Effect of Neutral Endopeptidase Inhibition on the Natriuresis and Renal Clearance of Atrial Natriuretic Peptide in Perfused Rat Kidney

Masayuki Yamaguchi,[1] Yukiya Hashimoto,[1] Hiroshi Itoh,[2] Kazuwa Nakao,[2] and Ken-ichi Inui[1,3]

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INTRODUCTION

Atrial natriuretic peptide (ANP) is a cardiac hormone with potent diuretic and natriuretic activities. It is eliminated by two pathways: degradation by neutral endopeptidase (NEP), and receptor-mediated endocytosis *via* a biologically "silent" C-receptor (1–3). NEP is involved in the clearance of circulating α-rat ANP (rat ANP (1–28), α-rANP) at pharmacological levels, whereas the C-receptor plays a major role in the clearance of lower or patho/physiological levels (less than several hundreds pM) of the peptide (3). On the other hand, the administration of NEP inhibitors, thiorphan and phosphoramidon, to the rats with cardiac failure induced a remarkable increase of sodium excretion without a significant increase in plasma α-rANP levels (4,5). However, the underlying mechanism of this natriuretic effect of NEP inhibition in the kidney is unknown.

ANP has not only natriuretic/diuretic but also vasodilatory activities. We previously reported that the decreased renal perfusion pressure caused by hypotension attenuates the natriuretic/diuretic action of α-rANP (6). In addition, NEP is involved in the metabolism of several other peptides including kinins, enkephalins, and neurotensins (5). Therefore, the pharmacodynamic analysis of ANP *in vivo* is complicated. In the present study, we used the perfused rat kidney to eliminate the hypotensive effect of α-rANP by keeping the perfusion pressure constant, and thus investigated the intrinsic natriuretic effect and renal clearance of α-rANP in the presence of an NEP inhibitor.

MATERIALS AND METHODS

Materials

α-rANP and phosphoramidon were obtained from Peptide Institute (Osaka, Japan). Sodium pentobarbital was purchased from Abbott (Chicago, IL, U.S.A). Bovine serum albumin was obtained from Organon Teknika (Boxtel, Holland). All other chemicals were of the highest purity available.

Perfused Kidney

The animal experiments were performed in accordance with *Guideline for Animal Experiments of Kyoto University.* The rat kidney was perfused as described previously (7). Briefly, male Wistar rats (300 ~ 350 g) were anesthetized with pentobarbital (50 mg/kg). After the right kidney was exposed, the ureter was cannulated for the urine collection using a PE-10 tube. Heparin solution (1000 IU/kg) was injected into the femoral vein, and a venous cannula (o.d. 2 mm, i.d. 0.8 mm) was placed in the vena cava just below the right renal vein. The renal artery was cannulated *via* the mesenteric artery using a 20 G needle, and the kidney was perfused without interrupting the renal blood flow. The perfusate was Krebs-Henseleit bicarbonate buffer containing 5% (w/v) bovine serum albumin, 40 mg/dl creatinine, 5 mM glucose, 3% mannitol, and 8 kinds of amino acids (8). The perfusate was aerated with 95% O_2 + 5% CO_2 and kept at 37°C. Renal perfusion pressure was maintained at 100 mmHg throughout the experiment.

Study Protocol

We examined the natriuretic action and clearance of α-rANP, and the effect of NEP inhibition on these parameters. After a short stabilizing period, we separated the 30-minute experimental period into two parts; the early period (first 15 minutes) was used for evaluation of the effect of α-rANP (period 1), and the later (another 15 minutes) was for the evaluation of the effect of an NEP inhibitor, phosphoramidon, on it (period 2). The phosphoramidon concentration used (20 μM) is shown to inhibit the NEP activity completely (3). Urine was collected at the end of each period to determine urine volume and the urinary excretions of creatinine, glucose, and sodium. Urine volume was deduced from the sample weight assuming a density of 1.0. The glomerular filtration rate (GFR) was calculated from the creatinine clearance. The outflow of the perfusate was also sampled to evaluate the renal extraction ratio of α-rANP, which was calculated as: 1 - (outflow perfusate concentration of α-rANP/perfusate concentration of α-rANP). These experiments were performed at the α-rANP concentrations of 200, 500, and 1000 pM.

Assay

The sodium concentration in the urine was determined using an ion meter (Horiba F-8AT, Kyoto, Japan) with an ion-specific electrode (Horiba Sera-100, Kyoto, Japan). The analyses of creatinine and glucose were performed by the Jaffé method and by an o-toluidine method, respectively. The concentration of α-rANP was determined by a specific radioimmunoassay (9).

Data Analysis

Values are expressed as mean ± S.E.M. The significance of differences between corresponding values of experimental periods was calculated by Wilcoxon's signed-rank test. Multiple

[1] Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

[2] Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606, Japan.

[3] To whom correspondence should be addressed. (e-mail: inui@kuhp.kyoto-u.ac.jp)

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Table I. Viability of the Perfused Rat Kidney in Control Rats

	Period	
	Period 1	Period 2
GFR (μl/min)	532 ± 124	640 ± 173
UFR (μl/min)	76.0 ± 18.3	85.1 ± 21.9
Fractional sodium reabsorption	0.942 ± 0.008	0.932 ± 0.015
Fractional glucose reabsorption	0.964 ± 0.004	0.956 ± 0.008

Note: Mean ± S.E.M. of five rats. GFR: glomerular filtration rate; UFR: urine flow rate.

comparison was performed using ANOVA followed by Scheffé's test. A P value less than 0.05 was considered significant.

RESULTS

The rat kidney was perfused at a constant pressure of 100 mmHg. Under these conditions, the flow rate of perfusate was 16.3 ± 0.4 ml/min ($n = 5$), and was not changed significantly during the experiment. Table I shows the viability of the perfused kidney in the absence of α-rANP. The GFR, urine flow rate (UFR), and fractional sodium reabsorption were not changed throughout the experiment. The reabsorption ratio of glomerular-filtrated glucose was more than 0.94 throughout the experiment. These values were not changed by the co-administration of phosphoramidon.

We studied the effect of neutral endopeptidase inhibition on the diuresis and natriuresis of α-rANP using the perfused rat kidney. Figure 1 shows the effect of α-rANP and phosphoramidon on the GFR in the rat kidney. GFR was not changed by α-rANP alone at any of the concentrations studied, and phosphoramidon did not increase GFR. On the other hand, the fractional sodium reabsorption was about 0.95 in the absence of α-rANP, and was decreased to 0.73 at the concentration of 1000 pM of α-rANP. Phosphoramidon decreased the fractional sodium reabsorption from 0.88 to 0.80 at the α-rANP concentration of 200 pM and from 0.78 to 0.69 at 500 pM. Figure 2 shows the effect of α-rANP on the urinary sodium excretion before and after the administration of phosphoramidon. Phosphoramidon induced increases in the urinary sodium excretion at the α-rANP concentrations of 200 pM and 500 pM; however, the effect was not observed at the α-rANP concentration of 1000 pM.

To evaluate the contribution of NEP to the renal elimination of α-rANP in the perfusate, we examined the effects of NEP inhibition on the renal clearance of α-rANP. Table II shows the effect of phosphoramidon on the extraction ratio of α-rANP in the perfused rat kidney. The renal extraction ratio of α-rANP was more than 0.16 at the α-rANP concentrations of 200 pM and 500 pM. In contrast, the extraction ratio at 1000 pM of α-rANP was less than 0.05. The renal extraction ratio of α-rANP was not changed by the co-administration of phosphoramidon at any of the concentrations studied.

DISCUSSION

The aim of this study was to investigate the effect of NEP inhibition on the natriuretic action and renal clearance of α-rANP using the isolated perfused rat kidney. The addition of α-rANP to the perfusate induced concentration-dependent increases in sodium excretion at the concentration range studied (200 ~ 1000 pM), and a potent NEP inhibitor, phosphoramidon, significantly increased the natriuretic action of 200 and 500 pM of α-rANP (Figure 2). In contrast, the renal clearance of α-rANP from the perfusate was not changed in the presence of phosphoramidon (Table II). These results suggested that the inhibition of metabolism of the filtered α-rANP at glomerulus is mainly responsible for the ANP-potentiating natriuretic effect of the NEP inhibitor.

The mechanism of the natriuretic action of ANP has been discussed extensively (2). Huang et al. (10) reported that when they used a high dosing rate of 1 μg/min/kg of ANP (393 pmol/min/kg), the natriuretic effect of ANP in normal rats was the result of an increase in the GFR as well as an increase in the filtration fraction of sodium. In our experiment, however, 200

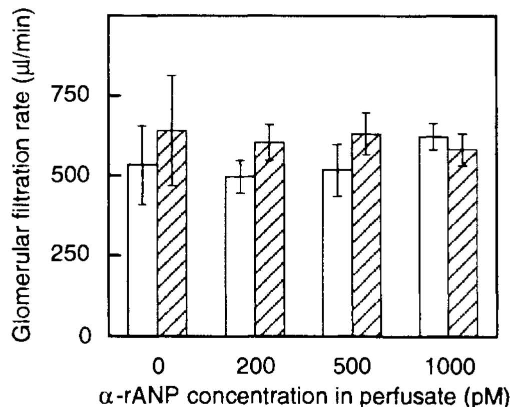

Fig. 1. Glomerular filtration rate before (open column, period 1) and after (hatched column, period 2) administration of phosphoramidon at various concentrations of α-rANP. Values are mean ± S.E.M. of five rats in each group.

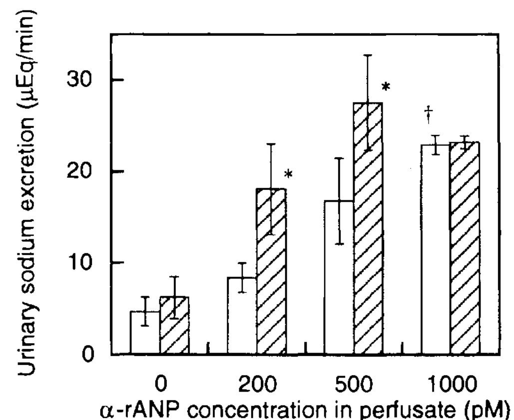

Fig. 2. Urinary sodium excretion before (open column, period 1) and after (hatched column, period 2) administration of phosphoramidon at various concentrations of α-rANP. Values are mean ± S.E.M. of five rats in each group. *Significantly different from period 1, $P < 0.05$ †Significantly different from vehicle control (0 pM of α-rANP), $P < 0.05$.

Table II. Effect of Phosphoramidon on the Extraction Ratio of α-rANP

α-rANP concenration in perfusate (pM)	α-rANP extraction	
	Period 1	Period 2
200	0.207 ± 0.039	0.215 ± 0.028
500	0.167 ± 0.037	0.178 ± 0.052
1000	0.042 ± 0.012	0.044 ± 0.019

Note: Mean ± S.E.M. of five rats in each group.

~ 1000 pM of α-rANP increased the natriuresis without changing the GFR (Figs. 1 and 2). This result suggests that the decrease in fractional sodium reabsorption is mainly responsible for the natriuresis of α-rANP at the concentration range studied. In the present study, the concentration dependence of the ANP-potentiating natriuretic effect of NEP inhibition was also investigated. Phosphoramidon increased the natriuretic action of ANP (200 ~ 500 pM) without changing the GFR (Figs. 1 and 2). On the other hand, phosphoramidon did not potentiate the natriuretic effect of 1000 pM of α-rANP. The UFR at 1000 pM of α-rANP was about 40% of the GFR; therefore, it is plausible that the diuretic/natriuretic effects of ANP were submaximal at the highest concentration used.

We previously reported that NEP plays a major role in the clearance of α-rANP at the pharmacological concentrations (3). However, in rats with myocardial infarction or aortovenocaval fistula, phosphoramidon induced a remarkable increase in natriuresis without showing a significant increase in the plasma concentration of α-rANP (5). Wilkins et al. (4) also reported that, using a rat aortovenocaval fistula model of cardiac failure which shows the increase of the plasma concentration of ANP (about 300 pM), the effect of thiorphan, another potent NEP inhibitor, on sodium excretion could not be explained solely in terms of an increase in circulating ANP levels. These results suggest that NEP inhibition in the systemic circulation is not responsible for the ANP-potentiating natriuretic action of an NEP inhibitor at the lower concentration range. However, in these *in vivo* study, we cannot neglect the possibility that the local metabolizing effect of α-rANP in the renal vascular side of the nephron is dramatically changed by an NEP inhibitor, without pharmacokinetic change of α-rANP in the whole body. In the present study, therefore, we evaluated the extraction ratio of α-rANP and effect of phosphoramidon on the extraction of it in the perfused rat kidney using a single pass condition.

The extraction ratio of α-rANP in the perfused kidney was decreased in a concentration-dependent manner (Table II), suggesting a saturable elimination pathway. In addition, phosphoramidon did not affect the renal extraction of α-rANP (Table II). Accordingly, NEP in the renal vascular side of the nephron was not significant for the metabolism of α-rANP. Maack et al. (1) reported that the co-administration of C-ANF_{4-23}, a C-receptor specific ligand, increased the natriuretic action of ANP in the isolated perfused rat kidney. The C-receptor is widely distributed in the body, and the receptor binding affinity of α-rANP for the C-receptor is about 6.6 pM (11). We also reported that the C-receptor is involved in the systemic clearance of the physiological levels of α-rANP (3). The present results suggested that the saturable elimination of α-rANP in the perfused kidney may be mainly *via* the C-receptor-mediated pathway, and also that NEP inhibition in the luminal side of the renal tubules may be mainly responsible for the ANP-potentiating action.

The localization of the biologically active receptors for ANP in the kidney has not been established. However, we found functional evidence of the existence of an ANP-A receptor, a biologically active receptor, not only at the basal but at the apical side of the LLC-PK_1 kidney epithelial cell line (12). The natriuretic peptide receptor localization in the kidney has recently been determined by immunohistochemistry (13). ANP-A receptors exist mainly at the basolateral side of inner medulla collecting duct. However, there are ANP-A receptors on the luminal side of the epithelial cells of the kidney, where ANP is thought to be impossible to access in the absence of inhibitors for NEP. That is, NEP is abundant in the brush border membrane of the proximal tubules, and the filtered natriuretic peptide at the glomerulus is completely degraded at this site (14).

In conclusion, we investigated the effect of neutral endopeptidase on renal clearance and action of ANP using perfused rat kidney, and demonstrated that the renal clearance of α-rANP is not changed by phosphoramidon. These results suggested that inhibition of metabolism of filtered α-rANP at glomerulus is mainly responsible for the ANP-potentiating natriuretic effect of the NEP inhibitor. These findings may provide new insights into the clinical use of NEP inhibitors and the underlying mechanism of NEP inhibition.

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