Effects of Neutral Endopeptidase Inhibition and Combined Angiotensin Converting Enzyme and Neutral Endopeptidase Inhibition on Angiotensin and Bradykinin Peptides in Rats

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ABSTRACT

The combination of neutral endopeptidase 24.11 (NEP) and angiotensin converting enzyme (ACE) inhibition is a candidate therapy for hypertension and cardiac failure. Given that NEP and ACE metabolize angiotensin (Ang) and bradykinin (BK) peptides, we investigated the effects of NEP inhibition and combined NEP and ACE inhibition on the levels of these peptides. We administered the NEP inhibitor ecadotril (0, 0.1, 1, 10, 100 mg/kg per day), either alone or together with the ACE inhibitor perindopril (0.2 mg/kg per day), to rats by 12 hourly gavage for 7 days. Ecadotril produced diuresis, natriuresis, increased urine cyclic guanosine monophosphate and BK-(1–9) levels, increased Ang II and Ang I levels in plasma, and increased Ang I levels in heart. Perindopril reduced Ang II levels in kidney, and increased BK-(1–9) levels in blood, kidney and aorta. Combined NEP/ACE inhibition produced the summation of these effects of separate NEP and ACE inhibition. In addition, perindopril potentiated the ecadotril-mediated diuresis, natriuresis and decrease in urine BK-(1–7)/BK-(1–9) ratio, which is an index of BK-(1–9) metabolism. Moreover, combined NEP/ACE inhibition increased Ang II levels in plasma and lung. These data indicate that summation of the effects of separate NEP and ACE inhibition provides the basis for the therapeutic efficacy of their combination. Whereas potentiation by perindopril of the diuretic and natriuretic effects of ecadotril may contribute to the therapeutic effects, increased Ang II levels in plasma and lung may compromise the therapeutic effects of combined NEP/ACE inhibition.

ACE (EC 3.4.25.1) and NEP 24.11 (EC 3.4.24.11) are two zinc containing metalloproteinases involved in the metabolism of a variety of biological peptides (Erdos, 1990; Roques et al., 1993). Both ACE and NEP have a widespread tissue distribution, including the heart, vascular endothelium and the brush border of proximal tubule cells of the kidney (Erdos, 1990; Roques et al., 1993; Bruneval et al., 1986; Graf et al., 1995). ACE inhibitors are clinically useful for the treatment of hypertension and cardiac failure (Hansson et al., 1993; Crozier et al., 1993). Moreover, NEP inhibitors have diuretic and natriuretic effects in man (Richards et al., 1990; Schmitt et al., 1994), and have beneficial effects in animal models of heart failure (Seymour et al., 1993; Rademaker et al., 1996; Willenbrock et al., 1996), and in patients with congestive cardiac failure (Elaner et al., 1992). Thus, inhibition of both ACE and NEP offers the possibility of improved therapy for hypertension and cardiac failure (Favrat et al., 1995; Trippodo et al., 1995).

Both ACE and NEP participate in the metabolism of angiotensin and bradykinin peptides, and NEP metabolizes ANP (Erdos, 1990; Roques et al., 1993). ACE converts the inactive Ang I to Ang II, and NEP metabolizes both Ang II and Ang I (Richards et al., 1992; Yamamoto et al., 1992) (fig. 1). Both enzymes metabolize bradykinin-(1–9) [BK-(1–9)] to bradykinin-(1–7) [BK-(1–7)] (Erdos, 1990; Roques et al., 1993), and also metabolize BK-(1–7) to smaller fragments (fig. 1). ACE inhibitors prevent the pressor response to Ang I, and both ACE and NEP inhibitors potentiate the depressor effects of BK-(1–9) (Ondetti et al., 1977; Yang et al., 1997). Moreover, NEP inhibition enhances the pressor response to Ang II and reduces the clearance of infused Ang II in man.

ABBREVIATIONS: ACE, angiotensin converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; ANOVA, analysis of variance; ANP, atrial natriuretic peptide; B2 receptor, type 2 bradykinin receptor; BK-(1–7), bradykinin-(1–7); BK-(1–8), bradykinin-(1–8); BK-(1–9), bradykinin-(1–9); ecadotril, N-(S)-[2-([acetylthio)methyl]-1-oxo-3-phenylpropyl]-glycine benzylester; EDTA, ethylenediaminetetra-acetate; GMP, guanosine monophosphate; GTC/TFA, 4 M guanidine thiocyanate, 1% trifluoroacetic acid; HPLC, high-performance liquid chromatography; NEP, neutral endopeptidase 24.11; RB104, 2-[3-iodo-4-hydroxy]phenylmethyl]-4-N-[3-(hydroxyamino-3-oxo-1-phenylmethyl)propyl]amino-4-oxobutanoic acid; RIA, radioimmunoassay; SHR, spontaneously hypertensive rat; Tris, tris-(hydroxymethyl)aminomethane.
peptides, blood pressure and cardiac hypertrophy (Campbell et al., 1994; Duncan et al., 1996). We were interested in the possible interactions between clinically relevant doses of ACE and NEP inhibitor. This dose of perindopril approximates recommended dosages for hypertension and cardiac failure (2–8 mg/day), although ACE inhibitors are frequently used in suboptimal dosage (Missouris and MacGregor, 1997; Poulier, 1993; Clark and Coats, 1995).

Materials and Methods

Animals. Male Sprague-Dawley rats (−300 g) were allowed free access to tap water and standard rat chow containing 0.25% sodium and 0.76% potassium (GR2, Clarke-King & Co., Gladstone, NSW, Australia). Two separate experiments were performed. Experiment 1 was a dose finding experiment, in which rats (n = 10 per group) were administered 0, 0.1, 1, 10 and 100 mg/kg per day ecadotril for 7 days. Body and tissue weights, blood pressure, blood bradykinin peptides, plasma renin, angiotensinogen, ACE and angiotensin peptides and tissue levels of angiotensin and bradykinin peptides were measured. In experiment 2, rats (n = 8 per group) were administered 0, 0.1, 1, 10 and 100 mg/kg per day ecadotril, either alone or together with 0.2 mg/kg per day perindopril. Plasma NEP, and urine volume, sodium, potassium, cyclic GMP and bradykinin peptides were measured in all rats of experiment 2. In addition, body and tissue weights, blood pressure, blood bradykinin peptides, plasma renin, angiotensinogen, ACE and angiotensin peptides, and tissue levels of angiotensin and bradykinin peptides were measured in rats administered 0 and 100 mg/kg per day ecadotril alone and in perindopril-treated rats administered 0, 0.1, 1, 10 and 100 mg/kg per day ecadotril. Ecadotril and perindopril were gifts from Bayer,AG, Wuppertal, Germany, and Institut de Recherches Internationales Servier, Paris, France, respectively. This study was performed in accordance with the guidelines of the Animal Experimentation Ethics Committee of St. Vincent’s Hospital.

Ecadotril and perindopril were administered in 0.5 ml 0.25% methylcellulose by 12 hourly gavage for 7 days. Blood pressure and body weight were measured on days 0, 1, 2 and 6. Systolic blood pressures were measured by the taill-cuff method (model 12-38L BP system, IITC Inc., Life Science Instrumentation, Woodland Hills, CA). On day 7, immediately after the last dose of drug, a water load of 20 ml/kg was administered by gavage and the rats were placed in metabolic cages for 6 hr. Urine was collected into containers cooled to the temperature of dry ice and stored at −80°C until assay for sodium, potassium, creatinine, cyclic GMP and bradykinin peptides. At the end of the 6-hr urine collection rats were killed by decapitation, trunk blood was collected for the measurement of plasma levels of renin, angiotensinogen, ACE, NEP and angiotensin peptides, and the left kidney, heart (cardiac ventricles), lung and aorta were rapidly removed, weighed and immediately homogenized in GTC/TFA for the measurement of tissue levels of angiotensin and bradykinin peptides. The right kidney was frozen in isopentane cooled to the temperature of dry ice for in vitro autoradiography. Blood bradykinin peptides were measured in separate groups of rats which were ad-

**TABLE 1**

Angiotensin and bradykinin peptide levels in plasma, blood, kidney, heart, aorta and lung of vehicle-treated rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ang II (fmol/g)</th>
<th>Ang I (fmol/g)</th>
<th>Ang II/Ang I Ratio (fmol/fmol)</th>
<th>BK-(1–7) (fmol/g)</th>
<th>BK-(1–9) (fmol/g)</th>
<th>BK-(1–7)/BK-(1–9) Ratio (fmol/fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>68 ± 8</td>
<td>17 ± 2</td>
<td>4.3 ± 0.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Blood</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.1 ± 0.8</td>
<td>2.3 ± 0.5</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>221 ± 31</td>
<td>53 ± 7</td>
<td>4.4 ± 0.4</td>
<td>49 ± 7</td>
<td>91 ± 19</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>Heart</td>
<td>12 ± 2</td>
<td>1.9 ± 0.4</td>
<td>9.6 ± 1.6</td>
<td>22 ± 2</td>
<td>22 ± 3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Aorta</td>
<td>50 ± 10</td>
<td>&lt;20</td>
<td>ND</td>
<td>15 ± 3</td>
<td>18 ± 6</td>
<td>2.4 ± 1.0</td>
</tr>
<tr>
<td>Lung</td>
<td>138 ± 18</td>
<td>8 ± 2</td>
<td>31 ± 6</td>
<td>51 ± 13</td>
<td>75 ± 14</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

Mean ± S.E., n = 16–23, except for aortic BK-(1–7) and BK-(1–9) levels and BK-(1–7)/BK-(1–9) ratio, where values are from experiment 2, n = 8; for experiment 1, aortic BK-(1–7) and BK-(1–9) levels were less than the minimum detectable (<12 fmol/g for each peptide). ND, Not determined.

**Fig. 1.** Diagrammatic representation of participation by neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) in the metabolism of bradykinin [BK-(1–9)] and angiotensin I (Ang I). The alternate pathway of conversion of Ang I to Ang II by serine protease activity is also shown.

(Richards et al., 1992). NEP inhibition also decreases Ang I conversion to Ang-(1–7) and increases plasma levels of Ang I and Ang II in rats infused with Ang I (Yamamoto et al., 1992).

Given the colocalization of ACE and NEP in many tissues, one would predict interactions between the effects of ACE and NEP inhibitors on angiotensin and bradykinin peptide levels during simultaneous ACE and NEP inhibition. Increased Ang I and Ang II levels in response to NEP inhibition may counteract the effects of simultaneous ACE inhibition on Ang II levels. Moreover, ACE and NEP inhibitors may have a synergistic effect on BK-(1–9) levels. The purpose of this study was to characterize the interactions between the effects of ACE and NEP inhibition on angiotensin and bradykinin peptides. We investigated the dose-related effects of the orally active prodrug of (S)-thiorphan (ecadotril, formerly called sinorphan) (Lecomte, 1990; Fournie-Zaluski et al., 1984) on circulating and tissue levels of Ang II, Ang I, BK-(1–7) and BK-(1–9) and urinary kinin levels in rats administered ecadotril alone and in rats simultaneously administered ecadotril and the ACE inhibitor perindopril. In addition to measurement of absolute peptide levels, we also calculated the Ang II/Ang I ratio, which provides an index of the rate of conversion of Ang I to Ang II, and the BK-(1–7)/BK-(1–9) ratio, which provides an index of the rate of BK-(1–9) metabolism to BK-(1–7). The dose of perindopril used in this study (0.2 mg/kg per day) was submaximal with respect to the effects of perindopril on angiotensin and bradykinin
ministered ecadotril and perindopril by identical protocols; 6 hr after the last dose of drug on day 7, rats were anesthetized with diethyl ether and 2 ml blood were collected from the inferior vena cava into syringes containing 10 ml GTC/TFA for the measurement of bradykinin peptides.

**Extraction and RIA of angiotensin and bradykinin peptides.** Plasma levels of Ang II and Ang I were measured as described previously (Campbell et al., 1995b). Briefly, trunk blood (2–3 ml) was rapidly collected into tubes containing 0.5 ml inhibitor solution (1 mM renin inhibitor acetyl-His-Pro-Phe-Val-Sta-Leu-Phe-NH₂ (Hui et al., 1988), 146 μM pepstatin, 50 mM 1,10-phenanthroline, 125 mM EDTA, 2 gliter neomycin sulfate, 2% dimethyl sulfoxide and 2% ethanol in water) at 4°C. The blood was centrifuged and the plasma (1–2 ml) was immediately extracted with Sep-Pak C₁₅ cartridges (Waters Chromatography Division, Milford, MA). Blood and tissues homogenized in GTC/TFA were processed as described previously and extracted with Sep-Pak C₁₅ cartridges (Campbell et al., 1995b). For the measurement of bradykinin peptides in urine, 1 ml freshly thawed urine was added to 10 ml GTC/TFA and extracted with Sep-Pak C₁₅ cartridges (Anastasopoulos et al., 1998). Peptides were acetylated and treated with piperidine before HPLC and assay of HPLC fractions by N-terminal directed RIA (Campbell et al., 1995b). Data were corrected for recovery as reported elsewhere (Campbell et al., 1995b; Anastasopoulos et al., 1998).

**Fig. 2.** Inhibition of NEP in vivo. Bar graphs show effects of ecadotril administration alone (open columns) and together with 0.2 mg/kg per day perindopril (closed columns) on ¹²⁵I-RB104 binding to kidney sections in vitro. Mean ± S.E. * P < .05, ** P < .01, compared with vehicle-treated control; †† P < .01, comparison of rats administered both ecadotril and perindopril with perindopril-treated control, n = 5 to 10 rats per group.

**Fig. 3.** Effects of ecadotril and perindopril on plasma NEP. Bar graphs show effects of ecadotril administration alone (open columns) and together with 0.2 mg/kg per day perindopril (closed columns) on plasma levels of active NEP, total NEP and the active NEP/total NEP ratio. Mean ± S.E. * P < .05, ** P < .01, compared with vehicle-treated control, n = 8 rats per group.

**Fig. 4.** Bar graphs show effects of ecadotril administration alone (open column) and together with 0.2 mg/kg per day perindopril (closed column) on systolic blood pressure, change in body weight, and heart weight/body weight ratio. Mean ± S.E. * P < .05, ** P < .01, compared with vehicle-treated control; †† P < .01, comparison of rats administered both ecadotril and perindopril with perindopril-treated control, n = 9 to 10 rats per group.
TABLE 2
Effects of ecadotril alone and ecadotril combined with perindopril on urinary volume, electrolytes and kinins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecadotril (mg/kg/Day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecadotril</td>
<td>20 ± 2</td>
<td>24 ± 2</td>
<td>29 ± 3a</td>
<td>23 ± 1</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Ecadotril + perindopril (0.2 mg/kg/day)</td>
<td>19 ± 1</td>
<td>22 ± 2</td>
<td>23 ± 1</td>
<td>24 ± 1b</td>
<td>24 ± 2a</td>
</tr>
<tr>
<td>Urinary sodium/creatinine ratio (mmol/mmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecadotril</td>
<td>318 ± 28</td>
<td>305 ± 22</td>
<td>411 ± 43</td>
<td>549 ± 60b</td>
<td>593 ± 36b</td>
</tr>
<tr>
<td>Ecadotril + perindopril (0.2 mg/kg/day)</td>
<td>284 ± 16</td>
<td>308 ± 22</td>
<td>380 ± 32</td>
<td>437 ± 25d</td>
<td>574 ± 39bd</td>
</tr>
<tr>
<td>Urinary potassium/creatinine ratio (mmol/mmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecadotril</td>
<td>6.7 ± 1</td>
<td>13.3 ± 1.9a</td>
<td>17.1 ± 2.6b</td>
<td>9.0 ± 1.5</td>
<td>12.8 ± 2.7</td>
</tr>
<tr>
<td>Ecadotril + perindopril (0.2 mg/kg/day)</td>
<td>10.8 ± 1.2</td>
<td>13.5 ± 2.5</td>
<td>18.0 ± 3.1b</td>
<td>17.9 ± 2.7b</td>
<td>18.9 ± 2.9b</td>
</tr>
<tr>
<td>Urine volume (μl/min)</td>
<td>20 ± 2</td>
<td>25 ± 2</td>
<td>27 ± 4</td>
<td>23 ± 3</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>Ecadotril</td>
<td>20 ± 2</td>
<td>27 ± 2d</td>
<td>29 ± 2d</td>
<td>26 ± 1b</td>
<td>29 ± 1b</td>
</tr>
<tr>
<td>Ecadotril + perindopril (0.2 mg/kg/day)</td>
<td>10.3 ± 5.7</td>
<td>8.1 ± 0.9</td>
<td>14.4 ± 4.6</td>
<td>3.0 ± 0.4</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Urine (1–7)/creatinine ratio (fmol/μmol)</td>
<td>6.4 ± 0.6</td>
<td>5.8 ± 1.2</td>
<td>6.0 ± 2.6</td>
<td>3.4 ± 0.8a</td>
<td>2.5 ± 0.5bd</td>
</tr>
<tr>
<td>Ecadotril</td>
<td>2.9 ± 1</td>
<td>1.4 ± 0.3</td>
<td>3.9 ± 1.4</td>
<td>1.6 ± 0.5</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Ecadotril + perindopril (0.2 mg/kg/day)</td>
<td>1.2 ± 0.2</td>
<td>1.8 ± 0.7</td>
<td>2.6 ± 0.8</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Urine (1–9)/creatinine ratio (fmol/μmol)</td>
<td>3.4 ± 1.7</td>
<td>3.4 ± 0.5</td>
<td>7.4 ± 2.6a</td>
<td>3.1 ± 0.3</td>
<td>7.8 ± 1.5b</td>
</tr>
<tr>
<td>Ecadotril</td>
<td>3.2 ± 0.6</td>
<td>2.4 ± 0.4</td>
<td>9.3 ± 2.7bd</td>
<td>4.6 ± 0.8</td>
<td>7.1 ± 1.7bd</td>
</tr>
<tr>
<td>Ecadotril + perindopril (0.2 mg/kg/day)</td>
<td>3.6 ± 0.7</td>
<td>2.5 ± 0.2</td>
<td>2.3 ± 0.5</td>
<td>1.1 ± 0.2b</td>
<td>0.7 ± 0.1bd</td>
</tr>
<tr>
<td>Urine (1–7)/BK/(1–9) ratio (fmol/fmol)</td>
<td>2.2 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>0.6 ± 0.1bd</td>
<td>0.7 ± 0.1bd</td>
<td>0.4 ± 0.1bd</td>
</tr>
</tbody>
</table>

Mean ± S.E.

P < .05, compared with vehicle-treated control.

P < .01, compared with vehicle-treated control.

P < .05, comparison of rats administered both ecadotril and perindopril with perindopril-treated control, n = 7–8.

P < .01, comparison of rats administered both ecadotril and perindopril with perindopril-treated control, n = 7–8.

Measurement of sodium, potassium, creatinine and cyclic GMP in urine. Urinary sodium, potassium and creatinine were measured by autoanalyzer by the Department of Chemical Pathology, St. Vincent's Hospital. Cyclic GMP was measured by RIA using reagents from Amersham International, Buckinghamshire, UK.

Measurement of renin, angiotensinogen, ACE, and NEP in plasma. Trunk blood for measurement of renin, angiotensinogen, ACE and NEP was collected into heparinized tubes on ice, then centrifuged and the plasma rapidly frozen on dry ice and stored at −80°C. The plasma concentrations of active renin and angiotensinogen were measured as described previously (Campbell et al., 1991). ACE enzymatic activity was measured using 3-(2-furylacryloyl)-l-phenylalanyl-glycyl-glycine as substrate (Johansen et al., 1987). NEP enzymatic activity was measured with succinyl-Ala-Ala-Phe-amidomethylcoumarin as substrate (Yandle et al., 1992); further incubation with aminopeptidase M released free amidomethylcoumarin that was measured fluorometrically. NEP assays were performed before and after dialysis to remove ecadotril. Plasma was dialyzed against 50 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.0, at 4°C overnight, then against five changes of 50 mM Tris-HCl, 0.1 M NaCl, 50 μM ZnCl₂, pH 8.0, at 4°C for 24 hr.

In vitro autoradiography. Cryostat sections of kidney (20 μm) were cut and mounted on gelatin-coated slides. In vitro autoradiography was performed after storage of the sections at −80°C. Precautions were taken to protect the slides from light at all stages. In vitro autoradiography of binding to NEP was performed using 125I-RB104 (Fournié-Zaluski et al., 1992). RB104 was a generous gift from Dr. B. Roques, Université René Descartes, Paris, France. The autoradiography buffer was 50 mM Tris-HCl, 50 μM ZnCl₂, pH 7.4. 125I-RB104 binding was performed using −0.6 × 10⁻¹⁰ M 125I-RB104 (250,000 cpm/ml) and nonspecific binding was assessed in the presence of 0.1 mM (S)-thiorphan. Binding to sections was quantified using a PhosphorImage (Molecular Dynamics, Sunnyvale, CA) with 125I-microscales (Amersham International).

Statistical analysis. Data are presented as means ± S.E. Both experiments 1 and 2 included vehicle-treated control rats. Comparisons with vehicle-treated rats were made by one-way ANOVA for experiments 1 and 2 separately, using Dunnett’s test for multiple comparisons with the vehicle-treated control. In addition, the effects of ecadotril in perindopril-treated rats in experiment 2 were analyzed using Dunnett’s test for multiple comparisons with the perindopril-treated control. Data were also analyzed for all groups by two-way ANOVA to assess the main effects of perindopril and ecadotril, and the significance of interactions between the two drugs. When more than half of the samples comprising a mean had values below the minimum detectable, the sample mean is shown as less than the minimum detectable. Where values were below the minimum detectable, they were set at half the minimum detectable for statistical calculations. Logarithmic transformation of the data was performed when required to obtain similar variances between groups. All tests were two-tailed. Differences were considered significant at P < .05. Statistical analyses were performed using SuperANOVA (Abacus Concepts, Inc., Berkeley, CA).

Results

As described in “Materials and Methods,” these data were obtained from two separate experiments. Plasma NEP, and urine volume, sodium, potassium, cyclic GMP and bradykinin peptides were measured in all rats of experiment 2, and these data are presented as absolute values. Blood pressure, change in body weight and heart weight/body weight ratio were very similar for the vehicle-treated rats from experiments 1 and 2, and these data are also presented as absolute values; otherwise data are presented as percentages of the mean of the relevant vehicle-treated control, to correct for differences between mean values for vehicle-treated control rats in experiments 1 and 2. Absolute values for pooled data for circulating and tissue levels of angiotensin and bradykinin peptides in vehicle-treated rats from experiments 1 and 2 are presented in table 1.
Inhibition of NEP. Ecadotril produced dose-related occupancy of renal NEP, as determined by binding of $^{125}$I-RB104 to kidney sections from rats treated with ecadotril alone, and from perindopril-treated rats (fig. 2). Perindopril did not influence $^{125}$I-RB104 binding.

We measured plasma levels of both active NEP (nondialyzed plasma) and total NEP (plasma dialyzed to remove ecadotril). Ecadotril did not decrease plasma active NEP; paradoxically, 0.1 mg/kg per day ecadotril increased NEP activity by 54% (fig. 3). Total NEP levels were also increased 42 to 57% by 0.1, 1 and 10 mg/kg per day ecadotril. Thus, the inhibition of plasma NEP activity was masked by the simultaneous induction of total NEP levels by ecadotril. When NEP activity was expressed as the active/total NEP ratio, 1 and 10 mg/kg per day ecadotril produced 21 and 33% inhibition, respectively. Perindopril was without effect on plasma levels of active and total NEP. When administered with perindopril, ecadotril had no effect on plasma levels of active NEP and produced a 27% increase in total NEP and 28% decrease in active/total NEP ratio at 100 mg/kg per day, effects that were not statistically significant when compared with the effects of perindopril alone.

Blood pressure, body weight, and heart weight/body weight ratio. Ecadotril at 10 and 100 mg/kg per day reduced systolic blood pressure by 12 to 14 mmHg on day 6 (fig. 4), but did not reduce blood pressure on day 1 and 2 (data not shown). Perindopril reduced blood pressure from day 1 (data not shown), and the decrease was 13 mmHg on day 6 (fig. 4); this decrease was of borderline statistical significance for perindopril alone ($P = .06$), but of statistical significance when combined with 1, 10 and 100 mg/kg per day ecadotril (fig. 4). Ecadotril did not produce any additional change in blood pressure of perindopril-treated rats.

Ecadotril had no effect on weight gain or on heart weight/body weight ratio in rats administered ecadotril alone (fig. 4). By contrast, perindopril produced statistically significant decreases in weight gain and heart weight/body weight ratio, effects that were prevented by administration of ecadotril to perindopril-treated rats (fig. 4).

Urine volume, electrolytes, cyclic GMP and bradykinin peptides. Perindopril alone had no effect on urine vol-
in urine kinin levels were associated with a marked suppression of the urine BK-(1–7)/BK-(1–9) ratio. Perindopril potentiated the effect of ecadotril on the urine BK-(1–7)/BK-(1–9) ratio, in that 10 mg/kg per day ecadotril was required to suppress the ratio in rats administered ecadotril alone, whereas 1 mg/kg per day ecadotril suppressed the ratio in perindopril-treated rats (table 2). Two-way ANOVA for all groups of rats showed a statistically significant interaction between the effects of ecadotril and perindopril on urine BK-(1–7)/BK-(1–9) ratio ($F_{4,70} = 3.5, P = .01$).

**Plasma renin, angiotensinogen, ACE and angiotensin peptides.** For pooled vehicle-treated rats from experiments 1 and 2, plasma renin levels were $16 \pm 3$ pmol Ang I/ml per hour, angiotensinogen levels were $406 \pm 15$ pmol/ml and ACE levels were $214 \pm 14$ U/liter (mean $\pm$ S.E., $n = 18$). Similar to the bimodal effect of ecadotril on urine volume and sodium/creatinine ratio, ecadotril had a dose-related bimodal effect on plasma renin and angiotensin peptides in rats administered ecadotril alone (figs. 5 and 6). Intermediate doses of ecadotril alone increased plasma Ang II and Ang I levels by approximately 2-fold, most likely due to the 2-fold increase in plasma renin levels, although the increase in plasma renin levels did not achieve statistical significance. Ecadotril alone at 10 mg/kg per day increased plasma ACE activity by 16% (fig. 5), but there was no effect on the plasma Ang II/Ang I ratio (fig. 6). Perindopril alone increased plasma renin levels 6-fold and reduced plasma angiotensinogen and ACE activity by 40% (fig. 5), accompanied by a 12-fold increase in Ang I levels and an 84% decrease in Ang II/Ang I ratio (fig. 6). When administered to perindopril-treated rats, ecadotril again displayed a dose-related bimodal effect. Intermediate doses of ecadotril tended to suppress plasma renin levels, associated with recovery of angiotensinogen levels in perindopril-treated rats (fig. 5). Ecadotril at 100 mg/kg per day increased plasma Ang II levels by 3-fold in perindopril-treated rats (fig. 6).

**Tissue angiotensin peptides.** Consistent with ACE inhibition, perindopril reduced the Ang II/Ang I ratio in kidney, heart and lung (fig. 7; table 3). For kidney, perindopril reduced Ang II levels without effect on Ang I levels (fig. 7), whereas for heart and lung, perindopril did not influence Ang II levels but increased Ang I levels (table 3). Perindopril did not modify angiotensin peptide levels in aorta (table 3).

Ecadotril did not modify angiotensin peptide levels in kidney, lung or aorta of rats administered ecadotril alone (fig. 7; table 3). However, ecadotril alone increased cardiac Ang I levels 3.5-fold, although without effect on cardiac Ang II levels, and the reduction in cardiac Ang II/Ang I ratio did not achieve statistical significance (table 3). Administration of ecadotril to perindopril-treated rats did not modify angiotensin peptide levels in kidney, heart or aorta, when compared to the effects of perindopril alone (fig. 7; table 3). However, ecadotril at 100 mg/kg per day increased lung Ang II levels in perindopril-treated rats (table 3).

**Blood bradykinin peptides.** Perindopril alone increased blood levels of BK-(1–9) and reduced the BK-(1–7)/BK-(1–9) ratio (fig. 8). Ecadotril reduced the BK-(1–7) levels and reduced the BK-(1–7)/BK-(1–9) ratio in rats administered 10 mg/kg per day ecadotril alone. Ecadotril alone did not increase blood BK-(1–9) levels; paradoxically, 1 mg/kg per day ecadotril reduced blood BK-(1–9) levels. Administration of ecadotril to perindopril-treated rats did not modify blood

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**Fig. 7.** Bar graphs show effects of ecadotril administration alone (open columns) and together with 0.2 mg/kg per day perindopril (closed columns) on kidney levels of Ang II and Ang I, and kidney Ang II/Ang I ratio. Mean $\pm$ S.E. * $P < .05$, ** $P < .01$, compared with vehicle-treated control, $n = 8$ to 10 rats per group.
brane kinin peptide levels, when compared to the effects of perindopril alone (fig. 8).

**Tissue bradykinin peptides.** Perindopril alone reduced the BK-(1–7)/BK-(1–9) ratio in kidney by 40% (fig. 9). Renal BK-(1–9) levels in rats administered perindopril alone were 2.6-fold higher than those for vehicle-treated rats; this was not statistically significant when analyzed by one-way ANOVA for experiment 2, but the main effect of perindopril was statistically significant when analyzed by two-way ANOVA of all groups (F = 19.8, P < .0001). Ecadotril reduced renal BK-(1–7) levels in rats administered ecadotril alone, but had no effect on renal BK-(1–9) levels or BK-(1–7)/BK-(1–9) ratio (fig. 9). Ecadotril did not modify renal bradykinin peptide levels or the renal BK-(1–7)/BK-(1–9) ratio in perindopril-treated rats, when compared to the effects of perindopril alone.

Perindopril did not affect bradykinin peptide levels in heart (fig. 10). The approximate 2-fold increase in cardiac BK-(1–9) levels in rats administered ecadotril alone was not statistically significant, but there was a statistically significant 50% reduction in cardiac BK-(1–7)/BK-(1–9) ratio in these rats. The combination of perindopril and ecadotril reduced cardiac BK-(1–7) levels, although not below the levels seen with perindopril alone. Moreover, the reduction in cardiac BK-(1–7)/BK-(1–9) ratio by the combination of perindopril and ecadotril was similar to that produced by ecadotril alone. The combination of perindopril and ecadotril had no effect on cardiac BK-(1–9) levels (fig. 10).

Perindopril increased BK-(1–9) levels in aorta (table 4); this increase was of borderline statistical significance in rats administered perindopril alone (P = .06), and the increase was statistically significant for perindopril-treated rats administered 0.1, 10 and 100 mg/kg per day ecadotril. The main effect of perindopril on aortic BK-(1–9) levels was statistically significant when analyzed by two-way ANOVA of all groups (F = 14.8, P = .0003). Ecadotril did not modify aortic bradykinin peptide levels in rats administered ecadotril alone. Moreover, the effects of the combination of perindopril and ecadotril on aortic bradykinin peptide levels were no different from the effects of perindopril alone (table 4).

Neither perindopril nor ecadotril alone affected bradykinin peptide levels in lung (table 4). The combination of perindopril and 100 mg/kg per day ecadotril increased the levels of both BK-(1–7) and BK-(1–9) in lung, in comparison with the effects of perindopril alone, but these levels were not different from the levels in vehicle-treated rats (table 4).

**Discussion**

The effects of combined NEP and ACE inhibition on angiotensin and bradykinin peptide levels largely represented the summation of the effects of separate NEP and ACE inhibition. NEP inhibition alone produced diuresis, natriuresis, increased urine cyclic GMP and BK-(1–9) levels, increased Ang II and Ang I levels in plasma and increased Ang I levels in heart. NEP inhibition also decreased BK-(1–7) and BK-(1–9) levels in blood and decreased the BK-(1–7)/BK-(1–9) ratio in urine, blood and heart. ACE inhibition alone reduced Ang II levels in kidney, and increased BK-(1–9) levels in blood, kidney and aorta. ACE inhibition also decreased Ang II/Ang I ratio in plasma, kidney, heart and lung, and decreased BK-(1–7)/BK-(1–9) ratio in blood and kidney. In addition to summation of the effects of separate NEP and ACE inhibition, interaction between the two inhibitors did occur. Perindopril potentiated the effects of ecadotril on diuresis, natriuresis and urine BK-(1–7)/BK-(1–9) ratio, and combined NEP/ACE inhibition increased Ang II levels in plasma and lung.

Our data illustrate tissue-specific responses of angiotensin and bradykinin peptide levels to NEP and ACE inhibition. We previously showed that the angiotensin and bradykinin systems are predominantly tissue-based systems (Campbell...
Differences between tissues in the angiotensin and bradykinin peptide responses to NEP and ACE inhibition are likely to represent differences in the relative contribution of NEP and ACE to the metabolism of these peptides in different tissues.

The dose of perindopril (0.2 mg/kg per day) used in our study was submaximal, and its effects were consistent with our previous study of the effects of a wide range of doses of perindopril on angiotensin and bradykinin peptide levels (Campbell et al., 1994). It is likely that different interactions between ecadotril and perindopril would have occurred for alternative doses of perindopril. We chose a submaximal dose of perindopril for these studies to enable the detection of possible additive effects of NEP and ACE inhibition. The dose of perindopril used in this study was similar to the doses prescribed for patients with hypertension and heart failure and was therefore an appropriate dose for study of the interactions of ACE and NEP inhibition.

Ecadotril is the orally active prodrug of (S)-thiorphan (Lecomte et al., 1990). (S)-thiorphan inhibits both NEP ($K_i = 54$ nM) and ACE ($K_i = 140$ nM) (Fournié-Zaluski et al., 1984; Roques et al., 1993). Given that we studied a 1000-fold range in ecadotril doses, with NEP inhibition evident at the lowest dose, one would anticipate that higher doses might have inhibited ACE. However, the present study showed no evidence of ACE inhibition, except perhaps in heart, where ecadotril increased Ang I levels by 3.5-fold, associated with a nonstatistically significant decrease in Ang II/Ang I ratio. We previously showed that the sulfhydryl-containing dual NEP/ACE inhibitor S21402-1, although of similar potency for NEP and ACE inhibition in vitro, was a much less potent ACE inhibitor than NEP inhibitor in vivo (Anastasopoulos et al., 1998). We speculated that modification of S21402-1 in vivo may have reduced the potency of ACE inhibition by S21402-1 (Anastasopoulos et al., 1998), and a similar mechanism may have reduced ACE inhibition by the sulfhydryl-containing (S)-thiorphan in this study.

It is well recognized that ACE inhibition increases total ACE enzyme levels (Boomsma et al., 1981). Helin et al. (1994)
reported that separate inhibition of ACE and NEP induced both enzymes, with varying induction in different tissues. NEP inhibition induced NEP in kidney and ACE in lung, whereas ACE inhibition induced NEP in lung and ACE in serum, lung and kidney (Helin et al., 1994). In our study we found that the induction of total plasma NEP masked the inhibition of plasma NEP activity by ecadotril, and may have masked to some extent the effects of NEP and ACE inhibition on metabolism of angiotensin and bradykinin peptides in blood and tissues. Even when expressed as the active NEP/total NEP ratio, the degree of inhibition of plasma NEP was much less than the inhibition of renal NEP, as indicated by $^{125}$I-RB104 binding to kidney sections. The greater inhibition of $^{125}$I-RB104 binding to kidney sections may have represented higher concentrations of (S)-thiorphan in urine, causing occupancy of proximal tubular brush border NEP, the main site of location of NEP in kidney (Roques et al., 1993). Plasma NEP activity was measured 6 hr after drug administration, and greater inhibition may have occurred before 6 hr. Previous studies in man showed maximal inhibition of plasma NEP activity and increase in plasma ANP levels at 1 to 2 hr after ecadotril administration, with return toward normal values by 6 to 8 hr (Lecomte et al., 1990; Dussaule et al., 1991). Moreover, Stasch et al. (1996) noted 75% inhibition of plasma NEP activity in rats at 1 hr, although the hypotensive effect of 100 mg/kg per day ecadotril was maintained beyond 6 hr after drug administration.

Many studies show that NEP inhibition potentiates plasma levels and effects of natriuretic peptide administration (Sybertz et al., 1990; Rademaker et al., 1996; Smits et al., 1990). Whereas the effects of NEP inhibition on endogenous plasma ANP levels depend on the experimental model (Stasch et al., 1996; Smits et al., 1990), NEP inhibition produces consistent increases in urine sodium and cyclic GMP excretion (Stasch et al., 1996; Dussaule et al., 1991), as shown in our study. Our data support previous studies demonstrating a major role for NEP in metabolism of kinins in urine (Ura et al., 1987). The potentiation by perindopril of the suppression of urine BK-(1–7)/BK-(1–9) ratio by ecadotril probably reflected the colocalization of NEP and ACE in the brush border of proximal tubular cells of the kidney (Roques et al., 1993; Bruneval et al., 1986). Thus, ACE may assume importance in BK-(1–9) metabolism in urine when NEP is inhibited.

Perindopril produced small decreases in blood pressure, body weight and heart weight/body weight ratio, in agreement with our previous finding in normotensive rats (Campbell et al., 1995a). We also found that the highest doses of ecadotril produced a similar decrease in blood pressure to that of perindopril, although the hypotensive effect was evident only after 2 days of ecadotril administration. In agreement with our study, Sybertz et al. (1990) reported that NEP inhibition failed to reduce blood pressure acutely, but reduced blood pressure of SHR after 3 days of NEP inhibition. The mechanism by which perindopril reduced body weight and heart weight/body weight ratio within 6 days is uncertain. Moreover, it is of interest that the highest doses of ecadotril prevented these effects of perindopril.

Ecadotril increased plasma levels of Ang II and Ang I, which we attribute to the associated rise in plasma renin, although the change in plasma renin did not achieve statistical significance. In agreement with our data, Wegner et al. (1996) observed an increase in plasma levels of renin and Ang I in rats administered 30 mg/kg per day ecadotril for 4 wk. It is of interest that urine volume and sodium, and plasma renin, Ang II and Ang I all suggested a bimodal dose-response to ecadotril in our study. Whereas urine volume and sodium were not increased by 10 and 100 mg/kg per day ecadotril alone, the diuretic and natriuretic responses to ecadotril were maintained at all doses of ecadotril in perindopril-treated rats. The mechanism for these bimodal responses is uncertain and may reflect changes in body fluid and sodium status, in addition to the effects of ANP on renin secretion. The increased plasma levels of Ang II and Ang I may have been due to a rise in plasma renin levels consequent to the natriuretic and diuretic response to intermediate doses of ecadotril. However, we cannot explain why the diuresis and natriuresis were not maintained for the highest doses of ecadotril alone. A similar bimodal dose-response to ecadotril was also seen in the perindopril-treated rats, where intermediate doses of ecadotril tended to suppress the renin response to perindopril, associated with an increase in

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Fig. 10. Bar graphs show effects of ecadotril administration alone (open columns) and ecadotril and perindopril (closed columns) on heart levels of BK-(1–7) and BK-(1–9), and heart BK-(1–7)/BK-(1–9) ratio. Mean ± S.E. * P < .05, ** P < .01, compared with vehicle-treated control, n = 8 to 12 rats per group.
plasma angiotensinogen levels toward normal. Whereas intermediate doses of ecadotril may have suppressed the renin response to perindopril by potentiating the inhibition of renin secretion by ANP, the mechanism of the restoration of the renin response at the highest dose of ecadotril is uncertain, and may have been due to potentiation of the natriuretic and diuretic responses by perindopril.

Despite marked effects on the levels of bradykinin peptides in urine, ecadotril had little effect on bradykinin peptide levels in kidney. By contrast, perindopril alone reduced the renal BK-(1–7)/BK-(1–9) ratio by 40%. The combination of ecadotril and perindopril reduced the BK-(1–7)/BK-(1–9) ratio to 60%, but this was not statistically significantly different from the effects of perindopril alone. These data indicate that the relative contribution of ACE and NEP to kinin metabolism in renal tissue was different from that of urine. Whereas NEP was the major pathway of BK-(1–9) metabolism in urine, ACE was the major pathway of BK-(1–9) metabolism in renal tissue. Moreover, the failure of combined NEP/ACE inhibition to completely suppress the BK-(1–7)/BK-(1–9) ratio in kidney and other tissues indicates that enzymes other than NEP and ACE play an important role in BK-(1–9) metabolism in tissue.

Apart from urine, the most consistent effects of ecadotril on kidney peptide levels were in the heart, where ecadotril alone reduced the BK-(1–7)/BK-(1–9) ratio by 50%. Although 0.2 mg/kg per day perindopril had little effect on cardiac bradykinin peptide levels in our study, we previously showed that higher doses of perindopril increased cardiac BK-(1–9) levels and reduced cardiac BK-(1–7)/BK-(1–9) ratio (Campbell et al., 1994). Our previous studies and our current data emphasize that both NEP and ACE have important roles in BK-(1–9) metabolism in heart. NEP immunostaining and activity have been found on the surface of cultured neonatal rat myocytes and endothelial cells of the human coronary vasculature (Piedimonte et al., 1994; Graf et al., 1995). Many studies demonstrate a role for kinins in mediating the cardiac effects of ACE inhibitors (Linz et al., 1995). Moreover, kinins may also mediate the cardiac effects of NEP inhibition. The type 2 BK-(1–9) (B2) receptor antagonist HOE 140 prevented the protective effects of NEP inhibition on ischemia reperfusion injury in the rat heart (Yang et al., 1997; Schriever et al., 1996) and on isoproterenol-induced myocardial hypoperfusion (Piedimonte et al., 1994).

Our study provides important insights into the mechanisms by which the combination of NEP and ACE inhibitors may provide superior therapy for hypertension and cardiac failure than either agent alone. Although the effects of combined NEP and ACE inhibition on angiotensin and bradykinin peptides represented mainly the effects of the most influential inhibitor alone in each specific tissue, interactions did occur between the effects of these two inhibitors on angiotensin and bradykinin peptides. Perindopril potentiated the effects of ecadotril on diuresis, natriuresis and urine BK-(1–7)/BK-(1–9) ratio. Moreover, combined NEP/ACE inhibition increased Ang II levels in plasma and lung. Whereas increased diuresis and natriuresis may contribute to the therapeutic effects, increased Ang II levels in plasma and lung may compromise the therapeutic effects of combined NEP/ACE inhibition. The cause of the increased Ang II levels in plasma and lung of perindopril-treated rats administered 100 mg/kg per day ecadotril is uncertain, given that ecadotril alone did not increase Ang II levels at this dose. NEP may play an important role in metabolism of circulating Ang II and Ang I (Richards et al., 1992; Yamamoto et al., 1992). During ACE inhibition, simultaneous NEP inhibition may lead to diversion of the elevated Ang I levels to conversion by incompletely inhibited ACE, or to alternative serine protease-mediated pathways of conversion to Ang II (fig. 1) (Campbell et al., 1994; Campbell, 1993) and, together with the inhibition of Ang II degradation by NEP, thus account for the increased Ang II levels in plasma and lung observed during combined NEP/ACE inhibition in our study.

### TABLE 4

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ecadotril (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Aorta BK-(1–7) (% of control)</td>
<td>100 ± 22</td>
</tr>
<tr>
<td>Ecadotril</td>
<td>138 ± 57</td>
</tr>
<tr>
<td>Aorta BK-(1–9) (% of control)</td>
<td>100 ± 34</td>
</tr>
<tr>
<td>Ecadotril</td>
<td>262 ± 69</td>
</tr>
</tbody>
</table>

Mean ± S.E. ND, Not determined. Two-way ANOVA for aortic BK-(1–9) levels of all groups showed a statistically significant main effect for perindopril (F1,49 = 14.8, P = .0003), n = 8–10 per group. For experiment 1, aortic BK-(1–7) and BK-(1–9) levels were less than the minimum detectable (<12 fmol/g for each peptide).

^a P < .01, compared with vehicle-treated control.

^b P < .05, comparison of rats administered both ecadotril and perindopril with perindopril-treated control.

^c P < .01, comparison of rats administered both ecadotril and perindopril with perindopril-treated control.
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