Differential tissue and enzyme inhibitory effects of the vasopeptidase inhibitor omapatrilat in the rat

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ABSTRACT

Vasopeptidase inhibitors simultaneously inhibit angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP). The present study characterized the tissue distributions of ACE and NEP and assessed the effects of the vasopeptidase inhibitor omapatrilat on ACE and NEP in rat tissues. In vivo ACE and NEP inhibition was studied by in vitro autoradiography and using the ACE inhibitor radioligand $^{125}$I-MK351A and the NEP inhibitor radioligand $^{125}$I-RB104 in rats that received oral omapatrilat (40 mg·day$^{-1}$·kg$^{-1}$) for 3 days. In vitro autoradiography was used to examine the distribution of ACE and NEP in the kidney, aorta, heart, adrenal gland, lung, intestine, liver, spleen and brain, and to assess enzyme inhibition after oral omapatrilat. Omapatrilat inhibited plasma ACE and increased plasma renin activity ($P < 0.01$). Tissue ACE was inhibited by 70–95% ($P < 0.01$), except in the brain, where ACE was not inhibited. NEP was inhibited by 87% in the kidney and by 20–40% in atria, aorta, adrenal gland, lung, intestine and blood; it was not inhibited in the brain, the ventricle or the spleen. Omapatrilat is a potent vasopeptidase inhibitor that significantly inhibits tissue ACE and NEP, with the degree of inhibition varying according to the enzyme and the tissue under assessment. The degree and site of tissue enzyme inhibition by vasopeptidase inhibitors may be relevant to end-organ protection as well as to the side-effect profiles of these agents.

INTRODUCTION

Vasopeptidase inhibitors simultaneously inhibit the enzymes angiotensin-converting enzyme (ACE) and neutral endopeptidase (NEP) [1]. NEP is the major enzyme responsible for the degradation of the natriuretic peptides, and its inhibition leads to increases in the levels of these vasorelaxant, diuretic and natriuretic peptides [2,3], while ACE inhibition prevents the formation of the vasoconstrictor, anti-natriuretic and trophic hormone angiotensin II [4].

In experimental studies, the vasopeptidase inhibitors have been shown to act as renoprotective agents in chronic renal failure [5], and as vasoprotective agents in endothelial dysfunction [6] and atherosclerosis [7]. The precise mechanism whereby vasopeptidase inhibitors mediate their beneficial effects is not known. Both NEP and ACE are ubiquitous exo-enzymes that are present not only in the kidney, but also in the heart, adrenal gland, brain, lung, intestine and spleen. Tissue ACE is now recognized as a key factor in cardiovascular disease, and evidence suggests that a principal target of ACE inhibitor action is at the tissue sites [8].

To date, the inhibitory effects of the vasopeptidase inhibitors on ACE and NEP at various tissue sites have not been fully explored, and it is not clear whether the vasopeptidase inhibitors may have not only beneficial, but also adverse, effects through their widespread enzyme

Key words: angiotensin-converting enzyme, neutral endopeptidase, omapatrilat, vasopeptidase inhibitor.
Abbreviations: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; NEP, neutral endopeptidase; PRA, plasma renin activity.
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inhibitory actions. Reports suggest that infusion of an NEP inhibitor into the rat hippocampus causes accumulation of amyloid-β peptide [9], while the presence of NEP in the colon may protect against inflammatory colitis [10]. Clinical trials in hypertensive patients using the vasopeptidase inhibitor omapatrilat have also raised concerns about angioedema, a serious and potentially fatal side effect [11].

The aim of the present study was to assess the tissue inhibitory actions of omapatrilat in the rat. We used in vitro autoradiography and the specific ACE and NEP inhibitor radioligands 125I-MK351A [12] and 125I-RB104 [13,14] respectively to assess the tissue distributions of ACE and NEP in the rat, and to measure the ability of omapatrilat to inhibit ACE and NEP in organs of cardiovascular relevance, such as the heart, aorta, kidney, adrenal and brain, as well as in the gastrointestinal tract, liver, spleen and lung.

METHODS

Experimental procedures were performed according to the National Health and Medical Research Council of Australia guidelines for animal experimentation. Adult male Sprague–Dawley rats (250–300 g) were obtained from the Austin Hospital animal house, and housed at 23–25 °C with a 12 h light/dark cycle and with access to standard rat chow (0.6 % NaCl; Norco, Melbourne, Australia) and tap water ad libitum. Omapatrilat (K, values: NEP, 9 nmol/l; ACE, 6 nmol/l [15]) was a gift from Bristol-Myers Squibb Pharmaceuticals (New Brunswick, NJ, U.S.A.).

In vivo effects of oral omapatrilat in rats

To assess the degree of inhibition by omapatrilat of tissue ACE and NEP in vivo, rats were gavaged with vehicle (5 % arabic gum) or omapatrilat (40 mg·day−1·kg−1) (n = 6 rats per group) for 3 days. This dose of omapatrilat reduces blood pressure and left ventricular hypertrophy in the spontaneously hypertensive rat, and these effects were associated with inhibition of renal ACE and NEP activity [16]. Rats were killed by decapitation 1 h after the final gavage, and trunk blood was collected into chilled heparin tubes containing 100 µl of sodium heparin (25 000 units/l) for the measurement of plasma ACE activity, and into EDTA/aprotinin tubes (500 kallikrein-inhibitory units/ml) for measurement of plasma renin activity (PRA) and atrial natriuretic peptide (ANP) levels. Kidney, brain, lung, aorta, liver, spleen, intestine, adrenal gland and kidneys were snap frozen at −40 °C and used to assess inhibition of tissue ACE and NEP activity by in vitro autoradiography. The heart was subdivided into atria and left ventricle before freezing. In addition, tissue levels of ANP and angiotensin II were assessed in the atria and ventricles.

Biochemical analysis

PRA and plasma and tissue ANP levels were measured by RIA as described previously [17,18]. Plasma ACE activity was measured by a fluorimetric assay [19].

In vitro autoradiography

In vitro autoradiography was performed on slide-mounted 20 µm tissue sections (n = 6 per group) using the specific radioligands 125I-RB104 for NEP [13,14] and 125I-MK351A for ACE [12], as described previously.

In brief, in vitro autoradiography for renal NEP was performed by preincubating slide-mounted 20 µm sections in 50 mmol/l Tris/HCl buffer, pH 7.4, for 50 min at room temperature, followed by incubation with 150 µl of buffer containing 125I-RB104 (≈ 0.04 µCi) for 2 h at room temperature. Non-specific binding was determined in the presence of 1 µmol/l SCH 42354. After incubation, sections were transferred through four successive 1 min washes of buffer at 4 °C, followed by a 5 s wash in distilled water. The sections were dried rapidly under a stream of cold air, loaded into X-ray cassettes together with a set of radioactivity standards, exposed to Agfa Scopix X-ray film for 3 days at room temperature, and then developed. Quantification of binding density was determined by computerized densitometry using radioactive standards, which were corrected for decay and fitted to calibration curves to convert the absorbance of the autoradiographs into units of d.p.m./mm². Results are expressed as a percentage of the binding observed in vehicle-treated rats.

For ACE autoradiography, slide-mounted sections were preincubated in 10 mmol/l sodium phosphate buffer containing 150 mmol/l NaCl, 2 g/l BSA and 50 µmol/l ZnCl₂, followed by a 1 h incubation in the same buffer containing 0.3 µCi/ml 125I-MK351A at room temperature. This was followed by four successive 1 min washes in buffer without BSA or radioligand at 4 °C to remove unbound radioligand. Non-specific binding was determined in the presence of 1 nmol EDTA. The sections were then processed as above.

Localization

To localize binding of the radioligand, in vitro autoradiography slides were dipped in LM-1 photographic emulsion (Amersham Biosciences), stored with desiccant for 14 days at 4 °C, developed in Kodak D19, fixed in Ilford Hypam, and stained with haematoxylin and eosin. Light microscopy was used to confirm the localization of ACE and NEP. Average labelling intensity values were estimated by two independent investigators in between four and six sections per tissue per rat.

Statistical analysis

Results are presented as means ± S.E.M., and data were analysed using one-way ANOVA and the Scheffe test where appropriate. Significant differences were obtained when P < 0.05.
RESULTS

Tissue ACE and NEP

Figure 1 shows computer-generated pseudo-colour images of the macroscopic autoradiographs for ACE and NEP in various rat tissues, and the effects of treatment with omapatrilat. Table 1 summarizes the localization of ACE and NEP as assessed with light microscopic autoradiography. ACE and NEP were widely distributed in all major organs, and in most tissues the ACE and NEP distribution was similar. However, the relative labelling intensity varied between the enzymes and also between tissues.
Figure 2  Density of ACE radiolabelling in tissues from vehicle- and omapatrilat-treated rats
Note the different scales for (A) and (B). Values are means ± S.E.M. (n = 6 per group); **P < 0.01 compared with vehicle-treated group. CN, caudate nucleus.

Table 1  Tissue distribution and localization of ACE and NEP assessed using light microscopy
The average labelling intensity values were estimated by two independent investigators in between four and six sections per tissue per rat. Key: −−, no binding; +, weak binding; ++, moderate binding; ++++, strong binding.

<table>
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<tr>
<th>Organ</th>
<th>Localization</th>
<th>ACE</th>
<th>NEP</th>
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<td>Kidney</td>
<td>Proximal tubule</td>
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<td>Heart</td>
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<td>Lung</td>
<td>Alveolar septa</td>
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<td>Intestine</td>
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Localization of ACE
The density of radiolabelling (d.p.m./mm²) of ACE is shown in Figure 2. ACE was found in all tissues, but more intense binding was noted in the kidney, intestine, brain, lung, aorta and adrenal medulla (Figure 2A), compared with the adrenal cortex, atria, ventricle, liver and spleen (Figure 2B; note the change in scale between Figures 2A and 2B). Light microscopy showed a high level of ACE in vascular endothelial cells, regardless of the organ of origin (Table 1). The aorta exhibited high levels of ACE in the endothelium and vasa vasorum of the adventitia, and in the heart both the ventricles and atria showed dense labelling of coronary and intramyocardial vessels and endocardium. In the lung, the endothelium of the pulmonary microvasculature and the endothelium and adventitia of large vessels were all densely labelled. In the kidney, ACE was localized to the proximal convoluted tubules of the juxta-medullary cortex and vessels of all regions. The dense fibrous capsule and the secretory cells, capillaries and venules of the medulla of the adrenal gland also labelled strongly for ACE. Within the brain there was strong labelling of the basal ganglia, including the caudate nucleus. The epithelial cells of the intestinal mucosa showed strong binding at the luminal extremities, and in the liver ACE was localized to the vessels of the portal tracts and fibrous tissue septa. The distribution of ACE in the spleen was confined mainly to the connective tissue trabeculae and the white pulp.

Localization of NEP
All tissues that demonstrated ACE labelling also exhibited varying degrees of NEP labelling (Figure 1), with the kidney having the highest level of NEP. The density of radiolabelling (d.p.m./mm²) for NEP is shown in Figure 3 (again note the change in scale between Figures 3A and 3B). The distribution of NEP labelling in tissues using light microscopic autoradiography, summarized in Table 1, was not as clear as with ACE labelling, due to the comparatively low level of labelling, which reflects the low levels of NEP itself. However, as with ACE, the proximal tubule brush border of the inner cortex of the kidney was strongly labelled. In the heart, both the atria and ventricles demonstrated silver grains over cardiomyocytes, and the alveolar septa of the lungs were also labelled. NEP labelling was evident in the epithelial cells of the intestinal mucosa and the caudate nucleus of the brain. Hepatocytes of the liver parenchyma also exhibited NEP labelling.

Effects of oral omapatrilat on plasma parameters and tissue ACE and NEP
The changes in plasma ACE activity, PRA and plasma ANP levels after 3 days of oral omapatrilat are shown in Table 2. Omapatrilat inhibited plasma ACE (P < 0.01) and increased PRA compared with vehicle (P < 0.01). The plasma ANP concentration was unchanged by omapatrilat. Omapatrilat increased ANP levels in the atria, and caused a small but non-significant increase in ventricular...
ANP. Angiotensin II was not detected in normal rat cardiac tissue, and there was no change with omapatrilat.

Treatment of rats with omapatrilat at 40 mg·day⁻¹·kg⁻¹ inhibited ACE by 70–95 % of control values in all tissues (P < 0.01), but did not inhibit ACE within the brain in the caudate nucleus (Figures 1 and 2; Table 3). Omapatrilat inhibited NEP by 87 % in the kidney and by 20–40 % in the aorta, liver, intestine, lung, adrenal gland and atrium (Figures 1 and 3; Table 3); NEP was not inhibited in the caudate nucleus, the ventricle or the spleen.

**DISCUSSION**

The present study used in vitro autoradiography and radiiodinated specific and selective ACE and NEP inhibitors to determine the tissue distributions of NEP and ACE. This approach also allowed us to quantify tissue ACE and NEP inhibition in specific cellular structures following oral administration of omapatrilat in the rat. The results provide in vivo evidence that the vasopeptidase inhibitor omapatrilat inhibits ACE and NEP at the site of highest abundance of both enzymes, namely the proximal tubules of the kidney, and also causes significant inhibition of ACE and NEP in other tissues.

We have shown previously that omapatrilat inhibits renal and left ventricular ACE [16], and the present study extends those findings to show that omapatrilat inhibits ACE by 70–95 % in the atria, aorta and adrenal gland, as well as the lung, intestine, liver and spleen. Omapatrilat inhibits ACE and NEP to a similar degree in the kidney (80–90 %), but is a less potent NEP inhibitor in general, with ∼20–40 % inhibition in the aorta, atrium, adrenal gland, liver, intestine and lung. There are a number of explanations for this variability; there may be differences in the concentrations of the enzymes, or in the affinity of the enzymes in different tissues. The precise localization of an enzyme within a tissue may also play a role in the level of inhibition, with varying penetration of the drug into different areas. For example, in the lung, ACE is localized predominantly to the vasculature, whereas NEP is found in the alveoli and bronchi, and the degree of inhibition varies from 20 % for NEP to 80 % for ACE.

The vasopeptidase inhibitors are under active investigation due to their effects in hypertension and heart failure.
Experimental data from our laboratory suggest that the vasopeptidase inhibitors have cardioprotective effects, i.e. regression of cardiac hypertrophy and decreasing fibrosis [20], and renoprotective actions, i.e. preventing progressive renal injury in chronic renal failure [5] and in diabetes [21]. Omapatrilat is also vasoprotective, improving both endothelial dysfunction [6] and atherosclerosis [7]. However, it is not clear precisely how the vasopeptidase inhibitors mediate their beneficial actions. Autoradiographic studies, which allow one to determine the localization of NEP and ACE in organs, and can be used to assess the tissue-specific inhibition of ACE and NEP, may be of use in this regard. It is known that the long-term benefits of ACE inhibitors in preventing or reversing end-organ damage depend on their ability to inhibit ACE in target organs [8], but it remains to be determined if differences in the degree or the site of NEP or ACE inhibition are of clinical importance for the vasopeptidase inhibitors.

Determining the tissue specificity of the vasopeptidase inhibitors may also be important in predicting their side-effect profiles. For example, bradykinin and substance P, which are abundant in the lung and are involved in neurogenic inflammation and plasma leakage into the airways, are metabolized by ACE and NEP. Although this gives rise to the theoretical concern that vasopeptidase inhibitors may increase cough, in heart failure the incidence of cough was similar in patients treated with either a selective ACE inhibitor (lisinopril) or omapatrilat (both 11%) [22]. A possible explanation may relate to the relative lack of NEP inhibition in the lung with omapatrilat.

Substance P also has pro-inflammatory effects in the intestine. Both ACE and NEP are expressed at the brush border of the intestine, where they maintain substance P and bradykinin at low levels. Studies in NEP knockout mice suggest that the absence of NEP exacerbates colitis due to reduced degradation of substance P [10]. In the rat, omapatrilat inhibited intestinal ACE by 99% and NEP by 37%. Whether such enzyme inhibition is related causally to the increase in diarrhoea in patients treated with omapatrilat compared with those given lisinopril (12% compared with 5%; \( P < 0.05 \)) is not known, particularly as NEP inhibition with racacodotril (acetorphan) has been found to be an effective and safe treatment for acute diarrhoea in humans [23].

It has also been shown that NEP-mediated proteolysis is the primary pathway for the elimination of insoluble amyloid-\( \beta \) peptide, suggesting that reduced hippocampal NEP activity may cause the accumulation of amyloid and possibly the induction of Alzheimer's disease [9]. Importantly, in the present study, omapatrilat did not penetrate the blood–brain barrier to inhibit ACE or NEP in a site of high abundance, the caudate nucleus, which makes it unlikely that omapatrilat inhibits ACE and NEP in the hippocampus. ACE and NEP are also present in areas outside the blood–brain barrier, such as the circumventricular organs, but these areas were not assessed in the present study. Studies with the ACE inhibitor ceronapril showed no inhibition of brain ACE, but inhibition in the circumventricular organs, with results after 2 weeks of chronic dosing being essentially the same as those obtained after a single dose [24]. However, large doses of perindopril (up to 16 mg/kg) did progressively inhibit ACE in brain structures, including the basal ganglia [25]. Given that vasopeptidase inhibitors will be used for long periods of time clinically, further studies are needed in order to assess more precisely their effects on cellular structures within the brain and outside the blood–brain barrier.

It is becoming clear that NEP and vasopeptidase inhibitors vary in their efficacy, and results from one study cannot be extrapolated to another. For example, in human heart failure the NEP inhibitor candoxatril increased exercise duration [26], whereas another, echadotril, had no effect on symptoms [27]. Differential tissue accessibility of different inhibitors may be one explanation. The relative degree of ACE compared with NEP inhibition also varies between vasopeptidase inhibitors; in the rat, omapatrilat has similar potency to inhibit renal NEP and ACE [16], whereas S21402 has greater efficacy to inhibit renal NEP compared with ACE [20]. Omapatrilat is a more potent inhibitor of vascular ACE than high-dose perindopril, quinapril, or benazapril, which result in only \( \sim 60\% \) inhibition [18], and also inhibits vascular NEP.

This may explain why, in the rat coronary bed, omapatrilat results in the greatest protection from bradykinin breakdown compared with ACE or NEP inhibitors alone [28], and why, in salt-sensitive hypertension, omapatrilat is superior to captopril in improving endothelium-dependent relaxation [6].

To date, few studies have investigated the effects of the vasopeptidase inhibitors on potential substrates, but this is of importance in determining the mechanism of action of these inhibitors. Both ACE and NEP are involved in the metabolism of angiotensin and bradykinin peptides; NEP metabolizes ANP as well as endothelin and adrenomedullin. In \textit{ex vivo} studies in human cardiac ventricular membranes, omapatrilat had greater protective effects on exogenous bradykinin metabolism than a pure ACE inhibitor [29], suggesting that the cardiac benefits of omapatrilat may be mediated in part through NEP inhibition. However, \textit{in vivo} in the rat, as shown in the present study, omapatrilat inhibited atrial but not ventricular NEP. The lack of effect of omapatrilat on ventricular NEP is mirrored by the lack of an increase in ventricular ANP, whereas atrial NEP inhibition was accompanied by increased atrial ANP levels. These data are of interest, given that, in a recent clinical trial, omapatrilat reduced the risk of death and hospitalization in chronic heart failure, but was not more effective than ACE inhibition alone [30]. Further studies are needed to determine the respective effects of NEP and ACE inhibition on the metabolism of potential substrates in various organs in different disease states.
In summary, omapatrilat is a vasopeptidase inhibitor that causes significant inhibition of tissue ACE and NEP, with the degree of inhibition varying according to the enzyme and the tissue under assessment. Both the degree and site of tissue enzyme inhibition by omapatrilat may be relevant to effects on various substrates, as well as to end-organ protection and side-effect profiles.

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