Enkephalinase Inhibition Increases Plasma Atrial Natriuretic Peptide Levels, Glomerular Filtration Rate, and Urinary Sodium Excretion in Rats With Reduced Renal Mass

Helen M. Lafferty, Mark Gunning, Patricio Silva, Mark B. Zimmerman, Barry M. Brenner, and Sharon Anderson

To investigate the in vivo effects of inhibition of endopeptidase 24.11, an enkephalinase enzyme shown to be involved in atrial natriuretic peptide (ANP) breakdown in vitro, we infused phosphoramidon, a specific inhibitor of endopeptidase 24.11, into rats with reduced renal mass (and chronic extracellular volume expansion) and into normal rats. Relative to baseline values in rats with remnant kidneys, phosphoramidon led to elevations of plasma ANP levels and concomitant increases in urinary sodium excretion, fractional excretion of sodium, glomerular filtration rate, filtration fraction, and urinary cyclic GMP excretion. Similar changes in renal function and urinary cyclic GMP excretion were obtained with thiorphan, another endopeptidase 24.11 inhibitor. These enhanced ANP levels and renal actions were not observed with phosphoramidon in normal rats. These results show that plasma ANP levels can be modulated in rats with reduced renal mass by inhibition of endopeptidase 24.11. (Circulation Research 1989;65:640-646)

Atrial natriuretic peptide (ANP) is a 28-amino acid peptide secreted by atrial myocytes in response to atrial distension. Its actions include elevation of glomerular filtration rate (GFR) and solute excretion, inhibition of renin and aldosterone release, relaxation of precontracted vascular smooth muscle, and reduction of systemic blood pressure. The hormone appears to be intimately involved in maintenance of extracellular fluid-volume homeostasis under conditions of acute volume disturbance and also in states of chronic volume overload in which an adaptive increase in sodium excretion is necessary to maintain extracellular volume homeostasis.

Recent studies have demonstrated the kidney to be a major site of degradation of ANP. The renal brush border is rich in endopeptidase 24.11, an enkephalinase enzyme, which degrades ANP in vitro. This in vitro degradation is completely inhibited by the addition of phosphoramidon, a specific inhibitor of endopeptidase 24.11.

The present studies were undertaken to investigate whether endopeptidase 24.11 inhibition in vivo would augment plasma ANP levels and thus enhance the hemodynamic and natriuretic actions of endogenous ANP. Therefore, we studied the effects of intravenous infusions of two different endopeptidase 24.11 inhibitors, phosphoramidon and thiorphan, on systemic and renal hemodynamic parameters in normal rats and in rats with five-sixths renal ablation, a model characterized by plasma volume expansion and elevated plasma ANP levels.

Materials and Methods

Adult male Munich-Wistar rats with initial weights of 220-260 g were used for these studies. Some of the rats were subjected to five-sixths renal ablation by removal of the right kidney and infarction of approximately two thirds of the left kidney by
ligation of two or three branches of the left renal artery. All groups were fed standard rat chow (Wayne Rodent Blox, Allied Mills, Chicago, Illinois) and allowed free access to water.

Renal Function Studies

Functional studies were performed 4 weeks after renal ablation. For these studies, rats were anesthetized with Inactin (100 mg/kg body wt i.p.) and placed on a temperature-regulated table. Immediately after the induction of anesthesia, the left femoral artery was catheterized with PE-10 polyethylene tubing, followed by a baseline collection of 210 μl blood for measurement of hematocrit (Hct) and inulin and para-aminohippurate (PAH) blanks and 300 μl blood for measurement of plasma ANP level. This arterial catheter was used for subsequent periodic blood sampling and estimation of mean arterial pressure (MAP), monitored with an electronic transducer connected to a direct-writing recorder. Polyethylene catheters were also inserted into the left and right jugular and right femoral veins for infusions of inulin, PAH, plasma, and experimental drugs, and the left ureter was catheterized with PE-10 polyethylene tubing. Since the plasma volume of rats prepared in this way is reduced by about 20%, the following protocol for maintaining the euvolemic state was used. After insertion of the jugular catheters, isoncotic rat plasma was infused for approximately 25 minutes in a total amount equal to 1% body weight; then the infusion rate was reduced to 0.58 ml/hr for the remainder of the experiment to maintain the baseline hematocrit at 36%. After induction of anesthesia the inulin was infused at 1.2 ml/hr from the start of the experiment at a concentration of 4% in 0.9% NaCl with PAH included at a concentration of 0.3%. Surgical preparation for the normal rats was identical; however, since pilot studies had established that the increases in urine volume and urinary sodium concentration were progressive from 20 minutes after infusion, for these parameters the results are of measurements taken at 100 minutes only.

Analytical

Inulin concentrations in plasma and urine were determined by a macroanthrone method. PAH concentrations in plasma and urine were measured by the method of Smith et al. Plasma and urinary sodium concentrations were measured by flame photometry. ANP levels were measured by a highly sensitive radioreceptor assay and ur cGMP levels were measured by radioimmunoassay (Biomedical Technologies, Stoughton, Massachusetts).

Statistical

Differences between baseline and experimental periods within the same animal were analyzed by the paired t test, and differences between groups were analyzed by comparison of percent changes with the unpaired t test. All results are presented as mean±SEM, and statistical significance is defined as p<0.05.

Table 1. Effect of Infusion of Phosphoramidon or Vehicle on Systemic and Renal Parameters in Rats With Reduced Renal Mass

<table>
<thead>
<tr>
<th>Drug</th>
<th>MAP (mm Hg)</th>
<th>U_nV (μeq/min)</th>
<th>FEna (%)</th>
<th>GFR (ml/min)</th>
<th>FF</th>
<th>pANP (pmol/l)</th>
<th>ur cGMP (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoramidon (n=10)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pre</td>
<td>162±10</td>
<td>0.63±0.15</td>
<td>0.72±0.17</td>
<td>0.63±0.05</td>
<td>0.32±0.01</td>
<td>56±3</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>Post</td>
<td>151±8</td>
<td>3.09±0.71*†</td>
<td>2.94±0.7*†</td>
<td>0.80±0.05*</td>
<td>0.37±0.02*†</td>
<td>169±28*†</td>
<td>27.5±5.2*†</td>
</tr>
<tr>
<td>Vehicle (n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Pre</td>
<td>150±11</td>
<td>0.28±0.06</td>
<td>0.33±0.08</td>
<td>0.60±0.07</td>
<td>0.34±0.02</td>
<td>54±3</td>
<td>6.3±1.9</td>
</tr>
<tr>
<td>Post</td>
<td>152±11</td>
<td>0.56±0.17</td>
<td>0.52±0.10</td>
<td>0.66±0.08</td>
<td>0.34±0.01</td>
<td>56±6</td>
<td>8.3±1.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MAP, mean arterial pressure; U_nV, urinary sodium excretion; FEna, fractional excretion of sodium; GFR, glomerular filtration rate; FF, filtration fraction; pANP, plasma atrial natriuretic peptide levels; ur cGMP, urinary cyclic GMP excretion; Pre, before infusion; Post, after infusion.

* p<0.05 vs. period 1, † p<0.05 vs. vehicle.
Results

Studies With Phosphoramidon and Thiophan in Rats With Renal Ablation

The effects of phosphoramidon infusion into five-sixths nephrectomized rats are summarized in Table 1 and Figures 1–3. After phosphoramidon infusion, there was a tendency for MAP to fall although the change did not reach statistical significance. Vehicle had no effect on MAP. Infusion of phosphoramidon resulted in a significant increase in UNaV, which significantly exceeded the small rise seen with vehicle alone (Figure 1). This natriuresis resulted from increases in both urine flow rate (from 11.9±1.9 to 29.1±4.6 µl/min) and urinary sodium concentration (from 48.2±8.9 to 101.3±10.5 meq/l), both being significantly greater than the small changes occurring during vehicle infusion alone, which averaged 9.4±1.4–12.4±1.2 µl/min for urine volume and 28.7±3.4–43.4±11.8 meq/l for urinary sodium concentration. Similarly, there was a four-fold rise in FENa, which was significantly greater than the rise after infusion of vehicle alone.

In addition to these natriuretic effects, phosphoramidon induced a significant rise in GFR, which was considerably greater than the small rise seen with vehicle alone, although the difference did not reach statistical significance. Phosphoramidon had no effect on renal plasma flow and thereby led to a significant increase in FF, whereas FF remained unchanged in the vehicle-treated group. In association with these renal effects, phosphoramidon infusion was associated with an increase in plasma ANP levels of more than 200%, but vehicle infusion was without effect (Figure 2). Excretion of ur cGMP also rose significantly after phosphoramidon infusion; the increase was significantly greater than that seen in vehicle-treated animals (Figure 3). Thus, infusion of phosphoramidon into rats with diminished renal mass resulted in a marked diuresis and natriuresis as well as a rise in GFR and FF, effects similar to those seen after infusion of ANP.19,20 These renal effects were associated with augmentation of already elevated plasma ANP levels and also of ur cGMP excretion.

Infusion of thiophan, another enkephalinase inhibitor, resulted in similar significant increases in UNaV, FENa, GFR, FF, and ur cGMP in rats with reduced renal mass when compared with vehicle-treated animals (Table 2).

Studies With Phosphoramidon in Normal Rats

The results of phosphoramidon and vehicle infusions into normal rats are shown in Table 3 and Figures 4–6. MAP remained unchanged in both groups. Although both UNaV (Figure 4) and FENa rose with phosphoramidon, the increases were smaller and more variable than the increases seen in the rats with renal ablation and did not differ significantly from the increases seen in the vehicle-treated normal animals. Comparable small rises in GFR were observed in both phosphoramidon- and vehicle-infused animals.

Plasma ANP levels rose from a baseline of 12±1 to 24±4 pmol/l after infusion of phosphoramidon; this increase was not different from the rise from 14±2 to 21±4 pmol/l observed in the vehicle-infused animals.
treated animals (Figure 5). Both groups showed a similar increase in ur cGMP (Figure 6). No significant differences were observed in any of the parameters measured between the phosphoramidon-treated and vehicle-treated groups. Thus, infusion of phosphoramidon into normal animals resulted in variable and small increases in UNaV and GFR that did not differ significantly from the increases seen in vehicle-treated animals and that were of lesser magnitude than the increases seen with endopeptidase 24.11 inhibitor in partially nephrectomized animals. In association with these changes, plasma ANP levels and ur cGMP excretion rose to a similar extent in both phosphoramidon-treated and vehicle-treated animals.

Discussion

ANP exerts its actions on the kidney by several mechanisms, including actions on the vasculature and direct effects on transporting epithelia. The precise mechanisms of action of ANP are currently being defined, and it appears that ANP mediates its effects by action on at least two independent receptor classes. Stimulation of the 130-kDa receptor leads to activation of particulate guanylate cyclase and generation of cGMP as a second messenger. This physiological activity of the 60-kDa receptor is not as yet clearly defined.

The present studies were undertaken to investigate whether in vivo endopeptidase 24.11 inhibition would potentiate ANP action and whether such augmentation of ANP action might have pathophysiological importance. Accordingly, we studied the effects of infusion of phosphoramidon and thiorphan, inhibitors of the enzyme endopeptidase 24.11, on renal function in rats with renal ablation and chronic extracellular volume expansion, as well as in normal rats. Endopeptidase 24.11, one of many peptidases located on the renal brush border, is involved in the breakdown of numerous vasoactive peptides, including angiotensins I, II, and III, oxytocin, and bradykinin; this enzyme hydrolyzes bonds involving the amino groups of hydrophobic residues provided they are not N- or C-terminal and contains a zinc atom at the active site. Endopeptidase 24.11 has been shown by Stephenson and Kenny to participate in ANP degradation at the renal brush border in vitro. In that study, ANP was incubated with either renal microvillus membrane preparations or purified endopeptidase 24.11, and in each case a single major breakdown product was observed, with an amino acid composition identical to ANP but fully separated from ANP by high-performance liquid chromatography, consistent with hydrolysis of a peptide bond within the ring structure of ANP. Breakdown of ANP was completely inhibited by the addition of phosphoramidon, a specific inhibitor of endopeptidase 24.11. Subsequently, Koehn et al confirmed these findings and, by identifying free-amino termini in the breakdown product, found that the hydrolysis occurred at the Cys 7-Phe 8 bond within the ring structure. Phosphoramidon and other inhibitors of the endopeptidase 24.11 enzyme have been designed with an aromatic moiety that will interact with the hydrophobic pocket of the enzyme, and a group such as thiol or phos-

<table>
<thead>
<tr>
<th>Drug</th>
<th>MAP (mm Hg)</th>
<th>UNaV (mg/min)</th>
<th>FENa (%)</th>
<th>GFR (ml/min)</th>
<th>FF</th>
<th>ur cGMP (pmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiorphan (n=7)</td>
<td>149±7</td>
<td>0.21±0.04</td>
<td>0.36±0.17</td>
<td>0.58±0.08</td>
<td>0.34±0.03</td>
<td>6.5±1.9</td>
</tr>
<tr>
<td>Post</td>
<td>154±9</td>
<td>1.65±0.42*</td>
<td>1.91±0.71**</td>
<td>0.78±0.11**</td>
<td>0.40±0.02*</td>
<td>16.6±6.5**</td>
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<tr>
<td>Vehicle (n=5)</td>
<td>139±8</td>
<td>0.23±0.05</td>
<td>0.24±0.06</td>
<td>0.76±0.15</td>
<td>0.31±0.03</td>
<td>6.5±0.8</td>
</tr>
<tr>
<td>Pre</td>
<td>137±11</td>
<td>0.42±0.17</td>
<td>0.36±0.10</td>
<td>0.78±0.17</td>
<td>0.32±0.02</td>
<td>8.4±1.9</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MAP, mean arterial pressure; UNaV, urinary sodium excretion; FENa, fractional excretion of sodium; GFR, glomerular filtration rate; FF, filtration fraction; pANP, plasma atrial natriuretic peptide levels; ur cGMP, urinary cyclic GMP excretion; Pre, before infusion; Post, after infusion.

*p<0.05 vs. period 1, **p<0.05 vs. vehicle.
Table 3. Effect of Infusion of Phosphoramidon or Vehicle on Systemic and Renal Parameters in Normal Rats

<table>
<thead>
<tr>
<th>Drug</th>
<th>MAP (mm Hg)</th>
<th>UNaV (μeq/min)</th>
<th>FENa (%)</th>
<th>GFR (ml/min)</th>
<th>FF</th>
<th>pANP (pmol/l)</th>
<th>ur cGMP (pmol/min)</th>
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<tbody>
<tr>
<td>Phosphoramidon</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Pre</td>
<td>120±5</td>
<td>0.61±0.28</td>
<td>0.38±0.17</td>
<td>1.11±0.09</td>
<td>0.28±0.02</td>
<td>12±1</td>
<td>5.2±1.2</td>
</tr>
<tr>
<td>Post</td>
<td>118±6</td>
<td>2.16±0.78</td>
<td>1.12±0.49</td>
<td>1.36±0.12*</td>
<td>0.30±0.03</td>
<td>24±4*</td>
<td>14.8±3.6*</td>
</tr>
<tr>
<td>Vehicle (n=6)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pre</td>
<td>125±5</td>
<td>0.54±0.16</td>
<td>0.39±0.10</td>
<td>0.97±0.06</td>
<td>0.28±0.01</td>
<td>14±2</td>
<td>2.9±0.6</td>
</tr>
<tr>
<td>Post</td>
<td>122±5</td>
<td>1.53±0.39*</td>
<td>0.92±0.14*</td>
<td>1.13±0.13</td>
<td>0.28±0.02</td>
<td>21±4</td>
<td>8.2±2.8*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MAP, mean arterial pressure; UNaV, urinary sodium excretion; FENa, fractional excretion of sodium; GFR, glomerular filtration rate; FF, filtration fraction; pANP, plasma atrial natriuretic peptide levels; ur cGMP, urinary cyclic GMP excretion; Pre, before infusion; Post, after infusion.

*p<0.05 vs. period 1; t p<0.05 vs. vehicle.

Phosphoramidon will interact with the zinc atom.27,28 Thiorphan is another such inhibitor; phosphoramidon is more specific for endopeptidase 24.11, and thiorphan has some capacity to inhibit angiotensin converting enzyme.29

In this study, phosphoramidon and thiorphan, when infused into rats with diminished renal mass, caused an impressive natriuresis and diuresis. This increase in salt and water excretion, if it occurred alone, could be explained by enhanced activity of any natriuretic peptide whose metabolism is known to be affected by endopeptidase 24.11. The natriuresis seen in the present study, however, occurred in combination with a rise in GFR and FF and an increase in ur cGMP excretion, effects similar to those seen after infusion of ANP.19,20,30 The data are consistent with the hypothesis that these compounds exert their in vivo effects by inhibiting the degradation of endogenous ANP by endopeptidase 24.11, thus allowing a higher plasma ANP concen-
ANP from the circulation of bilaterally nephrectomized rats was delayed in the presence of the enkephalinase inhibitor; this delayed disappearance resulted in the appearance of ANP in the urine. This finding is consistent with inhibition of ANP breakdown at the proximal brush border with excretion of the intact peptide, rather than an increase in plasma levels of ANP. The enzyme is also located at other sites, including intestine, adrenal glands, lymph nodes, pancreas, and salivary glands. Accordingly, we postulate that the enzyme is inhibited at another location, either intrarenal or extrarenal. Recent work by other investigators lends support to the possibility that the enzyme is located at an extrarenal site. SCH 39370, another specific inhibitor of endopeptidase 24.11, failed to inhibit ANP breakdown in two-kidney rats but resulted in the appearance of ANP in the urine. This finding is consistent with inhibition of ANP breakdown at the proximal brush border with excretion of intact peptide and no augmentation of circulating ANP level. Furthermore, disappearance of infused ANP from the circulation of bilaterally nephrectomized rats was delayed in the presence of the enkephalinase inhibitor; this delayed disappearance is consistent with an extrarenal site of inhibition of ANP breakdown. The possibility that the vasculature may be a site of endopeptidase action and ANP breakdown is suggested by the finding that isolated carotid artery segments incubated with ANP release amino acid sequences not accounted for by the action of carboxypeptidase enzymes alone; thus, the possibility of endopeptidase activity in these vessels is suggested.

The observed differences between phosphoramidon effects in normal and partially nephrectomized rats raise several interesting points. As previously noted, baseline plasma ANP levels were higher in the rats with reduced renal mass than in the normal animals; these higher levels reflect the chronic volume overload that characterizes this model. The failure of enkephalinase inhibition to alter UNaV, FEna, GFR, and FF in normal animals may signify that a high baseline plasma ANP level, and thus a high level of enzyme activity, is necessary in order for enzyme inhibition to significantly augment plasma ANP levels and physiological effects. Indeed, differing baseline plasma ANP levels may explain why the present study and the study of Ura et al show differences in phosphoramidon effects in normal rats. In the latter study, phosphoramidon infusion resulted in significant increases in UNaV. However, the volume replacement used by these authors was significantly greater than that used in our study. The greater fluid replacement may have resulted in acute volume expansion and elevation of plasma ANP levels (not measured in their study), leading to the high baseline UNaV values obtained and rendering the response to phosphoramidon similar to that observed in our rats with reduced renal mass. In our study, the increases in plasma ANP levels, along with the increases in UNaV and FEna seen in normal rats receiving either phosphoramidon or vehicle, presumably reflect only the response to the small volume load incurred during the course of the infusion. Alternatively, it is possible that a response to phosphoramidon would be observed in normal animals with the use of much higher doses. However, work by other investigators also suggests that baseline ANP level and enzyme activity may be important factors in determining the effectiveness of enzyme inhibition. Potentiation of the effects of exogenously infused ANP has been observed with infusions of the endopeptidase inhibitors SQ 29,072, thiorphan, and phosphoramidon (M.B. Zimmerman, unpublished observations); this occurrence suggests that, when ANP level (and therefore enzyme activity) is high enough, inhibition of enzyme activity will augment plasma ANP levels and physiological effects. Together, these findings provide strong evidence that endopeptidase 24.11 is involved in ANP breakdown in vivo, that ambient ANP level and enzyme activity are determinants of enzyme inhibitor efficacy, and that ANP breakdown (and inhibition thereof) may occur at extrarenal sites.

In conclusion, inhibition of the enzyme endopeptidase 24.11 in rats with reduced renal mass, chronic extracellular volume expansion, and elevated plasma ANP levels results in further elevation of ANP levels and enhanced ANP actions. This elevation is presumably an effect of inhibition of ANP degradation. The absence of a response in normal animals suggests that a high baseline ANP level may be necessary for the inhibitors to exert their effects.

Acknowledgments
We are grateful to L.E. Clarey, S.J. Downes, S.L. Riley, K.J. Sandquist, and J.L. Troy for expert technical assistance.

References

**KEY WORDS** • atrial natriuretic peptide • phosphoramidon • cyclic GMP • natriuresis
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