Inhibition of neutral endopeptidase, the degradative enzyme for natriuretic peptides, in rat kidney after oral SCH 42495

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1. Inhibition of neutral endopeptidase (NEP), the degradative enzyme for atrial natriuretic peptide, was studied in vitro and in vivo using a previously characterized NEP inhibitor radioligand, 125I-labelled RB104.

2. SCH 42354, the active di-acid of the ethylester prodrug, SCH 42495, caused a concentration-dependent displacement of 125I-labelled RB104 from rat renal NEP. The concentration of SCH 42354 that displaced 50% of radioligand bound to the enzyme NEP (IC50) was 3.3 ± 0.1 nmol/l (mean ± SEM). Enalaprilat, an angiotensin converting enzyme inhibitor, did not displace 125I-labelled RB104 in concentrations up to 10 μmol/l.

3. In adult normotensive Sprague–Dawley rats, oral SCH 42495 (3–300 mg/kg) caused a significant inhibition of renal NEP (P<0.001). SCH 42495 had no effect on renal or plasma angiotensin converting enzyme activity, but high-dose SCH 42495 (300 mg/kg) caused a significant increase in plasma renin activity (P<0.01).

4. In a time course study, oral SCH 42495 (30 mg/kg) caused rapid (within 30 min) and significant inhibition of renal NEP for up to 48 h (P<0.001). No changes in plasma atrial natriuretic peptide or plasma angiotensin converting enzyme activity were seen.

5. These data provide evidence that short-term administration of the NEP inhibitor SCH 42495 results in inhibition of renal NEP and does not inhibit the circulating or the tissue renin–angiotensin system. The NEP inhibitor radioligand 125I-labelled RB104, is a useful tool to study tissue NEP inhibition after administration of NEP inhibitors.

INTRODUCTION

Atrial natriuretic peptide (ANP) causes natriuresis, diuresis, vasodilatation and inhibition of the renin–angiotensin system [1]. The therapeutic usefulness of ANP is limited by a short plasma half-life due to inactivation by two main mechanisms, receptor-mediated endocytosis via the clearance receptor [2] and hydrolysis by neutral endopeptidase (NEP, EC 3.4.24.11) [3, 4]. Although the relative importance of the enzymic and non-enzymic pathways is debated, with reports that when circulating ANP levels are in the physiological range the clearance receptor plays the major role in degradation [5, 6], as well as reports that both pathways play an equal role [7, 8], orally effective NEP inhibitors are now under evaluation as potential therapeutic agents in the treatment of hypertension and heart failure.

SCH 42495 is the pro-drug of the free acid SCH 42354 and is a potent and selective inhibitor of NEP which reduces blood pressure in volume-dependent models of hypertension [9, 10], produces beneficial effects on cardiovascular remodelling in genetic hypertension [11, 12] and after chronic hypoxia [13], and prevents myointimal proliferation after vascular injury [14]. NEP inhibitors also lower blood pressure in essential hypertension [15, 16] and have favourable effects in human congestive heart failure [17].

The precise mechanism and site of action of NEP inhibitors is not fully understood. NEP is a ubiquitous ecto-enzyme found in highest abundance in the brush-border membranes of the proximal tubules but present also in the lung, brain, intestine, spleen, endothelial cells and neutrophils [3, 4]. Inhibition of renal NEP prevents degradation of ANP filtered by the glomerulus, enabling filtered ANP to reach receptors in the distal segment of the nephron to cause a natriuresis and diuresis. As NEP in the kidney is located intra-luminally, it does not play a role in the clearance of ANP from plasma, and inhibition of NEP in extra-renal tissues is likely to account for changes in circulating ANP. Studies in vivo in man and experimental animals use haemodynamic measurements plus assessment of diuresis and natriuresis and urinary/plasma ANP and cyclic GMP as indicators that NEP has been inhibited after administration of an NEP inhibitor.

Key words: autoradiography; 125I-labelled RB104, neutral endopeptidase, SCH 42495.

Abbreviations: ACE, angiotensin converting enzyme; ANP, atrial natriuretic peptide; DAPNG, N-dansyl-D-alanyl-glycyl-L-4-nitrophenylalanyl-glycine; IVA, in vitro autoradiography; NEP, neutral endopeptidase; PRA, plasma renin activity; RIBA, radioinhibitory binding assay; SD, Sprague–Dawley; TFA, trifluoroacetic acid.

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To date there is no direct evidence, in vivo, that administration of an NEP inhibitor results in inhibition of tissue NEP. This paper describes the use of in vitro autoradiography (IVA) and radioinhibitory binding assays (RIBA) to localize NEP in the kidney and to assess the renal NEP inhibitory effects of SCH 42495, a potent and selective inhibitor of NEP, using 125I-labelled RB104, the most potent radio-labelled NEP inhibitor available to date ($K_d = 0.03$ nmol/l) [18].

**METHODS**

**Animals**

Experimental procedures were approved by the Austin Hospital Animal Research Ethics Committee and performed according to the National Health and Medical Research Council of Australia guidelines for animal experimentation. Female Sprague-Dawley (SD) rats (200–250 g) were used in all studies. Rats were housed at 23–25°C in a 12 h/12 h light-dark cycle, with food containing 0.4–0.6% NaCl (Norco) and water ad libitum.

**Materials**

SCH 42495 was a gift from E. Sybertz (Schering-Plough Corporation). RB104 {2-[(3-iodo-4-hydroxy)-phenylmethyl]-4-N-[3-hydroxyamino-3-0~0- 1 -phenyl-phenylalanyl-glycine (DAPNG) were obtained from Sigma Chemical Co. BSA was obtained from CSL Ltd. All other reagents were obtained from either BDH or from Ajax Chemicals.

**Membrane preparations**

Renal membranes for NEP enzymic assay and RIBA were prepared as previously described [19, 20]. Briefly, kidneys were diced, suspended in 4 ml of ice-cold 50 mmol Tris/HCl buffer, pH 7.4, and homogenized for 3 x 20 s with a polytron homogenizer (Janke and Kunkel, Ultra-Turrax) at 13500 rev./min. The homogenate was centrifuged for 5 min at 3000 rev./min, the pellet discarded and the supernatant re-centrifuged for 60 min at 18 000 rev./min. The resulting pellet was resuspended in ice-cold 50mm Tris/HCl buffer, pH 7.4, and then 10 ml of 0.1% TFA. The reactants were eluted from the Sep-Pak cartridge using 10 ml of 0.1% TFA followed by 50 ml of methanol/water containing 0.1% TFA in a gradient from 40% to 100% methanol. Fractions of the largest peaks were then tested for binding activity using renal autoradiography, and the best binding fractions with the lowest non-specific binding were pooled and used for the present studies.

**In vitro autoradiography for renal NEP**

Slide-mounted sections (20 μm in thickness) were preincubated 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-labelled RB104 (~0.04 μCi, 75 000 c.p.m.) applied directly to each section for 2 h at room temperature. Non-specific binding was determined in the presence of 100 mmol/l EDTA and 2.5 mmol/l phenanthroline or 1 μmol/l SCH 42354.

After incubation, sections were transferred through four successive 1 min washes of buffer at 4°C followed by 5 s in distilled water. The sections were rapidly dried under a stream of cold air, loaded into X-ray cassettes and exposed to Agfa Scopix X-ray film for 3 days at room temperature. After exposure, the sections were stained with haematoxylin and eosin and examined with the autoradiographs for anatomical localization of 125I-labelled RB104.

The optical density of the autoradiographs was calibrated in terms of radioactive density in d.p.m./mm² by reference to standards carried through the procedure and quantified by computerized densitometry.

**In vitro autoradiography for angiotensin converting enzyme (ACE)**

The radioligand used to study ACE was 125I-labelled 351A; 351A is a tyrosyl derivative of the specific ACE inhibitor lisinopril. It was radioiodinated with 125I using chloramine T and purified by
Tissue neutral endopeptidase inhibition in the rat

SP-Sepharose C25 chromatography as previously described [23].

Briefly, slide-mounted sections (20 μm) were pre-incubated 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 with 0.3 μCi/ml (230 pmol/l) 125I-labelled 351A 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. The sections were then processed as above. Non-specific binding was determined in the presence of 1 mmol EDTA.

RIBA for renal NEP

Preliminary experiments determined the optimal conditions for 125I-labelled RB104 binding to rat renal membranes and the conditions necessary for approximately 30% binding of 125I-labelled RB104 were used: 25 μg/100 μl of renal membranes were incubated with 125I-labelled RB104 (~0.02 μCi, 40 000 c.p.m.) and 12.5 mmol/l Tris/HCl buffer for 1 h at 35°C. Bound and free 125I-labelled RB104 were separated by centrifugation (4°C, 13 000 rev./min, 10 min) and the 100 μl supernatant was counted in a gamma counter (LKB 1260 Mutigamma II). Specific binding was determined as specific minus non-specific binding determined in the presence of 2.5 mmol/l phenanthroline or 1 μmol/l SCH 42354.

Radioinhibitory binding studies for renal ACE

125I-labelled 351A was used as the radioligand to quantify ACE in renal membranes using an RIBA as described previously [23].

Plasma assays

Plasma ACE activity was measured in a fluorimetric assay using a synthetic tripeptide substrate [23]. Plasma ANP was measured after florisil extraction by RIA [24, 25]. Plasma renin activity (PRA) was measured as described previously [26]. Plasma sodium was measured using a flame photometer (Instrumentation Laboratories, Milan, Italy).

In vitro inhibition by SCH 42354 of NEP binding

The in vitro inhibition by SCH 42354 of selective NEP binding in rat kidney was determined by incubating renal membranes from untreated SD rats in an RIBA as described above. Serial dilutions of SCH 42354 or the ACE inhibitor enalaprilat were added to the reaction mixture to obtain a final concentration in the range 100 pmol/l to 10 μmol/l.

Specific binding was calculated as total minus non-specific binding.

In vivo dose response with SCH 42495

SD rats were gavaged with vehicle (5% arabic gum) or SCH 42495 (0.3, 1, 3, 30 and 300 mg/kg) and killed 1 h post-gavage (n = 6 per time point). Trunk blood was collected into pre-chilled tubes containing heparin, or EDTA/aprotinin (500 kalli-krein inhibitor units/ml) for the measurement of plasma sodium concentration and plasma ACE activity, and PRA respectively. Kidneys were snap-frozen in isopentane at −40°C and used for the autoradiographic localization of NEP and ACE. The degree of NEP or ACE inhibition after oral SCH 42495 was expressed as the percentage of binding in tissue from vehicle-treated rats.

Time course

SD rats were gavaged with vehicle (5% arabic gum) or SCH 42495 (30 mg/kg) and killed at 0, 0.5, 1, 2, 4, 8, 12, 24 and 48 h post-gavage (n = 4 per time point). Trunk blood was collected as above for the measurement of plasma sodium, plasma ACE activity and plasma ANP concentration. Kidneys were snap-frozen in isopentane at −40°C and later used to prepare kidney membranes for measurement of NEP by enzymic and radioinhibitory binding assays. In a separate study, the same protocol was followed but kidneys alone were collected for autoradiographic localization of NEP. The degree of NEP inhibition after oral SCH 42495 was expressed as the percentage of binding in tissue from vehicle-treated rats.

Statistics

The results are presented as means±SEM. The data have been analysed using analysis of variance and Scheffe test where appropriate. Differences were considered significant when P<0.05.

RESULTS

RIBA

SCH 42354, the active di-acid, caused a concentration-dependent displacement of specific 125I-labelled RB104 binding from rat renal membrane NEP (Figure 1). The concentration of SCH 42354 that displaced 50% of specific NEP binding (IC₅₀) was 3.3±0.1 nmol/l (mean±SEM; n = 3). The IC₅₀ obtained using 125I-labelled RB104 is in agreement with published results using an enzymic assay and rabbit renal membranes (IC₅₀ = 8.3±4 nmol/l) [9]. In contrast, enalaprilat had no effect on selective NEP radioligand binding in rat renal membranes.
I25-labelled RB104 binding to rat kidney are presented in Figure 3. The autoradiographs represent binding to NEP (Figure 3A) and NEP inhibition after oral SCH 42495 (Figure 3B). Non-specific binding was not detectable and is not shown. A high density of NEP binding was seen in the outer stripe of the outer medulla and the inner cortex, consistent with binding to the deep proximal tubule. No binding was detected in the glomeruli. Quantitative values obtained from computer analysis of the autoradiographs are shown in Figure 4. Renal NEP was significantly inhibited after oral SCH 42495 doses of 3 mg/kg and above ($P < 0.001$). In contrast, SCH 42495 caused no inhibition of renal ACE assessed using IVA (results not shown).

**Time course**

The time course of changes in plasma ANP and ACE activity after gavage with SCH 42495 (30 mg/kg) are shown in Figure 5. Oral SCH 42495 significantly reduced plasma sodium at 24 h and 48 h after dosing (time zero, sodium 131 ± 1 mmol/l; 24 h, 126 ± 1 mmol/l; 48 h, 125 ± 1 mmol/l, $P < 0.05$) but had no effect on plasma ANP concentration (Figure 5a) or plasma ACE activity (Figure 5b). Figure 6 shows the percentage of NEP inhibition compared with vehicle-treated rats measured by enzymic assay (Figure 6A), by quantitative computer analysis of renal autoradiographs (Figure 6B) and by radioligand binding (Figure 6C). All three methods showed significant renal NEP inhibition within 30 min of drug administration which persisted for up to 48 h ($P < 0.001$).

**DISCUSSION**

This study provides direct *in vivo* evidence that the NEP inhibitor SCH 42495 causes inhibition of NEP in the site of highest abundance of NEP, namely the proximal tubules of the kidney. The radioiodinated NEP inhibitor $^{125}$I-labelled RB104 was used for *in vitro* autoradiographic and radioinhibitory binding studies to localize and quantify renal NEP and assess the degree of inhibition after oral administration of the selective NEP inhibitor SCH 42495.

NEP activity is usually measured by enzymic kinetic techniques [20, 22] using synthetic substrates, but tissue preparations often contain non-specific peptidases that destroy the reaction product of NEP or the substrate used to assess NEP activity. The technique of radioinhibitor binding to assess NEP is not affected by these problems. The use of IVA allows precise determination of NEP and its inhibition in specific cellular structures in complex organs, such as the brain and the kidney, where NEP is localized to discrete anatomical areas. Our results using $^{125}$I-labelled RB104 confirm earlier work using $^{125}$I-labelled SCH 47896 showing that a high density
of NEP binding sites are present in the inner cortex and outer stripe of the outer medulla [20]. Neither this nor our previous study [20] showed binding in rat glomeruli. There is some debate as to the presence of glomerular NEP, and our results contrast with those of Landry et al. [27], who found NEP in both tubules and glomeruli in the dog, also using $^{125}$I-labelled RB104. It is possible that inter-species differences in the localization of NEP exist.

A single dose of oral SCH 42495 potently inhibited renal NEP and was devoid of plasma or tissue ACE inhibitory activity even at high doses. The significant increase in PRA with high-dose SCH 42495 may reflect stimulation of the renin–angiotensin system as a counter-regulatory mechanism to maintain blood pressure. The onset of action of SCH 42495 was rapid and long lasting, with renal NEP inhibition persisting for up to 48 h.

No increase in circulating ANP was observed, but any explanation for this must remain speculative as measurements of blood pressure and renal function were not made in this study. It is possible that a reduction in circulating volume has occurred after SCH 42495, which would reduce secretion of ANP and offset any rise in plasma ANP caused by reduced clearance; alternatively the lack of an

Fig. 3. Computer-generated images of autoradiographs of $^{125}$I-labelled RB104 binding to rat kidney. Black represents a high level of binding and grey low to undetectable levels of binding. The autoradiographs represent specific NEP binding (A) and NEP inhibition after oral SCH 42495 (B). Non-specific binding was not detectable and is not shown.
Fig. 4. Quantitative values obtained from computer analysis of the autoradiographs shown in Figure 3. Renal NEP was significantly inhibited after oral SCH 42495 by doses of 3 mg/kg and above (P<0.001).

increase in plasma ANP may reflect greater occupancy of the clearance receptor, which would mask any increase in circulating ANP due to NEP inhibition.

A review of the literature shows that NEP inhibitors have variable effects on plasma ANP levels in normotensive animals. Reports in rats [28, 29], sheep [8] and man [30, 31] showed no effect of NEP inhibitors on plasma ANP levels, despite their producing biological responses; other studies show a clear rise in plasma ANP with NEP inhibition in rats [9], sheep [32, 33] and man [17, 34, 35]. A more consistent increase in plasma ANP has been observed when NEP inhibitors are given in the setting of elevated circulating ANP levels.

However, even in normotensive animals, the biological consequences of NEP inhibition persist well beyond any effect on plasma ANP [31, 32], suggesting that inhibition of tissue NEP and consequent changes in tissue levels of ANP may play a role in
the mechanism of action of the NEP inhibitors. If NEP inhibition does provide a chronic increase in ANP tissue bioactivity this could form the basis of a novel approach to the treatment of cardiovascular disease. Certainly, chronic treatment with SCH 42495 in spontaneously hypertensive rats caused regression of left ventricular hypertrophy despite no change in blood pressure or circulating ANP [11]. This result suggests that ventricular NEP inhibition and local ANP accumulation may counteract the growth-promoting effects of angiotensin II. By relating the degree of tissue inhibition to physiological effects it may be possible to determine the role of local natriuretic peptide systems in the beneficial effects of NEP inhibitors.

This work did not assess whether NEP is differentially inhibited in different tissues, nor whether the pattern of NEP inhibition differs with chronic administration or with different NEP inhibitors, but such studies are important. Differential tissue accessibility by different NEP inhibitors may explain the variability in their efficacy.

In summary, the NEP inhibitor SCH 42495 causes a rapid and prolonged suppression of renal NEP, with no inhibition of the circulating or the tissue renin–angiotensin system. The radiolabelled NEP inhibitor 125I-labelled RB104 can be used for in vitro autoradiography to map the anatomical localization of NEP in the kidney and other tissues, as well as in radioligand binding assays. As NEP inhibitors are under active investigation as new therapeutic agents in hypertension and heart failure, the renal and extra-renal sites of action of NEP inhibitors require clarification. The techniques described in this paper can be used to assess the degree of NEP inhibition in various tissues after administration of NEP inhibitors, which may prove useful in determining their mechanism of action.

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REFERENCES