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Enzymatic inactivation of major circulating forms of atrial and brain natriuretic peptides

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Abstract

We compared the enzymatic inactivation of major circulating forms of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). Both ANP and BNP induced a significant increase in cyclic GMP (cGMP) formation in cultured epithelial cell line derived from porcine kidney, LLC-PK$_1$. The cGMP formation stimulated by ANP in LLC-PK$_1$ cells was significantly decreased by pre-treatment of the peptide with rat renal brush-border membranes, and the inactivation of ANP was inhibited by neutral endopeptidase inhibitors, phosphoramidon and *S*-thiorphan. BNP exhibited greater resistance to enzymatic inactivation than did ANP. In addition, phosphoramidon potentiated the natriuresis with a low dose (7.5 pmol min^{-1} kg^{-1}) of ANP but not of BNP in rats. These results suggest that enzymatic degradation of natriuretic peptides is highly dependent on peptide structure, and that the affinity of BNP to neutral endopeptidase is less than that of ANP.

Keywords: ANP (Atrial natriuretic peptide); BNP (Brain natriuretic peptide); Kidney epithelial cell line (LLC-PK$_1$); Neutral endopeptidase; Phosphoramidon; Thiorphan

1. Introduction

The vasodilating and natriuretic actions of atrial natriuretic peptide (ANP) are therapeutic for some cardiovascular disorders including hypertension and congestive heart failure. Clinical application of ANP is, however, limited because of its short biological half-life and poor bio-availability following oral administration (Wilkins et al., 1990). On the other hand, endogenous circulating ANP levels are elevated in several diseases for which ANP is considered of clinical benefit. An alternative to administering exogenous ANP is to enhance the activity of the endogenous circulating peptide by inhibiting its metabolism by neutral endopeptidase (EC 3.4.24.11). Endogenous ANP increases in cardiac failure rats produced by aortocaval fistula or myocardial infarction as compared to control rats, and treatment with the neutral endopeptidase inhibitors, thiorphan and phosphoramidon, has significant natriuretic effect in cardiac failure rats only (Wilkins et al., 1990; Yasuhara et al., 1994). However, the circulating ANP concentration is increased only slightly by the administration of neutral endopeptidase inhibitors. The findings suggest that the natriuretic response to neutral endopeptidase inhibitors in cardiac failure rats cannot be explained simply in terms of an increase in circulating ANP levels, and that ANP filtered at the glomerulus is mainly responsible for the ANP-potentiating natriuretic effect of neutral endopeptidase inhibitors. (Wilkins et al., 1990; Yasuhara et al., 1994; Yamaguchi et al., 1998).

Neutral endopeptidase is involved in the metabolism of several other peptides including kinins, enkephalins, neurotensins, and brain natriuretic peptide (BNP) (Kenny and Stephenson, 1988; Norman et al., 1991). It should be noted that circulating BNP levels are also elevated in disease states in which the renal responses to neutral endopeptidase inhibitors are more pronounced (Mukoyama et al., 1990a; Hama et al., 1995). BNP was initially isolated from

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porcine brain, but has been shown to be synthesized in, and secreted from, the hearts of rats and humans (Ogawa et al., 1990; Mukoyama et al., 1990b). Lang et al. (1992) reported that endogenous human BNP concentrations are increased by treatment with candoxatril in patients with chronic heart failure. Seymour et al. (1992) reported that the neutral endopeptidase inhibitor, SQ28603 (*N*-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]-beta-alanine), significantly potentiates the renal and depressor activities of the N-terminal-truncated 32-amino acid rat BNP analogue (rat BNP-[14-45]) in conscious spontaneously hypertensive rats (SHR). In addition, administration of neutral endopeptidase inhibitors reportedly increases the endogenous plasma levels of BNP in hypertensive or cardiac failure rats (Hirata et al., 1994; Wegner et al., 1996). These findings raise the possibility that inhibition by neutral endopeptidase may be partly BNP-dependent; however, the metabolizing activity of neutral endopeptidase for BNP has not been elucidated (Hashimoto et al., 1993; Hashimoto et al., 1994a).

In the present study, we compared the enzymatic inactivation of a major circulating form of BNP (32-amino acid human BNP or 45-amino acid rat BNP) with that of a 28-amino acid α-ANP. That is, we examined the enzymatic inactivation of the natriuretic peptides by neutral endopeptidase in isolated rat renal brush-border membranes. The biological activity of the peptides was assessed from the cyclic GMP (cGMP) formation on the A-type natriuretic peptide receptor in the kidney epithelial cell line, LLC-PK_1 (Suga et al., 1992b; Hashimoto et al., 1994b). In addition, we evaluated the effect of a neutral endopeptidase inhibitor on the renal actions of ANP and BNP in rats, where the natriuretic peptide was administered exogenously at the low dose-rate (7.5 pmol min^{-1} kg^{-1}) to increase the circulating peptide concentrations slightly.

2. Materials and methods

2.1. Materials

Phosphoramidon, α-human ANP, α-rat ANP, human BNP and rat BNP were purchased from the Peptide Institute (Osaka, Japan). *S*-Thiorphan was kindly donated by Shionogi (Osaka, Japan). Sodium pentobarbital was purchased from Abbott (Chicago, IL). All other chemicals were of the highest purity available.

2.2. Cell culture

LLC-PK_1 cells obtained from the American Type Culture Collection (ATCC CRL-1392) were cultured in plastic dishes (Corning Glass Works, Corning, NY, USA) in medium 199 (Flow Laboratories, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Microbiological Associates, Bethesda, MD, USA) without antibiotics, in an atmosphere of 5% CO_2–95% air at 37°C, and were subcultured every 4–5 days using 0.02% EDTA and 0.05% trypsin. The cells were used between passages 219 and 225.

To prepare cell monolayers cultured on permeable supports, the cells were seeded at a density of 4×10^5 cells cm^{-2} on polycarbonate membrane filters (3-μm pores) inside a Transwell™ cell culture chamber (4.71 cm^2 growth area, Coster, Cambridge, MA, USA), and the chambers were placed in 6-well cluster plates with 2.6 ml of the outside (basolateral side) medium and 1.5 ml of the inside (apical side) medium (Hashimoto et al., 1994b). In separate experiments, 35-mm plastic dishes (Iwaki Glass, Tokyo, Japan) were inoculated with 4×10^5 cells in 3 ml of complete culture medium. The medium was replaced by fresh medium every 2–4 days, and the cells were used on day 7.

2.3. Animals

Male Wistar rats, weighing 250–350 g, were used. Prior to the experiments, the rats were housed in a temperature- and humidity-controlled room with free access to water and standard rat chow. The animal experiments were performed in accordance with the guidelines for animal experiments of Kyoto University.

2.4. Cyclic GMP formation stimulated by ANP and BNP in LLC-PK_1 cell monolayers

Cyclic GMP formation stimulated by ANP and BNP in LLC-PK1 monolayers was measured at 37°C (Hashimoto et al., 1994b). Briefly, the cell monolayers in Transwell™ chambers or in 35-mm dishes were washed twice with buffer A (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Hepes/Tris, pH 7.4 and 10 mM D-glucose), and then preincubated at 37°C for 5 min with buffer A containing 0.2% bovine serum albumin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX), 1 μM phosphoramidon, and 1 μM captopril. The formation of cGMP was initiated by addition of a solution containing a natriuretic peptide to the apical side (inside a Transwell™ chamber or a 35-mm dish) or basolateral side (outside a Transwell™ chamber) of the cell monolayer. After incubation at 37°C for 5 min, the reaction was terminated by the addition of ice-cold trichloroacetic acid solution to the medium to give a final medium trichloroacetic acid concentration of 6%. The medium was collected after extraction for 30 min on ice and the residue was treated for 30 min with ice-cold 6% trichloroacetic acid. The combined supernatant was washed three times with water-saturated ethyl ether. The amount of cGMP was determined with radioimmunoassay using a commercial kit (Amersham, Buckinghamshire, UK). Net cGMP formation was evaluated by subtracting the amount

of cGMP formed after the vehicle treatment from that formed after the peptide treatment.

2.5. Inactivation of ANP and BNP by rat renal brush-border membranes

Brush-border membranes were isolated from the rat renal cortex as described previously (Hori et al., 1985). A 900-μl aliquot of brush-border membrane suspension containing 300 μg of protein in buffer A was incubated with ANP/BNP (100 μl of 300 nM solution) at 37°C. After the appropriate time, the reactions were terminated by the addition of an ice-cold stop solution consisting of 100 μl buffer A containing 2.2% bovine serum albumin, 5.5 mM IBMX, 11 μM phosphoramidon, and 11 μM captopril. In a separate experiment, the reaction was inhibited by phosphoramidon (10 μM) or *S*-thiorphan (10 μM). The cyclic GMP-generating activity of natriuretic peptides in the reaction mixture (1.1 ml) was measured using LLC-PK_1 cells cultured in 35-mm plastic dishes, as described above (Section 2.4).

2.6. Effect of phosphoramidon on natriuresis of ANP and BNP in rats

The rats were anesthetized with 35 mg kg^{-1} sodium pentobarbital. α-Rat ANP or rat BNP was dissolved in phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and 2% mannitol, and 7.5 pmol min^{-1} kg^{-1} was infused via a femoral vein at a constant rate of 110 μl min^{-1} throughout the experiment, including a 45-min stabilizing period. The administration protocol of phosphoramidon consisted of a loading dose of 1.96 mmol kg^{-1}, followed by a maintenance dose of 165 nmol min^{-1} kg^{-1} (Hashimoto et al., 1994a). A catheter was placed into the bladder for urine collection, and the urinary sodium concentration was determined using an ion meter (Horiba F-8AT, Kyoto, Japan) with an ion-specific electrode (Horiba Sera-100, Kyoto, Japan).

2.7. Data analysis

The data are given as means ± S.E. Statistical analysis was performed with Scheffé's test. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Cyclic GMP formation stimulated by ANP and BNP in LLC-PK_1 cells

Fig. 1 summarizes the net cGMP formation stimulated by natriuretic peptide (100 nM) addition to the apical or basolateral side of LLC-PK_1 cell monolayers in Transwell™ chambers. ANP and BNP induced a significant

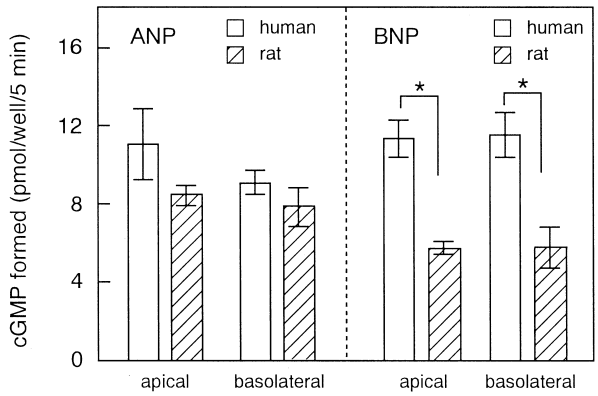

Fig. 1. cGMP formation stimulated by ANP (left panel) and BNP (right panel) added to the apical and basolateral side of the LLC-PK_1 cell monolayer at a peptide concentration of 100 nM. Mean ± S.E. for four monolayers. * Significantly different ($P < 0.05$ by Scheffé's test).

increase in cGMP formation in LLC-PK_1 kidney epithelial cells. In addition, natriuretic peptide-induced cGMP formation on the apical receptor in LLC-PK_1 cells was similar to that on the basolateral receptor (Fig. 1). The extent of cGMP formation stimulated by human BNP on the apical and basolateral receptors in LLC-PK_1 cells was slightly greater than that stimulated by α-human ANP, whereas the extent of cGMP formation stimulated by rat BNP was slightly less than that stimulated by α-rat ANP (Fig. 1). There were significant differences in cGMP formation activity between human and rat BNP ($P < 0.05$).

3.2. Inactivation of ANP and BNP by rat renal brush-border membranes

We examined the enzymatic inactivation of natriuretic peptides by neutral endopeptidase in rat renal brush-border membranes, where the biological activity of the peptides was assessed on the basis of the cGMP formation on the apical receptor in LLC-PK_1 cells cultured in plastic dishes. Fig. 2 shows the residual cGMP formation activity of ANP as a function of time of pre-treatment with rat renal brush-border membranes (Fig. 2). Both α-human ANP and α-rat ANP were significantly and time dependently inactivated by pre-treatment with brush-border membranes. The amount of cGMP stimulated by residual ANP after 3 min of pre-treatment with brush-border membranes was less than 30% of the control level (Fig. 2).

We further compared the inactivation of BNP by rat renal brush-border membranes with that of ANP. Fig. 3 shows the net cGMP production stimulated by α-human ANP (left panel) and human BNP (right panel) after 3 min of pre-treatment with rat renal brush-border membranes in the presence or absence of neutral endopeptidase inhibitors. Cyclic GMP formation stimulated by α-human ANP in LLC-PK_1 cells decreased to about 30% of the control level after pre-treatment with brush-border membranes. The addition of the neutral endopeptidase in-

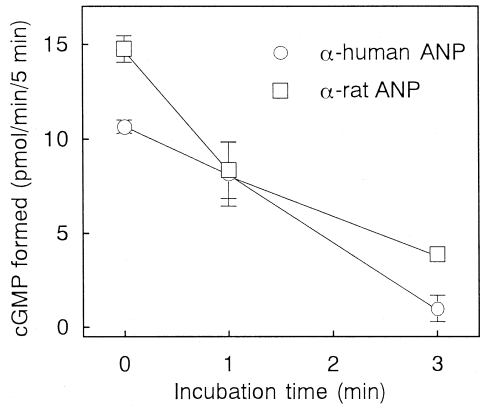

Fig. 2. Residual cGMP-formation activity of α-human ANP (circles) and α-rat ANP (squares) as a function of time of pre-treatment with rat renal brush-border membranes. Values are means for four culture dishes, and bars represent S.E.

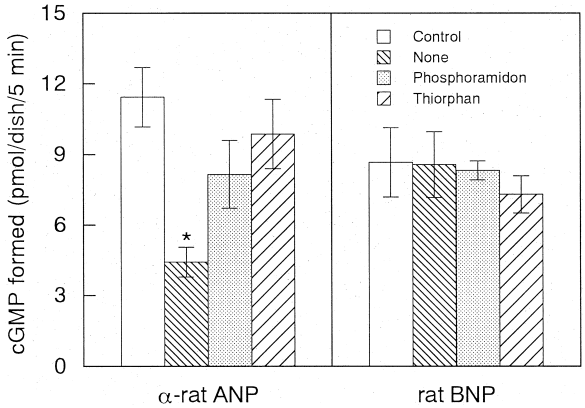

Fig. 4. Effect of neutral endopeptidase inhibitors on inactivation of α-rat ANP (left panel) and rat BNP (right panel) by rat renal brush-border membranes. Values are means for four culture dishes, and bars represent S.E. * Significantly different from control ($P < 0.05$ by Scheffé's test).

hibitors, phosphoramidon and *S*-thiorphan, almost completely inhibited the inactivation of α-human ANP by brush-border membranes. In contrast, pre-treatment (3 min) of human BNP with brush-border membranes had no influence on the cGMP formation stimulated by human BNP in the presence or absence of neutral endopeptidase inhibitors (Fig. 3).

We also investigated the inactivation of α-rat ANP and rat BNP by rat renal brush-border membranes. Fig. 4 shows the net cGMP production stimulated by α-rat ANP (left panel) and rat BNP (right panel) after 3 min of pre-treatment with brush-border membranes in the presence or absence of neutral endopeptidase inhibitors. α-Rat ANP was strongly inactivated by pre-treatment with brush-border membranes, and the decrease in cGMP production in LLC-PK_1 cells was inhibited by neutral endopeptidase inhibitors. Pre-treatment (3 min) of rat BNP with brush-border membranes had no influence on the cGMP formation stimulated by rat BNP in the presence or absence of neutral endopeptidase inhibitors (Fig. 4).

3.3. Effect of phosphoramidon on natriuresis of ANP and BNP in rats

In order to assess the natriuretic peptide-potentiating actions of neutral endopeptidase inhibition in vivo, we examined the effects of phosphoramidon on the natriuresis of α-rat ANP and rat BNP administered exogenously at a dose-rate of 7.5 pmol min^{-1} kg^{-1} in rats. Fig. 5 shows the time course of urinary sodium excretion in normal rats given α-rat ANP or rat BNP. α-rat ANP and rat BNP at this dose-rate did not have any natriuretic effects before the infusion of phosphoramidon. Following administration of phosphoramidon, urinary sodium excretion was significantly elevated in rats treated with α-rat ANP, but not in rat BNP-treated rats.

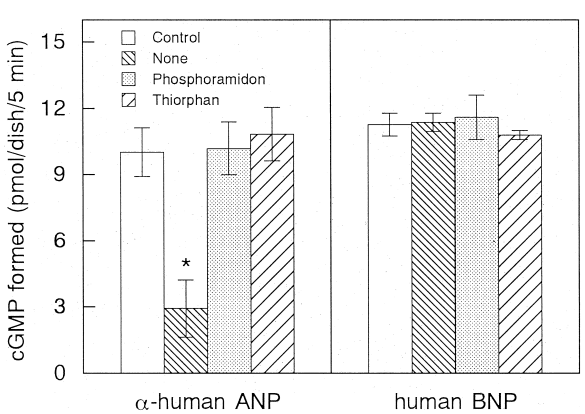

Fig. 3. Effect of neutral endopeptidase inhibitors on inactivation of α-human ANP (left panel) and human BNP (right panel) by rat renal brush-border membranes. Values are means for four culture dishes, and bars represent S.E. * Significantly different from control ($P < 0.05$ by Scheffé's test).

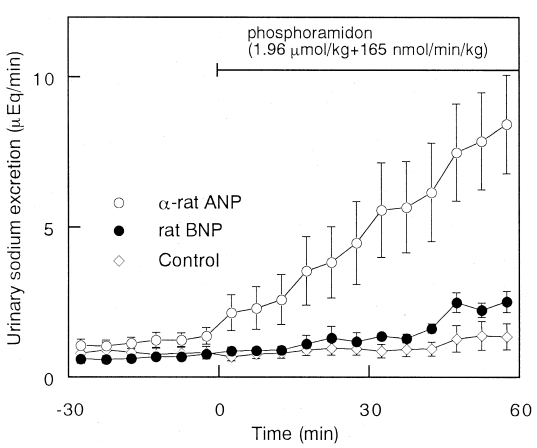

Fig. 5. Time course of mean (±S.E.) urinary sodium excretion following the administration of phosphoramidon in normal control (open diamond), α-rat ANP-treated (open circles) and rat BNP-treated (filled circles) rats. Values are means for 4–15 rats, and bars represent S.E.

4. Discussion

The structures of the mammalian ANP are highly conserved, the 28-amino acid α-rat ANP differing from α-human ANP by only one residue, Ile^{12} for Met^{12}. The ring structure, which is constructed with a disulfide bond at Cys^{7}–Cys^{23}, is indispensable for the biological actions of ANP. The enzymatic degradation of α-rat ANP and α-human ANP is mainly mediated by neutral endopeptidase, and the major target of neutral endopeptidase is the Cys^{7}–Phe^{8} bond in the ring structure (Kenny and Stephenson, 1988). BNP has striking homology to ANP for the amino acid sequence of the ring structure and very similar pharmacologic actions. However, BNP shows species differences in its sequences in the N- and C-terminal parts: the predominant circulating molecular forms are the 45-amino acid rat BNP and 32-amino acid human BNP in rats and humans, respectively (Ogawa et al., 1990; Mukoyama et al., 1990b). In addition, porcine, rat, and human BNP have a homologous Cys–Phe bond which is the corresponding site of proteolysis for α-rat ANP and α-human ANP. However, the proteolytic sites for purified rat neutral endopeptidase for 26-amino acid porcine BNP, 32-amino acid rat BNP-(14-45), and human BNP are completely different from one another (Norman et al., 1991). We now examined the inactivation of a major circulating form of natriuretic peptide by neutral endopeptidase in isolated rat renal brush-border membranes, where the biological activity of the peptides was assessed from the cGMP formation in LLC-PK_1 renal epithelial cells.

Regarding the molecular mechanism of the natriuretic peptide response, there is increasing evidence that cGMP serves as an intracellular mediator of the biological action of the peptide. Molecular cloning has shown that the particulate A-type receptor with high efficacy for ANP and BNP possesses an extracellular binding domain and an intracellular guanylate cyclase domain (Chinkers et al., 1989). The presence of another subtype (B-type receptor) with low efficacy for ANP and BNP but with high efficacy for the recently isolated C-type natriuretic peptide has also been demonstrated (Koller et al., 1991). In our previous study, we reported that the biologically active A-type receptor with high efficacy for α-human ANP is expressed in the apical and basolateral membranes of the renal epithelial cell line, LLC-PK_1 (Hashimoto et al., 1994b). In the present study, we demonstrated that human and rat BNP as well as ANP induce a significant increase in cGMP formation in LLC-PK_1 kidney epithelial cells, though there was a difference in the cGMP formation activity between human and rat BNP. In addition, the natriuretic peptide-induced cGMP formation on the apical receptor in LLC-PK_1 cells was similar to that on the basolateral receptor (Fig. 1). Therefore, in subsequent experiments, we utilized the LLC-PK_1 cells grown in 35-mm tissue culture dishes to evaluate the biological activity of ANP and BNP on the apical A-type receptor.

In the present study, α-human ANP and α-rat ANP were strongly inactivated by neutral endopeptidase in rat renal brush-border membranes (Fig. 2), and neutral endopeptidase inhibitors protected α-human ANP and α-rat ANP from enzymatic inactivation (Figs. 3 and 4). In contrast, cGMP formation stimulated by human BNP and rat BNP was not influenced by pre-treatment of the peptides with neutral endopeptidase in rat renal brush-border membranes (Figs. 3 and 4). These results indicate that BNP is more resistant than ANP to inactivation by neutral endopeptidase. The findings are consistent with in vitro data showing that purified porcine neutral endopeptidase hydrolyzes the N-terminal-elongated α-human ANP analogue, urodilatin (Thr-Ala-Pro-Arg-α-human ANP), and human BNP more slowly than α-human ANP (Kenny et al., 1993). In addition, results of recent studies have suggested that the degradation of natriuretic peptides by neutral endopeptidase seems to be highly dependent on structural differences. For example, Gagelmann et al. (1988) reported that α-human ANP is extensively degraded by a membrane preparation from dog kidney cortex, and that urodilatin is much more resistant to neutral endopeptidase than is α-human ANP.

In the previous study, we showed that phosphoramidon does not potentiate the renal actions of pharmacological doses (30–100 pmol min^{-1} kg^{-1}) of rat BNP in rats, but does potentiate the actions of α-rat ANP (Hashimoto et al., 1993). In addition, the clearance of rat BNP given intravenously (600 pmol min^{-1} kg^{-1} for 2 min) is decreased only slightly by the coadministration of phosphoramidon in rats (Hashimoto et al., 1994a). In contrast, Seymour et al. (1992) reported that SQ28603 significantly potentiates the renal and depressor activities of an N-terminal-truncated 32-amino acid rat BNP analogue (rat BNP-[14-45]), given at an intravenous bolus dose of 3 nmol kg^{-1} to conscious SHR. In the present study, therefore, we further evaluated the effect of phosphoramidon on the renal actions of a major circulating form of natriuretic peptides in normal rats, where α-rat ANP and 45-amino acid rat BNP were administered exogenously at a low dose-rate (7.5 pmol min^{-1} kg^{-1}) to increase the circulating peptide concentrations slightly. Coadministration of phosphoramidon induced a significant increase in sodium excretion in α-rat ANP-treated rats, whereas the neutral endopeptidase inhibitor did not affect natriuresis of rat BNP (Fig. 5). These findings suggest that enzymatic degradation of natriuretic peptides is highly dependent on the structures of the peptides.

In conclusion, this study demonstrated different mechanisms involved in the enzymatic inactivation of ANP and BNP. The results also indicated that the affinity of BNP to neutral endopeptidase is less than that of ANP. The findings may help us to understand the pharmacokinetic differences between ANP and BNP and the underlying mechanism of the renal actions of neutral endopeptidase inhibitors.

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