Combined inhibition of angiotensin II and endothelin suppresses the brain natriuretic peptide response to developing heart failure

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ABSTRACT

Blockade of AngII (angiotensin II) and ET (endothelin)-1, established and potential therapeutic strategies respectively, for heart failure, may have an adverse effect on the cardiac secretion of the natriuretic peptides, hormones with actions beneficial in this disease. The present study investigates the roles of AngII and ET-1 in regulating the stretch-induced release of the natriuretic peptides during the development of heart failure. On seven separate days, eight sheep underwent incremental left ventricular pacing (155, 190 and 225 beats/min for 90 min each) with concurrent infusions of a vehicle control, AngII, ET-1, AngII + ET-1, losartan [AT1 (AngII type 1) receptor antagonist], bosentan (ET\textsubscript{A}/ET\textsubscript{B} receptor antagonist) or losartan + bosentan. Pacing-induced rises in LAP (left atrial pressure) were amplified by the simultaneous administration of separate AngII and ET-1, and attenuated following blockade of the peptides, with maximum effects observed during combined treatments. Although these changes in atrial pressure were paralleled by concomitant alterations in circulating levels of both ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide), the plasma natriuretic peptide/atrial pressure relationship tended to be augmented by AngII and ET-1 and diminished by their blockade. A significant difference was demonstrated between the enhanced plasma BNP response to increasing LAP during combined AngII + ET-1 administration and decreased response during losartan + bosentan treatment ($P < 0.05$). A similar, but non-significant, trend was evident for ANP. The present study indicates dual AngII/ET-1 blockade diminishes BNP (and to a lesser extent ANP) secretion in developing heart failure, suggesting that augmentation of the natriuretic peptide system during the combination of these therapies may be of benefit.

INTRODUCTION

In HF (heart failure), the inability of the heart to pump sufficient blood is aggravated by the reflex activation of a number of vasopressor/volume-retaining factors, which ultimately act to further overload the already failing heart. Inhibition of one of these factors, AngII (angiotensin II), has over the past decade become one of the front-line therapies used in the management of patients with HF [1]. Recently, the potential benefits of blockade of the vasoconstrictor peptide ET (endothelin)-1 have been trialled in HF, with mixed results to date. Although some trials suggest ET antagonism may improve haemodynamics and symptoms and possibly reduce morbidity [2,3], the two longer-term trials found an increased early risk of worsening HF [4,5] with no overall mortality...
benefits [4]. Any new therapy is likely to be used in conjunction with blockade of AngII, and a number of studies in both experimental [6,7] and human [8,9] HF have investigated the combination of these two approaches to anti-failure treatment and found additive salutary systemic and cardiac haemodynamic effects.

A pivotal compensatory response to HF is the activation of the cardiac natriuretic peptides [ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide)], which have beneficial vasodilating, natriuretic and renin- and aldosterone-suppressant actions [10,11]. Although cardiac transmural pressure is considered to be the main determinant of both ANP and BNP secretion from the heart [12], increasing evidence indicates that AngII and ET-1 both increase the basal, as well as stretch-induced, secretion of the natriuretic peptides from cardiac tissue [13–17]. Conversely, secretion of ANP and BNP is reduced by antagonism of AngII [14] and ET-1 [17–19].

Given the broad spectrum of compensatory effects the natriuretic peptides exhibit in HF, any attenuation of their secretion is potentially significant.

The present study sets out to investigate for the first time the effects of separate and combined administration of AngII and ET-1 and their receptor antagonists on stretch-induced ANP and BNP secretion during developing HF in sheep.

**MATERIALS AND METHODS**

**Surgical preparation**

Eight Coopworth ewes (45–56 kg) were instrumented via a left lateral thoracotomy [20]. Briefly, under general anaesthesia (induced by 17 mg of intravenous thiopentone/kg of body weight; maintained with 2.5% halothane, 2 litres/min nitrous oxide and 2 litres/min oxygen) two catheters were inserted into the left atrium for blood sampling and LAP (left atrial pressure) determination. A Konigsberg pressure-tip transducer was inserted in the aorta to record MAP (mean arterial pressure), an electromagnetic flow probe was placed around the ascending aorta to measure CO (cardiac output), a 7 French Swan-Ganz catheter was inserted in the pulmonary artery for infusions and a 7 French His-bundle electrode was stitched subepicardially to the wall of the left ventricle for LV (left ventricular) pacing. Animals recovered for 14 days before commencing the study protocol. During the experiments the animals were held in metabolic cages, had free access to water and ate a diet containing 80 mmol sodium/day and 200 mmol potassium/day.

**Study protocol**

On seven separate days (at least 1 day apart), the sheep underwent LV pacing at 155, 190 and 225 beats/min for 90 min at each rate. On initiation of pacing, the animals received in balanced random order constant infusions of (i) a vehicle control (50 ml of 0.9% saline), (ii) AngII (2 ng·kg⁻¹·min⁻¹), (iii) ET-1 (0.6 ng·kg⁻¹·min⁻¹), (iv) AngII + ET-1 [doses as for (i) and (ii)], (v) losartan [AT1 (AngII type 1) receptor antagonist; 2 mg·kg⁻¹·h⁻¹], (vi) bosentan (ETₐ/ETₐ receptor antagonist; 2.5 mg·kg⁻¹·h⁻¹) or (vii) losartan + bosentan [doses as for (v) and (vi)] for the 4.5 h study period.

Haemodynamic measurements (MAP, LAP and CO) were made at 15 min intervals in the hour prior to infusions (pre-pacing baseline) and every 30 min during each pacing period. Measurements were determined by on-line computer-assisted analysis using established methods [21]. Blood samples, collected immediately following haemodynamic measurements, were collected into tubes on ice, centrifuged at 4°C and stored at either −20°C or −80°C before assay for ANP, BNP, cGMP, ET-1, PRA (plasma renin activity) and AngII [20–23]. The investigation protocol was approved by the local Animal Ethics Committee.

**Materials**

AngII and ET-1 were purchased from American Peptide Company, Sunnyvale, CA, U.S.A. Bosentan was supplied by La Roche, Basel, Switzerland. Losartan was supplied by Du Pont Pharmaceuticals, Wilmington, Delaware, U.S.A.

Doses of AngII and ET-1 were selected based on dose–response pilot studies (results not shown), whereby similar and significant rises in LAP were induced by each peptide with minimum disruption in blood pressure. The losartan and bosentan doses selected produced approximate mirror image changes in LAP. These doses are clinically relevant in that plasma AngII and ET-1 concentrations achieved during infusion of the respective peptides lie within the pathophysiological ranges observed in HF, whereas administration of the receptor antagonists produced significant changes in haemodynamic function.

**Statistics**

Results are expressed as means ± S.E.M. Baseline values represent the mean of measurements made in the hour immediately pre-pacing/pre-treatment. Statistical analysis of the response to pacing across the different treatments was performed by repeated measures ANOVA. The relationship between LAP and the natriuretic peptides across the different treatments was analysed using a general linear model. Statistical significance was assumed when P < 0.05.

**RESULTS**

**LV pacing**

Stepped increments in LV pacing (155, 190 and 225 beats/min) in conjunction with the vehicle control infusion
induced rate-dependent falls in CO and MAP, and increases in LAP, plasma ANP, BNP and cGMP (all P < 0.001). Significant rises in plasma concentrations of PRA and AngII were evident at the highest pacing rate (both P < 0.001), whereas ET-1 levels were unaltered by pacing for the period of this study (Figures 1–3, Table 1). These haemodynamic and hormonal changes are consistent with those observed previously during the development of pacing-induced congestive HF [20,24].

**Plasma AngII and ET-1**

Incremental LV pacing with concomitant infusion of AngII, both alone and in combination with ET-1, approximately doubled plasma levels of the peptide relative to control (both P < 0.001; Figure 1). Plasma AngII concentrations were also elevated (although at a more gradual rate) following administration of bosentan (approx. 2-fold; P < 0.01), losartan (>3-fold; P < 0.001) and, most markedly, dual blockade of the two peptide systems (>6-fold; P < 0.001). ET-1 infusion alone decreased AngII levels compared with control (P < 0.05). PRA responses to the different treatments paralleled those of plasma AngII, except where AngII itself was infused, in which case PRA levels fell below those of the vehicle control phase (AngII, P < 0.05; AngII + ET-1, P < 0.001; Table 1).

Plasma levels of ET-1 were increased similarly by exogenous ET-1 infused separately and together with AngII (approx. 2-fold; both P < 0.001), and further still by bosentan, both alone and in combination with losartan (approx. 3-fold; both P < 0.001; Figure 1). ET-1 concentrations were not altered by individual AngII or losartan treatments.

### Haemodynamics

Administration of AngII and ET-1 tended to accentuate decreases in CO associated with rapid LV pacing (not significant), whereas this effect was significantly attenuated by the concurrent administration of bosentan, losartan and the combination of the two agents (all P < 0.05; Figure 2). Blood pressure decreases observed during pacing alone were augmented by treatment with bosentan (P < 0.05) and losartan + bosentan (P < 0.01), and were largely eliminated by AngII (separately and together with ET-1; both P < 0.001; Figure 2). ET-1 alone tended to elevate, and losartan decrease, blood pressure relative to vehicle control data (both 0.1 > P > 0.05).

### LAP and plasma natriuretic peptides

Pacing-induced rises in LAP were amplified by the concomitant infusion of AngII or ET-1 alone (both...
Mean LAP and ANP and BNP responses to 4.5 h AngII and ET-1 agonism and antagonism in pacing-induced heart failure

**Table 1** AngII and ET-1 agonism and antagonism in pacing-induced heart failure

Mean ± S.E.M. PRA and cGMP responses in eight sheep before (pre-pace baseline) and during stepped increments in LV pacing with concomitant infusions of vehicle, AngII, ET-1, AngII + ET-1, losartan, bosentan and losartan + bosentan (Los + Bos) are shown. *P < 0.01 and **P < 0.001 between baseline and paced data on the vehicle control day; †P < 0.05, ††P < 0.01 and †††P < 0.001 compared with the vehicle control.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Pre-pace baseline</th>
<th>155</th>
<th>190</th>
<th>225</th>
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<tbody>
<tr>
<td>PRA (nmol·L⁻¹·h⁻¹)</td>
<td>Vehicle</td>
<td>0.28 ± 0.03</td>
<td>0.24 ± 0.04</td>
<td>0.31 ± 0.05</td>
<td>0.51 ± 0.06**</td>
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<td>AngII</td>
<td>0.24 ± 0.02</td>
<td>0.16 ± 0.02†</td>
<td>0.20 ± 0.04†</td>
<td>0.24 ± 0.07††</td>
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<tr>
<td></td>
<td>ET-1</td>
<td>0.26 ± 0.05</td>
<td>0.17 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>0.24 ± 0.05††</td>
</tr>
<tr>
<td></td>
<td>AngII + ET-1</td>
<td>0.30 ± 0.04</td>
<td>0.18 ± 0.03</td>
<td>0.22 ± 0.02†</td>
<td>0.22 ± 0.02††</td>
</tr>
<tr>
<td></td>
<td>Losartan</td>
<td>0.26 ± 0.03</td>
<td>0.71 ± 0.10††</td>
<td>0.88 ± 0.22††</td>
<td>1.43 ± 0.14†††</td>
</tr>
<tr>
<td></td>
<td>Bosentan</td>
<td>0.27 ± 0.05</td>
<td>0.32 ± 0.06</td>
<td>0.51 ± 0.07††</td>
<td>0.82 ± 0.13†††</td>
</tr>
<tr>
<td></td>
<td>Los + Bos</td>
<td>0.30 ± 0.05</td>
<td>1.24 ± 0.18††</td>
<td>2.44 ± 0.30†††</td>
<td>3.24 ± 0.49†††</td>
</tr>
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</table>

| cGMP (nmol/L)       | Vehicle            | 11.4 ± 1.2        | 12.5 ± 2.2 | 36.6 ± 8.3* | 49.9 ± 10.6** |
|                     | AngII              | 11.8 ± 1.4        | 14.3 ± 1.1 | 42.6 ± 3.7 | 56.3 ± 4.9 |
|                     | ET-1               | 11.4 ± 1.1        | 12.0 ± 0.9 | 43.7 ± 9.3 | 48.6 ± 7.1 |
|                     | AngII + ET-1       | 12.0 ± 0.8        | 14.3 ± 1.3 | 68.8 ± 13.7††† | 66.7 ± 11.0† |
|                     | Losartan           | 12.5 ± 1.1        | 14.8 ± 1.1 | 28.9 ± 2.7 | 49.0 ± 6.6 |
|                     | Bosentan           | 12.2 ± 0.9        | 13.9 ± 1.0 | 31.5 ± 4.4 | 47.5 ± 10.2 |
|                     | Los + Bos          | 11.0 ± 0.9        | 13.8 ± 1.4 | 30.7 ± 4.4 | 40.2 ± 7.7 |

*P < 0.05, †P < 0.01, ††P < 0.001 compared with the vehicle control.
A number of studies suggest that the influence of AngII on natriuretic peptide secretion is minimal compared with that of ET-1. Magga et al. [25] demonstrated that bolus administration of bosentan, but not losartan, blocked the rise in BNP mRNA levels produced by pressure load in the atria of rats. However, Gigante et al. [26] have found that losartan decreased ANP expression by 42% in the atria of salt-restricted rats. Interestingly, neither study observed an effect of receptor blockade on the ventricular levels of either ANP or BNP mRNA, suggesting that endogenous ET-1 and AngII may differentially regulate natriuretic peptide gene expression in atrial and ventricular tissues. The findings of the present study indicate that AngII still plays a significant role in the regulation of natriuretic peptide secretion, since the maximum impact on ANP and BNP secretory responses was observed during dual agonism and antagonism of the ET-1 and AngII systems. A similar finding was reported by Leskinen et al. [19] in volume-loaded rats, where the effect of combined administration of bosentan and losartan on volume expansion-induced ANP release was more marked than that of losartan alone. Indeed, these authors [19] found that combined inhibition of these two peptides almost completely blocked the ANP response to acute volume load in these animals. There is some evidence suggesting that the two peptides may act in series to mediate stretch-dependent activation of the cardiac natriuretic peptides. Liang and Gardner [27] found that, although both Ang II and ET-1 increased BNP gene promoter activity in strained cultured neonatal rat ventricular myocytes, the AngII response was blocked by ET-1 antagonism (BQ-123), whereas the ET-1 response was unaffected by losartan. This implies that the secretagogue activities of the peptides may be inter-related, a concept in keeping with the profoundly integrated nature of these two peptide systems. Not only is AngII reported to up-regulate prepro-ET-1 mRNA and augment ET-1 production and ECE (ET-converting enzyme) activity in vivo [28,29], but ET-1, in turn, has been shown to stimulate both vascular ACE (angiotensin-converting enzyme) activity [30] and AngII release [31]. These data suggest that a positive feedback loop links these two systems in disease states such as chronic HF.

Despite the evidence of a positive feedback relationship between ET-1 and AngII, we found plasma ET-1 levels were not increased relative to control by administration of AngII (although events at the tissue level are unknown), and circulating levels of AngII were actually decreased following infusion of ET-1. It is likely that the concomitant decrease in PRA during ET-1 administration (presumably due to the suppressive effect of raised arterial pressures as well as a possible direct inhibitory effect of ET-1 on renin release [32]) accounts for this AngII decrease. Antagonism of the respective receptors of both AngII and ET-1 elevated circulating levels of each peptide. During losartan treatment, AngII levels
rose in concert with those of PRA, reflecting the renin stimulus of decreased renal perfusion pressure and loss of negative feedback by AngII. The elevation of plasma ET-1 with bosentan treatment, a consequence observed previously in patients with HF [33], is consistent with the significant role the ET\(_B\) receptor is reported to play in the clearance of the peptide [34]. Bosentan administration was also associated with stimulation of circulating renin–angiotensin in the present study. Activation of this system by bosentan was not previously observed by Krum et al. [35] in patients with hypertension, although it should be noted that a much lower dose of bosentan was used, the agent was taken orally rather than intravenously (resulting in decreased absolute bioavailability) and, most importantly, starting blood pressures were markedly elevated relative to those in the present study. Indeed, Berthold et al. [36], measuring renin release at various renal perfusion pressures in dogs, found that ET\(_A\) receptor blockade had little effect on renin release at normal renal perfusion pressure, but strongly enhanced the sensitivity of pressure-dependent renin release at lower pressures.

The large rises in plasma renin and AngII concentrations observed in response to combined losartan/bosentan administration in the present study are presumably due to the cumulative renin-stimulatory effects of inhibiting both peptide systems simultaneously. These include marked falls in blood pressure (and therefore renal perfusion pressure), which were additive of pressure decreases induced by each agent given separately, and the loss of both negative feedback by AngII (via losartan) and inhibition by ET-1 (via bosentan) [32]. In addition, the natriuretic peptides, which also inhibit renin release, were significantly lower during combined receptor antagonism than during any other treatment. An obvious consequence of such a large increase in plasma AngII in the presence of selective AT1 receptor blockade is increased stimulation of the AT2 (AngII type 2) receptor. Activation of this receptor is reported to inhibit cell growth and proliferation, promote apoptosis and possibly induce vasodilation [37], effects which oppose those of the AT1 receptor. Most evidence to date indicates that the stimulatory effect of AngII on natriuretic peptide gene expression [27] and release [14] is mediated via the AT1 receptor. However, Gigante et al. [26] found that, whereas AT1, but not AT2, receptor blockade decreased ANP mRNA levels in the atria of salt-restricted rats, concomitant treatment with the two antagonists resulted in further significant inhibition of ANP expression. These authors [26] concluded that the AngII receptor subtypes mediate the regulation of atrial ANP mRNA though a synergistic action. It is uncertain what impact increased AT2 receptor activation may have had on the results of the present study. Of interest, another study has demonstrated that activation of the AT2 receptor suppresses both basal and ANP-stimulated cellular cGMP concentrations [38]. Clearly, the interaction between the natriuretic peptide system and the two AngII receptor subtypes require further investigation.

Given the integrated nature and vasoconstricting/volume-retaining/proliferative activities of these two peptide systems, dual antagonism is a logical therapeutic strategy for HF. Indeed, combined administration of losartan and bosentan in the present study produced maximum falls in blood pressure and was most effective in attenuating the falls in CO and rises in cardiac preload associated with the development of HF. These data are in agreement with previous studies in both experimental and human HF demonstrating that these two anti-failure treatments have additive cardiac haemodynamic (and tissue) effects. In rats with HF induced by coronary artery ligation, Mulder et al. [7] reported that dual ET and AngII blockade decreased LV systolic pressure and LVEDP (LV end-diastolic pressure) more than either treatment alone, whereas Fraccarollo et al. [6] demonstrated that only the combined treatments significantly decreased LVEDP, improved LV dP/dt(max) and prevented cardiac hypertrophy. In HF patients already on ACE inhibition therapy, additional short-term oral ET receptor antagonism induced further decreases in systemic vascular resistance, MAP and right atrial pressure and improvements in cardiac output [8,9]. These findings support a significant role for both AngII and ET in the control of cardiac function and vascular tone in HF.

Although the present and previous studies [6–9] have demonstrated that combined inhibition of AngII and ET-1 has additional beneficial cardiac and haemodynamic effects in HF over those of monotherapy, the present study also demonstrates dual blockade has a blunting effect on the secretory response of the natriuretic peptides to increasing intra-cardiac pressures during developing HF. This is of major concern given the salutary effects these peptides exhibit in states of cardiac and volume overload, such as HF. Administration of either ANP or BNP in this setting is reported to decrease peripheral vascular resistance, arterial and central filling pressures and plasma renin and aldosterone concentrations, improve CO and induce natriuresis and diuresis [10,11]. The natriuretic peptides have also been shown to inhibit sympathetic nerve activity [39] and both AngII- and ET-1-induced proliferation of vascular smooth muscle cells and cardiomyocytes [40]. Conversely, blockade of the endogenous natriuretic peptides is associated with increases in systemic vascular resistance, blood pressure [41], plasma renin–angiotensin–aldosterone, ET, noradrenaline [42,43] and cellular proliferation [44], and decreases in urine output and sodium excretion [41,42]. Not surprisingly, inhibition of this system significantly accelerates the development of HF [42,43,45]. Indeed, it is possible that the increased early risk of worsening HF (as a consequence of fluid retention) observed in a significant percentage of patients during
recent clinical trials of bosentan [4,5], as well as the lack of mortality benefits, may reflect a suppression of natriuretic peptide production due to loss of ET-1 secretagogue activity. The negative impact of ET-1 blockade on the natriuretic peptide response to stretch may be even more critical in severe HF, since ET appears to play an increased role in augmenting natriuretic peptide secretion at higher intra-cardiac pressures [16]. Given the results of the present study showing combined ET-1 and AngII blockade blunts stretch-induced natriuretic peptide secretion in HF more than ET-1 inhibition alone, dual ET-1/AngII inhibition therapy might be expected to have a more significant effect. Another conceivable complication arising from the blunting of ANP and BNP stretch-induced secretion during combined ET-1 and AngII inhibition is the potential impact on interpretation of plasma levels of the natriuretic peptide as an index of cardiac function [46] and as a guide to adjustment of pharmacotherapy for HF [47].

The results of the present study, together with those of existing reports demonstrating the beneficial actions of ANP and BNP in HF and the detrimental consequences associated with their blockade, suggest that the response of the natriuretic peptide system during the combination of these therapies may influence the clinical response. Maintenance or augmentation of plasma natriuretic peptides during such dual therapy might be beneficial. Indeed, a recent study [48] examining the effects of a composite neutral endopeptidase (the enzyme responsible for cleavage of the natriuretic peptides), ACE and ECE inhibitor in rats with chronic HF has reported improvements in cardiac pre- and after-load, LV remodelling and LV function greater than that induced by the agents separately. The therapeutic potential of such a three-pronged approach awaits the results of future clinical trials.

In conclusion, the present study demonstrates for the first time that dual AngII/ET-1 inhibition therapy diminishes the BNP (and to a lesser extent ANP) secretory response to increasing intra-cardiac pressure in developing HF. The effects of chronic ET-1/Ang-II blockade, which may differ from those of acute blockade (as performed in the present study), require investigation.

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