Long-Acting Natriuretic Peptide, Vessel Dilator, and Kaliuretic Peptide Enhance the Urinary Excretion Rate of β_2-Microglobulin

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The atrial natriuretic peptide (ANP) gene synthesizes a 126–amino acid (aa) prohormone from which four peptide hormones are derived. These 4 peptide hormones consisting of aa 1 to 30 (ie, long-acting natriuretic peptide [LANP]), aa 31 to 67 (vessel dilator), aa 79 to 98 (kaliuretic peptide), and aa 99 to 126 (ie, ANP) have diuretic, natriuretic, and/or kaliuretic properties. ANP has been reported to have its natriuretic and protein-excreting effects via both the proximal and distal tubules, but where in the kidney the other three peptide hormones have their natriuretic and/or diuretic effects is unknown. Further, it has never been investigated as to whether these three other peptide hormones enhance protein excretion. The present investigation was designed to determine (1) if these atrial peptides enhance protein excretion and (2) if their effects involve the proximal tubules of healthy humans by examining the excretion rate of β_2-microglobulin, a marker of proximal tubule function. Twenty-four healthy human subjects were studied following the infusion of 100 ng/kg body weight/min for 60 minutes of each of the respective peptides. LANP enhanced the excretion rate of β_2-microglobulin 2-fold within 20 minutes of beginning its infusion ($P < .05$) and was 2.5-fold higher than the preinfusion excretion rate at the end of the infusion. The excretion rate of β_2-microglobulin continued to be significantly ($P < .01$) increased for 3 hours after cessation of the LANP infusion, with the maximal excretion rate (ie, 3.8-fold increase) at 2.5 hours after stopping the infusion. Vessel dilator showed a more marked enhancement of β_2-microglobulin during its infusion, with the excretion rate increasing 2.5-fold at 20 minutes, and was increased 4-fold ($P < .01$) at the end of the infusion. With cessation of the vessel dilator infusion, the excretion rate of β_2-microglobulin decreased but was still elevated 2-fold ($P < .05$) 3 hours after stopping the infusion. Kaliuretic peptide enhanced the β_2-microglobulin excretion rate a maximal 3-fold, which occurred at the end of its infusion. The β_2-microglobulin excretion secondary to kaliuretic peptide remained 2-fold ($P < .05$) above baseline during the 3-hour postinfusion period. These peptide hormones similarly enhanced the albumin and total protein excretion rates 2- to 4-fold. These results indicate that LANP, vessel dilator, and kaliuretic peptide each (1) enhance protein excretion in healthy humans and (2) inhibit proximal tubular protein reabsorption.

THE ATRIAL NATRIURETIC PEPTIDE (ANP) hormonal system appears to play an important role in sodium and water homeostasis. The ANP hormonal system consists of a 126–amino acid (aa) prohormone synthesized within myocytes of the heart and stored in granules within the heart for release into the circulation.[1-4] This hormonal system contains several peptides from the same 126-aa prohormone with blood pressure–lowering, natriuretic, diuretic, and/or kaliuretic properties[5-7] (Fig 1). Thus, peptides consisting of aa 1 to 30 (ie, long-acting natriuretic peptide [LANP]), aa 31 to 67 (vessel dilator), aa 79 to 98 (kaliuretic peptide), and aa 99 to 126 (ANP) of the ANP prohormone each have blood pressure–lowering, diuretic, natriuretic, and/or kaliuretic properties in both humans[6,7] and animals.[5,8] The ANP prohormone is partially proteolytically cleaved within the heart, and a 98-aa amino terminus and 28-aa carboxyl terminus (ie, ANP) of this prohormone are released into the circulation.[9-12] In the circulation, vessel dilator, LANP, and kaliuretic peptide circulate as distinct entities after proteolytic cleavage from the rest of the amino terminus of the ANP prohormone by proteases[6,13-15] (Fig 1).

Vessel dilator, LANP, and ANP bind to specific receptors[16-18] and subsequently enhance the activity of the membranous form of guanylate cyclase (EC 4.6.1.2) as part of their mechanism(s) of action.[8,19] The enhancement of guanylate cyclase activity by each of the respective ANPs increases the intracellular messenger cyclic guanosine monophosphate,[8,19,20] which causes vasodilation. Vessel dilator and LANP also inhibit renal Na^+-K^+-adenosine triphosphatase (ATPase) as part of their natriuretic mechanism(s) of action.[21,22] In contradistinction, ANP does not have any effect on renal Na^+-K^+-ATPase.[21-24] Vessel dilator and LANP each enhance prostaglandin E_2 synthesis both in vitro[21,22] and in vivo,[25] which appears to be the mediator of inhibition of Na^+-K^+-ATPase by these peptides.[21,22] Vessel dilator, LANP, kaliuretic peptide, and ANP are released simultaneously with central hypervolemia.[15,26] These peptide hormones are also released simultaneously in vitro from isolated perfused atria by atrial distention.[27]

These peptide hormones have been found to localize to the sub–brush border of the pars convoluta and pars recta of the proximal tubules of the human kidney with immunoperoxidase staining.[28,29] Immunofluorescent studies revealed that each of these peptides had a strong inclination for the perinuclear region in the proximal and distal tubules.[28,29] Vessel dilator, LANP, and ANP also localized with both immunoperoxidase and immunofluorescent staining to the cortical collecting ducts, glomeruli, and peritubular and interstitial blood vessels.[28,29] In diabetic animals, these four peptides collectively may contribute to the protein hyperfiltration that occurs in early diabetes mellitus.[30] ANP has been reported to have its natriuretic and protein-excreting effects via both the proximal[31,32] and distal[32] tubules,

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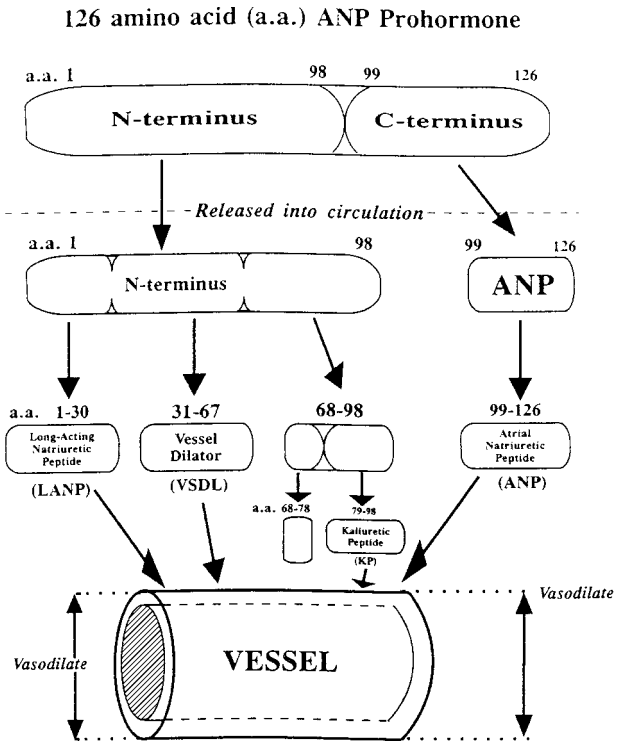

Fig 1. LANP consisting of aa 1-30, vessel dilator, aa 31-67, and kaliuretic peptide, aa 79-98, originate from the amino terminus of the 126-aa ANP prohormone, whereas ANP consisting of aa 99-126 originates from the carboxy terminus of this prohormone. Each of these peptide hormones circulate as distinct entities and have diuretic, natriuretic, and/or kaliuretic properties.[6,7] Each of these peptide hormones also decrease blood pressure via vasodilation of blood vessels.[6,7]

but where in the kidney the other three peptide hormones have their natriuretic, diuretic, and potential enhancement of protein excretion effects has never been investigated. The present investigation was designed to determine whether their effects involve enhancing the excretion rate of albumin and total protein and involve the proximal tubules of healthy humans by examining the excretion rate of β_2-microglobulin, a marker of proximal tubular function.[32,33]

SUBJECTS AND METHODS

Healthy Subjects

Twenty-four healthy subjects (12 men and 12 women aged 20 to 58 years; mean, 32 years; all normotensive with blood pressure <125/80 mm Hg) were studied. These subjects had a heart rate of 56 to 80 bpm, with a respiration rate between 12 and 14 per minute. They were divided into 4 similar groups based on age, sex, weight, blood pressure, and heart rate. The characteristics of each individual in this investigation have been published[6] in an investigation of potassium and sodium excretion following administration of each of the atrial peptides. None of the subjects had any known disease and, importantly, none of the subjects had any abnormality of sodium or water metabolism. None were using any medication. Informed consent was obtained from each subject after the nature and possible consequences of the studies were fully explained. This study was approved by the Institutional Review Board of the University of South Florida Health Sciences Center and the Research Committee of the James A. Haley Veterans Hospital. It was also approved by the US Food and Drug Administration (FDA IND No. 32,119).

Experimental Protocol

The experimental protocol consisted of a 60-minute baseline period preceding any infusion. A total volume of 20 mL normal saline (0.9% sodium chloride with or without peptides) was infused by a constant-rate infusion pump over a 60-minute period. Urine samples were obtained every 20 minutes during the infusion and at 30-minute intervals during the 1-hour baseline and 3-hour postinfusion periods. The control subjects received vehicle only but otherwise adhered to an identical protocol of a 1-hour equilibration period followed by a 1-hour infusion period with a 3-hour recovery period of evaluation. One hundred nanograms per kilogram of body weight per minute was chosen for the infusion dosage of the ANPs because the rate of release of vessel dilator, LANP, and kaliuretic peptide from the atrium of the heart with physiologic stimuli is 138 to 292 ng/kg body weight/min.[34] Molar equivalents of a 100-ng/kg body weight dose are 29, 26, and 46 pmol/kg body weight for LANP, vessel dilator, and kaliuretic peptide, respectively. Thus, the concentrations used in this investigation are in the physiologic range based on their release rates.

Each of the subjects ingested their usual diet until the evening before the study. All subjects were studied in the morning after an overnight fast, beginning their baseline period at 8 AM. Each individual was studied in the seated position. To maintain a similar plasma volume throughout the study, water was given orally in milliliters for each 1 mL urine output at the above-mentioned time periods. Each subject received only one peptide infusion.

Purity of ANPs

The human forms of LANP, vessel dilator, and kaliuretic peptide were synthesized by Peninsula Laboratories (Belmont, CA). Before use in these studies, samples of these commercially synthesized peptides were subjected to high-performance liquid chromatography to determine the purity using a Novapak C_{18} (5 μm) cartridge column as described previously.[35] After determining that the peptides were pure, they were dissolved in 0.9% saline solution in the hospital pharmacy, where pyrogen and sterility testing were performed before dispensing the 100-ng/kg body weight concentrations of each peptide into two 10-mL syringes. Each 10-mL syringe was infused over a 30-minute period. After completing the experiments, the syringes and infusion catheters were examined by radioimmunoassays for each of the respective peptides to determine the amount of the peptides that remained within the syringes or tubing. Approximately 5% of each peptide remained on the walls of the syringes and tubing after completion of the infusion.

β_2-Microglobulin

β_2-Microglobulin was analyzed with a solid-phase immunoradiometric assay using a ^{125}I-labeled anti-β_2-microglobulin monoclonal antibody in liquid phase and a polyclonal anti-β_2-microglobulin antibody immobilized to the walls of polystyrene tubes (Diagnostic Products, Los Angeles, CA). β_2-Microglobulin was determined in urine samples at 0, 30, 60 (beginning of peptide infusion), 80, 100, 120 (end of peptide infusion), 150, 180, 210, 240, 270, and 300 minutes in each subject (N = 24). The detection limit (ie, sensitivity) of the assay is 1.8 ng/mL. This assay is highly specific and does not recognize human immunoglobulin G, whose CH3 region resembles β_2-microglobulin in structure and aa sequence. Ten microliters of each of the timed, undiluted urine samples were added to the polyclonal antibody–coated polystyrene tubes and gently shaken with a rack shaker (model 51401-00; Cole-Parmer Instrument, Niles, IL) for 30 minutes. After the addition of β_2-microglobulin buffer followed by washing, the monoclonal ^{125}I-β_2-microglobulin antibody was added and the tubes were gently shaken for

30 minutes before decanting. The tubes were then analyzed in a gamma counter (Gamma Trac 1193; TM Analytic, Elk Grove Village, IL) for radioactivity. The intraassay and interassay coefficients of variation were 3.2% and 7%, respectively. Serial dilution of the urine samples showed an excellent parallelism of the standards and unknowns in this assay.

Albumin and Total Protein

Albumin content was measured using a Beckman (Brea, CA) Array 360 system that determines microquantities of albumin (ie, microalbumin) in urine by rate nephelometry using a goat antibody to human albumin for the antigen-antibody reaction. The analytic range of this assay measures albumin accurately between 0.2 and 864 ng/dL. (All of the samples measured were within this range.) The albumin intraassay coefficient of variation was 2.6%, and the interassay coefficient of variation was 7.6%. The excretion rate for albumin was then calculated by dividing the measured amount of albumin in the urine at each time point by the number of minutes required to produce the urine at the respective time points.

The total protein concentration in urine was measured by the BioRad Protein Assay Method (BioRad, Hercules, CA) using a microtiter plate procedure (MRX Microplate Reader; Dynatech Laboratories, Chantilly, VA) with a Revelation Version 2.0 computer program (Dynatech). This protein assay is based on the method of Bradford[36] as described previously from our laboratory.[16] Albumin and total protein levels were measured in urine samples at the same periods already outlined for β_2-microglobulin. Each sample was examined in triplicate, with a total protein intraassay coefficient of variation of 4.5% and interassay coefficient of variation of 9.8%.

Statistics

All data are expressed as the mean ± SEM. Statistical analysis was performed using Student's *t* test. For statistical significance, a *P* value less than .05 was required.

RESULTS

LANP enhanced the excretion rate of β_2-microglobulin 2-fold within 20 minutes of beginning its infusion (Fig 2). The excretion rate was 2.5-fold higher than the preinfusion excretion rate at the end of LANP infusion. The excretion rate of

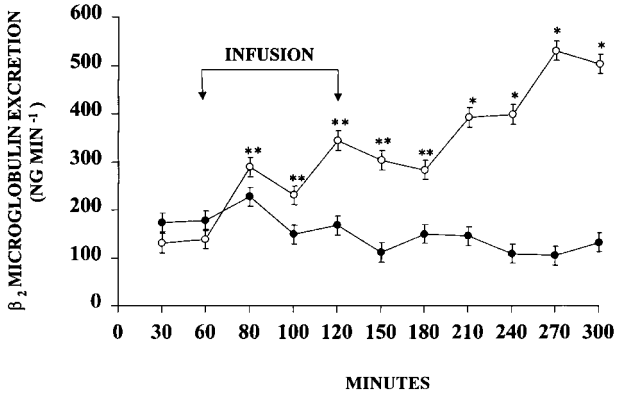

Fig 2. LANP (○) enhances the excretion rate (ng · min⁻¹) of β_2-microglobulin in healthy human subjects. The increase in the β_2-microglobulin excretion rate secondary to LANP was significant at $P < .05$ (**) during the infusion and $P < .01$ (*) for 3 hours after the infusion *v* the preinfusion rate and healthy controls (●) who received saline only, evaluated by Student's *t* test (n = 6 per group).

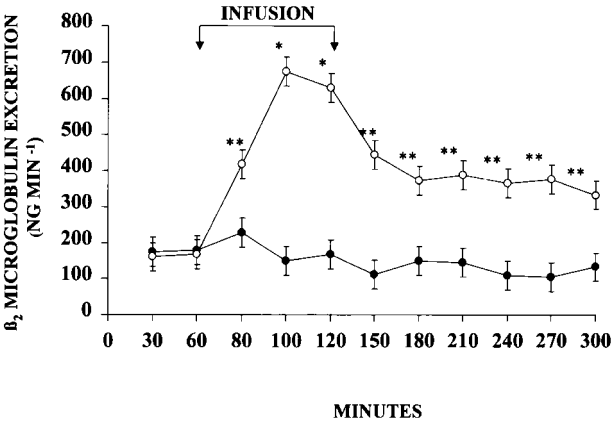

Fig 3. Vessel dilator (○) enhances the excretion of β_2-microglobulin in healthy subjects a maximum of 4-fold. The increase in the β_2-microglobulin excretion rate secondary to vessel dilator was significant at $P < .01$ (*) during the infusion and $P < .05$ (**) for 3 hours after the infusion *v* the preinfusion values and healthy controls (●), evaluated by Student's *t* test (n = 6 per group).

β_2-microglobulin increased from 139 ± 20 ng · min^{-1} immediately before the infusion to 345 ± 40 ng · min^{-1} at the end of the 60-minute infusion. The excretion rate of β_2-microglobulin continued to be significantly increased ($P < .01$) for 3 hours after cessation of the LANP infusion (Fig 2). The maximal excretion rate of β_2-microglobulin (ie, 533 ± 42 ng · min^{-1}, 3.8-fold increase) occurred 2.5 hours after stopping the LANP infusion. In the control subjects (who received saline only), the β_2-microglobulin excretion rate remained stable throughout the 300-minute experimental period (ie, they did not have any enhanced excretion). The excretion rate of β_2-microglobulin was thus increased ($P < .01$) compared with the 60-minute preinfusion (ie, baseline) period in subjects who received LANP and also significantly increased ($P < .01$) compared with subjects who did not receive LANP.

Vessel dilator also enhanced the excretion rate of β_2-microglobulin (Fig 3), but its pattern of enhancement was different than that of LANP (Fig 2). Vessel dilator showed a more marked enhancement of the excretion rate of β_2-microglobulin (2.5-fold at 20 minutes and 4-fold increased at the end of the infusion) during its infusion compared with LANP. With the cessation of vessel dilator infusion, the excretion rate of β_2-microglobulin decreased, but it was still increased 2-fold ($P < .05$) 3 hours after stopping the infusion compared with the preinfusion values and with human controls who received saline only (Fig 3).

Kaliuretic peptide enhanced the β_2-microglobulin excretion rate a maximum of 3-fold ($P < .01$), which occurred at the end of the infusion (Fig 4). There was a marked increase ($P < .01$) in the excretion rate of β_2-microglobulin within 20 minutes of beginning the kaliuretic peptide infusion. Upon stopping the kaliuretic peptide infusion, the excretion rate of β_2-microglobulin decreased to 2-fold above baseline ($P < .05$), but it remained 2-fold above baseline during the 3-hour postinfusion period (Fig 4). The β_2-microglobulin excretion rate secondary to kaliuretic peptide was also significantly ($P < .01$) increased

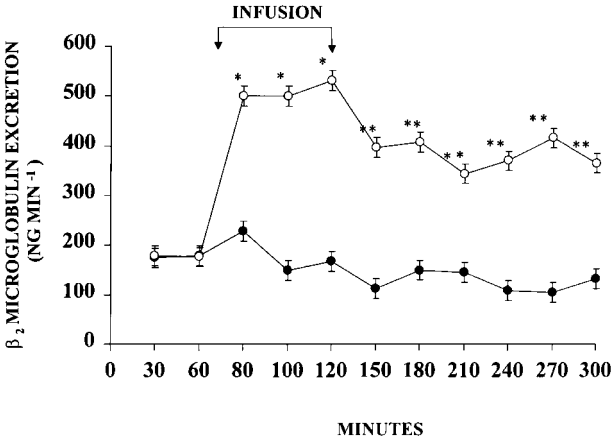

Fig 4. Kaliuretic peptide (○) enhances the β_2-microglobulin excretion rate 3-fold (at the end of infusion). The enhancement of the β_2-microglobulin excretion rate was significant at $P < .01$ (*) during the infusion and $P < .05$ (**) for 3 hours after the infusion *v* preinfusion values and healthy controls (●), evaluated by Student's *t* test (n = 6 per group).

compared with control subjects who received saline only. With respect to the ability to maximally enhance the excretion rate of β_2-microglobulin, the order is vessel dilator > LANP > kaliuretic peptide.

LANP, vessel dilator, and kaliuretic peptide also enhanced the albumin excretion rate (milligrams per deciliter per minute) and total protein excretion rate (micrograms per milliliter per minute) 2- to 4-fold in these same healthy individuals. With respect to the ability to maximally enhance the excretion rate of albumin and the excretion rate of total protein, the order is vessel dilator > LANP > kaliuretic peptide.

DISCUSSION

β_2-Microglobulin is a low–molecular-weight (11,800-dalton) protein that is relatively specific for proximal tubular function, since 99.9% of filtered β_2-microglobulin is reabsorbed by the proximal tubule.[37,38] β_2-Microglobulin has been identified as the light chain of HLA-A, -B, and -C major histocompatibility complex antigen.[38] β_2-Microglobulin is 100 aa in length and, because of its low molecular weight, is free-filtered through the glomerular membrane.[38] An increase in urinary excretion indicates a decrease in tubular reabsorption of this protein.[38]

In the present study, LANP, vessel dilator, and kaliuretic peptide enhanced the excretion rate of β_2-microglobulin 3- to 4-fold and the excretion rate of albumin and total protein 2- to 4-fold. None of these three peptide hormones have been previously investigated in terms of whether they might enhance protein excretion. The finding that all three enhance the excretion rate of albumin, total protein, and β_2-microglobulin indicates that each of these peptide hormones have the ability to enhance protein excretion. The enhanced excretion of proteins (β_2-microglobulin and albumin) in early diabetes mellitus may thus be secondary to the collective effects of these three peptide hormones and ANP to decrease proximal tubule protein reabsorption, since all four are increased 2- to 6-fold in the circulation in spontaneously diabetic rats compared with normoglycemic Wistar rats from which the spontaneously diabetic rats were derived.[30]

The ability of these three peptide hormones to enhance β_2-microglobulin excretion indicates that they inhibit proximal tubular reabsorption of this protein. Whether their ability to inhibit proximal tubular reabsorption of this protein is a direct effect or is due to an enhanced glomerular filtration rate (GFR) can be deduced by the knowledge that LANP, vessel dilator, and kaliuretic peptide did not increase the GFR in the subjects of the present investigation.[6] Thus, there was no increase in the GFR in these healthy human subjects[6] when β_2-microglobulin was simultaneously increased by LANP, vessel dilator, and kaliuretic peptide. This information suggests that their effects on the proximal tubule are direct. These findings are distinctly different versus ANP, since its effect on β_2-microglobulin is thought not to be directly on the proximal tubule but rather via increasing the GFR.[32] It is possible that although LANP, vessel dilator, and kaliuretic peptide do not increase the GFR,[6] their effects may occur partially via increasing the macromolecular permeability of glomerular cells. With respect to this possibility, LANP, vessel dilator, and ANP localize with both immunoperoxidase and immunofluorescent staining to glomeruli, peritubular and interstitial blood vessels, as well as the sub–brush border of pars convoluta and pars recta of the proximal tubules of human kidneys.[29] The immunoperoxidase staining of ANP was particularly striking in the endothelium of interstitial arteries and vasa recta.[29] In the glomeruli, prominent staining was noted in the peripheral glomerular capillary wall and in some of the visceral epithelial cells. These peptide hormones each localized to the glomerulus wall, with the intensity of staining in the order of ANP > vessel dilator > LANP.[29] In proximal tubules, the order of intensity of immunoperoxidase staining was reversed, with vessel dilator = LANP > ANP.[29]

Potassium excretion appears to be mainly a distal nephron function.[39] In the healthy human subjects of the investigation, while vessel dilator was enhancing β_2-microglobulin excretion, there was little (1 subject) or no enhancement (remaining subjects) of potassium excretion.[6] These findings together would suggest that vessel dilator's natriuretic effects are mainly due to its interaction with the proximal tubule with little or no effect on the distal tubule. LANP and kaliuretic peptide, on the other hand, enhanced potassium excretion in the present subjects[6] at the same time they enhanced β_2-microglobulin secretion, suggesting that they affect both proximal and distal tubules. Vessel dilator is thus distinguished from LANP, kaliuretic peptide, and ANP[32] in that its protein excretion–enhancing and natriuretic effects appear to be mediated by proximal tubular inhibition of reabsorption without any significant distal tubular effects.

We have recently found that vessel dilator is important for preserving the integrity of proximal tubules in ischemic renal failure.[40] In an animal model of ischemic renal failure, vessel dilator, even when administered 2 days after acute renal failure was established, decreased acute tubular necrosis to less than 5%, compared with 25% to 75% necrotic tubules in animals that did not receive vessel dilator.[40] This amelioration of acute tubular necrosis with vessel dilator was associated with a decrease in mortality from 88% (untreated animals) to 14% in vessel dilator–treated animals.[40] This information would sug-

gest that vessel dilator may be important for maintaining the integrity of the proximal tubules, as well as modulating their function, as found in the present investigation.

In summary, LANP, vessel dilator, and kaliuretic peptide significantly enhanced protein excretion, and this enhancement of protein excretion lasted for 3 hours after stopping the respective infusions. Their ability to enhance the excretion rate of β_2-microglobulin indicates that the mechanism of their increase in protein excretion is a decreased proximal tubular protein reabsorption.

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