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Maximizing the natriuretic effect of endogenous atriopeptin in a rat model of heart failure

(neutral endopeptidase/cGMP/phosphodiesterase)

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ABSTRACT **The effect of pharmacological manipulation of atriopeptin (AP) activity on sodium excretion and blood pressure was examined in the rat aortovenocaval (A-V) fistula model of cardiac failure. Introduction of an A-V shunt led to a marked and sustained elevation of plasma AP immunoreactivity and urinary cGMP levels. Further elevation of plasma AP levels by infusion of exogenous peptide induced modest increases in urinary sodium and cGMP excretion and a decrease in blood pressure but these responses were significantly attenuated compared to sham-operated animals. In contrast, low-dose infusion of M+B 22948 (a cGMP phosphodiesterase inhibitor) or thiorphan [a neutral endopeptidase (membrane metallo-endopeptidase, EC 3.4.24.11) inhibitor] induced a natriuresis in A-V fistula rats, which exceeded that seen in control animals given these compounds and matched the peak natriuresis produced in sham-operated animals by high doses of AP. In the doses used, these compounds had little effect on blood pressure. The greater renal efficacy of M+B 22948 in A-V fistula rats is consistent with postreceptor facilitation of AP activity. The effect of thiorphan on sodium excretion was accompanied by a pronounced increase in urinary cGMP and AP immunoreactivity excretion (and was attenuated by anti-AP monoclonal antibody) but could not be explained solely in terms of an increase in circulating AP levels. It is proposed that thiorphan allows filtered AP to reach renal tubule sites that are normally inaccessible to the peptide and are thus protected from down-regulation by high circulating AP levels. The implication of these observations for patients in cardiac failure is the potential for using pharmacological agents to maximize the response to endogenous AP without compromising cardiac function.**

The cardiac atria synthesize and store a peptide with natriuretic–diuretic and vasorelaxant properties, known as atriopeptin (AP) or atrial natriuretic factor [ANF-(99–126)], which is thought to participate in the regulation of blood volume and blood pressure (1, 2). Raised plasma AP immunoreactivity (AP_{ir}) levels have been reported in patients with congestive cardiac failure and the increase appears to correlate with the severity of the disease (3, 4). It is likely that the increase in plasma AP concentration represents a compensatory response, lessening cardiac workload by facilitating sodium and water excretion and reducing peripheral vascular resistance. This interpretation has stimulated studies to evaluate the therapeutic value of further increases in plasma AP levels in patients with heart failure. Studies in which the peptide has been infused have shown an enhancement of sodium and water excretion and an improvement in ventricular performance, as judged by changes in atrial and ventricular pressure and stroke volume (5–7). Unfortunately, the short half-life of the peptide, the necessity for parenteral administration, and occasional excessive drops in blood pressure limit the usefulness of the peptide itself as a therapeutic agent.

An alternative to exogenous AP is to enhance the activity of endogenous circulating peptide. One approach to this goal is to inhibit degradation of cGMP. There is increasing evidence that cGMP acts as a second messenger for AP. For example, particulate guanylate cyclase functions as receptor for AP (8), and the peptide is a potent stimulator of cGMP production in vascular and renal tissue (9, 10). We have shown that M+B 22948, a selective cGMP phosphodiesterase inhibitor, greatly potentiates the natriuretic response to coadministered AP and enhances the natriuretic activity of endogenous AP during acute volume expansion (11).

Another approach to enhancing AP activity is to inhibit metabolism of the peptide. *In vitro* studies have demonstrated that AP is a substrate for neutral endopeptidase (NEP; membrane metallo-endopeptidase, EC 3.4.24.11) (12. 13). The major cleavage site is the Cys^{105}-Phe^{106} bond, which results in an inactive metabolite. The enzyme is widely distributed in the body but is most abundant in the renal cortex, in particular the proximal tubule (14). Several inhibitors of NEP are known; one such compound is thiorphan (DL-3-mercapto-2-benzylpropanoyl-glycine). Given alone to rats, it is natriuretic and diuretic (15). Coadministered with AP, it has been reported to enhance plasma AP_{ir} levels and to potentiate the renal response to the peptide (15).

Accordingly, we examined the effect of M+B 22948 and thiorphan on urinary sodium excretion and blood pressure in an animal model of heart failure. An aortovenocaval (A-V) shunt was made in rats distal to the renal arteries to create a model of high-output cardiac failure with chronically elevated plasma AP_{ir} and urinary cGMP levels. We reasoned that if M+B 22948 and thiorphan operated by enhancing AP activity, these compounds would be more effective in A-V fistula animals than in sham-operated controls. To evaluate further the efficacy and mechanism of action of these pharmacological manipulations, we compared the response of these compounds with that produced by infusion of exogenous peptide and investigated the effect of anti-AP monoclonal antibody on the action of thiorphan.

MATERIALS AND METHODS

Surgical Procedures and Infusion Protocol. A-V fistula surgery was performed on male Sprague–Dawley rats (250–

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Abbreviations: AP, atriopeptin; AP_{ir}, atriopeptin immunoreactivity; A-V fistula, aortovenocaval fistula; pBNP, porcine brain natriuretic peptide; NEP, neutral endopeptidase.

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300 g), anesthetized with ether. The fistula (1–1.5 mm long) was made through a side to side anastomosis between aorta and vena cava ≈10 mm distal to the renal arteries. Sham operations were performed by exposing and temporarily clamping (≈5 min) aorta and inferior vena cava without cutting or suturing. After the operation, the animals were placed in cages with free access to water and were fed a standard rat diet. The 1-month mortality rate for this procedure was ≈10%.

Infusions of AP-(103–126), M+B 22948, and thiorphan were carried out in rats 7–14 days after surgery. Under ether anesthesia, polyethylene catheters (PE50) were inserted into the femoral vein, carotid artery, and bladder. The animals were then allowed to regain consciousness in individual restraining cages. An infusion of 5% dextrose/0.225% sodium chloride (0.07 $ml \cdot kg^{-1} \cdot min^{-1}$) via the femoral vein was started and continued for the rest of the study period. Observations were begun 90 min from the start of the infusion. All drugs were administered in vehicle without altering the infusion rate. Urine was collected into preweighed tubes and blood pressure was measured via the carotid artery every 15 min.

Assays. Plasma and urine samples were assayed directly for AP concentration by ELISA using guinea pig antiserum as described (16). AP_{ir} was determined by comparison with a standard curve prepared using AP-(103–126). The intra- and interassay variations were 3% and 4%, respectively.

Urine cGMP concentration was measured by radioimmunoassay as described (11). Urine volume was measured gravimetrically and sodium concentration was measured by flame photometer (IL 943).

Plasma AP_{ir} and Urinary cGMP Levels after A-V Fistula Surgery. Rats (n = 10; controls and fistulas) were placed in metabolic cages for 24 hr without access to food or water before and 1, 2, and 4 weeks after surgery. Urinary cGMP excretion was estimated from a 24-hr urine collection. On removal from the cages, a blood sample was taken from the tail vein of each rat while conscious but restrained.

Infusion of Exogenous AP-(103–126). A-V fistula rats and their controls (n = 8 in each group) were challenged with increasing doses of AP-(103–126). After two baseline collections, animals received four doses of AP-(103–126) (100, 300, 600, and 1000 $mg \cdot kg^{-1} \cdot min^{-1}$) in sequential 15-min infusions. Blood was collected for estimation of plasma AP_{ir} before and at the end of each dose of AP. Urine volume and sodium concentration were measured and aliquots were stored at −20°C for AP_{ir} and cGMP assay.

Infusion of M+B 22948. A-V fistula rats and their controls (n = 5 in each group) received an infusion of M+B 22948 (33 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 60 min via the femoral vein. The infusions began after two 15-min baseline collections. Urine volume and sodium concentration were measured and samples were stored at −20°C for cGMP assay.

Infusion of Thiorphan. A-V fistula rats and their controls (n = 6 in each group) received an infusion of thiorphan (0.5 $mg \cdot kg^{-1} \cdot min^{-1}$) through the femoral vein over 30 min. The infusions were started after two 15-min baseline measurements. Blood was collected for plasma AP_{ir} before and 15, 45, and 90 min after the start of thiorphan. Urine volume and sodium concentration were measured and samples were stored at −20°C for AP_{ir} and cGMP assay.

Anti-AP Monoclonal Antibody and Response to Thiorphan. The effect of an inactivating monoclonal antibody directed against AP-(99–126) on the response to a bolus injection of thiorphan in A-V fistula rats was studied. The development and characterization of this antibody has been described (11). Ten milligrams of monoclonal antibody (n = 4) or bovine immunoglobulin (n = 4) was infused over 15 min in 1 ml of 0.9% sodium chloride 60 min prior to commencing baseline observations. After two 15-min baseline measurements, animals received thiorphan at 30 mg/kg in 0.9% saline (1 ml) by bolus injection. Observations were continued for 45 min. At the end of the experiment, the animals that received anti-AP monoclonal antibody were challenged with an infusion of AP-(103–126) (600 $mg \cdot kg^{-1} \cdot min^{-1}$) over 15 min and then porcine brain natriuretic peptide (pBNP) (600 $mg \cdot kg^{-1} \cdot min^{-1}$) in a 15-min infusion.

Statistics. Data are presented as means ± SEM. Comparisons within groups were made by Student's paired t test and comparisons between groups were made by Student's unpaired t test.

RESULTS

Plasma AP_{ir} and Urinary cGMP Levels after A-V Fistula Surgery. Mean basal plasma AP_{ir} concentration in the control group was 145 ± 60 pg/ml and did not change significantly in the 28-day period after surgery (Table 1). There was a small nonsignificant increase in 24-hr urinary cGMP excretion during this period.

After A-V fistula surgery, plasma AP_{ir} levels increased 8-fold and remained elevated. A marked and sustained increase in 24-hr urinary cGMP excretion was also recorded; the excretion rate in fistula rats was ≈3-fold greater than controls at each time point after surgery.

Response to Infusion of AP-(103–126). The sham-operated rats showed a typical bell-shaped curve in urinary sodium excretion in response to increasing doses of AP-(103–126) (Fig. 1). The attenuation of natriuresis at higher doses of AP is most likely due to the accompanying decrease in blood pressure. Nonetheless, the peak increase in sodium excretion was twice ($P < 0.05$) that achieved in the A-V fistula rats. The blunted natriuresis in these animals was accompanied by a smaller decrease in blood pressure. Basal urinary cGMP excretion was elevated in A-V fistula rats ($P < 0.05$), consistent with the higher basal plasma AP_{ir} levels in these animals. However, despite maintaining equal or higher

Table 1. Plasma AP_{ir} levels and 24-hr urinary cGMP excretion in sham-operated and A-V fistula rats

	Day			
	0	7	14	28
Plasma AP_{ir}, pg/ml				
Sham	145 ± 62	160 ± 48	166 ± 66	172 ± 55
A-V fistula	181 ± 51	1368 ± 324*†	1604 ± 404*†	1160 ± 349*†
Urinary cGMP, nmol per 24 hr				
Sham	36 ± 8	48 ± 10	60 ± 12	68 ± 15
A-V fistula	32 ± 7	150 ± 30*†	175 ± 38*†	162 ± 34*†

Results are expressed as means ± SEM (n = 10 in each group).
*$P < 0.05$ compared to baseline.
†$P < 0.05$ compared to time-controlled sham-operated animals.

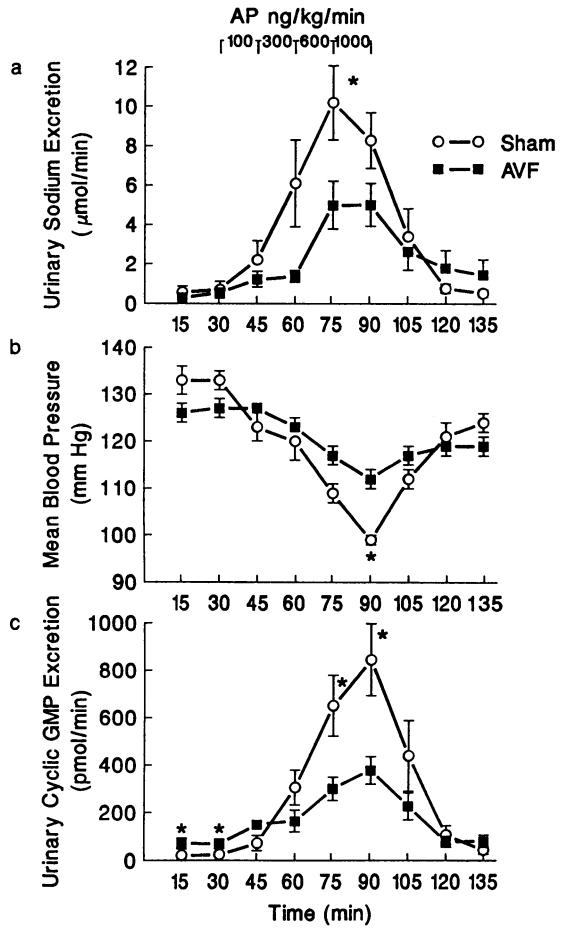

FIG. 1. Effect of increasing doses (100–1000 ng·kg^{-1}·min^{-1}) of AP-(103–126) on sodium excretion (*a*), mean carotid blood pressure (*b*), and urinary cGMP excretion (*c*) in A-V fistula (AVF) vs. sham-operated rats. Means ± SEM. *, $P < 0.05$ compared with sham-operated control.

plasma AP_{ir} levels during the AP infusion (Fig. 2), urinary cGMP excretion fell behind that seen in sham-operated animals. An important observation was that no significant change in urinary AP_{ir} levels was recorded in either group of animals despite the high plasma AP_{ir} levels achieved (Fig. 2).

Response to Infusion of M+B 22948. The A-V fistula rats were clearly more sensitive to the natriuretic effects of low-dose infusion of M+B 22948 than the sham-operated animals (Fig. 3); a 7-fold increase in sodium excretion was seen in the fistula animals, compared to a 3-fold increase in the controls ($P < 0.05$). Basal urinary cGMP excretion was higher in the A-V fistula rats ($P < 0.05$) and doubled during infusion of the cyclic phosphodiesterase inhibitor; a very much smaller, nonsignificant increase in urinary cGMP levels was seen in the control group. Mean blood pressure was significantly lower ($P < 0.05$) in the fistula group compared to controls at all time points during the study (116 ± 3 vs. 139 ± 4 mmHg), but neither group showed any change in response to infusion of the phosphodiesterase inhibitor.

Response to Infusion of Thiorphan. There was a striking difference between A-V fistula rats and sham-operated rats in their response to infusion of thiorphan (Fig. 4). A gradual nonsignificant increase in urinary sodium excretion was observed in the control group. In contrast, a rapid 6-fold increase was produced in the fistula animals ($P < 0.01$), which diminished gradually after the infusion was stopped. The natriuresis in the fistula animals was accompanied by a pronounced increase in urinary cGMP excretion but there was no change in blood pressure. A modest, transient but

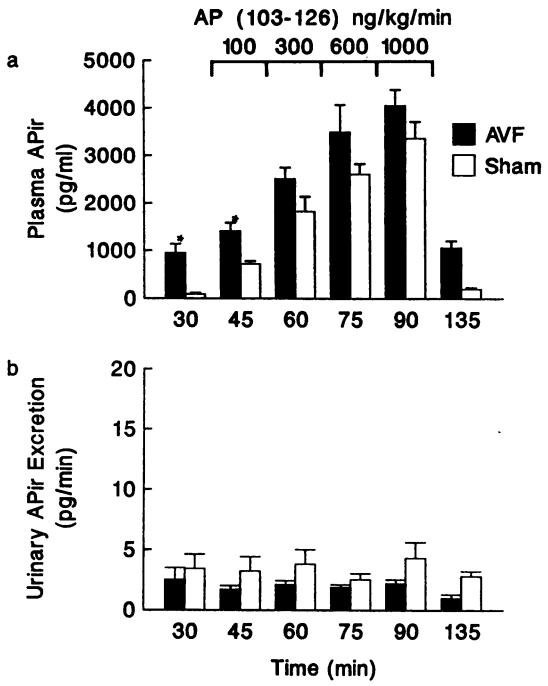

FIG. 2. Effect of increasing doses (100–1000 ng·kg^{-1}·min^{-1}) of AP-(103–126) on plasma AP_{ir} (*a*) and urinary AP_{ir} (*b*) excretion in A-V fistula (AVF) vs. sham-operated rats. Means ± SEM. *, $P < 0.05$ compared with sham-operated control.

significant ($P < 0.05$) increase in plasma AP_{ir} levels was seen in the A-V fistula rats (Fig. 5); a small, nonsignificant increase in plasma AP_{ir} concentration was seen in the control group. More impressive were the changes in urinary AP_{ir} levels, which in the A-V fistula rats were rapid, very pronounced ($P < 0.01$), and, as with the natriuresis, diminished gradually after the infusion was stopped.

Effect of Anti-ANF Monoclonal Antibody Thiorphan Response. Pretreatment with the monoclonal antibody against AP inhibited the increase in urinary sodium and cGMP excretion induced by thiorphan (Table 2). Thus a 7-fold

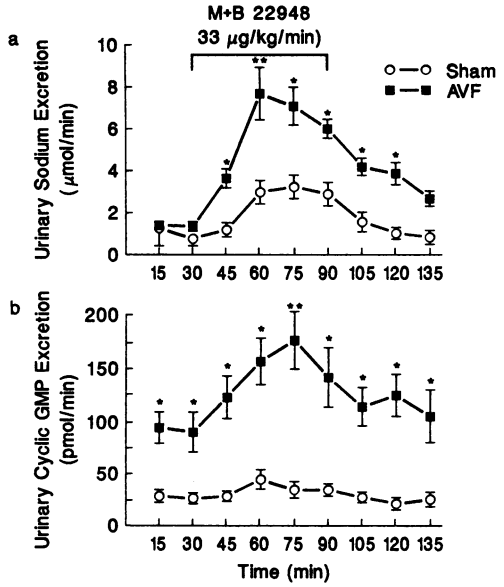

FIG. 3. Effect of M+B 22948 infusion (33 μg·kg^{-1}·min^{-1}) on sodium excretion (*a*) and urinary cGMP excretion (*b*) in A-V fistula (AVF) vs. sham-operated rats. Means ± SEM. *, $P < 0.05$; **, $P < 0.01$ compared with sham-operated control.

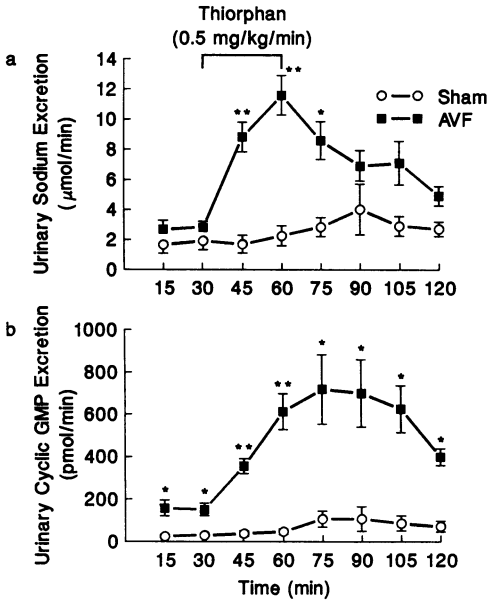

FIG. 4. Effect of thiorphan infusion (0.5 $mg \cdot kg^{-1} \cdot min^{-1}$) on sodium excretion (*a*) and urinary cGMP excretion (*b*) in A-V fistula (AVF) vs. sham-operated rats. Means ± SEM. *, $P < 0.05$; **, $P < 0.01$ compared with sham-operated control.

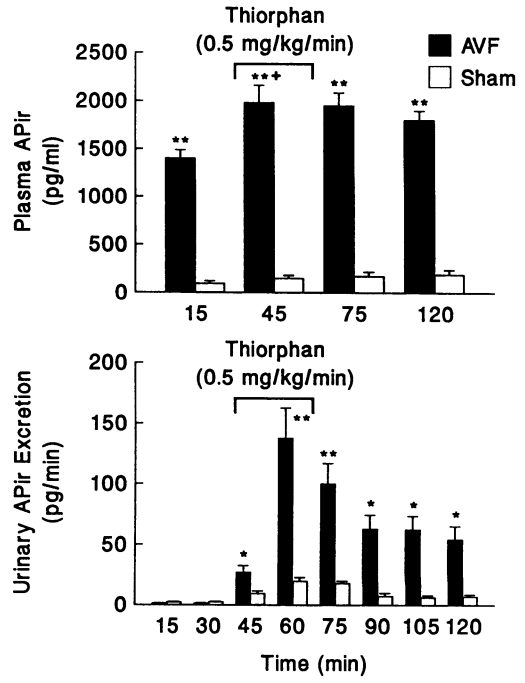

FIG. 5. Effect of thiorphan infusion (0.5 $mg \cdot kg^{-1} \cdot min^{-1}$) on plasma AP_{ir} (*a*), and urinary AP_{ir} excretion (*b*) in A-V fistula (AVF) vs. sham-operated rats. Means ± SEM. *, $P < 0.05$; **, $P < 0.01$ compared with sham-operated control. +, $P < 0.05$ compared with baseline.

increase in sodium excretion was seen in the fistula rats that received bovine immunoglobulin ($P < 0.01$) compared to a transient 2-fold increase in the group that received inactivating anti-atrial natriuretic factor monoclonal antibody. This antibody blocked any further increase in sodium excretion in response to a challenge infusion of AP-(103–126), but the animals were able to respond to pBNP, a structurally related natriuretic peptide.

DISCUSSION

Elevated plasma AP_{ir} concentration is a consistent finding in cardiac failure (3, 4). While reduced clearance may be a contributory factor, there is ample evidence for increased cardiac AP synthesis, most notably in the ventricle (17–20). Furthermore, the increased urinary cGMP excretion is functionally significant. cGMP has been shown to act as a second messenger for AP, particularly in the renal actions of the peptide (11, 21–23), and urinary cGMP levels may be used as a marker of AP activity (24, 25). Taken together, the high plasma AP_{ir} levels with recruitment of the ventricle for AP synthesis and the increased urinary cGMP levels suggest an active role for AP in heart failure.

However, further increases in plasma AP_{ir} concentration in A-V fistula rats by infusion of exogenous peptide led to an attenuated increase in urinary sodium and cGMP excretion and a smaller decrease in blood pressure compared to the sham-operated animals. These findings confirm and extend those of others, who have reported blunting of the natriuretic effect of AP in animal models of low-output heart failure (26, 27) and patients with congestive cardiac disease (5). This resistance to AP may be due to a number of factors that operate to retain sodium in heart failure; for example, increased activity of the renin-aldosterone system (28), increased renal sympathetic nerve activity (29), and reduction in renal perfusion pressure (30) have all been shown to oppose the action of the peptide. In addition, our observation that the increase in urinary cGMP excretion was also diminished in the A-V fistula animal provides evidence that down-regulation of AP receptors may be another contributory factor.

In contrast, both M+B 22948 and thiorphan were very effective in stimulating a natriuresis in the A-V fistula rat. The 3-fold increase in sodium excretion during low-dose M+B 22948 infusion in the sham-operated animals is comparable with our previous findings with this compound in normal conscious rats (11). The 7-fold increase in sodium excretion in the A-V fistula animals with M+B 22948 represents a significantly greater natriuresis and was achieved with a minimal decrease in blood pressure. The selectivity of M+B 22948 for cGMP phosphodiesterase (31), the accompanying increase in urinary cGMP excretion, and its greater potency

Table 2. Effect of monoclonal antibody (mAb) to AP on response to thiorphan (30 mg/kg), AP-(103–126) (600 $ng \cdot kg^{-1} \cdot min^{-1}$), and pBNP (600 $ng \cdot kg^{-1} \cdot min^{-1}$) in A-V fistula rats

	Basal	Thiorphan	AP-(103–126)	pBNP
$U_{Na}V$, μmol/min				
Control	1.0 ± 0.4	7.27 ± 1.19*	—	—
mAb	1.5 ± 0.6	3.2 ± 1.5	3.3 ± 1.1	7.1 ± 2.7*
$U_{cGMP}V$, pmol/min				
Control	101 ± 23	624 ± 157*	—	—
mAb	86 ± 12	101 ± 13	110 ± 17	360 ± 54*

Controls received bovine immunoglobulin. Results are expressed as means ± SEM ($n = 4$ in each group). $U_{NA}V$ = ([Na] · vol)/time (min) = urinary Na excretion per min. $U_{cGMP}V$ = urinary cGMP excretion per min.

*$P < 0.05$ compared to baseline.

in this model of stimulated cGMP production suggest that the compound was acting by potentiation of cGMP.

The increase in sodium excretion in the A-V fistula animals during infusion of thiorphan was particularly impressive. A small nonsignificant increase in sodium excretion was observed in the sham-operated animals, whereas the peak natriuresis in the A-V fistula rats matched that seen in the sham-operated rats given high doses of exogenous peptide. The natriuresis in both groups was accompanied by an increase in plasma AP_{ir} concentration and urinary cGMP excretion, compatible with an inhibitory effect of thiorphan on AP metabolism. However, the magnitude of the increase, even in A-V fistula animals, was only a fraction of that achieved during the AP infusion experiments. Given the resistance of this model of heart failure to the effect of infused peptide, these data suggest that the natriuretic response to thiorphan cannot be explained simply in terms of an increase in circulating AP_{ir} levels.

Nonetheless, the effect of thiorphan was markedly attenuated by pretreatment with monoclonal antibody directed against AP, evidence that AP is a prime factor in initiating the natriuretic response to the drug. An interesting observation in healthy rats is that coadministration of thiorphan with AP potentiates the natriuretic but not the hypotensive response to the peptide (15). Similarly, in A-V fistula rats, thiorphan enhanced the natriuretic activity of AP with little effect on blood pressure. An attractive hypothesis that would explain the preferential enhancement of the natriuretic action of AP and the greater potency of thiorphan in heart failure compared to infusions of the peptide is that the compound protects filtered AP from degradation in the kidney and enables the peptide to reach normally inaccessible renal tubule sites. Consistent with this idea is the appearance of AP_{ir} in the urine of animals treated with thiorphan. Very little AP_{ir} was detected in the urine of sham-operated or A-V fistula rats under basal conditions, or even when plasma AP_{ir} levels were greatly elevated by infusion of the peptide. Clearly, the proximal tubule, which is replete in NEP, is capable of degrading any AP that is normally filtered by the kidney. However, inhibition of the enzyme with thiorphan resulted in the prompt appearance of large amounts of AP_{ir} in the urine, an effect more pronounced in the A-V fistula rats. Two locations immediately suggest themselves as tubule sites where AP may exert its natriuretic effect. Receptors for AP have been demonstrated in inner medullary collecting ducts (25); whether these are sited at the luminal or basal membrane is not clear, but if luminal, they may be capable of responding to increased filtered load of peptide. Another potential site of action of filtered AP is the proximal tubule, where an interaction between AP and angiotensin II favoring natriuresis has been described (32, 33).

In summary, the studies reported here suggest that inhibition of cGMP degradation and NEP activity are more effective than infusion of exogenous peptide as a means of maximizing AP activity in heart failure. Furthermore, these manipulations appear to focus the activity of AP on the kidney rather than enhance the hypotensive actions of the peptide. The implication for the patient with cardiac failure is not only the avoidance of the problems of peptide administration but also the possibility of increasing sodium excretion without compromising cardiac function. It is tempting to speculate on another advantage of these manipulations; that is, the greater efficacy of these inhibitors in the presence of elevated circulating AP levels suggests that as diuresis proceeds and AP levels fall, they become less potent, thus safeguarding the patient from overdiuresis with all its attendant adverse consequences.

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