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Clearance of brain natriuretic peptide in patients with chronic heart failure: indirect evidence for a neutral endopeptidase mechanism but against an atrial natriuretic peptide clearance receptor mechanism

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1. Brain natriuretic peptide is a new natriuretic hormone with striking similarity to atrial natriuretic peptide, but there are no previous data concerning its clearance in man. Two pathways of clearance for atrial natriuretic peptide are recognized: degradation by neutral endopeptidase and binding to atrial natriuretic peptide clearance receptors. We have examined the effect of candoxatril, an inhibitor of neutral endopeptidase (dose range 10–200 mg), and the effect of an infusion of a pharmacological dose [45 μg (90 μg in two patients)] of synthetic human atrial natriuretic peptide on plasma human brain natriuretic peptide-like immunoreactivity levels in seven patients with mild to moderate chronic heart failure.

2. Plasma human brain natriuretic peptide-like immunoreactivity levels were elevated in all patients (mean ± SEM 22.0 ± 6.2 pmol/l) compared with healthy control subjects (1.3 ± 0.2 pmol/l, $n = 11$).

3. In all patients, candoxatril increased both plasma atrial natriuretic peptide ($P < 0.05$) and plasma human brain natriuretic peptide-like immunoreactivity ($P < 0.05$) levels.

4. By contrast, an exogenous infusion of atrial natriuretic peptide had no effect on plasma human brain natriuretic peptide-like immunoreactivity levels despite increasing the plasma atrial natriuretic peptide concentration to 424 ± 74 pmol/l, which is a level of atrial natriuretic peptide which would have 'swamped' all atrial natriuretic peptide clearance receptors.

5. We have therefore shown that plasma human brain natriuretic peptide-like immunoreactivity levels in chronic heart failure are increased by a neutral endopeptidase inhibitor, but are unchanged by an exogenous infusion of atrial natriuretic peptide. Our results suggest that in patients with chronic heart failure, degradation by neutral endopeptidase is an important pathway for clearance of brain natriuretic peptide. By an indirect approach, we did not find any evidence of a role for atrial natriuretic peptide clearance receptors in the metabolism of brain natriuretic peptide in these patients. Although this is in agreement with work *in vitro*, there could be alternative explanations for the lack of a change in circulating human brain natriuretic peptide-like immunoreactivity during exogenous administration of atrial natriuretic peptide.

INTRODUCTION

Brain natriuretic peptide (BNP) is a new natriuretic peptide which was first isolated from porcine brain [1]. BNP has striking similarity with atrial natriuretic peptide (ANP) both in amino acid sequence and in biological actions [1–3] and has either 26 or 32 amino acid residues: porcine (p) BNP-26 and pBNP-32, respectively [4]. BNP is also synthesized in, and secreted into the circulation from, the porcine heart [5]. Rat BNP (rBNP), a 45-amino acid residue peptide, has also been isolated from the heart [6].

Until recently, information on BNP in man has been scarce, mainly because human BNP (hBNP) immunoreactivity was not detected with early antisera which were raised against pBNP or rBNP. However, the complementary DNA sequence encoding for the hBNP precursor, hBNP (1–108), was recently elucidated [7]. Since then hBNP has been isolated from the human atrium and found to comprise 32 amino acid residues, which are identical to the sequence (77–108) of the hBNP precursor. Furthermore, hBNP-like immunoreactivity (hBNP-li) has now been detected in plasma [8, 9] and found to be grossly elevated in patients with chronic heart failure [9]. The control of synthesis and the biological actions of BNP have been studied in animals [1–3], but as yet there is no information about the clearance of BNP in man. We have therefore conducted a study to examine the clearance of BNP in patients with chronic heart failure.

Two important pathways of clearance for ANP are recognized: binding to ANP 'C' or clearance (non-guanylate cyclase-linked) receptors and degradation by the enzyme neutral endopeptidase (EC 3.4.24.11, endo-

Key words: atrial natriuretic peptide, brain natriuretic peptide, congestive heart failure, endopeptidase 24.11.
Abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; h, human; hBNP-li, human brain natriuretic peptide-like immunoreactivity; p, porcine; r, rat.
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peptidase 24.11, atriopeptidase) [10, 11]. Our study had two parts: the first part was designed to examine the role of neutral endopeptidase in BNP clearance, and the second part examined the role of ANP clearance receptors in BNP clearance. In the first part, we determined whether candoxatril (UK-79,300; Pfizer Central Research, Sandwich, Kent, U.K.) altered plasma hBNP-li levels in patients with chronic heart failure. Candoxatril is an orally active pro-drug and is the indanyl ester of candoxatrilat (UK-73,967), the active enantiomer of UK-69,578. Candoxatrilat is a specific inhibitor of neutral endopeptidase. To examine the role of clearance receptors in BNP clearance in patients with chronic heart failure, one would ideally examine the effect of the administration of a clearance receptor ligand on plasma hBNP-li levels in these patients. However, because of ethical constraints regarding the administration of a clearance receptor ligand in these patients, we have had to resort to an indirect approach to address this issue. Therefore in the second part, we have examined the effect of raising plasma ANP levels by an infusion of exogenous ANP on plasma hBNP-li levels in patients with chronic heart failure. We raised plasma ANP levels so that ANP clearance receptors were fully occupied. A subsidiary aim of the second part of the study was to examine whether there is a negative feedback mechanism whereby ANP inhibited BNP release. It was recently postulated that the myocardial ANP clearance receptors might mediate a negative feedback inhibition of ANP release [12].

METHODS

Patients and protocol

Seven male patients (age 65 ± 2 years, mean ± SEM) with stable and treated mild to moderate chronic heart failure (New York Heart Association II–III) were studied on 4 days at least 4 days apart. All had impaired left ventricular function (mean radionuclide ejection fraction 35%, range 22–44%) and reduced exercise capacity and were on regular anti-cardiac failure therapy: diuretics (six), angiotensin-converting enzyme inhibitors (five), long-acting nitrates (five) and calcium antagonists (four). All gave their voluntary written informed consent before participation in this investigation, which was approved by the hospital ethical committee.

On arrival at the clinical laboratory at 08.30 hours after an overnight fast, a standard light breakfast was provided. The patients' daily morning medication was withdrawn on the day of the study. Patients were requested to rest in the semi-recumbent position and an intravenous cannula was inserted in each forearm for infusion and venous sampling. After an hour's rest (0 min), each patient swallowed candoxatril tablets in doses of 10 mg, 50 mg or 200 mg or placebo in a single-blind randomized manner. Blood samples were taken immediately before dosing (0 min) and at + 120 min after dosing.

On the placebo investigational day alone, patients were studied for a further 30 min when an infusion of 45 μg of synthetic human (h) ANP (99–126) (Bissendorf GmbH, Wedemark, F.R.G.) (90 μg in two patients) was commenced at + 120 min and continued until + 150 min. Blood samples were taken at + 120 min, + 135 min and at + 150 min. Heart rate and blood pressure were measured at frequent intervals using a semi-automatic sphygmomanometer (Dinamap Vital Signs Monitor 1846; Critikon, Tampa, FL, U.S.A.).

Sample collection and analysis

Venous blood was collected into chilled tubes (volume, preservative) for measurement of plasma hBNP-li and ANP levels [5 ml, EDTA (potassium salt) and 4000 kallikrein inhibitory units of aprotonin (Trasylol; Bayer, U.K. Ltd, Newbury, Berks, U.K.)], plasma renin activity [5 ml, EDTA (potassium salt)] and plasma aldosterone level (5 ml, lithium heparin). All samples were placed on ice, were centrifuged at 4°C and the plasma was separated off and stored at −20°C until assayed. All samples were measured together, in one assay run, for any given variable.

Plasma hBNP-li was measured by r.i.a. as previously described [13], after plasma extraction, using a commercially available r.i.a. kit (Peninsula Laboratories Inc, Belmont, CA, U.S.A.). Plasma samples (1 ml) were acidified with 1 ml of 0.1% trifluoroacetic acid and passed through Sep-Pak C_8 cartridges (Amersham International Ltd, Amersham, Bucks, U.K.). The cartridges were pre-activated with buffer (4 ml) containing 60% acetonitrile/0.1% trifluoroacetic acid and buffer containing 0.1% trifluoroacetic acid. After each aliquot the cartridges were washed with 0.1% trifluoroacetic acid (10 ml) and the BNP was eluted with 3 ml of 60% acetonitrile/0.1% trifluoroacetic acid solution. The eluate was evaporated under vacuum and the precipitate was resuspended in 250 μl of BNP assay buffer.

The anti-hBNP-32 antibody used was generated against synthetic hBNP-32 (Peninsula Laboratories). The antibody showed the following cross-reactivity: hBNP-32, 100%; rBNP-32, 0.04%; rBNP-45, pBNP-26, 0%; α-ANP (1–28), 0%; angiotensin II, endothelin-1 and vasopressin, 0%.

A portion (100 μl) of reconstituted BNP extract was incubated in duplicate with 100 μl of anti-hBNP-32 antibody, which had been reconstituted with 13 ml of r.i.a. buffer, at 4°C overnight. Then ^{125}I-hBNP-32 (between 10000 and 20000 c.p.m.) was added, mixed and incubated at 4°C overnight. Standard curves were constructed with standard hBNP-32 (Peninsula Laboratories) in r.i.a. buffer over the concentration range 0.5–128 pg/tube. Precipitation was with 100 μl of diluted goat anti-rabbit IgG serum and 100 μl of diluted normal rabbit serum. Precipitates were allowed to form for 2 h at room temperature before 0.5 ml of r.i.a. buffer was added. The pellets were separated by centrifugation at 1700 *g* for 20 min, supernatant was removed and the radioactivity in the pellets was counted in a LKB Clini Gamma counter. The plasma hBNP-li level was estimated by direct comparison with the standard curve and was corrected for percentage extraction. The intra-subject and inter-subject coefficients

of variation for this assay were 5.8% and 14.8%, respectively. The percentage recovery of hBNP in this assay was 70.6%. The limit of detection for this assay was 0.5 pmol/l.

Plasma ANP was measured by r.i.a. after plasma extraction by the method of Richards *et al.* [14]. The intra-subject and inter-subject coefficients of variation for this assay were 4.9% and 7.8%, respectively. Plasma renin activity was measured by r.i.a. of angiotensin I generated by a 90 min incubation at 37°C using a commercially available kit (CIS U.K., High Wycombe, Bucks, U.K.). The intra-subject and inter-subject coefficients of variation were 4.4% and 9.8%, respectively. Plasma aldosterone was measured with a commercially available r.i.a. kit (Serono Laboratories Ltd, Woking, Surrey, U.K.). The intra-subject and inter-subject coefficients of variation for this method were 6.6% and 14.7%, respectively.

Statistical evaluation

All values are expressed as means ± SEM. All variables were analysed by analysis of variance, and both linear and quadratic dose–response relationships within the active doses were examined on a log-dose scale. Individual comparisons between active treatment and placebo were made using Dunnett's *t*-statistic [15]. A two-tailed *P* value of less than 0.05 was taken as significant.

RESULTS

All patients completed the study. The baseline plasma hBNP-li and ANP levels were 22.0 ± 6.2 pmol/l and 42.3 ± 16.1 pmol/l, respectively. In 11 healthy male subjects aged 20–23 years, the peripheral plasma hBNP-li and ANP levels were 1.3 ± 0.2 pmol/l and 5.6 ± 1.7 pmol/l, respectively. These plasma hBNP-li levels correspond well with two previously quoted values for BNP in normal subjects, 0.9 pmol/l and 1.5 pmol/l [8, 9]. The values in our patients with chronic heart failure also correspond well with those previously quoted for patients with chronic heart failure [9].

Effect of a neutral endopeptidase inhibitor

In all patients, candoxatril raised plasma ANP and hBNP-li levels (Fig. 1). There was no apparent dose–response relationship in these increments in plasma ANP and hBNP-li levels. Plasma renin activity tended to fall after both candoxatril and placebo, but no significant difference was observed (Table 1). Similarly, the plasma aldosterone level tended to fall after both candoxatril and placebo, but candoxatril at the highest dose (200 mg) caused a significant fall in the plasma aldosterone level ($P < 0.05$, Table 1).

In this short study, candoxatril had no effect on blood pressure even at the highest dose (mean arterial pressure: placebo, from 102 ± 6 mmHg to 101 ± 7 mmHg, not significant; candoxatril 10 mg, from 98 ± 6 mmHg to 98 ± 5 mmHg, not significant; candoxatril 50 mg, from 92 ± 4 mmHg to 98 ± 5 mmHg, not significant; candoxatril 200 mg, from 98 ± 5 mmHg to 98 ± 5 mmHg, not significant).

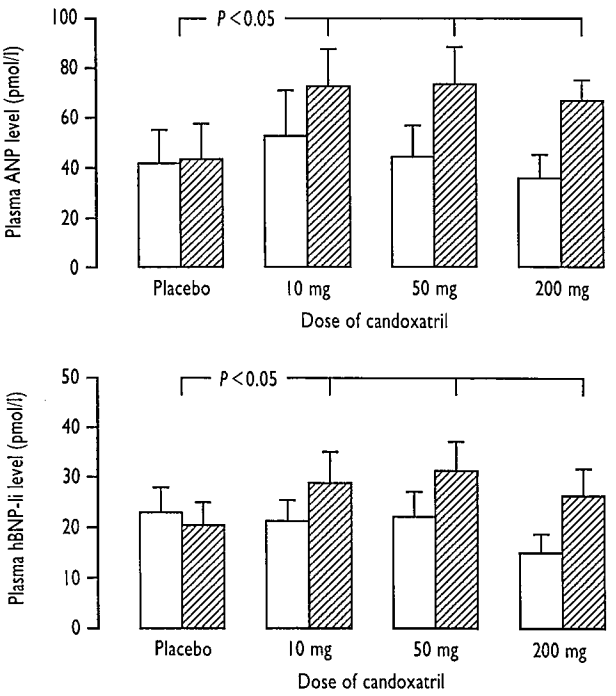

Fig. 1. Effect of candoxatril on plasma hBNP-li and ANP levels in patients with chronic heart failure. Plasma hBNP-li and ANP levels in patients with chronic heart failure ($n = 7$) are shown before (□) and 120 min after (▨) single oral doses of candoxatril (0 mg, 10 mg, 50 mg and 200 mg). Values are expressed as means ± SEM. In all patients, candoxatril raised plasma ANP ($P < 0.05$ versus placebo by analysis of variance) and hBNP-li ($P < 0.05$ versus placebo by analysis of variance) levels. There was no apparent dose–response relationship in these increments in plasma ANP and hBNP-li levels.

Effect of infusion of hANP (99–126)

Infusion of hANP (99–126) caused a significant rise in plasma ANP levels on the placebo study day (Fig. 2). However, infusion of hANP (99–126) did not alter plasma hBNP-li levels throughout the infusion period (Fig. 2).

As expected, infusion of hANP (99–126) caused a significant fall in the plasma aldosterone level (from 219 ± 33 to 185 ± 25 pmol/l, $P < 0.05$). Plasma renin activity was, however, unchanged by hANP (99–126) with values of 0.48 ± 0.12 ng s^{-1} l^{-1} before infusion and 0.45 ± 0.13 ng s^{-1} l^{-1} at the end of the infusion (not significant). Blood pressure remained unchanged during the infusion of hANP (99–126) (before infusion mean arterial pressure was 101 ± 7 mmHg and at the end of the infusion mean arterial pressure was 98 ± 4 mmHg).

DISCUSSION

This study had three major findings. First, we showed that plasma hBNP-li, like ANP, is elevated in patients with chronic heart failure. Secondly, we showed that can-

Table 1. Effects of candoxatril on plasma renin activity and plasma aldosterone level in patients with chronic heart failure ($n=7$). Blood samples were taken immediately before dosing and at +120 min after dosing with candoxatril. Statistical significance (Dunnet's t-statistic): $^*P<0.05$.

	Placebo	Candoxatril		
		10 mg	50 mg	200 mg
Plasma renin activity (ng s^{-1} l^{-1})				
Before candoxatril	0.56±0.12	0.67±0.08	0.63±0.14	0.56±0.04
After candoxatril	0.48±0.12	0.43±0.05	0.42±0.08	0.43±0.08
Plasma aldosterone level (pmol/l)				
Before candoxatril	255±30	341±108	385±91	383±88
After candoxatril	219±33	203±53	288±72	202±36*

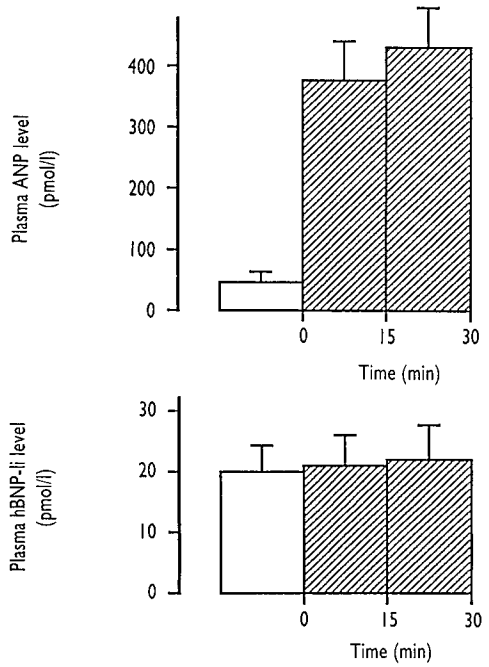

Fig. 2. Effect of exogenous ANP infusion on plasma hBNP-li and ANP levels in patients with chronic heart failure. Plasma hBNP-li and ANP levels in patients with chronic heart failure ($n=7$) are shown before (□) and during (▨) a 30 min infusion of 45 μg of synthetic ANP (99–126) (90 μg in two patients). Values are expressed as means±SEM. Infusion of hANP (99–126) caused a significant rise in plasma ANP levels ($P<0.001$ by analysis of variation). Infusion of hANP (99–126) did not alter plasma hBNP-li levels.

doxatril, a specific neutral endopeptidase inhibitor, not only increased plasma ANP levels but also plasma hBNP-li levels in patients with chronic heart failure. Finally, we showed that a pharmacological dose of exogenous infused hANP (99–126) does not alter plasma hBNP-li levels in these patients.

The clearance of BNP has been little studied. Because of its striking similarity in amino acid sequence to ANP, it is conceivable that its disposal may be similar to that of ANP. Two principal mechanisms have been suggested to be important in the clearance of ANP. First, ANP has been shown to be inactivated by enzymic cleavage through the zinc-containing enzyme neutral endopeptidase, which is present in the brush border of the proximal tubule of the kidney [11]. Secondly, it is believed that ANP binds to a non-guanylate cyclase-linked ANP receptor, the so-called ANP 'C' or clearance receptor, which appears to have a clearance role [10]. Renal cortical membranes with endopeptidase activity have been shown to cleave pBNP-26 at several different sites [16]. hANP (99–126), on the other hand, is cleaved only at one site, between cysteine-105 and phenylalanine-106. The results from our study suggest that neutral endopeptidase plays an important role in the metabolism of BNP. Because of its striking similarities to ANP in biological actions [1–3], it is conceivable that some of the natriuretic and haemodynamic effects of candoxatril [17] in chronic heart failure may be due to an increase in plasma BNP levels.

At a receptor level, BNP and ANP appear to interact with the same biologically active receptor in that the simultaneous addition of BNP and ANP at their maximally effective concentrations did not have an additive effect [18]. However, at the molecular level, two distinct biologically active and guanylate cyclase-linked ANP receptors have been identified, one (GC-A) responding equally to both ANP and BNP, the other (GC-B) being preferentially stimulated by BNP [19]. Our study does not address the question of how ANP and BNP interact with regard to these biologically active receptors. Our study is concerned with the ANP/BNP interaction at the ANP clearance receptor. In previous work, Maack *et al.* [10] infused a clearance receptor ligand into intact rats and found that the endogenous plasma ANP immunoreactivity increased with accompanying natriuresis. This leads to the proposition that the ANP clearance receptors are specific for ANP clearance, serving as a hormonal buffer system to regulate plasma levels of ANP. Our study is somewhat similar to that of Maack *et al.* [10], except, for ethical reasons previously alluded to, that we have infused exogenous hANP (99–126) instead of a clearance receptor ligand. We found that when ANP clearance receptors were occupied by exogenous ANP, endogenous hBNP-li levels did not change. The final plasma ANP level achieved at the end of the infusion of hANP (99–126) was 424±74 pmol/l (1314±229 pg/ml). The dissocia-

tion constant for ANP towards its ANP clearance receptor has been estimated by different workers to be between 1 and 100 pmol/l [20]. Therefore, our exogenous ANP infusion is liable to have occupied most available ANP clearance receptors, especially in view of previous data showing that, in chronic heart failure, ANP clearance receptors, at least in the platelet, have the same dissociation constant (10 pmol/l) as in normal subjects [21]. Therefore, our findings would suggest that ANP clearance receptors are of little or no importance in the clearance of BNP, at least in patients with chronic heart failure. However, it must be stressed that this is only indirect evidence and there may be alternative explanations for the lack of change in circulating hBNP-li during exogenous administration of ANP. First, it may be argued that, during exogenous ANP infusion, the activity of neutral endopeptidase may increase to compensate for the reduced number of available clearance receptors. However, one could then argue that the opposite did not occur in the first part of the study, i.e. when neutral endopeptidase was blocked, clearance receptors did not increase to compensate. Another possible explanation is that the affinity of clearance receptors for BNP is such that exogenous administration of ANP cannot displace BNP from the receptor. However, in a recent study *in vitro* Mukoyama *et al.* [22] have categorically shown that the binding affinity of hBNP for clearance receptors is 14 times lower than that of ANP. In fact, the same workers also showed that exogenously infused hBNP is cleared from the circulation more slowly than ANP, suggesting that BNP clearance may differ from that of ANP, and they attributed this to the lower binding affinity of hBNP for clearance receptors. Our results would support this, and leave open the possibility that there are separate BNP clearance receptors. Obviously any evidence for separate BNP clearance receptors in man must remain speculative and cannot be directly inferred from this study. Clearly, more studies are required to examine this possibility, for example with studies examining the effect of selective ligands for clearance receptors on plasma BNP levels in animal studies.

In conclusion, we have shown that plasma hBNP-li levels in chronic heart failure are increased by candoxatril, a neutral endopeptidase inhibitor, but are unchanged by an exogenous ANP infusion. Our results thus raise the possibility that the increase in plasma BNP levels may contribute to some of the biological effects of neutral endopeptidase inhibitors. In addition, our study provides indirect evidence that in patients with chronic heart failure, neutral endopeptidase is of more importance in BNP clearance than are the ANP non-guanylate cyclase-linked or clearance receptors. Although this agrees with work *in vitro*, there could be alternative explanations for the lack of a change in circulating hBNP-li levels during exogenous administration of ANP.

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