Delayed metabolism of human brain natriuretic peptide reflects resistance to neutral endopeptidase

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Abstract

Metabolism of natriuretic peptides is regulated by two degradative pathways: uptake by the clearance receptor (natriuretic peptide receptor C – NPR–C) and hydrolysis by neutral endopeptidase (NEP). Affinity studies favour a dominant role of NPR–C in hormone degradation in several species but do not account for the efficacy of NEP inhibitors *in vivo*, nor the uniquely prolonged half life ($t_{1/2}$) of human brain natriuretic peptide (hBNP). Postulating that (1) delayed metabolism of hBNP reflects resistance to NEP and (2) interactions between NPR–C and NEP increase enzyme activity, we have used purified ovine and human NEP, plus ovine lung plasma membranes to study the relative importance of receptor and enzyme pathways. We have also related the findings to hormone metabolism

in vivo. Binding affinities of atrial natriuretic peptide (ANP), hBNP and ovine BNP (oBNP) to oNPR–C were similar ($K_d=8\text{--}16\text{ pM}$). In contrast, unlike ANP and oBNP, hBNP was not significantly degraded by purified oNEP or plasma membranes. Despite similar (and high) affinity of oNPR–C for oBNP and hBNP, the $t_{1/2}$ of hBNP (12.7 min) was more than fourfold that of oBNP (2.6 min). Although we found no evidence for receptor–enzyme interaction, our results show that the delayed metabolism of hBNP reflects resistance to NEP. These findings have important implications for future treatment strategies in human disease.

Journal of Endocrinology (2000) **167**, 239–246

Introduction

The natriuretic peptides atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are circulating hormones of cardiac origin involved in the regulation of extracellular fluid and blood pressure homeostasis (Espiner *et al.* 1995). Several effects of ANP and BNP, including natriuresis, vasodepression, inhibition of renin and aldosterone and shrinkage of plasma volume, are related to the natriuretic peptide hormone concentration in blood, which is itself the net outcome of cardiac secretion and disappearance from plasma (Espiner *et al.* 1995). Regulation of natriuretic peptide secretion from the heart has been studied extensively but much less is known of the mechanisms affecting disappearance of these hormones from the circulation. Total body clearance of ANP or BNP appears to be determined by two major pathways: binding and internalisation via the non guanyl cyclase linked natriuretic peptide receptor C (NPR–C) (Maack *et al.* 1993) and enzymatic degradation by the extracellular membrane-bound metalloproteinase neutral endopeptidase (NEP) EC 24.11 (Kenny *et al.* 1993). The relative importance of these two degradative pathways is

controversial (Espiner *et al.* 1995). Early studies in rats on ANP disappearance from plasma (Okolicany *et al.* 1992) showed that the clearance receptor pathway was the most important degradative route since not only was there an excess of spare (unoccupied) receptors at physiological plasma concentrations (Maack *et al.* 1993) but, more importantly, the affinity of ANP for NPR–C (affinity constant, K_d , picomolar) (Suga *et al.* 1992), was several orders of magnitude greater than that reported for the purified NEP (K_m or K_i , micromolar) extracted from tissues (Kenny *et al.* 1993). Surprisingly, however, specific inhibitors of NEP increase endogenous levels of ANP in several species (Ruskoaho 1992), suggesting an important role for the enzyme in the regulation of natriuretic peptides even at physiological concentrations. How the enzyme impacts on the regulation of natriuretic peptides, in view of the above affinities, is quite unexplained.

Despite the physiological importance (Espiner *et al.* 1995) and predictive value (Richards *et al.* 1999) of BNP in human cardiovascular disorders, the metabolism of BNP has been even less well studied than that of ANP. What is clear, however, is the uniquely prolonged disappearance rate ($t_{1/2}$) of human BNP-32 (hBNP) from plasma in

humans (Holmes *et al.* 1993), compared with the uniformly rapid clearance of other natriuretic peptides in humans and in other species (Ruskoaho *et al.* 1992, Charles *et al.* 1996a), findings which remain unexplained. The aim of the present study was therefore to explore mechanisms accounting for the uniquely delayed metabolism of hBNP. Although not studied previously there is circumstantial evidence that the difference in the metabolism of species-specific BNP forms *in vivo* reflects the summative effects of receptor binding and enzyme hydrolysis (Charles *et al.* 1996b, Rademaker *et al.* 1997). Concerning the former, the affinity of NPR-C for BNP is predicted by the amino acid sequence of the receptor: Ileu at position 188 (as in hNPR-C) reduces the affinity for hBNP (but not for ANP or ovine/porcine BNP) when compared with rat NPR-C where alanine substitutes for Ileu (Engel *et al.* 1994). These findings may explain in part the shorter $t_{1/2}$ of ovine BNP (oBNP) in humans (McGregor *et al.* 1990) when compared with hBNP. Whether variations in the affinity of the enzyme for BNP forms (or turnover rate) affect half life is unknown. Compared with oBNP, hBNP is a less favoured substrate for both porcine (Kenny *et al.* 1993) and human (Watanabe *et al.* 1997) endopeptidase, raising the possibility that the uniquely prolonged $t_{1/2}$ of hBNP in humans reflects lower affinities for both NPR-C and enzyme. Because in sheep oBNP is rapidly metabolised (Charles *et al.* 1996a) – suggesting the presence of Ala at 188 in the ovine NPR-C, as in rat – we predicted that comparative studies of BNP metabolism in sheep may distinguish the separate effects of receptor and enzyme. Accordingly we have used purified ovine and human NEP, and freshly prepared cell membranes from ovine lungs – tissue known to be important in natriuretic peptide clearance and metabolism (Bates *et al.* 1989, Hollister *et al.* 1989) – to characterise the relative importance of the receptor and enzymatic pathways, and to relate the findings to the disappearance of ANP/BNP from plasma *in vivo* in two species (human and sheep) exhibiting differing patterns of BNP metabolism. We have also tested the hypothesis that interactions occur between the receptor and enzyme such that the hormone is more effectively cleaved by NEP once bound to NPR-C. Our findings show for the first time that the uniquely delayed metabolism of hBNP is in fact related to its resistance to the enzyme. No support for receptor–enzyme interaction was found. Taken together, these findings have important implications for future treatment strategies enhancing BNP bioactivity in humans.

Materials and Methods

Reagents

Tris (Tris (hydroxymethyl) methylamine), TFA (trifluoroacetic acid) and sucrose were purchased from BDH

Chemicals (Palmerston North, New Zealand). ANP, hBNP (human BNP-32), oBNP (=porcine BNP-26), C-ANP(4–23) (Des[Gln¹⁸, Ser¹⁹, Gly²⁰, Leu²¹, Gly²²]-ANF 4–23-NH₂ (rat)) and phosphoramidon were purchased from Peninsula Laboratories, San Carlos, CA, USA. Leupeptin, phenylmethylsulphonyl fluoride (PMSF) and polyethylenimine were purchased from Sigma Chemicals, St Louis, MO, USA. SCH39370 and SCH32615 were kindly provided by Schering Plough, Bloomfield, NJ, USA.

Preparation of ovine lung plasma membranes

Ovine lung membranes were prepared according to the method of Maeda (Maeda *et al.* 1983). In brief, tissue was homogenised in two volumes (wt/vol.) of ‘homogenisation buffer’ (50 mM Tris–HCl, 0.15 M NaCl, 0.1 mM PMSF, 5 mM MgCl₂, 1 µg/ml leupeptin; pH 7.4), centrifuged at 9500 g and the supernatant then layered over a sucrose pad (41%) and centrifuged (8500 g) for 1 h. The membrane layer at the interface was then aspirated, centrifuged at 140 000 g and the resulting pellet re-suspended. After further washes the product was suspended in ‘homogenisation buffer’, aliquoted and stored at –80 °C. Purification of the lung membrane preparation was monitored by measuring the specific activity of plasma membrane markers (NEP and angiotensin converting enzyme (ACE)), and glucose 6-phosphatase as a marker of endoplasmic reticulum. From crude homogenate to final purified membrane preparation, there was a fourfold increase in ACE, a 13-fold increase in NEP and a fall to zero (undetectable) in glucose 6-phosphatase.

Natriuretic peptide receptor assay

Lung membranes (0.02 mg protein/ml final concentration) were equilibrated for 20–24 h at 4 °C with 15 000 c.p.m. ¹²⁵I-ANP and unlabelled peptide ANP, hBNP, oBNP, C-ANP(4–23), each 5–15 000 pg in radio-receptor assay buffer (‘homogenisation buffer’ with 2×10^{-5} M phosphoramidon and 0.1% BSA). Bound radioactivity was collected onto polyethylenimine-treated glass fibre paper (Whatman GF/C paper pre-soaked in 1% polyethylenimine for 120 min), washed with 0.15 M NaCl, and counted in a gamma counter. Non-specific binding was measured in the presence of an excess of ANP (15 000 pg). Displacement curves were fitted, and K_d values calculated, using the computer program LIGAND (Munson & Rodbard 1980). A single receptor site analysis gave the best fit and was used for determining all K_d values.

Monoclonal antibody to NPR-C

Purified monoclonal antibody (Porter *et al.* 1993) (CR.203.5A, Scios Nova, CA, USA) to the hNPR-C was

donated by Dr Gordon Porter, Scios Nova, Mountain View, CA, USA. Antibodies were raised using a recombinant vaccinia virus expressing hNPR-C cDNA, and assessed for ability to inhibit ^{125}I -ANP binding to a purified preparation of truncated hNPR-C ($\text{IC}_{50} = 4.7 \times 10^{-5} \text{ mg/ml}$).

Purified endopeptidase 24.11

Purified preparations of ovine and hNEP from kidney extracts (Kittelberger & Neale 1990) were kindly donated by Drs Paul Davis and R Kittelberger, Wellington School of Medicine, Wellington, New Zealand. These preparations gave single bands on gel electrophoresis and both enzymes were recognised by antiserum to NEP. Further studies in our laboratory revealed that both enzyme preparations exhibited NEP activity when measured in our NEP assay (Yandle *et al.* 1992) and were inhibited by specific inhibitors of NEP, including phosphoramidon, SCH 39370 (Yandle *et al.* 1992) and SCH32615 (Charles *et al.* 1996b).

HPLC analysis

Incubates were centrifuged and the supernatant injected on to a 22.5 cm Brownlee RP300 cartridge column at 40 °C with a flow rate of 1 ml/min. Peptides were eluted with a gradient of 15–45% acetonitrile in 0.1% TFA over 30 min. Eluted peptides were detected using u.v. absorbance at 210 nm. Where ^{125}I -ANP was included, the eluate was also collected in fractions (0.5 ml) which were counted in a gamma counter.

Hydrolysis of ANP by NEP, and influence of receptor occupancy

The rate of hydrolysis of ANP by intact lung membranes was measured in the presence of substrate concentrations close to the enzyme K_m . Degradation rates were determined after HPLC from the reduction of intact ^{125}I -ANP radioactivity, and by reduction in the peak area of intact unlabelled ANP after separation from metabolites using HPLC with u.v. absorbance. Experimental conditions were established to achieve linear degradation rates of ANP within the incubation period. In brief, lung membrane preparations (0.003–0.02 mg protein/ml) were incubated at 37 °C in incubation buffer (50 mM Tris HCl, 0.15 M NaCl, 5 mM MgCl_2 , 0.1% BSA, pH 7.4) containing unlabelled ANP (1–150 μM) and labelled ANP in constant ratio ($25 \times 10^6 \text{ c.p.m./}\mu\text{M}$). After 30 min incubation, the reaction was stopped by the addition to incubates (100 μl) of 20 μl of 0.3% TFA in 12% acetonitrile, after which solutions were frozen immediately and stored at –80 °C. Degradation of ANP due to enzymes other than NEP was negligible (<2%), as

demonstrated in control incubations in the presence of phosphoramidon, and no hydrolysis was observed in the absence of plasma membranes.

The extent of hydrolysis at each peptide concentration was limited to <20% except in the instance of the lowest concentration (1 μM ANP) where hydrolysis was <28%. The decrease in ANP under the above conditions (as observed by HPLC analysis) was linear over the time period examined (30 min, $r = -0.99$). The K_m for ANP was calculated from disappearance of the ANP peak as assessed by HPLC using linear regression analysis of $1/V$ vs $1/s$ plots where V is the degradation rate and s is the substrate concentration.

Comparative rates of natriuretic peptide degradation

Comparative degradation rates of the natriuretic peptides (oBNP, hBNP and ANP) were determined by incubating the peptides with ovine lung membrane preparations as well as with purified forms of sheep and human NEP. Peptides (10 μM) were incubated with lung membrane preparations or with purified enzyme in incubation buffer as described above. The concentration of NEP in each of the above preparations was standardised according to activity as measured by a fluorometric assay (Yandle *et al.* 1992). After timed incubations (ANP 30 min, hBNP 120 min, oBNP 60 min at 37 °C), the incubation reaction was stopped by the addition of 0.3% TFA in 12% acetonitrile, and samples stored at –80 °C prior to HPLC analysis as described above. Specificity of the enzyme hydrolysis was established using phosphoramidon in 'control' incubations for each peptide studied and hydrolysis solely due to NEP was calculated by subtraction. A NEP unit was defined as the amount of NEP needed to release 1 nM/ml per min of free amidomethylcoumarin (AMC) from the synthetic substrate glutaryl-Ala-Ala-Phe-AMC.

Disappearance of BNP from plasma in vivo

Two 50 kg Coopworth ewes were surgically prepared with a Swan–Ganz catheter. The sheep received infusions of both hBNP and o (porcine) BNP (Bachem Torrance CA, USA) with a 2 h 'washout' between infusions. We have previously shown that the 26 amino acid sequence of oBNP is identical to porcine BNP (Aitken *et al.* 1994). Infusions of both hBNP and oBNP were administered at a dose of 3.2 pM/kg per min for 120 min via the distal (pulmonary artery) port of the Swan–Ganz catheter. Administered doses were confirmed by assay of an aliquot of the infusate. Blood samples (10 ml) were drawn from the proximal port 15 min before and immediately prior to commencement of infusions, at 15 min intervals throughout infusion, and then at 1 min intervals for 10 min immediately post-infusion and 12.5, 15, 20, 25, 30, 45 and 60 min post-infusion. Samples were drawn on ice, centrifuged and plasma stored at –80 °C prior to assay for

Figure 1 Inhibition of binding of radiolabelled ANP in ovine lung membranes by ANP ($\blacktriangle$) and the NPR-C specific ligand C-ANP(4-23) ($\square$).

hBNP (Yandle *et al.* 1993) and oBNP (Charles *et al.* 1996a). No significant cross-reactivity of either human or oBNP was detected in ovine or human assays respectively.

Single exponential decay curves ($f(x) = ae^{-bx} + c$) were fitted to the data and the half life calculated from $t_{1/2} = 0.69/b$.

All experiments on animals or animal tissues were undertaken after approval by the Christchurch School of Medicine Animal Ethics Committee (Christchurch, New Zealand).

Results

Receptor binding

After 24 h incubation at 4 °C, net binding of ^{125}I -ANP in lung membrane preparations averaged 26% and 6% in the presence and absence of phosphoramidon respectively. HPLC analysis of incubation products confirmed that labelled ANP was >90% degraded in the absence of phosphoramidon, and that degradation was prevented by its inclusion. All binding studies were therefore undertaken in the presence of phosphoramidon.

As shown in Fig. 1 addition of the specific NPR-C ligand, C-ANP(4-23) progressively reduced the net binding of ^{125}I -ANP. Approximately 70% displacement of labelled ANP was observed at C-ANP(4-23) concentrations of 1000 pM/l. Similarly, the addition of monoclonal antibody to hNPR-C greatly reduced net

Figure 2 Inhibition of binding of radiolabelled ANP by ovine (porcine) BNP ($\bullet$), ANP ($\blacktriangle$) and human BNP ($\blacksquare$) in ovine lung membranes.

binding from 29% (control) to 9% (data not shown), whereas a non-immune extract had no effect. Taken together these findings indicate that 70% of the binding of labelled ANP in the ovine lung membrane preparation is attributable to NPR-C receptors.

Representative binding displacement curves for ANP, hBNP and oBNP are shown in Fig. 2. Displacement curves (undertaken on three different occasions) were similar for all three peptides which completely displaced labelled ANP at high concentrations. Calculated K_d values were 8, 10 and 16 pM respectively for hBNP, ANP and oBNP.

NEP

Assay conditions were established that gave linear reaction rates over the substrate concentration ranges used, and reaction velocities (calculated on the basis of decrease in radioactivity associated with the ANP peak), were in close agreement with values obtained from u.v. absorption measurements. Interference by receptor binding in these determinations is unlikely as the substrate (ANP) concentrations (1–150 μM) – at which the K_m measurement was made – were more than sufficient to saturate NPR-C receptors (ANP concentration 10^6 fold that of the receptor K_d concentration).

The K_m for the ovine enzyme acting on ANP in intact lung membranes was 10 μM , similar to that reported for the solubilized enzyme (Kenny *et al.* 1993), and therefore

Table 1 Relationship of $t_{1/2}$ to NEP and NPR-C characteristics in sheep and humans

	Sheep			Humans		
	NEP ^a (pmol/min)	NPR-C ^b (k_d , pM)	$t_{1/2}$ (min)	NEP ^a (pmol/min)	NPR-C ^c (k_d , pM)	$t_{1/2}$ (min)
ANP	0.24	10	3	0.48	10	3.1
oBNP	0.08	16	2.6	0.20	11	3.1
hBNP	0.01	8	12.7	0.02	140	22

^aDegradation rate per unit of purified enzyme.^bMeasured in plasma membranes from sheep lung.^cMeasured in human vascular smooth muscle cells (taken from Suga *et al.* 1992).The half lives ($t_{1/2}$) in sheep have been taken from the current study (oBNP and hBNP) and Charles *et al.* 1996a (ANP), and in humans from Yandle *et al.* 1986 (ANP), McGregor *et al.* 1990 (oBNP) and Holmes *et al.* 1993 (hBNP).

was inconsistent with a preferential action of NEP on the receptor-bound hormone.

Comparative rates of natriuretic peptide degradation

The exceptionally small amounts of purified ovine and human NEP available precluded full determination of K_{cat} and K_m . Measurement of the enzyme degradation rate on standardised preparations, however, enabled a direct comparison between the ovine and human enzyme. When peptides were incubated with purified sheep NEP or human NEP, rates of degradation of hBNP differed markedly from those of ANP or oBNP (Table 1). Human BNP was not significantly degraded by either preparation of NEP. Similar findings were obtained with oNEP purified from sheep lung membranes. Absolute and relative degradation rates in sheep membrane preparations for ANP (0.53 pmol/min), oBNP (0.10 pmol/min) and hBNP (0.02 pmol/min) were in close agreement with those observed with the purified enzyme. ANP was consistently degraded more rapidly than oBNP by sheep NEP and by the human purified enzyme. These findings indicate that the unique structure of hBNP rather than the species form of the enzyme confers resistance to hydrolysis by NEP.

Disappearance of BNP from plasma in vivo

The disappearance rate ($t_{1/2}$) of oBNP was 3.0 min and 2.1 (median 2.6) min in two different sheep and similar to that previously reported in sheep (Charles *et al.* 1996a). In the same two sheep, $t_{1/2}$ of hBNP was 13.8 min and 11.5 (median 12.7) min respectively (Fig. 3, Table 1). As previously reported (McGregor *et al.* 1990), $t_{1/2}$ of oBNP in humans was 3.1 min.

Discussion

BNP secretion, a product largely of the ventricles, has been the focus of increasing interest for several reasons.

First, studies using rat cardiomyocytes show that BNP gene transcript activation and turnover are rapid (Hanford *et al.* 1994, Magga *et al.* 1997), suggesting that in contrast to ANP, BNP functions as a rapid response gene and may have unique paracrine functions. Increases in BNP transcripts in the human ventricle of patients with congestive heart failure or cardiac hypertrophy (Gerbes *et al.* 1994) exceed those of ANP. Further, the increase in plasma BNP concentrations above resting levels are proportionally greater in subjects with heart failure or following myocardial infarction than those of ANP (Espiner *et al.* 1995). Recent studies from several laboratories indicate that plasma BNP better reflects the severity of left ventricular dysfunction (Richards *et al.* 1999) as well as prognosis after myocardial infarction (Richards *et al.* 1998). Biological activity of BNP in humans is at least comparable to that of ANP, and in some studies more so (Pidgeon *et al.* 1996). Taken together these findings constitute compelling reasons for detailed study of BNP's metabolism, particularly since in humans the disappearance rate of hBNP from plasma is uniquely prolonged.

In previous *in vivo* studies in sheep (Charles *et al.* 1996a) we have shown that the $t_{1/2}$ of all three natriuretic peptides (ANP, oBNP and CNP) are similar (2–3 min) whereas in humans $t_{1/2}$ of hBNP (22 min) is some sevenfold greater (Holmes *et al.* 1993) than ANP, CNP (Espiner *et al.* 1995) or oBNP (McGregor *et al.* 1990). We reasoned that the uniquely prolonged half life of hBNP in humans may reflect a lower affinity of both NPR-C and NEP for hBNP, and have used the sheep (in which species endogenous natriuretic peptides are rapidly metabolised) to distinguish the separate contributions of enzyme and receptor to the metabolism of hBNP. Using ovine lung membranes and purified NEP we measured binding affinities and rates of hydrolysis of ANP and BNP forms, and related the findings to $t_{1/2}$ studies in sheep and humans. The binding affinity of ANP and oBNP for oNPR-C in lung membrane preparations was high (K_d 10–16 pM), and similar to that found for these hormones in rat, bovine and human tissue preparations of NPR-C (Suga *et al.* 1992). However, in contrast to the lower binding affinity

Figure 3 Disappearance of oBNP and hBNP from plasma in sheep 1 (left panels) and sheep 2 (right panels).

of hBNP for the hNPR-C (K_d 140 pM (Suga *et al.* 1992)), oNPR-C binds hBNP with high affinity (K_d 8 pM). These findings suggest that the oNPR-C retains an alanine residue at position 188, as reported for rat NPR-C (Engel *et al.* 1994). Interestingly, purified oNEP and ovine lung membrane NEP preparations both degraded ANP and oBNP but did not significantly degrade hBNP. The purified human enzyme (fully active against ANP and oBNP) also failed to degrade hBNP. From these findings we conclude that whereas in humans there is concordance in receptor and enzyme affinity (i.e. high affinity of both enzyme and receptor for ANP/oBNP, and low affinity of both for hBNP) this is not so in sheep where hBNP is bound with high affinity to oNPR-C yet is not a good substrate for ovine NEP. These combinations of findings have enabled us to interpret the separate effects of NPR-C and NEP *in vivo* for the first time. As shown in Table 1, whereas $t_{1/2}$ of ovine natriuretic peptides in sheep is short (2–3 min), the $t_{1/2}$ of hBNP is some fourfold increased. Our previous studies in humans showed a similar $t_{1/2}$ for ANP and oBNP (3 min) but a prolonged $t_{1/2}$ (22 min) for hBNP.

Thus whereas in the human the lower affinity of NPR-C and NEP for hBNP both contribute to the much prolonged $t_{1/2}$ of hBNP, the current data suggests that despite high affinity of oNPR-C for hBNP the reduced ability of NEP to hydrolyse hBNP is associated with a relatively prolonged $t_{1/2}$ of hBNP *in vivo*. In fact, the $t_{1/2}$ for hBNP in sheep (12.7 min) lies midway between that of oBNP (2.6 min) and hBNP in humans (22 min), indicating that both degradative systems have the potential to participate equally in natriuretic peptide metabolism. Our findings also predict that the natriuretic peptide response to NEP inhibition will differ in the two species. This has recently been confirmed *in vivo* where, in contrast to the proportionate and equal response of both plasma ANP and BNP in sheep (Charles *et al.* 1996b, Rademaker *et al.* 1997), the response of plasma BNP to NEP inhibition in humans is much less than that of ANP (Lainchbury *et al.* 1999).

A second and related aim of our study was to examine the possibility of interactions between receptor and enzyme which, by increasing efficacy of NEP in tissues, may account for the unexpected activity of NEP inhibitors

in vivo (Charles *et al.* 1991, Richards 1994). For example, local interaction between receptor and enzyme at the cell membrane – as reported to occur in the instance of substance P (Okamoto *et al.* 1994) – could make receptor-bound hormone more susceptible to hydrolysis by NEP. Using sheep lung membranes (containing both high concentrations of NPR-C and an active NEP system) we found no evidence for such an interaction. Thus under conditions where NPR-C receptors were fully saturated, the K_m of the enzyme (10 μ mol) was comparable to that (30 μ mol) determined for the solubilised enzyme (Kenny *et al.* 1993). While not excluding an interaction between the receptor (including NPR-A) and enzyme in whole cell preparations, or an effect on catalytic rate, our findings point to other mechanisms. For example, preferential access of circulating hormone to NEP in the vascular endothelium (Llorens-Cortes *et al.* 1992) may help to explain the efficacy of NEP inhibitors *in vivo*.

Taken together, the above findings have important implications for treatment strategies aimed at increasing plasma BNP bioactivity in humans. In particular, our findings indicate that the highly effective combination of NPR-C and NEP blockade in sheep (Rademaker *et al.* 1997) – inducing marked increases in both endogenous ANP and BNP and consequential biological effects – would not be expected in humans. For these reasons it will be important in humans to focus on other therapeutic strategies independent of alterations in NEP activity, for example measures exploiting the enhanced activity of hBNP analogues (Schoenfeld *et al.* 1997).

Acknowledgements

We thank Dr Gordon Porter, Scios Nova Inc., Mountain View, California for the monoclonal antibody to the human NPR-C, and Dr Paul Davis, School of Medicine, Wellington, New Zealand for gifts of purified renal endopeptidase. This work was supported by grants from the Health Research Council of New Zealand, the National Heart Foundation of New Zealand and the New Zealand Lottery Grants Board.

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Received 21 March 2000

Accepted 28 June 2000