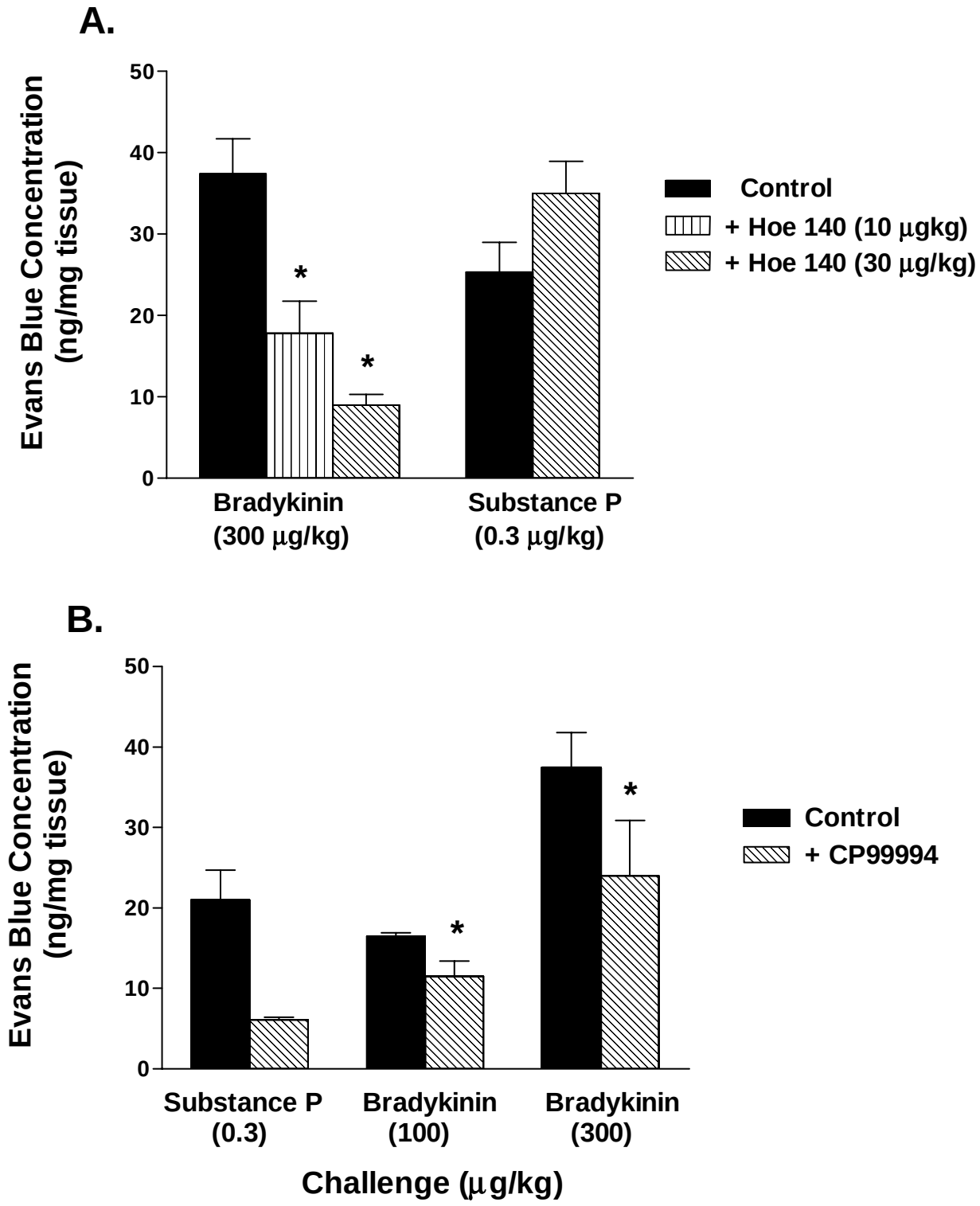

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