Atrial Natriuretic Peptides Negatively and Positively Modulate Circulating Endothelin in Humans

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The present investigation was designed to examine the effect of four atrial peptide hormones with vasodilatory properties on the circulating immunoreactive (ir) levels of the vasoconstrictive peptide endothelin (ET) in 36 healthy human subjects. Circulating levels of human ET and cyclic guanosine monophosphate ([cGMP] a potential mediator of the effects of atrial peptides) were measured every 30 minutes during 1-hour preinfusion, 1-hour infusion, and 3-hour postinfusion periods. Atrial natriuretic factor ([ANF] amino acid (aa) 99 to 126 of the 126-aa ANF prohormone) and kaliuretic peptide (aa 79 to 98 of this same prohormone) significantly ($P < .05$) decreased circulating ET concentrations. Kaliuretic peptide effects were early and ANF effects were delayed until kaliuretic peptide effects began to wane. Long-acting natriuretic peptide (LANP), consisting of aa 1 to 30 of the ANF prohormone, on the other hand, significantly ($P < .05$) increased ET circulating concentrations during a 1-hour infusion period. The increase in ET in the circulation secondary to LANP became nonsignificant, although it was still elevated, within 30 minutes of ceasing LANP infusion. Vessel dilator, consisting of aa 31 to 67 of the ANF prohormone, and infusion of vehicle alone did not significantly change circulating levels of ET during the 5 hours of this investigation. ANF infusion increased plasma cGMP sevenfold, but plasma cGMP had decreased to only onefold above normal during the period that ANF had an effect on circulating ET levels. There was not any significant increase or decrease in plasma cGMP concentrations secondary to the other atrial peptide hormones. These data suggest that kaliuretic peptide and ANF negatively modulate circulating ET concentrations, while LANP, which is released simultaneously with ANF in response to physiologic stimuli, positively modulates circulating ET concentrations to help maintain circulating ET within a narrow normal range. The data from the present investigation would further suggest that circulating cGMP levels do not mediate the various atrial peptide effects on circulating ET levels.

ENDOTHELIN (ET)-1 is a 21–amino acid (aa) peptide produced and released by endothelial cells.[1,2] ET-1 is a potent vasoconstrictive agent[1-5] that circulates in plasma at low concentrations (ie, 10^{-15} mol/mL [fmol]/mL).[6-8]

Atrial natriuretic factor (ANF), a 28-aa vasodilatory peptide from the C-terminal end of the ANF prohormone (ie, aa 99 to 126 of the 126-aa prohormone), has been reported to inhibit ET-1 secretion from cultured cells.[9-13] Thus, ANF has been found to inhibit ET-1 secretion from cultured human[9,10] and bovine[11] endothelial cells, isolated porcine aorta,[12] and cultured rat mesangial cells.[13] Evidence that the in vitro effect of ANF has any physiological or in vivo significance by decreasing basal plasma ET levels has not yet been demonstrated.

In addition to ANF, there are three other peptide hormones contained within the ANF prohormone.[14-16] These peptides consisting of aa 1 to 30 (ie, long-acting natriuretic peptide [LANP]), aa 31 to 67 (vessel dilator), and aa 79 to 98 (kaliuretic peptide) of the 126-aa ANF prohormone have vasodilatory properties equal to those of ANF in humans[16] and animals.[17] These peptides and ANF can overcome the vasoconstriction of aorta secondary to phenylephrine in vitro.[18] Vessel dilator, LANP, kaliuretic peptide, and ANF circulate in humans[15,19] and are released simultaneously with central hypervolemia induced by head-out of water immersion[20] or rapid ventricular pacing.[21] Since each of these atrial peptides circulate and have physiologic effects opposite to ET effects, the present investigation was designed to determine if one or more of these peptides influence the circulating concentration of ET-1.

SUBJECTS AND METHODS

Healthy Volunteers

Thirty-six healthy subjects (18 men and 18 women aged 20 to 58 years [mean, 32 years], all normotensive with blood pressures <125/80 mm Hg) were studied. These volunteers had heart rates ranging from 56 to 80 beats per minute, with respiration rates between 12 and 14 per minute. They were divided into six similar groups based on age, weight, blood pressure, heart rate, and sex (three women and three men in each group). None of the volunteers had any known disease. Of importance, none had any abnormality of sodium or water metabolism. None were taking any medication. Informed consent was obtained from each of the volunteers after the nature and possible consequences of the studies were fully explained. The hemodynamic and renal effects measured simultaneously with ET and cyclic guanosine monophosphate (cGMP) levels secondary to infusion of the respective atrial peptides have been published previously.[16] 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. This study was also approved by the US Food and Drug Administration (FDA IND No. 32,119).

Experimental Protocol

After obtaining written informed consent, an Insyte-w 20-gauge, 1.5-in catheter was placed into the forearm of each subject for infusion and blood sampling. A 60-minute baseline period preceded each infusion. A total volume of 20 mL normal saline (0.9% sodium chloride with or without peptides) was infused by a

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constant-rate infusion pump over a 60-minute period. Blood samples were obtained every 30 minutes during infusion, 1-hour baseline, and 3-hour postinfusion periods. One hundred nanograms per kilogram body weight per minute was chosen for the infusion dosage of these atrial natriuretic peptides, because the rate of release of *N*-terminal ANF prohormone peptides from the atrium of the heart with physiological stimuli is 138 to 292 ng/kg body weight/min, whereas the release rate of ANF from the atrium is 76 ng/kg body weight/min.[22] Molar equivalents of a 100-ng/kg body weight dose are 32, 29, 26, and 46 pmol/kg body weight for ANF, LANP, vessel dilator, and kaliuretic peptide, respectively. Thus, the concentrations used in this investigation are in the physiological range, based on the release rates for *N*-terminal ANF prohormone peptides, and slightly above the physiological range for ANF.

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 the baseline period at 8:00 AM. Each volunteer was studied in the seated position. After completion of the 60-minute baseline period, to maintain a similar plasma volume throughout the study, orange juice (Na^+ 0.001 mmol/L, K^+ 0.046 mmol/L) was given orally in milliliters for each milliliter of urine output at the above times. Each volunteer received only one peptide infusion.

Purity of Atrial Natriuretic Peptides

The human forms of LANP, vessel dilator, kaliuretic peptide, and ANF 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 (HPLC) to determine purity using a Novapak C_{18} (5-μm) cartridge column. The flow rate for HPLC was 1 mL/min with 0.1% trifluoroacetate solvent in pump A and 60% acetonitrile in 0.1% trifluoroacetate in pump B, with a gradient of 0% to 60% acetonitrile achieved in 40 minutes. This evaluation verified purity and authenticity when compared with the known HPLC elution profile[23] of these peptides. After determining that the respective 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 experiment, each of the syringes and the infusion catheter were examined by radioimmunoassays[16,23] to determine the amount of the respective peptides that may have remained within syringes or tubing. Approximately 5% of each peptide remained on the walls of the syringes and tubing after completion of the infusion.

ET Radioimmunoassay

Blood samples were obtained every 30 minutes during the 60-minute preinfusion (ie, baseline period), the 60-minute infusion of atrial peptides or vehicle control, and the 3-hour postinfusion period. Each blood sample was collected into chilled 5-mL EDTA tubes to prevent proteolytic breakdown of any peptides that might be present. The samples were transported on ice and immediately centrifuged at 3,000 × *g* for 15 minutes. After centrifugation, each sample was extracted with 100% ethanol (1:2 dilution), vortexed, and allowed to stand at 4°C for 30 minutes as described in detail previously.[23] ET level was measured in plasma with an ET-1,2 (high-sensitivity) [^{125}I] assay kit with Amerlex-M magnetic separation (Amersham, Arlington Heights, IL).

cGMP Measurement

cGMP level was measured in plasma as previously described.[24,25] Plasma collected as described earlier for the ET assay every 30 minutes before and during infusion and for 3 hours postinfusion was also evaluated for cGMP. Any possible increases or decreases in cGMP secondary to the respective atrial natriuretic peptide infusion were evaluated with the cGMP assay, stopped after 5 minutes of incubation by addition of 0.5 mL 50% methanol containing 10% perchloric acid at −20°C. Approximately 2,000 cpm [^3H]cGMP was added to the supernatant to monitor recovery. Each sample for measurement of cGMP was lyophilized, and then redissolved in 0.5 mL 50-mmol/L acetate buffer (pH 6.2), and aliquots were assayed. 8-[^3H]cGMP and antibodies to cGMP were obtained from Amersham. Results were corrected for recovery and expressed in picomoles of cGMP per milliliter of plasma.

Analytical Methods

Data obtained in this investigation are presented as the mean ± SD. Measurements obtained in the same subject over time were evaluated by repeated-measures ANOVA. Duncan's multiple-range test (MRT) was used after ANOVA to evaluate which means of ET and cGMP were significantly different from baseline and from each other. Statistical significance required a *P* value less than .05 (95% confidence limits).

RESULTS

ANF decreased plasma ET levels (Fig 1). The decrease in plasma ET was not significant during ANF infusion, but instead was a delayed effect, with the most significant decrease ($P < .05$) being 90 minutes after ceasing the infusion (Fig 1). Plasma ET concentration then returned to the normal range at 150 minutes after ceasing the infusion, and remained in the normal range throughout the rest of the postinfusion period (Fig 1).

Kaliuretic peptide also decreased plasma ET levels (Fig 1). Infusion of kaliuretic peptide, as opposed to ANF, significantly ($P < .05$) decreased plasma ET levels during

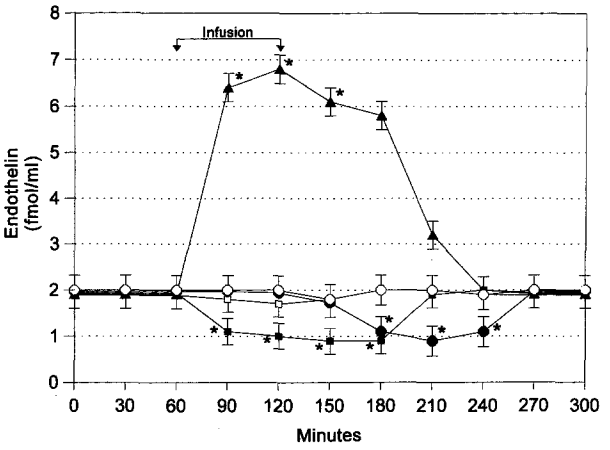

Fig 1. ANF (●) and kaliuretic peptide (■) decrease circulating ET and LANP (▲) increases circulating ET levels when each of these peptides are infused at 100 ng/kg body weight/min. The respective decreases or increases in plasma ET secondary to these peptides were significant at *$P < .05$ when evaluated by ANOVA followed by Duncan's MRT. Vessel dilator (○) and vehicle (□) only (ie, control) had no significant effect on plasma ET levels when evaluated by ANOVA followed by MRT. n = 6 per group.

its infusion (Fig 1). Circulating ET concentration was still significantly decreased 60 minutes after stopping the kaliuretic peptide infusion, but by 90 minutes, ET concentration had returned to the normal range and remained there throughout the rest of the 3-hour postinfusion period (Fig 1).

LANP, as opposed to ANF and kaliuretic peptide, significantly ($P < .05$) increased circulating ET concentration from 2 ± 0.1 to 6.8 ± 0.3 fmol/mL (Fig 1). This increase in circulating ET concentration was significant only during the infusion of LANP (Fig 1). Thus, within 30 minutes of cessation of LANP infusion, the plasma concentration of ET had decreased to the extent that it was no longer significantly elevated as compared with its basal value (Fig 1). Vessel dilator did not have any significant effect on plasma ET concentrations (Fig 1). Likewise, in subjects who received vehicle only, plasma ET levels did not vary significantly over the 5-hour experimental period in healthy human subjects (Fig 1). During the respective infusions, circulating levels of the atrial peptides increased equivalently.[15]

Vessel dilator, LANP, kaliuretic peptide, and ANF infusions decreased systolic and diastolic blood pressure 10/6.3, 7.9/5, 7.4/6, and 8.8/6 mm Hg, respectively ($P < .05$). Vessel dilator, LANP, ANF, and kaliuretic peptide increased urine flow fourfold to 12-fold. Urine flow secondary to vessel dilator, LANP, and kaliuretic peptide was still significantly increased ($P < .01$) 2 to 3 hours after stopping the respective infusions, but by 2 hours after ANF infusion, urine flow was significantly increased in only one of six subjects. Sodium excretion increased threefold to eightfold, threefold to sixfold, none to twofold (not significant), and threefold to 11-fold, respectively, with LANP, vessel dilator, kaliuretic peptide, and ANF. Heart rate did not change during infusion of any of these atrial peptides.

Controversy exists regarding the ANF in vitro inhibition of ET synthesis of whether it is mediated by cGMP.[9,26] Because of this controversy, simultaneous plasma cGMP measurements along with plasma ET levels were incorporated into the present investigation. ANF at a 100-ng/kg body weight/min concentration increased cGMP in the plasma sevenfold by the end of the 60-minute infusion (Fig 2). The increase in plasma cGMP secondary to ANF, although decreasing with cessation of the infusion, remained significantly ($P < .05$) elevated 3 hours after stopping the ANF infusion (Fig 2). Vessel dilator, kaliuretic peptide, and LANP did not significantly increase or decrease plasma cGMP concentrations (Fig 2). Thus, neither during the respective infusions or in 3 hours of follow-up study after cessation of the infusions did LANP, vessel dilator, or kaliuretic peptide increase or decrease the concentration of cGMP within the circulation. In control subjects, who received vehicle only, there was no significant variation in plasma cGMP concentration throughout the 5-hour investigation period.

DISCUSSION

Infusion of ANF resulted in a decrease in the circulating concentration of ET-1. The present in vivo findings are consistent with in vitro experimental results, which have found that ANF inhibits ET-1 secretion from cultured endothelial and mesangial cells.[9-13] The present findings are also consistent with the finding that ANF inhibits ET-1–induced renal vasoconstriction[27] and decreases the pressor response to infusion of ET-1.[28] There has been one previous investigation of infusing ANF and measuring plasma ET levels.[29] During a 1-hour total investigation period that incorporated a 15-minute infusion of ANF with a 45-minute postinfusion period, ANF did not significantly affect basal ET levels.[29] The present investigation confirms that during the period of the previous investigation, ANF did not affect basal plasma ET levels. ANF was found in the present investigation to have a delayed onset of action, with its first significant effect on circulating ET levels at 60 minutes postinfusion. ANF effects on ET were sustained, with significantly decreased ET levels for another 60 minutes once the effect on circulating ET levels became significant. Thus, the ANF effect on circulating ET is both delayed and sustained for 1 hour once the decreased levels occur. Whether the effects of ANF on circulating ET concentrations were direct or secondary could not be determined in the present investigation.

The ability of ANF to inhibit the circulating concentration of ET-1 suggests that there may be feedback relationship between ANF and ET. Intravenous injection of ET-1 increases circulating ANF levels.[30,31] The present investigation would suggest that once ET-1 increases plasma ANF, ANF in turn inhibits the synthesis of ET-1, resulting in the observed decrease of ET-1 in the circulation secondary to ANF. Since ET-1 is not stored within the secreting granules in the cytoplasm of endothelial cells,[2,32] any stimuli that result in decreased or increased levels of ET-1 in the circulation must affect synthesis if the decrease or increase of ET-1 in the circulation is sustained, as was found in the

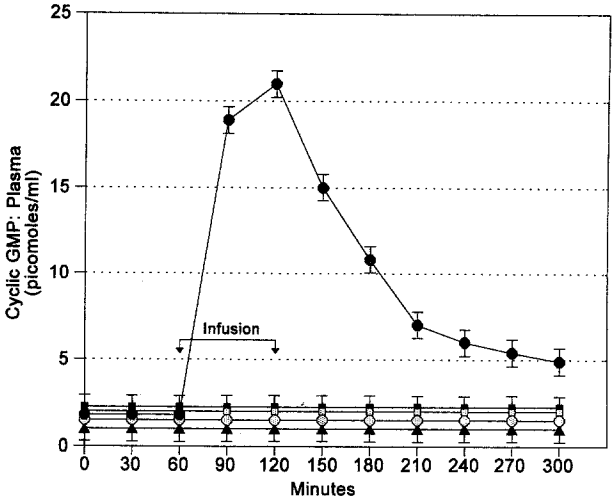

Fig 2. ANF (●) increases plasma cGMP concentrations, and vessel dilator (○), LANP (▲), and kaliuretic peptide (■) do not, when infused at 100 ng/kg body weight/min for 60 minutes. The increase in plasma cGMP secondary to ANF was significant at $P < .01$ at the end of the infusion and was still significant at $P < .05$ 2 hours after stopping the infusion when evaluated by ANOVA followed by MRT. There was also no change in plasma cGMP in controls (□). n = 6 per group.

present investigation. Thus, since the half-life of ET is short and it is not stored within the body,[2,32] in order to have a sustained decrease in the circulating concentration of ET secondary to ANF as found in the present investigation, a decreased synthesis of ET must have occurred. The circulating concentration of ET is believed to be a reliable indicator of the amount of de novo ET synthesis that is occurring.[2,32] Previous in vitro studies[9-13] suggest that this is what happens, ie, ANF inhibits the synthesis of ET.

Kaliuretic peptide also decreased circulating ET-1 levels when infused into normal human subjects. The effect of kaliuretic peptide on ET-1 levels is not mediated by ANF, since previous studies have demonstrated that kaliuretic peptide decreases ANF release via a negative-feedback mechanism.[15] Thus, during infusion of kaliuretic peptide, when circulating levels of ET-1 are decreasing, ANF circulating levels are also significantly decreasing.[15] Since ET-1 is not stored, but rather has to be synthesized before being released, kaliuretic peptide effects are also on ET-1 synthesis rather than release. The time course of the kaliuretic peptide inhibition of circulating ET levels was markedly different from that of ANF. Kaliuretic peptide inhibition began early (ie, it was significant at the first time point in the infusion). After the ANF effect on circulating ET levels became significant at 60 minutes postinfusion, the kaliuretic peptide inhibiting effect on circulating ET levels ceased. Thus, kaliuretic peptide and ANF, which exist adjacent to each other in the ANF prohormone (ie, aa 79 to 98 and 99 to 126, respectively), appear to act in concert with respect to decreasing circulating ET levels, with kaliuretic peptide early effects followed in sequence by ANF effects.

An interesting and unexpected finding in the present investigation was that LANP increased the circulating concentration of ET. This peptide hormone consisting of the first 30 aa of the ANF prohormone appears to increase ET synthesis, since ET is not stored. Importantly, the findings of the present investigation elucidate a mechanism for maintaining circulating ET concentrations within a narrow normal range. Since LANP is released simultaneously with ANF and the other atrial peptides in response to physiologic stimuli,[20] large swings in circulating concentrations of ET do not occur, because the peptides causing ET concentrations to decrease in the circulation are balanced by another peptide's (LANP) modifying this decrease. One would suspect that the overall effect of physiologic stimuli to the release of atrial peptides would result in a decrease in the circulating concentration of ET, since two of the atrial peptides' effects result in a decrease in the circulating concentration of ET, while only one of the atrial peptides is balancing their effects by simultaneously working to enhance the circulating concentration of ET. It is important to note in this regard that the LANP effect of increasing ET levels ceased before the ANF effects of decreasing circulating ET began. Thus, at least the delayed effect to physiological release of atrial peptides would be a decrease in the circulating ET concentration, since this decrease occurred after the stimuli (LANP) to increase ET levels had ceased to have an effect.

It has been suggested that the ANF mechanism of action of inhibiting ET synthesis in vitro involves the intracellular messenger, cGMP.[9,33] The present investigation demonstrates that an increase in plasma cGMP per se is not necessary to decrease the circulating concentration of ET. Infusion of ANF increased cGMP in the plasma of each healthy individual, while kaliuretic peptide, which also decreased the ET-1 circulating concentration, had no effect on plasma cGMP levels. Data from the present investigation also demonstrate that one cannot use plasma cGMP measurements to reflect changes in circulating ET concentrations, since two of three peptide hormones that were found to change plasma ET levels had no effect on plasma cGMP concentrations.

The present investigation, which demonstrates that circulating cGMP concentration is not, however, directly related to circulating ET concentration, does not rule out the possibility that intracellular increases in cGMP might influence ET synthesis. However, there is an in vitro study indicating that ANF inhibition of ET-1 production is not mediated by cGMP,[11] similar to the findings of the present in vivo study. In the study reported by Hu et al,[11] it was found that the ANF analog, C-ANF (aa 102 to 121 of the prohormone), which does not increase cGMP, inhibited intracellular ET production to the same extent as did ANF. Taken together, these studies indicate that cGMP is not the mediator of atrial peptide effects on ET synthesis or on circulating levels of ET.

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