Effect of endopeptidase-24.11 inhibition and of atrial natriuretic peptide clearance receptor ligand on the response to rat brain natriuretic peptide in the conscious rat

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1 The present studies examined the effect of (a) a specific endopeptidase-24.11 (E-24.11) inhibitor (candoxatrilat) and (b) a ligand for the atrial natriuretic peptide (ANP) clearance receptor (SC 46542) on the renal and blood pressure response to brain natriuretic peptide (BNP) in conscious rats.

2 Infusion of BNP 200 ng kg⁻¹ min⁻¹ for 60 min produced a small rise in urinary sodium and guanosine 3':5'-cyclic monophosphate (cyclic GMP) excretion with a non-significant fall in mean arterial blood pressure.

3 Candoxatrilat (3 mg kg⁻¹) alone had no significant effect on sodium excretion or blood pressure but markedly potentiated the natriuretic response to BNP.

4 Similarly SC 46542 (68 µg kg⁻¹; 6.8 µg kg⁻¹ min⁻¹) which produced no significant effect on its own, potentiated the natriuresis-induced by BNP, although the effect was of shorter duration compared to that of candoxatrilat.

5 The data indicate two approaches to the potentiation of the renal activity of BNP and suggest that BNP may mediate some of the activity of E-24.11 inhibitors reported in cardiac failure.

Keywords: Brain natriuretic peptide; endopeptidase 24.11; clearance receptor

Introduction

First isolated from porcine brain (Sudoh *et al.*, 1989), it is now clear that brain natriuretic peptide (BNP) is most abundant in cardiac tissue (Saito *et al.*, 1989; Aburaya *et al.*, 1989; Kambayashi *et al.*, 1989). The molecular form of the cardiac peptide varies considerably between species. In man, BNP is synthesized as a 108 amino acid prohormone; the C-terminal 32 residues are cleaved and secreted into the circulation (Sudoh *et al.*, 1989; Kambayashi *et al.*, 1990b). Two forms of BNP are found in the rat heart; pro-BNP 1-95 and BNP-45 (Aburaya *et al.*, 1989; Nakao *et al.*, 1990). The latter has been shown to be the major storage, secretory and circulating form of BNP in rat plasma (Nakao *et al.*, 1990; Aburaya *et al.*, 1991).

BNP displays considerable sequence homology to atrial natriuretic peptide (ANP) and has a similar profile of biological activity (Sudoh *et al.*, 1988; Kambayashi *et al.*, 1990a; Kita *et al.*, 1991). Both peptides exhibit a 17 amino acid ring formed by a disulphide bond and both produce a natriuresis and a reduction in blood pressure when infused into animals and man (McGregor *et al.*, 1990; Yoshimura *et al.*, 1991). It has been proposed that the two peptides act in concert to regulate sodium balance and blood pressure.

In addition to filtration at the glomerulus, two other pathways are thought to participate in the clearance of ANP; namely, metabolism by endopeptidase-24.11 (E-24.11) and receptor-mediated endocytosis (Ruskoaho, 1992). E-24.11 opens the 17 amino acid ring and inactivates ANP (Stephenson & Kenny, 1987; Olins *et al.*, 1989). The clearance receptors for ANP are distinct from guanylyl cyclase linked receptors for this peptide in that they have not been clearly linked to any second messenger system but have been shown to internalise ANP in cell culture (Maack, 1992). Inhibition of either pathway alone has been shown to enhance the natriuretic response to ANP (Wilkins *et al.*, 1992) and this observation has therapeutic potential: E-24.11 inhibitors are currently under evaluation as novel agents for treating patients with cardiac failure (Northridge *et al.*, 1989).

There is increasing evidence that BNP is also cleared by

E-24.11 and may therefore contribute to the pharmacological actions of E-24.11 inhibitors. Rat and human BNP are hydrolysed by this enzyme, although the cleavage site differs between the two peptides (Norman *et al.*, 1991). Recently, Seymour *et al.* (1992) have reported that co-infusion of the E-24.11 inhibitor, SQ 28,603, enhances the natriuretic response to rat BNP-32 in the spontaneously hypertensive rat and human BNP-32 in the cynomolgus monkey. It must be noted, however, that SQ-28,603 is not specific for E-24.11 as it has also been reported to inhibit the transformation of big endothelin-1 (1-38) to endothelin-1 (1-21) (Gardiner *et al.*, 1992), a property not shared by all E-24.11 inhibitors (McMahon *et al.*, 1991; Pollock & Opgenorth, 1991).

The role of receptor-mediated clearance of BNP *in vivo* also needs to be addressed. Studies *in vitro* have shown that human BNP exhibits a 14 fold lower affinity for the human clearance receptor than ANP (Nakao *et al.*, 1991). Similarly, rat BNP-45 has a 6 fold lower affinity than rat ANP for the rat clearance receptor (Suga *et al.*, 1992). It has been suggested that this may contribute to the longer half-life of circulating BNP compared to ANP. However, there are no studies of the effect of clearance receptor ligands on the biological activity of BNP *in vivo*.

Accordingly, the present study examined the effect of a specific E-24.11 inhibitor, candoxatrilat, and the clearance receptor ligand, SC 46542, on the renal and hypotensive response to an exogenous infusion of rat BNP-45 in the conscious rat.

Methods

Infusion protocol

Studies were performed on male Wistar rats (275–300 g). Under Hypnorm (fentanyl/fluanisone) anaesthesia, cannulae (portex 0.58 mm) were placed in a femoral artery, femoral vein and bladder. The animals were allowed to regain consciousness in individual restraining cages. An infusion of sodium chloride 150 mM (2.38 ml h⁻¹) containing [³H]-inulin

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(Amersham) $2 \mu\text{Ci ml}^{-1}$ was given via the femoral vein, which was also used to administer drugs, and continued for the study duration. Observations were started 3 h after beginning the infusion. Urine was collected at 15 min intervals into preweighed tubes for determination of volume, sodium, guanosine 3':5'-cyclic monophosphate (cyclic GMP) and [^3H]-inulin concentrations. Blood pressure was measured via the femoral artery every 15 min (MacLab instruments). Arterial blood samples (200 μl) were taken into lithium-haematocrit tubes for [^3H]-inulin measurement 30 and 90 min from the start of observations.

Studies

Effect of candoxatrilat To investigate the effect of E-24.11 inhibition on the response to BNP, animals were divided into one of the following treatment groups: (a) BNP-45, 200 $\text{ng kg}^{-1} \text{min}^{-1}$ for 60 min ($n = 6$); (b) saline vehicle alone ($n = 6$); (c) candoxatrilat 3 mg kg^{-1} (in 100 μl saline) as a single bolus injection over 1 min ($n = 6$); (d) candoxatrilat 3 mg kg^{-1} (in 100 μl saline) at the start of the 60 min BNP-45 infusion ($n = 7$). The treatments were commenced after two baseline urine collections. The dose of BNP was chosen as one which would produce only a small natriuresis when given alone. Groups (a) and (b) received 100 μl saline in place of candoxatrilat at the appropriate time. Doses of $\geq 3 \text{ mg kg}^{-1}$ candoxatrilat represent the top of the dose-response curve for natriuresis in hydrated rats (Shepperson *et al.*, 1991; Wilkins *et al.*, 1992).

Effect of SC 46542 To investigate the effect of a clearance receptor ligand on the response to BNP, the following additional treatment groups were studied: a) SC 46542 68 $\mu\text{g kg}^{-1}$ bolus (in 100 μl saline); followed by 6.8 $\mu\text{g kg}^{-1} \text{min}^{-1}$ for 60 min ($n = 6$); b) as in a) but combined with BNP-45 200 $\text{ng kg}^{-1} \text{min}^{-1}$ ($n = 6$); c) SC 46542 680 $\mu\text{g kg}^{-1}$ bolus (in 100 μl saline); then 6.8 $\mu\text{g kg}^{-1} \text{min}^{-1}$ for 60 min ($n = 7$); d) as in c) but combined with BNP-45 200 $\text{ng kg}^{-1} \text{min}^{-1}$ ($n = 7$).

Assays

Urine volume was measured gravimetrically. Sodium concentration was measured by flame photometry (Corning 480). [^3H]-inulin level were determined by liquid scintillation counting in Insta-gel (Packard). Urinary cyclic GMP concentration was measured by radioimmunoassay on appropriately diluted samples as previously described (Wilkins *et al.*, 1990a).

Drugs and peptides

Rat BNP-45 was purchased from Peninsula Laboratories. Candoxatrilat is a specific E-24.11 inhibitor (Danilewicz *et al.*, 1989) and was a gift from Pfizer UK. SC 46542 is des(Phe 106 Gly 107 Ala 115 Gln 116)-ANP(5-28) and was synthesized by Monsanto Company, St Louis, U.S.A.; it binds specifically to the ANP clearance receptor (Koepke *et al.*, 1989).

Statistics

Data are presented as mean $\pm$ s.e.mean. Blood pressure and renal responses were examined by analysis of variance with respect to treatment and time (urine collection). Statistical significance was assessed by Scheffé's test. Statistical significance was assumed when the P value was <0.05 . All calculations were made with Complete Statistical System (StatSoft) software.

Results

Sodium excretion

Infusion of BNP 200 $\text{ng kg}^{-1} \text{min}^{-1}$ produced a modest 2 to 3

fold increase in urinary sodium excretion compared to baseline but this did not differ significantly from the gradual 2 fold rise seen in the vehicle-treated group during the period of observation (Figure 1). Candoxatrilat alone had no significant effect on sodium excretion with respect to baseline or to vehicle controls (Figure 1a). When co-infused with BNP, however, there was a 5 fold increase in the natriuretic response; both the peak and duration of the natriuresis were significantly greater than seen with BNP alone ($P < 0.01$). Similarly, infusion of SC 46542 (68 $\mu\text{g kg}^{-1}$, followed by 6.8 $\mu\text{g kg}^{-1} \text{min}^{-1}$) alone did not significantly affect sodium excretion but enhanced the response to BNP (Figure 1b). There were significant differences in the time-course of the response to the two combination treatments. The peak response to candoxatrilat and BNP occurred slightly later than that with SC 46542 and BNP, but was more prolonged. Increasing the dose of SC 46542 10 fold (680 $\mu\text{g kg}^{-1}$, then 6.8 $\mu\text{g kg}^{-1} \text{min}^{-1}$) produced no further potentiation of the effect of BNP beyond that seen with the lower dose of clearance receptor ligand.

Cyclic GMP excretion

BNP alone produced a significant rise in urinary cyclic GMP excretion compared to vehicle treated controls ($P < 0.01$, Figure 2). This response was enhanced by candoxatrilat and

Figure 1 (a) Effect of brain natriuretic peptide (BNP) infusion (200 $\text{ng kg}^{-1} \text{min}^{-1}$, $\square$), candoxatrilat (3 mg kg^{-1} , $\blacksquare$), vehicle (saline, $\blacklozenge$) and BNP after candoxatrilat ($\blacksquare$) on urinary sodium excretion in conscious rats. (b) Effect of SC 46542 (68 $\mu\text{g kg}^{-1}$ bolus, then 6.8 $\mu\text{g kg}^{-1} \text{min}^{-1}$, $\triangle$), BNP (200 $\text{ng kg}^{-1} \text{min}^{-1}$, $\square$), vehicle (saline, $\blacklozenge$) and BNP with SC 46542 ($\blacktriangledown$) on urinary sodium excretion in conscious rats. Data are mean with s.e.mean. * $P < 0.01$, compared to BNP infusion alone group and $\dagger P < 0.01$ compared to vehicle. BNP in combination with candoxatrilat is significantly different ($P < 0.01$) from the SC 46542 and BNP combination at times 45, 75, 90, 105 min.

Figure 2 Urinary cyclic GMP excretion during treatment with: (a) vehicle (open columns), candoxatrilat (3 mg kg^{-1}) alone (hatched columns), BNP $200 \text{ ng kg}^{-1} \text{ min}^{-1}$ infusion (cross hatched columns), and candoxatrilat (3 mg kg^{-1}) prior to BNP infusion (solid columns); and (b) vehicle, (open columns), SC 46542 ($68 \mu\text{g}$ bolus then $6.8 \mu\text{g kg}^{-1} \text{ min}^{-1}$) alone (hatched columns) BNP $200 \text{ ng kg}^{-1} \text{ min}^{-1}$ infusion (cross hatched columns), and the combination of SC 46542 and BNP (stippled columns). Data are mean with s.e.mean. $\dagger P < 0.01$ compared to vehicle and $* P < 0.05$ compared to BNP alone.

by SC 46542. Neither candoxatrilat nor SC 46542 alone significantly affected cyclic GMP excretion.

GFR and blood pressure There was a small rise in GFR on initiating the BNP infusion in all the treatment groups, but in no group was it sustained after the first 15 min of the infusion (Table 1). This transient rise in GFR is probably an artifact due to the wash-out of $[^3\text{H}]$ -inulin from the 'dead-

space', constituted by the bladder stump and cannula, following the rapid increase in urine flow and is unlikely to be representative of increased glomerular filtration.

Mean arterial blood pressure showed a gradual downward drift during infusion of BNP alone compared to the vehicle-treated group, but this did not reach statistical significance (Table 1). Neither candoxatrilat nor SC 46542 alone significantly affected blood pressure; in combination with BNP, a significant ($P < 0.05$) fall in blood pressure was observed at the end of the peptide infusion (Table 1).

Discussion

Inhibition of E-24.11 and infusion of a specific ligand for the ANP clearance receptor are two approaches used to inhibit the clearance of ANP and enhance its natriuretic action (Wilkins *et al.*, 1992). This study shows that both manipulations also potentiate the renal response to BNP in the rat.

Several authors have shown that BNP is a substrate for E-24.11 *in vitro* (Vogt-Schaden *et al.*, 1989; Norman *et al.*, 1991). The site of hydrolysis of BNP differs from that for ANP and, like the molecular form, varies between species. E-24.11 cleaves ANP at the Cys-Phe bond within the ring in all mammalian species studied to date but this bond is not the primary site of hydrolysis in BNP. Instead, rat BNP-32 is hydrolysed at the Arg-Leu bond within the ring and the Arg-Leu bond in the C-terminus. The primary site of attack on human BNP-32 is the Met-Val bond in the N-terminal sequence followed by the Arg-Ile bond in the ring.

The 17 amino acid ring is essential for the biological activity of ANP and BNP, and so hydrolysis of bonds within the ring would be expected to inactivate the peptide. Recently, Seymour *et al.* (1992) reported that the E-24.11 inhibitor, SQ 28,603, potentiates the renal response to human BNP-32 in cynomolgus monkeys and rat BNP-32 in the SHR. The structural diversity of BNP means that it is essential to use the peptide appropriate to the test species (Kambayashi *et al.*, 1990a). We have examined the effect of E-24.11 inhibition on the response to rat BNP-45, the circulating form of the peptide in this species. Our observations support and extend those of Seymour *et al.* (1992) and strengthen the thesis that E-24.11 is an important enzyme in the inactivation and clearance of circulating BNP.

Data on the contribution of the ANP clearance receptor to the regulation of BNP activity are limited. Nakao *et al.* (1991) have reported that the binding affinity of human BNP for human ANP clearance receptors is only 7% that of human ANP. These authors suggest that the clearance receptor is less active in removing BNP than ANP from the circulation and that this contributes to its longer half-life *in*

Table 1 Glomerular filtration rate and mean arterial blood pressure

	Time (min)							
	15	30	45	60	75	90	105	120
GFR (ml min^{-1})								
Saline	3.7 ± 0.3	3.8 ± 0.3	4.1 ± 0.2	3.7 ± 0.2	3.6 ± 0.4	3.5 ± 0.2	3.4 ± 0.3	3.3 ± 0.6
BNP	2.9 ± 0.3	3.4 ± 0.3	4.1 ± 0.4	3.2 ± 0.2	2.9 ± 0.2	2.9 ± 0.2	2.3 ± 0.3	3.2 ± 0.3
BNP + candoxatrilat	3.3 ± 0.3	3.1 ± 0.2	3.9 ± 0.3	3.2 ± 0.1	3.1 ± 0.2	3.1 ± 0.2	2.7 ± 0.2	3.1 ± 0.2
BNP + SC 46542	3.4 ± 0.2	3.4 ± 0.2	3.9 ± 0.4	3.1 ± 0.1	3.1 ± 0.1	2.9 ± 0.3	2.5 ± 0.3	3.1 ± 0.3
BP (mmHg)								
Saline	108 ± 2	107 ± 2	108 ± 2	109 ± 3	110 ± 4	111 ± 4	111 ± 4	114 ± 5
BNP	110 ± 3	111 ± 3	110 ± 3	109 ± 2	105 ± 3	105 ± 3	106 ± 4	108 ± 3
BNP + candoxatrilat	107 ± 2	110 ± 2	110 ± 3	109 ± 3	107 ± 2	$104 \pm 2^*$	106 ± 3	106 ± 3
BNP + SC 46542	113 ± 5	113 ± 4	112 ± 4	108 ± 4	105 ± 4	$104 \pm 3^*$	104 ± 4	104 ± 3

Data are mean $\pm$ s.e.mean. Brain natriuretic peptide (BNP) was infused from 30 ± 90 min. Candoxatrilat (3 mg kg^{-1}) was given prior to BNP $200 \text{ ng kg}^{-1} \text{ min}^{-1}$. SC 46542 was given as $68 \mu\text{g kg}^{-1}$ bolus then $6.8 \mu\text{g kg}^{-1} \text{ min}^{-1}$ during BNP $200 \text{ ng kg}^{-1} \text{ min}^{-1}$.

$*P < 0.05$ compared to saline.

vivo compared to ANP. This is difficult to test in man with currently available agents. In an attempt to circumvent this problem, Lang *et al.* (1992) infused ANP into patients with heart failure in a dose which they calculated would 'swamp' the ANP clearance receptor and saw no change in plasma BNP levels. Our data support a role for the clearance receptor in the regulation of BNP activity *in vivo* in the rat. Nonetheless, a useful comparison can be made between the present experiments and a comparable study with ANP, in which an approximately equimolar dose of ANP was used in place of BNP (Wilkins *et al.*, 1992). In the present study, maximum potentiation of the natriuretic effect of BNP with SC 46542 was obtained with the lower dose used ($68 \mu\text{g kg}^{-1}$, $6.8 \mu\text{g kg}^{-1} \text{ min}^{-1}$) and the response was similar, although of shorter duration, to that produced by candoxatrilat (3 mg kg^{-1}). In the ANP study, this dose of SC 46542 caused sub-maximal potentiation; a 10 fold higher dose of SC 46542 ($680 \mu\text{g kg}^{-1}$, $68 \mu\text{g kg}^{-1} \text{ min}^{-1}$) produced significantly greater potentiation of the natriuretic effect of ANP than the lower dose and exceeded the maximal response that could be obtained with candoxatrilat (Wilkins *et al.*, 1992). These observations would be consistent with the idea that SC 46542 is more effective at competing with BNP than ANP for the clearance receptor and that, compared to E-24.11, the clearance receptor plays a smaller role in clearing BNP than ANP.

The effects of candoxatrilat and SC 46542 on the hypotensive response to BNP were small, and observed at the end of the BNP infusion, when the animals are significantly hypovolaemic from the diuretic actions of these treatments, and perhaps increased extravasation of fluid. Candoxatrilat and

SC 46542 have little effect on the fall in blood pressure during ANP infusion (Wilkins *et al.*, 1992), and Seymour *et al.* (1992) observed no potentiation of the depressor effect of BNP in the monkey or SHR with their E-24.11 inhibitor. The kidneys are a rich source of both E-24.11 and clearance receptors and play an important role in clearing ANP. It seems that inhibition of these clearance pathways preferentially enhance the local effects of ANP and BNP on the kidney, rather than their systemic actions.

The present study is clinically relevant as E-24.11 inhibitors are undergoing trials in the treatment of heart failure. Plasma BNP levels are elevated in this condition (Mukoyama *et al.*, 1991). Indeed the incremental rise is greater than that seen with ANP. As the two peptides are equipotent (Kambayashi *et al.*, 1990a) this suggests that BNP plays as important a role in this condition as ANP. Interestingly, several authors have commented on the observation that E-24.11 inhibitors are more effective natriuretic agents in heart failure than would be predicted from the rise in plasma ANP level (Wilkins *et al.*, 1990b; Caverio *et al.*, 1990). Given that the response to BNP is also potentiated by E-24.11 inhibitors, a component of the response to these drugs in heart failure may be due to BNP.

In summary, this study suggests that BNP can be potentiated by pharmacological manipulations used to inhibit the clearance of ANP and may contribute to the therapeutic effect of these manipulations in heart failure.

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