Neutral endopeptidase inhibition potentiates the natriuretic actions of adrenomedullin

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ADRENOMEDULLIN (ADM) is a potent renal vasodilating and natriuretic peptide initially isolated from human pheochromocytoma. Recent investigations have revealed that exogenous ADM increases urinary sodium excretion (7), which is mediated in part by the renal prostaglandin system and nitric oxide (6, 13). These previous studies suggest that the natriuretic response is also a result of increases in renal blood flow (RBF) and glomerular filtration rate (GFR) together with decreases in tubular sodium reabsorption. In addition, studies have established that the biological actions of ADM may also involve activation of receptors coupled to adenylate cyclase with cAMP generation (3, 5).

Studies have revealed that ADM is a 52 amino acid peptide with one intramolecular disulfide bond and a carboxy-terminal amide structure whose precursor is a 185 amino acid protein (preproADM) (10). After signal peptide cleavage, preproADM is processed to a 164 amino acid (proADM) and then to mature ADM (1—52).

Neutral endopeptidase 24.11 (NEP) is a membrane-bound metalloprotease that cleaves endogenous peptides at the amino side of hydrophobic residues. This ectoenzyme is located in greatest abundance in the kidney (9). NEP substrates include peptides such as atrial natriuretic peptide (ANP), bradykinin, and substance P (14). Indeed, inhibition of renal NEP has resulted in potentiation of the natriuresis secondary to exogenous administration of factors such as ANP (15). These studies underscore the importance of NEP as a regulator of sodium excretion. Although recent investigations have reported that the N-terminal region of proADM is cleaved in vitro by NEP (16), to date, the pathway for degradation of mature ADM remains to be defined.

The current study was designed to test the hypothesis that NEP inhibition in vivo would potentiate the renal natriuretic actions of exogenous ADM and that systemic NEP inhibition would increase plasma ADM levels. We therefore investigated bilateral renal hemodynamic and excretory function in normal anesthetized dogs in which one kidney was exposed to locally increased concentration of ADM while the contralateral kidney served as control before and during acute systemic NEP inhibition. We also determined plasma ADM before and during systemic NEP inhibition.

METHODS

Study protocol. Studies were performed in six male mongrel dogs weighing between 20 and 24 kg. Dogs were maintained on a normal sodium diet with standard dog chow (lab canine diet 5006; Purina Mills, St. Louis, MO) with free access to tap water. All studies conformed to the guidelines of the American Physiological Society and were approved by the Mayo Clinical Animal Care and Use Committee.

On the evening before the experiment, 300 mg of lithium carbonate was administered orally for the assessment of renal tubular function, and dogs were fasted overnight. On the day of the acute experiment, all dogs were anesthetized with pentobarbital sodium given intravenously (30 mg/kg). Supplemental nonhypotensive doses of pentobarbital sodium were given as needed during the experiment. After tracheal intubation, dogs were mechanically ventilated (Harvard res-
NEP INHIBITION POTENTIATES NATRIURETIC ACTIONS OF ADM

Bilateral flank incisions were made, and both kidneys were exposed via a retroperitoneal approach. Both ureters were cannulated with polyethylene catheters (PE-200) for timed urine collection, and a calibrated noncannulating electromagnetic flow probe was placed carefully around each renal artery and connected to a flowmeter (model FM 5010: Carolina Medical Electronics, King, NC) for continuous monitoring of RBF. In addition, a curved 23-gauge needle, attached to polyethylene tubing (PE-50), was inserted into each renal artery proximal to the flow probe. An infusion of normal saline (1 ml/min) was initiated to maintain the patency of the needles. Finally, the right femoral vein was cannulated with a polyethylene catheter (PE-240) for direct arterial blood pressure measurement and arterial blood sampling.

After completion of the surgical preparation, a priming dose of inulin (ICN Biomedicals, Cleveland, OH) dissolved in isotonic saline solution was injected, followed by a constant infusion of 1 ml/min to achieve a steady-state plasma inulin concentration between 40 and 50 mg/dl. The dogs were placed in dorsal suspension and allowed to equilibrate for 60 min without intervention. Body temperature was maintained by external warming (infrared heating lamp, heating pad model K-1-3; Gorman-Rupp Industries Division, Bellville, OH).

After an equilibration period of 60 min, a 30-min baseline clearance (Baseline) was performed. This was followed by a 15-min lead-in period during which ADM (1–52, human; Phoenix Pharmaceuticals, Mountain View, CA) infusion at 1 µg·kg⁻¹·min⁻¹ was begun intravenously, after which the second 30-min clearance period (ADM clearance) was performed. The contralateral kidney received a vehicle infusion (normal saline, 1 ml/min) and served as the control kidney during the experiment.

After the second clearance period, an intravenous bolus injection of Candoxatrilat at a dose of 240 µg/kg was administered, followed by continuous infusion of Candoxatrilat at a dose of 8 µg·kg⁻¹·min⁻¹. After a 15-min lead-in period with infusion of the NEP inhibitor, a 60-min NEP inhibition (ADM + NEPI) clearance was performed. At the end of the third clearance, the ADM and NEP inhibitor infusions were stopped.

Analytical methods. Plasma for electrolyte and inulin measurements was obtained from blood collected in heparinized tubes. Plasma and urine electrolytes including lithium were measured by flame-emission spectrophotometry (model IL 943 flame photometer; Instrumentation Laboratory, Lexington, MA). Plasma and urine inulin concentrations were measured by the anthrone method, and GFR was measured by the clearance of inulin. The lithium clearance technique was employed to estimate the proximal and distal fractional reabsorption of sodium. Proximal fractional reabsorption of sodium was calculated by the following formula: \[
\text{PFR}_{Na, \%} = \frac{\text{UNa} - \text{V}}{\text{INa} - \text{V}} \times 100
\]
and DFR of sodium was calculated by the following formula: \[
\text{DFR}_{Na, \%} = \frac{\text{UNa} - \text{V}}{\text{INa} - \text{V}} \times 100
\]

Results of quantitative studies are expressed as means ± SE. Statistical comparisons were performed by using the one-way ANOVA followed by post hoc Bonferroni test. Comparison between kidneys was performed by using Student’s paired t-test. Statistical significance was accepted for \(P < 0.05\).

**RESULTS**

The bilateral renal responses to unilateral infusion of ADM before and during systemic NEP inhibition are reported in Table 1. In response to ADM infusion, GFR in the ADM-infused kidney did not change, whereas RBF increased from baseline and renal vascular resistance decreased compared with the control kidney.

<table>
<thead>
<tr>
<th>Table 1. Renal hemodynamic and excretory function</th>
<th>Baseline</th>
<th>ADM</th>
<th>ADM + NEPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADM kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>39.1 ± 4.6</td>
<td>38.8 ± 6.5</td>
<td>53.9 ± 9.9</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>198 ± 40</td>
<td>288 ± 56*</td>
<td>305 ± 59*</td>
</tr>
<tr>
<td>RVR, RU</td>
<td>0.91 ± 0.26</td>
<td>0.61 ± 0.15*</td>
<td>0.59 ± 0.14*</td>
</tr>
<tr>
<td>UV, ml/min</td>
<td>0.20 ± 0.03</td>
<td>1.06 ± 0.17*</td>
<td>2.02 ± 0.36*†‡</td>
</tr>
<tr>
<td>UNa, µeq/min</td>
<td>41.2 ± 13.1</td>
<td>227.1 ± 40.9*†</td>
<td>410.5 ± 79.1*†‡</td>
</tr>
<tr>
<td>PFR_{Na, %}</td>
<td>74.9 ± 5.5</td>
<td>52.6 ± 7.8*†</td>
<td>55.6 ± 7.5</td>
</tr>
<tr>
<td>DFR_{Na, %}</td>
<td>97.7 ± 0.7</td>
<td>90.7 ± 1.2*†</td>
<td>87.2 ± 1.4*†</td>
</tr>
<tr>
<td>U_{cAMP, V, pmol·ml⁻¹·min⁻¹}</td>
<td>1,022 ± 167</td>
<td>1,196 ± 210</td>
<td>1,070 ± 183</td>
</tr>
<tr>
<td>U_{cGMP, V, pmol·ml⁻¹·min⁻¹}</td>
<td>263 ± 76</td>
<td>125 ± 39*</td>
<td>145 ± 47*</td>
</tr>
<tr>
<td>Control kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR, ml/min</td>
<td>39.7 ± 3.7</td>
<td>36.3 ± 3.9</td>
<td>48.3 ± 4.8</td>
</tr>
<tr>
<td>RBF, ml/min</td>
<td>189 ± 33</td>
<td>186 ± 32</td>
<td>177 ± 36</td>
</tr>
<tr>
<td>RVR, RU</td>
<td>0.87 ± 0.2</td>
<td>0.87 ± 0.2</td>
<td>0.99 ± 0.22</td>
</tr>
<tr>
<td>UV, ml/min</td>
<td>0.13 ± 0.02</td>
<td>0.17 ± 0.03</td>
<td>0.30 ± 0.10</td>
</tr>
<tr>
<td>UNa, µeq/min</td>
<td>25.5 ± 13.6</td>
<td>32.5 ± 17.7</td>
<td>79 ± 37</td>
</tr>
<tr>
<td>PFR_{Na, %}</td>
<td>78.3 ± 5.3</td>
<td>75.3 ± 4.3</td>
<td>72.2 ± 4.7</td>
</tr>
<tr>
<td>DFR_{Na, %}</td>
<td>98.3 ± 0.6</td>
<td>97.9 ± 0.9</td>
<td>96.3 ± 1.5</td>
</tr>
<tr>
<td>U_{cAMP, V, pmol·ml⁻¹·min⁻¹}</td>
<td>803 ± 91</td>
<td>804 ± 188</td>
<td>966 ± 195</td>
</tr>
<tr>
<td>U_{cGMP, V, pmol·ml⁻¹·min⁻¹}</td>
<td>232 ± 33</td>
<td>187 ± 24</td>
<td>232 ± 42</td>
</tr>
</tbody>
</table>

Values are means ± SE. ADM + NEPI, ADM + NEP inhibition clearance; ADM, adrenomedullin; NEP, neutral endopeptidase 24.11; GFR, glomerular filtration rate; RBF, renal blood flow; RVR, renal vascular resistance; UV, urine flow; UNa, urinary sodium excretion; PFR_{Na, \%}, proximal fractional reabsorption of sodium; DFR_{Na, \%}, distal fractional reabsorption of sodium; U_{cAMP, V}, urinary cAMP excretion; and U_{cGMP, V}, urinary cGMP excretion. *\(P < 0.05\) vs. baseline. †\(P < 0.05\) vs. ADM clearance. ‡\(P < 0.05\) vs. control kidney.
Urine flow and urinary sodium excretion increased from baseline and were greater than that observed in the control kidney. Proximal and distal fractional reabsorption of sodium decreased in the ADM-infused kidney compared with the control kidney. There was no change in urinary cAMP excretion in either kidney, with no increase in urinary cGMP excretion.

Systemic NEP inhibition further increased urine flow and urinary sodium excretion in the ADM-infused kidney. This increase remained higher compared with the contralateral control kidney. There was no change in urinary cAMP excretion in either kidney, with no increase in urinary cGMP excretion.

DISCUSSION

The current investigation was designed to test the hypothesis that systemic NEP inhibition potentiates the renal actions of the newly identified peptide ADM. The present findings confirm our hypothesis and report that NEP inhibition potentiates the renal natriuretic and diuretic responses to intrarenally administered ADM in normal anesthetized dogs. The increase in sodium excretion by NEP inhibition occurred in the absence of an increase in GFR or further increase in RBF, indicating that a decrease in tubular sodium reabsorption is the mechanism for natriuresis. The increase in plasma ADM during systemic NEP inhibition supports the conclusion that ADM is a substrate for NEP.

The current study confirms previous investigations that ADM is a natriuretic peptide (2, 4, 7, 22). In the present study, low intrarenal concentrations of ADM markedly increased sodium excretion in the absence of an increase in plasma or urinary ADM. This natriuretic response was associated with an increase in RBF in the absence of an increase in GFR. Therefore, alterations in peritubular physical factors secondary to renal vasodilatation may have resulted in decreasing peritubular gradient for sodium reabsorption. Alternatively, a direct tubular action of ADM is another mechanism that must be considered.

The studies have established that the biological actions of ADM may also involve activation of receptors coupled to adenylate cyclase and with cAMP generation (3, 5). In the current investigation, however, we observed no increase in urinary cAMP excretion during intrarenal ADM infusion and systemic NEP inhibition. This lack of increase in urinary cAMP may be explained by two alternative mechanisms. The first is that the concentration of infused ADM was low and, when
infused intrarenally, had no effects on the contralateral kidney. Therefore, we may have activated adenylate cyclase receptors, but at a threshold level which did not result in the release of cellular cAMP into the urine. An alternative explanation is that nonadenylate cyclase receptors or nonadenylate cyclase mechanisms could also be involved in mediating the renal actions of ADM. This is a likely possibility based upon reports that suggest that the biological properties of ADM in the myocardium may also not be dependent upon activation of adenylate cyclase (21).

NEP is an ectoenzyme found in greatest abundance in the kidney, specifically in the brush-border vesicles of proximal tubules (9, 19). Repeated studies have established that other natriuretic peptides with disulfide rings such as ANP are degraded by NEP (9, 15, 23). As we have demonstrated in the current study, a significant increase in plasma ADM during systemic NEP inhibition supports the conclusion that ADM is an additional substrate for NEP. This conclusion is also supported by the study of Nagatomo et al. (16) in which proADM NH2-terminal 20 peptide was rapidly cleaved by NEP. In addition, Lewis et al. (11) reported that ADM is also degraded in vitro by ovine adrenal, lung, and kidney preparations and that this effect was prevented by metalloprotease inhibitors. In the latter study however, phosphoramidon, a specific inhibitor of NEP, did not alter ADM degradation (11). There are two important differences between the current study and that by Lewis et al. (11). First of all, different NEP inhibitors that may possess different potencies or specificities were used in the two studies. The additional variable is species, as the Lewis study was performed utilizing membrane preparations from ovine adrenal, kidney, and lung, whereas our investigation was performed in the intact canine. Therefore, the discrepancy between the two studies could be related to significant differences in the pharmacology of two NEP inhibitors or to the differences between the canine and ovine species.

The current study was designed to address the specific contribution of systemic NEP inhibition in potentiating the renal actions of ADM. We therefore administered ADM intrarenally at a concentration that did not result in a spillover to the other kidney or increase plasma levels so as to address the local actions of ADM independent of any changes in circulating hormones, such as ANP, which would have equally affected both kidneys. In the current study, sodium excretion was significantly potentiated only in the kidney that received ADM with no increase in urinary cGMP excretion. Such a response should exclude any contribution of ANP to the natriuretic response, as this natriuresis occurred only in the ADM-infused kidney during systemic inhibition of NEP with no increase in urinary cGMP excretion, a marker for ANP activation. We conclude that NEP inhibition specifically potentiates the natriuretic actions of ADM.

The current study has important therapeutic implications. First, intrarenal ADM administration was natriuretic and diuretic, and this action was potentiated by systemic NEP inhibition. Several studies have reported that NEP inhibition in heart failure and renal failure results in natriuresis (1, 12, 17, 18). Such natriuretic responses to NEP inhibition in cardiorenal disease states have been attributed to potentiation of ANP and kinins (12). The present study suggests that the natriuretic response to NEP inhibition in such disease states may also involve inhibition of ADM degradation. Based upon current findings, a role for ADM in the natriuretic response to NEP inhibition in these disease states should be considered.

In conclusion, the present investigation is the first to demonstrate that NEP inhibition potentiates the natriuretic and diuretic responses to intrarenal ADM. This potentiation occurs secondary to a decrease in tubular sodium reabsorption in association with renal vasodilation. Lastly, the increase in plasma ADM during systemic NEP inhibition supports the conclusion that ADM is a substrate for this ectoenzyme.

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