Increased urinary excretion of uroguanylin in patients with congestive heart failure

STEVEN L. CARRITHERS,1,2 SAMMY L. EBER,3 LEONARD R. FORTE,3 AND RICHARD N. GREENBERG1,2
1 Division of Infectious Diseases, Department of Medicine, University of Kentucky, and 2 Lexington Veterans Affairs Medical Center, Lexington, Kentucky 40536; and 3 Department of Pharmacology, Truman Veterans Affairs Medical Center, and Missouri University, Columbia, Missouri 65212

Increased urinary excretion of uroguanylin in patients with congestive heart failure. Am. J. Physiol. Heart Circ. Physiol. 278: H538–H547, 2000.—Uroguanylin is a small-molecular-weight peptide that activates membrane-bound receptor-guanylate cyclases in the intestine, kidney, and other epithelia. Uroguanylin has been shown to participate in the regulation of salt and water homeostasis in mammals via cGMP-mediated processes, bearing a distinct similarity to the action of the atriopeptins, which play a defined role in natriuresis and act as prognostic indicators of severe congestive heart failure (CHF). The objectives of this study were to measure the urinary levels of uroguanylin and the circulating plasma levels of atrial natriuretic peptide (ANP) in healthy individuals (n = 53) and patients with CHF (n = 16). Urinary excretion of uroguanylin was measured by a cGMP accumulation bioassay employing human T84 intestinal cells. In individuals without CHF, the concentration of uroguanylin bioactivity was 1.31 ± 0.27 nmol cGMP/ml urine and 1.73 ± 0.25 μmol cGMP/24-h urine collection. The urinary bioactivity of uroguanylin in males (1.74 ± 0.55 nmol cGMP/ml urine; n = 27) tended to be higher than the excretion levels in females (0.94 ± 0.16 nmol cGMP/ml urine; n = 26) over a 24-h period but did not achieve statistical significance. Both male and female groups showed 24-h temporal diurnal variations with the highest uroguanylin levels observed between the hours of 8:00 AM and 2:00 PM. The circulating level of ANP was 12.1 ± 1.6 pg/ml plasma and did not significantly vary with respect to male/female population or diurnal variation. In patients with CHF, the concentration of plasma ANP and urinary uroguanylin bioactivity increased substantially (7.5-fold and 70-fold, respectively, both P < 0.001) compared with healthy levels. Uroguanylin was purified from the urine of CHF patients and shown to be the bioactive, COOH-terminal, 16 amino acid portion of the human prouroguanylin protein. The increased urinary uroguanylin excretion observed during CHF may be an adaptive response to this cardiovascular pathophysiology.

guanylin; atrial natriuretic peptide; guanosine 3′,5′-cyclic monophosphate; guanylate cyclase

REGULATION OF ELECTROLYTE and water transport is critical to the maintenance of fluid volume. Recently, the cGMP signal transduction pathway has been shown to be an important mechanism controlling renal function with respect to urinary excretion of sodium, potassium, and chloride (9, 10). The atrial natriuretic peptide family of paracrine and endocrine hormones is involved in this regulation of salt and water homeostasis (5). These peptides include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP), each of which has been found to have natriuretic, diuretic, and vasodilatory properties in humans (10, 34, 46). ANP, the peptide that has been the most extensively studied in this family (4, 10, 40), elicits its actions by activating transmembrane guanylate cyclase-A (GC-A) receptors and increasing intracellular cGMP. This cyclic nucleotide directly mediates fluid and electrolyte transport in cells by activating protein kinases, thus modulating the phosphorylation state of specific chloride, potassium, and sodium channels (10). Although ANP is produced mainly in the cardiac atria, receptors for this peptide have been identified in the aorta, kidney, vascular smooth muscle, central nervous system, and adrenal gland (10, 41). ANP is also produced in other organs involved in salt and water homeostasis, such as the kidney and intestine (5, 20, 41).

ANP and related peptides have provided important insights into the pathophysiology and potential treatment of congestive heart failure (CHF), hypertension, and other disorders exhibiting abnormal fluid and electrolyte balance (4, 5, 9, 34). In persons with CHF, a disorder that disrupts the interplay of neurohormonal and hemodynamic forces regulating electrolytic and water transport, ANP increases in the circulation proportionally to the severity of the sodium and water retention (1, 4, 9, 40, 41). Most studies report a close correlation between plasma levels of ANP and severity of CHF [New York Heart Association functional (NYHA) class; 4, 25, 32, 42]. Similarly, the concentration of plasma cGMP, and to a lesser degree, urinary cGMP, also relate to the severity of this disorder (25). However, a close relationship between increased plasma/urinary cGMP and circulating ANP in CHF is not always observed (27, 47). Therefore, additional factors and tissues may be involved in producing the cGMP associated with sodium and water retention during CHF. Uroguanylin and guanylin are newly identified endogenous peptides that were originally isolated from urine.
and intestine, respectively (7, 30). Like ANP, uroguanylin and guanylin elicit a natriuresis by activating specific guanylate cyclase receptors in the kidney (15, 16). Both peptides are synthesized as prohormones and require proteolytic processing into small COOH-terminal 14–16 amino acid peptides that bind to and regulate guanylate cyclase-C (GC-C) in kidney and intestinal epithelial cells (16, 17). This membrane receptor protein was originally identified as an intestinal receptor for the Escherichia coli heat-stable toxin (STa) peptides, which are secreted intraluminaly by enteric bacteria that cause Travelers’ diarrhea (15, 16). Bacterial STa peptides are related in structure to uroguanylin and guanylin. Thus this toxin acts as a molecular mimic and superagonist of the endogenous peptide hormones (15–18). These peptides constitute a family of GC-C-activating peptides whereby binding of these agonists to an extracellular domain of the receptor activates the intracellular catalytic domain of GC-C producing cGMP within target cells. The intracellular cGMP, in turn, stimulates transepithelial chloride secretion in the intestine by regulating the phosphorylation state and chloride channel activity of the cystic fibrosis transmembrane conductance regulator (8, 15, 17). Other biological consequences of uroguanylin and guanylin activation include bicarbonate secretion (14–16), inhibition of taurine transport (15), and kaliuresis (19).

Uroguanylin has been implicated to have both paracrine and endocrine functions because mRNA transcripts for both the peptide hormone and its receptor are present in the gut, kidney, reproductive system, and brain (12, 13, 43). In addition, uroguanylin circulates in the plasma as a prohormone and active peptide hormone (12, 28, 38). However, there is no information on the physiological role that uroguanylin may play in salt and water transport. As an initial study, we wanted to know if uroguanylin levels were elevated in patients with conditions involving abnormal fluid and electrolyte homeostasis, like CHF. Although this peptide hormone was originally purified from urine (30), few studies have described the presence of uroguanylin excretion patterns in normal individuals and patients with disease. The objectives of this study were 1) to identify a difference, if any, of uroguanylin excretion in patients with CHF compared with that in healthy subjects who served as a control population, and 2) to evaluate urinary uroguanylin levels in relation to age, gender, diurnal variation, and daily variation. The circulating ANP levels were measured as an indicator of CHF. Uroguanylin measurements were also compared with the plasma ANP concentration from each patient.

**METHODS**

Study design and clinical specimens. The University of Kentucky Institutional Review Board approved this study, which involves the use of human subjects. The volunteers gave their written informed consent for participation. Fifty-three healthy volunteers participated in the initial screen for this study: 27 male (age, 26.1 ± 4.5 yr; range 21–40 yr) and 26 female (age, 31.6 ± 6.7 yr; range 22–48 yr). A venous blood sample (10 ml) was taken from each individual between the hours of 8:00 AM and 2:00 PM, and analyzed for serum sodium, potassium, creatinine, and plasma ANP space concentrations (described below). The urine from each individual was collected for 24 h and measured for uroguanylin (described below). Of the 53 volunteers who participated in the initial collections, 24 (8 male, 16 female) returned 7–10 days later for a secondary screening. Blood samples and urine collections were taken again from each individual who participated in the first screening as performed previously.

A second protocol included in this study involved a total 24-h urine collection from patients with CHF. Entry criteria included a diagnosis of clinically acute or chronic CHF as a result of severe left ventricular dysfunction (ejection fraction ≤40%), NYHA Class II or III (6), and no other significant medical conditions. Sixteen CHF patients (8 male, 8 female) were included in this report (Table 1). Their clinical and hemodynamic situations were stable with no change in medication during the last month. Patients with significant concomitant disease such as infection, pulmonary disease, malignancy, or collagen vascular disease were not included. Serum creatinine, sodium, and potassium values were obtained from the patients’ medical records at the time of urine collection. The blood samples taken from CHF patients were also assayed for plasma ANP concentrations.

**Cell culture.** The human colon carcinoma cell line T84 (ATCC; Bethesda, MD) was used in these studies. Cells were cultured as described previously (2, 21, 26).

**ANP measurement.** ANP was measured in blood samples from the healthy individuals and patients with CHF by methods previously described (4, 41), utilizing a competitive radioimmunoassay (RIA; Peninsula Laboratories; Belmont, CA). The ANP-RIA measures only ANP-99–126 (41). Blood samples (20 ml) were collected via peripheral venipuncture in sterile polypropylene tubes containing EDTA (1 mg/ml of blood) and stored immediately on ice. Serum creatinine, electrolytes, and osmolality were determined before further preparation. Sample blood was centrifuged at 1,600 g for 15 min at 4°C, and the supernatant was stored at −70°C until use (stability up to 1 mo). Aliquots of plasma were diluted in a 1:1 ratio with 1% trifluoroacetic acid (TFA) followed by centrifugation of the sample at 15,000 g for 20 min at 4°C. The supernatant was loaded onto a precultivated C18-reversed phase (RP) Sep-Pak column (Waters; Milford, MA) that was conditioned with 1% TFA after a 60% acetonitrile initial wash. The column was then washed twice with 1% TFA to remove plasma debris followed by elution of the peptide with 3.0 ml 60% acetonitrile containing 1% TFA. The eluate was evaporated to dryness and resuspended in the RIA buffer supplied in the ANP-RIA kit. Aliquots (100–200 µl) of this supernatant were centrifuged for 10 min at 10,000 g, and the supernatant was assayed for ANP as described below.

**Data analysis.** ANP concentrations were measured by radioimmunoassay (RIA) using antiserum to ANP-109–126 (Peninsula Laboratories). ANP was purified from bovine brain, and the receptor protein was originally identified as an intestinal receptor for the E. coli heat-stable toxin (STa) peptides, which are secreted intraluminaly by enteric bacteria that cause Travelers’ diarrhea (15, 16). Bacterial STa peptides are related in structure to uroguanylin and guanylin. Thus this toxin acts as a molecular mimic and superagonist of the endogenous peptide hormones (15–18). These peptides constitute a family of GC-C-activating peptides whereby binding of these agonists to an extracellular domain of the receptor activates the intracellular catalytic domain of GC-C producing cGMP within target cells. The intracellular cGMP, in turn, stimulates transepithelial chloride secretion in the intestine by regulating the phosphorylation state and chloride channel activity of the cystic fibrosis transmembrane conductance regulator (8, 15, 17). Other biological consequences of uroguanylin and guanylin activation include bicarbonate secretion (14–16), inhibition of taurine transport (15), and kaliuresis (19).

**Table 1. Clinical characteristics of patients with congestive heart failure**

<table>
<thead>
<tr>
<th>Patient Profile</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>61.4 ± 8.0</td>
<td>63.9 ± 4.4</td>
</tr>
<tr>
<td>(49–93)</td>
<td>(41–81)</td>
<td></td>
</tr>
<tr>
<td>NYHA Class II/III</td>
<td>3.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>1.9 ± 0.4</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>(1.5–3.4)</td>
<td>(1.5–4.4)</td>
<td></td>
</tr>
<tr>
<td>Serum sodium, meq/l</td>
<td>137.4 ± 1.9</td>
<td>139.7 ± 1.3</td>
</tr>
<tr>
<td>(131–142)</td>
<td>(134–148)</td>
<td></td>
</tr>
<tr>
<td>Serum potassium, meq/l</td>
<td>4.2 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>(4.0–4.8)</td>
<td>(3.4–5.1)</td>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE; n = 8 male and 8 female patients. NYHA, New York Heart Association. Numbers within parentheses indicate ranges.
Collections were from healthy individuals was collected in four 6-h fractions. The intra- and interassay coefficients of variation for the RIA were 5.8 and 7.0%, respectively. ANP levels were measured from this prepared sample. The percent recovery for ANP was ~86 ± 5%. All determinations were performed in duplicate or triplicate. The intra- and interassay coefficients of variation for the RIA were 5.8 and 7.0%, respectively.

Urine parameters and preparation. A 24-h urine collection from healthy individuals was collected in four 6-h fractions. Collections were from 1) 2:00 AM-8:00 AM, 2) 8:00 AM-2:00 PM, 3) 2:00 PM-8:00 PM, and 4) 8:00 PM-2:00 AM. Each 6-h urine sample was measured for total volume and separated in 30-ml sample aliquots. The urine collected from CHF patients was a 24-h pooled fraction, which was also separated into 30-ml aliquots. To these aliquots, 30 µl of 1% TFA were added followed by vortexing and centrifugation at 20,000 g for 20 min at 4°C. The supernatant was applied to a preconditioned C₁₈-RP Sep-Pak. Equilibration of the Sep-Pak column consisted of a 10-ml wash with acetonitrile in 0.1% TFA, followed by 0.1% TFA in water. After sample loading, the column was washed with 20 ml 0.1% TFA, and the uroguanylin fraction was eluted with 8 ml of 40% acetonitrile containing 0.1% TFA. The eluate was equally split into two fractions, desalted, and lyophilized. One tube was resuspended in 1 ml of DMEM/F-12 media (GIBCO; Gaithersburg, MD) containing 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.5) and acetyl-1 mM of the phosphodiesterase inhibitor IBMX (21, 23). The remaining fraction was stored at -20°C.

cGMP accumulation assay in T84 cells. The detection and quantification of uroguanylin extracted from urine samples were performed using a de novo T84 cell stimulation bioassay by previously described methods (7, 21). The bioactivity (represented as pmol cGMP produced·min⁻¹·ml⁻¹) was a direct measure of the amount of GC-C-stimulating peptide present in a particular sample. Uroguanylin bioactivity was assessed at acidic pH (pH 5.5; 21). Briefly, the sample was layered onto the T84 cell monolayers (in a 24-well plate) for 40 min at 37°C. The medium was removed, and the cells were washed twice with serum-free media. Cells were lysed, and peptide-induced intracellular cGMP was measured using a specific RIA (α-cGMP antibody was kindly provided by Dr. Donald C. Robertson, University of Idaho; 2, 21). Protein was measured (Bio-Rad; Hercules, CA) using bovine serum albumin as standard; one confluent T84 cell well is ~1 mg/ml protein. The effects observed on cGMP accumulation in this assay under acidic conditions reflect primarily, if not completely, the bioactivity of uroguanylin in the urine (21). Sensitivity for this bioassay approaches 1 pmol cGMP·well⁻¹·mg protein⁻¹, which is similar to the sensitivity found previously (31).

In this report, urine bioactivity is referred to as uroguanylin-like cGMP accumulation activity because all urine samples were not purified by HPLC. The effects of other known urine peptides and atriopeptins were compared with those of synthetic uroguanylin and guanylin (3, 7, 26, 39, 45). Under acidic conditions employed in this bioassay, guanylin, the other known endogenous GC-C activating peptide, is ~150 times less potent than uroguanylin (21, 22, 30, 31). Peptides of the ANP family (ANP, BNP, CNP, urodilatin) at 1 µM concentrations do not stimulate increases in intracellular cGMP in this assay and neither does sodium nitroprusside (1 mM). Lack of atriopeptin-induced cGMP accumulation is due to the lack of GC-A and GC-B receptors on the surface of T84 cells (3, 39, 44, 45). Urinary cGMP does not contribute to this assay either, because it is removed during the preparative process (7, 30). Only intracellular cGMP is measured in this assay. Thus the T84 cell cGMP accumulation bioassay performed under acidic conditions provides a very specific and sensitive model system for the measurement of uroguanylin-induced cGMP agonist activity. In selected urine samples from CHF patients, the cGMP-stimulating peptide was purified and sequenced (described below). STa was employed as a standard for the activation of GC-C in T84 cells under acidic and alkaline conditions. All assays were performed in duplicate or triplicate, and results are expressed as the means ± SE of at least three experiments.

Purification of uroguanylin. Urine samples from healthy individuals and patients with CHF were collected and prepared as described above. The reconstituted Sep-Pak fractions were subjected to a series of four RP-HPLC steps under different acetonitrile gradient conditions. Fractions from each column chromatography step were evaluated for bioactivity as described above employing the cGMP accumulation T84 cell assay under acidic (pH 5.5) and alkaline (pH 8.0) conditions (21). The 40% acetonitrile-0.1% TFA fraction eluted from the Sep-Pak preparation cartridge was dried under vacuum and reconstituted in HPLC buffer A (0.1% TFA). Approximately 1 ml of sample was applied to a C₁₈-RP-Bondapack column (5 mm × 30 cm, Waters) preequilibrated with buffer A. A linear gradient of 0–100% buffer B (80% acetonitrile, 0.1% TFA) was run at 2 ml/min over 100 min, while absorbance was monitored at 214 and 280 nm. The bioactive fractions were collected, combined, dried, and resuspended in 5 ml of buffer A. About 1 ml of sample was again applied to a C₁₈-RP-Bondapack column and fractionation proceeded isocratically for 10 min with 15% buffer B, followed by a linear gradient to 35% buffer B for 100 min, and a final 15 min at 100% buffer B. Two-milliliter fractions were collected, and aliquots of each were assayed. Bioactive fractions were combined (from all HPLC runs for the respective urine sample), lyophilized to eliminate the acetonitrile and TFA, and resuspended in 3 ml of buffer A. One-milliliter samples were applied to a third RP-HPLC run consisting of the following parameters: isocratic elution for 5 min to 10% buffer B; linear gradient for 90 min to 25% buffer B; linear gradient for 50 min to 55% buffer B; and 10 min 100% buffer B. Bioactive uroguanylin fractions were identified (retention time ~72 min, ~21.9% acetonitrile), pooled, dried, and reapplied to the RP-column under the same conditions. Urine from CHF patients and healthy individuals were prepared exactly the same way throughout the four-step HPLC procedure. For control and recovery purposes, synthetic opossum uroguanylin was subjected to the same procedure. Final recovery of uroguanylin was ~60% throughout this preparative and analytical purification. The purified fraction containing the highest cGMP-activating potential at pH 5.5 was reduced and alkylated by 2 M guanidine HCl, desalted by RP-HPLC, and sequenced by the University of Kentucky Sequence Core Facility, and subjected to electrospray ionization mass spectrometry (37).

Suckling mouse intestinal fluid assay. Uroguanylin and uroguanylin-like preparations from urine extracts were tested for their ability to induce intestinal fluid accumulation in newborn mice. Because uroguanylin was originally identified as an intestinal hormone that causes chloride secretion and diminished sodium reabsorption leading to fluid accumulation in the lumen (16, 19), the suckling mouse assay was employed. NIH Institute of Cancer Research/Harlan Sprague-Dawley (ICR/HSD) suckling mice 2–4 days old were gavaged with 0.1 ml of test solutions as described previously (19). An intestinal weight-to-carcass weight ratio of 0.0875 represents one unit of activity. STa, opossum uroguanylin, and opossum guanylin have been shown to induce diarrhea in suckling mice with 3.75, 40, and 150 ng, respectively (19).
UROGUANYLIN INCREASES DURING CHF

RESULTS

To establish the conditions and activation by which uroguanylin acts on GC-C in T84 cells, a series of experiments were performed to show the concentration response for stimulation of cGMP accumulation (Fig. 1). Uroguanylin stimulates T84 cells and cGMP accumulation in a dose-dependent fashion at pH 5.5 and 8.0. However, a greater increase in cellular cGMP levels was observed at pH 5.5. The concentrations of uroguanylin yielding half-maximum activation of GC-C (K_a as determined by the method of Hanes (24)) were K_a (pH 5.5) = 0.25 ± 0.03 µM and K_a (pH 8.0) = 1.41 ± 0.20 µM. These values are similar to the EC_50 concentrations determined previously for uroguanylin at pH 5.5 and pH 8.0 (23). For comparison, STA was evaluated for the activation of T84 cells under the same conditions (Fig. 1). STA was more potent at both pH 5.5 (K_a = 16.75 ± 1.93 nM) and pH 8.0 (K_a = 109.90 ± 13.28 nM; Hanes plot for STA not shown). Incidentally, guanylin is more active (3-fold) at pH 8.0 than uroguanylin at the same pH (data not shown; 21). Under acidic conditions, uroguanylin was 100-fold more potent than guanylin. These results confirm what has previously been demonstrated (21), that uroguanylin- and guanylin-sensitive pH conditions can be used to identify these peptides in various biological samples such as urine.

Figure 2 shows that concentrations of uroguanylin-like excretion and plasma ANP in patients with stage II/III CHF are elevated 70-fold and 7.5-fold, respectively, above levels obtained from healthy volunteers (both P < 0.001). In this small group of patients with CHF (n = 16), there does not seem to be a connection between increased uroguanylin levels and 1) any drug therapy such as diuretics, intravenous dobutamine, or angiotensin-converting enzyme inhibitors, 2) mechanical ventilation, 3) hemodynamic status such as low/high cardiac output, hypotension, sepsis, and 4) demographic factors such as age or gender.

To confirm that the urinary cGMP-activating potential observed in the bioassay was uroguanylin, urine samples from CHF patients and healthy individuals were subjected to a series of reverse-phase HPLC fractionation steps. These experiments were performed to show that the purified uroguanylin fraction from the HPLC was the sole agent responsible for the cGMP agonist activity found in the urine extracts. Figure 3 shows that the cGMP-activating peptide eluted from C_{18} RP-HPLC elutes at the same retention time as the standard synthetic uroguanylin. The fractions from the...
urine sample(s) of CHF patients demonstrating the highest bioactivity were collected, pooled, and prepared for sequencing. The sequence was NDDCEL CVN VACTGCL. This peptide, which appears to be >95% homogenous, is the COOH-terminal, 16 amino acid portion of human prouroguanylin. The peptide is fairly acidic, with a theoretical pI of 3.49, and the predicted molecular weight of the reduced peptide is 1671.89. Electrospray mass spectrometric analysis of the peptide yielded an observed molecular size of 1667.1 atomic mass units. These molecular weights correspond to the theoretical weights derived from the sequence if two disulfide bonds link the four cysteines, and therefore indicate that the full sequence of this urinary peptide was determined by NH2-terminal protein sequence analysis.

To determine whether the presence of an increased uroguanylin peak in the HPLC analysis is actually due to uroguanylin-induced cGMP accumulation in the T84 cell bioactivity, additional patients with varied urinary uroguanylin bioactivities were tested. One would predict a low peak of activity for uroguanylin with HPLC in patients with low uroguanylin-like bioactivity. Table 2 shows the data for urines from five patients with CHF that were assayed by the cGMP accumulation bioactivity assay and by HPLC. Uroguanylin-like bioactivity corresponds to the HPLC-purified uroguanylin peak from the same urine samples. Thus the presence of the increased uroguanylin peak by HPLC suggests that the increased cGMP generation from human CHF urine sample is linked to the uroguanylin system. This is a crucial piece of data in the absence of quantitative data with RIA. The increased uroguanylin excretion levels observed in the urine of patients with CHF do not seem to have a relationship with serum sodium, potassium, or plasma ANP concentrations. In addition, neither age nor gender appears to affect urinary uroguanylin levels. However, in the limited subset of patients analyzed by both the bioactivity assay and by HPLC, the severity

---

**Fig. 3.** Reverse-phase HPLC elution and activity profile for uroguanylin purified from representative 24-h urine samples collected from a patient with CHF and a healthy individual. Fractions containing bioactive uroguanylin were pooled and concentrated from one C18 reversed phase (RP) Sep-Pak and three consecutive C18 RP-HPLC steps as described in METHODS. This profile represents final purification step of uroguanylin from urine by C18 RP-HPLC. Each profile (A, B, C) shows ultraviolet light-absorbing (214 nm) trace (top solid line), acetonitrile gradient used to elute column (middle dashed line), and bioassay responses to select individual column fractions performed under acidic and alkaline conditions (pH 5.5, solid circles; pH 8.0, shaded bars). A: HPLC fractionation of one representative urine sample from a CHF patient. B: representative fractionation of a urine sample from a healthy male individual. Urine was treated exactly like urine sample shown in A throughout all preparative and analytic procedures. C: purified opossum uroguanylin standard elution/activity profile by RP-HPLC under same conditions as A and B. Recovery of uroguanylin for final HPLC step is 86.9 ± 3.1% and for entire Sep-Pak/4-step RP-column purification procedure (as described in METHODS) is 57.2 ± 6.9%. Peak activity fraction in C eluting at a retention time similar to that of purified uroguanylin in C (RT ~72 min) was collected, reduced, alkylated by 2.0 M guanidine HCl, and desalted by RP-HPLC. Sequence (underlined) from this peak fraction is shown in A. This sequence corresponds to human uroguanylin.
Uroguanylin bioactivity was measured from the urine sample as described in METHODS. RF indicates the response factors determined from the quantitative integration of uroguanylin peak areas described in Fig. 3. CHF, congestive heart failure.

Table 2. Uroguanylin bioactivity corresponds to HPLC-purified peptide in same set of urine samples from patients with CHF

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, yr</th>
<th>NYHA Class</th>
<th>Serum Concentration</th>
<th>Uroguanylin Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Creatinine, mg/dl</td>
<td>Sodium, meq/l</td>
</tr>
<tr>
<td>Male</td>
<td>66</td>
<td>III</td>
<td>3.4</td>
<td>142</td>
</tr>
<tr>
<td>Female</td>
<td>41</td>
<td>III</td>
<td>3.0</td>
<td>144</td>
</tr>
<tr>
<td>Female</td>
<td>77</td>
<td>II</td>
<td>1.7</td>
<td>135</td>
</tr>
<tr>
<td>Male</td>
<td>93</td>
<td>II</td>
<td>1.8</td>
<td>142</td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>II</td>
<td>0.6</td>
<td>136</td>
</tr>
</tbody>
</table>

Uroguanylin bioactivity was expressed as means ± SE of peptide-induced formation of nmol cGMP produced/ml urine. Total bioactivity (µmol cGMP) = (urine volume collected for 24 h) × (nmol cGMP/ml); n = 27 males and 26 females. Uroguanylin-like excretion in male and female population. Excretion of uroguanylin is also highest during the morning-daytime hours. The male excretion levels for uroguanylin were significantly higher during the hours of 8:00 AM and 2:00 PM (P ≤ 0.05). The uroguanylin-like excretion values over a 7-day period were reproduced consistently with those individuals who underwent the 24-h urine analysis twice (P ≤ 0.05 as determined by paired t-test; data not shown). Differences of circulating ANP were not observed between the healthy male and female subjects (Table 3). The overall mean ANP concentration in the

Table 3. Urinary levels of bioactive uroguanylin and circulating plasma levels of ANP from healthy individuals

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uroguanylin</td>
<td>1.74 ± 0.55</td>
<td>0.94 ± 0.16</td>
<td>2.15 ± 0.50</td>
<td>1.38 ± 0.20</td>
</tr>
<tr>
<td>ANP</td>
<td>12.6 ± 2.10</td>
<td>11.5 ± 1.30</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Uroguanylin-like bioactivity was measured in the urine as described in METHODS under acidic conditions and reported as means ± SE of peptide-induced formation of nmol cGMP produced/ml urine. Total bioactivity (µmol cGMP) = (urine volume collected for 24 h) × (nmol cGMP/ml); n = 27 males and 26 females. Atrial natriuretic peptide (ANP) was measured in the plasma of subjects by RIA (41) (expressed as pg peptide/ml). Differences of circulating ANP levels with respect to gender were not observed. Average ANP levels for all normal individuals was 12.1 ± 1.6 pg/ml. NA, not assessed.
healthy individuals was 12.1 ± 1.6 pg/ml, which is consistent with the levels previously observed in healthy control individuals (20, 25, 27, 32).

DISCUSSION

This study represents the first physiological demonstration that bioactive uroguanylin is increased in the urine of patients with CHF. A 70-fold increase (P = 0.001) was observed over the uroguanylin peptide activity levels for healthy individuals. This finding of markedly elevated levels of uroguanylin peptides in patients with severe cardiac failure is clearly of interest. CHF represents a cardiovascular disorder in which salt and fluid homeostasis plays a significant pathophysiological role causing abnormal cardiac output, sodium retention by the kidney, and edema (1, 9, 36). Patients with heart failure commonly have edema that may also influence the production of intestinal and cardiac natriuretic peptides such as uroguanylin and ANP (1). Because uroguanylin and ANP are natriuretic peptides, their action on the kidney and the subsequent increases in urinary sodium and potassium excretion may be an adaptive response to the salt and fluid retention of CHF. Indeed, increases in specific natriuretic peptides, like ANP, are established prognostic indicators of edematous states (i.e., CHF, nephrotic syndrome, etc.; 9, 34, 36, 41). It is tempting to speculate possible roles of uroguanylin in the pathophysiology of CHF; in both situations, gut function as well as fluid homeostasis may be abnormal. The increase of uroguanylin activity may be a physiological response to salt retention, edema, or vascular flow. Also, it may be postulated that uroguanylin is not the only endogenous natriuretic peptide mediated by GC-C in mammals during CHF. A disorder associated with imbalances of salt and water may signal an increase in additional factors, such as guanylin, that aid in homeostasis. Alternatively, uroguanylin may stimulate or enhance the action of specific natriuretic and relaxing agents (e.g., ANP, EDRF) secreted by the kidney or heart that, in turn, elicit specific actions of uroguanylin.

Our data reflect 1) uroguanylin excretion from a healthy human control population with respect to age, gender, and diurnal and daily variations, and 2) the determination of uroguanylin-like excretion in patients with CHF. Excreted levels of bioactive uroguanylin were measured by a cGMP accumulation T84 cell bioassay (7, 21). This bioassay has been optimized to take advantage of conditions in which uroguanylin may be quantitated by measuring specific binding and activation of GC-C in T84 cells de novo (7, 21, 22, 30). Relative potencies of uroguanylin extracted from the urine samples were assessed by the ability of the peptide to stimulate intracellular cGMP accumulation in T84 intestinal cell monolayers at pH 5.5 (21). In our studies, we confirmed that this cGMP-activating peptide found in the urine is, indeed, uroguanylin by HPLC purification techniques (Fig. 3) and physical characterization (i.e., mass, pl). The peptide sequence reflected the COOH-terminal 16 amino acids of the human prouroguanylin sequence, which is the bioactive moiety of the circulating prohormone. Uroguanylin activity (and not prouroguanylin) can be quantitated by the T84 cell bioassay under acidic conditions. By demonstrating the effect of the peptide in vivo using the suckling mouse assay (Fig. 4), we can conclude that the purified human uroguanylin from CHF urine is authentic and acts as an intestinal secretogogue. This urinary peptide, which is increased during CHF, appears to be the sole agent responsible for the observed cGMP accumulation in the model T84 cell intestinal epithelium and the fluid secretion in the suckling mouse.

The fact that this cGMP agonist from urine was purified, sequenced, and shown conclusively to be uroguanylin is stronger evidence than RIA data. Immunoassay results are dependent on the antisera made against a specific immunogen. For uroguanylin, RIA data are weaker than actual purification and sequencing techniques because of possible cross-reactivity of antisera with members of the guanylin/uroguanylin peptide family. However, in the absence of a quantitative RIA for human uroguanylin, we asked the question if the uroguanylin peak observed in the patient’s urine samples during HPLC mimicked the level of cGMP generation observed in the T84 cell bioassay. Table 2 shows that a low peak of uroguanylin corresponds to a low cGMP bioactivity in the same urine sample. The
low number of patients in this study makes it difficult to relate uroguanylin excretion with the patient’s age or gender, but studies evaluating the staging of CHF by urinary uroguanylin levels may be warranted because higher uroguanylin bioactivities are observed in those patients with more severe heart failure. Considerable evidence exists suggesting that uroguanylin plays an important role in sodium and water homeostasis (15, 16, 19). This peptide elicits a natriuresis, kaliuresis, and diuresis in a de novo isolated rat kidney perfusion model (14) and in an in vivo renal function sealed-mouse assay (19). Uroguanylin is believed to act by activating transmembrane guanylate cyclase receptors in a signaling mechanism similar to that of ANP (15). ANP binds to GC-A, GC-B, and the natriuretic peptide clearance receptor, whereas uroguanylin binds only to GC-C. Because ANP levels are markedly elevated in patients with CHF, and both ANP and uroguanylin are proposed to aid in the control of salt and water balance in the body, we sought to measure uroguanylin levels in patients with CHF. We did this indirectly by measuring uroguanylin bioactivity in urine samples collected from healthy individuals and patients with CHF. However, it was beyond the scope of this pilot study to examine the underlying mechanisms for the observed changes in uroguanylin bioactivity. The intent of this report was to describe the increases of uroguanylin excretion in patients with CHF, and in doing that, draw some correlations to healthy uroguanylin levels with respect to age, gender, temporal variations, and ANP levels.

The source of the increased urinary uroguanylin associated with CHF is unknown. The uroguanylin isolated from the urine may be derived from the plasma (12) as a result of the filtration of the bioactive 16-amino acid moiety and its prohormone into the tubular filtrate. Also, uroguanylin could be produced locally and secreted into the nephron by cells lining the lumen. The peptide could then act on its target cells in the renal tubules (14, 15, 17). We demonstrated that bioactive uroguanylin infused into the circulation of rats and mice appears in the urine (unpublished observations). This suggests that uroguanylin is filtered by the kidney. Prouroguanylin is synthesized in the intestine and heart by chromaffin cells and secreted into the lumen and circulation (12, 15, 16, 22). The levels of circulating prohormone are unknown in healthy individuals and in patients with disease, but proteolytic processing to the active peptide must take place before binding to its receptor. This proteolytic processing requires chymotrypsin or chymotrypsin-like proteases (which can be found in high concentration in the proximal small intestine (15) and the nephron (12, 15, 17)). Activation of the receptor-guanylate cyclase molecules embedded in apical membranes of kidney target cells results in the increased urinary excretion of sodium, potassium, and water (14, 19, 35). During pathophysiological states of sodium retention (e.g., CHF, renal failure) or when dietary salt exceeds the physiological requirements, expression of uroguanylin (and related peptides, i.e., guanylin; 7) and its receptor (13) may be upregulated. In the colon, the uroguanylin/guanylin signaling pathway is downregulated as an adaptive response to salt restriction (33). Alternatively, the fluid and sodium retention associated with CHF may play a role in the release of uroguanylin from the myocardium into the plasma serving as a compensatory and/or adaptive response to this pathophysiological circumstance. Thus increased levels of uroguanylin excretion observed in patients with CHF may be due to increased circulation prouroguanylin (which then can be proteolytically cleaved into the COOH-terminal bioactive moiety in the bloodstream, nephron, or intestine) or increased expression of a uroguanylin-converting enzyme(s) in the circulation or nephron. Finally, increased secretion of locally produced uroguanylin in the renal tubule may also account for increased uroguanylin levels in the urine.

Uroguanylin has been purified from intestinal segments from mammals, with the highest expression found in the small intestine and the plasma (12, 16). Messenger RNA for the preprouroguanylin protein has been found in other regions of the digestive system (including the stomach), the central nervous system, the reproductive system, the lymphoid system, and the kidney (13). In addition, the uroguanylin/guanylin (GC-C) have also been identified in the heart. Low levels of GC-C mRNA have also been identified in fetal, neonatal, and regenerating liver, testis, placenta, brain, and kidney (15, 17, 28, 43). Thus the uroguanylin signaling mechanism may play a role in cardiac and renal function, especially during periods of abnormal salt and water retention associated with CHF.

The studies presented herein describe the first working baseline of bioactive uroguanylin-like excretion levels in humans (n = 53). It was essential that some baseline knowledge of healthy human uroguanylin excretion be established to allow appropriate conclusions to be drawn concerning the presence and levels of this peptide in patients with disease. The total uroguanylin-like activity from urine samples collected and pooled from four 6-hour urine fractions in a 24-hour period was 1.73 ± 0.26 µmol cGMP. The urinary cGMP agonist did not vary with respect to age, but gender differences (Table 3) and a diurnal circadian fluctuation within a 24-hour period (Fig. 5) were observed. Neither gender nor age affected the levels of uroguanylin excretion in patients with CHF. Because only 24-hour urine samples were used from the patients, no data on diurnal temporal variations were available. The data from the healthy control population present some interesting questions regarding the purpose for salt and water balance during the day. The highest uroguanylin-like bioactiv-
ity for males and females is shown between the hours of 8:00 AM and 2:00 PM (Fig. 5). These data may suggest that during early daytime hours, the period in the day that commonly includes meals with excess sodium, compensatory mechanisms are required to achieve salt homeostasis, and one or more additional natriuretic factors (i.e., uroguanylin) help stimulate renal sodium excretion. Nighttime hours exhibit low uroguanylin levels, which may imply that this peptide is downregulated during the sodium/calorie-deprived period of the day. Thus uroguanylin excretion may be directly proportional to the salt intake (28). Indeed, it has been shown that sodium excretion is highest between the hours of 9:00 AM and 1:00 PM for a healthy population. The correlation detected between the excretion of uroguanylin and the excretion of sodium is also consistent with the hypothesis that uroguanylin may contribute to the natriuretic response elicited by the ingestion of salt. So the increase in urinary uroguanylin in healthy individuals, which may be derived from the circulation or through local production by renal cells, may serve as part of the mechanism to control sodium surfeit during mealtimes.

Urodilatin, a renal analog of ANP that has been found to help modulate sodium excretion in humans, also has been shown to follow the same diurnal excretion pattern. However, as shown previously, urodilatin does not contribute to the specific uroguanylin-induced cGMP accumulation T84 cell bioassay employed in this report (3, 7, 39, 45). Therefore, both uroguanylin and urodilatin may contribute to sodium excretion during sodium surfeit, although a link between the two peptides has not been investigated.

There is a continuing search for more effective treatments for patients with heart failure. One of the many consequences of ventricular dysfunction is sodium retention by the kidney leading to fluid overload with resulting pulmonary and peripheral congestion (36). Diuretic therapy is a mainstay in the treatment of CHF but is often limited by resulting electrolyte abnormalities and renal dysfunction (9, 34, 36, 41). Uroguanylin has been found to elicit a natriuresis by direct effects on the kidney, and this peptide may be involved in the physiological management of fluid balance (14, 19). This peptide hormone acts in a similar fashion to the action of the natriuretic peptides, which are currently under extensive investigation. However, before this study no data existed concerning the urinary levels of uroguanylin in normal individuals and patients with cardiovascular disease. Because of the known sources of uroguanylin and its message, and the fact that increases are observed during sodium retention or overload, this peptide may contribute to the regulation of sodium excretion in health and disease through an endocrine axis among the intestine, heart, and kidney. The results from this study regarding uroguanylin excretion may, in the long term, assist in understanding the pathophysiology of fluid overload conditions as well as in the development of more effective monitoring and therapy in the maintenance of proper salt and water balance in patients with heart failure.

The authors gratefully acknowledge the expert technical assistance from Jessica Crytzer, Scott F. Musson, Brett R. Johnson, W. E. Scott, Jason Chang, and Rajesh G. Shah. Also, we appreciate the intellectual participation and advice from Drs. Andrew M. Cross, J. R., and Peter M. Sapin from the Department of Medicine and Cardiology, University of Kentucky Chandler Medical Center. The authors also thank the Lexington VA Medical Center for space allocated to Drs. Greenberg and Carrithers.

This research was supported by a grant from the Kentucky Affiliate of the American Heart Association (R. N. Greenberg).

Address for reprint requests and other correspondence: R. N. Greenberg, Dept. of Medicine, Division of Infectious Diseases, Univ. of Kentucky Chandler Medical Center, 800 Rose St., MN-672, Lexington, KY 40536-0084 (E-mail: RNGREE01@POP.UKY.EDU).

Received 28 April 1999; accepted in final form 30 August 1999.

REFERENCES