Presence of Angiotensin Peptides in Human Urine

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Immunoreactive angiotensin I and angiotensin II were found in human urine that was purified on octadecasilyl-silica cartridges. The daily excretion of angiotensin I and II in healthy volunteers was 189.00 (SE 38.36) and 17.54 (SE 3.07) pmol/24 h or 148.09 (SE 32.22) and 12.82 (SE 2.34) pmol/L, respectively (n = 12). No circadian rhythm was observed in the excretion patterns of angiotensin I and II. In vitro degradation of angiotensin I or II could not be detected in acidified urine samples. A marked increase in the excretion of angiotensin I and II could be demonstrated in patients with anaphylactoid reactions to drugs and food additives after oral challenge. Immunoreactive angiotensin I and II could be characterized by HPLC as ile<sup>9</sup>-angiotensin I, ile<sup>9</sup>-angiotensin II, and angiotensin II metabolites.

Additional Keyphrases: chromatography, reversed-phase · radioimmunoassay · anaphylaxis · sample handling

Angiotensin II (Ang II) is the biologically active component of the renin–angiotensin system (RAS).<sup>1</sup> It is generated from angiotensin I (Ang I) by angiotensin-converting enzyme. Peptidases metabolize Ang I and Ang II to various fragments. Ang II was first isolated and purified from plasma >30 years ago (1). Since then, Ang II and other RAS components have been identified in the adrenal gland, testis, kidney, blood vessels, heart, and brain (2, 3). Ang II, a potent vasoconstrictor, is involved in a wide range of biological processes, including blood pressure control and volume homeostasis (4). The RAS is involved in stress and hemorrhage (5, 6). Experiments with dogs demonstrated that plasma Ang II increased severalfold in response to hemorrhage, thus indicating a role of Ang II in regulating hemodynamics during hemorrhagic hypovolemia and hypotension (6). Although angiotensin peptides can be identified in blood, it is unclear whether they can be excreted by the kidney and, therefore, be detected in urine. Our purpose here was to measure and characterize angiotensins in human urine by radioimmunoassay (RIA) and high-performance liquid chromatography (HPLC). We investigated the possibility of altered urinary excretion of these peptides in patients with anaphylactoid reactions to drugs and food additives.

Materials and Methods

Subjects. The control group comprised 12 healthy volunteers, six men and six women, mean age 31 years (range 23–54 years). Five patients with severe anaphylactoid reactions (AR) to drugs and food additives also participated; severity of clinical symptoms was classified according to the four-grade rating system of Ring and Messmer (7): Patient 1—a 50-year-old woman, AR to cyanocobalamin, grade III; patient 2—a 33-year-old man, AR to propylphenazine, grade II; patient 3—a 64-year-old woman, AR to acetylsalicylic acid, grade I; patient 4—a 57-year-old man, AR to acetylsalicylic acid, grade I; and patient 5—a 42-year-old woman, AR to sodium benzoate, grade II.

Oral provocation test. To diagnose allergies, we took the patient's history and performed skin and oral provocation tests as described previously (8). Briefly, substances were administered orally in gelatin capsules, with the first dose given at 1000 h. We administered cyanocobalamin at 20, 100, and 200 μg; propylphenazine at 5, 20, and 100 mg; acetylsalicylic acid at 50, 250, 500, and 1000 mg; and sodium benzoate at 50, 250, and 500 mg. The oral provocations were performed under medical supervision with the subject not knowing what substance was being tested. Patients were instructed to report any adverse reactions and were examined carefully for symptoms during the test period.

Collection of samples. Patients and control subjects collected their urine over a 24-h period. The urine was collected in a 1-L graduated plastic flask each time the individual urinated. The volume of each urine fraction varied from 100 to 500 ml. The time of collection and the volume of each urine sample were recorded. We poured 40 mL of the collected urine into a 50-mL plastic tube containing 0.32 mL of 7 mol/L HCl. The pH of the acidified urine samples was 2.5. We mixed and froze the samples and stored them at −80 °C.

Urine purification. We thawed urine samples and centrifuged them at 1000 × g for 10 min at 4 °C. The pellet was discarded and 20 mL of the supernate was purified on octadecasilyl-silica cartridges (Sep Pak C<sub>18</sub>; Millipore Waters, Eschborn, FRG). This procedure has been used successfully for purifying and enriching angiotensin peptides in human sweat and is described in detail elsewhere (9). After preparing the cartridges, we applied the samples and washed the cartridges. We eluted the retained angiotensin peptides with a mixture of methanol/water/trifluoroacetic acid (80/19.9/0.1, by vol), then dried the samples by centrifugation under reduced pressure. The dried samples were dissolved in 1 mL of RIA buffer (see below) and centrifuged at 10 000 × g for 2 min at room temperature. Portions were taken for the radioimmunological measurement of the pep-
tides. Blank values for the cartridges were determined by applying identical volumes of distilled water instead of urine. To determine the analytical recovery of this technique, we added $^{125}$I-labeled Ang I or $^{125}$I-labeled Ang II to the urine samples.

**Incubation of Ang I and Ang II.** Urine samples were collected from six volunteers, three men and three women, as described above. Synthetic Ile$^8$-Ang II (0.1 mL of a 1 mmol/L solution in isotonic saline (NaCl, 150 mmol/L)) was incubated with 0.7 mL of acidified urine at 37°C for 0, 1, 5, 10, 20, 30, and 60 min. At each interval, 0.1 mL of the incubate was withdrawn, mixed with 0.1 mL of 0.1 mol/L trifluoroacetic acid, and boiled for 5 min. After centrifugation at 10,000 × g, for 2 min at ambient temperature, 0.1-mL samples were analyzed by HPLC.

Likewise, urine samples from the same subjects were collected with and without the addition of the HCl. Untreated urine had a mean pH of 5.5; the acidified urine was pH 2.5. Both untreated and acidified urines were incubated with $^{125}$I-labeled Ang I or $^{125}$I-labeled Ang II as described above. After the incubation was stopped, the samples were centrifuged at 10,000 × g for 2 min at ambient temperature, and 0.15-mL samples were analyzed by HPLC.

**Radioimmunoassays.** RIAs of Ang I and Ang II were performed as described previously (10). Briefly, antisera to Ang I and Ang II were produced in New Zealand White rabbits. $^{125}$I-labeled Ang I and Ang II were purchased from Amersham (Amersham Buchler, Braunschweig, FRG). Ang I and Ang II antisera were used at a final dilution of 1:200,000 with 50% of the wells containing 10 and 10 fmol for Ang I and Ang II, respectively. Both assays were carried out in 0.05 mol/L Tris·HCl buffer (pH 7.2) containing bovine serum albumin, 1 g/L. The detection limit for both assays was 1.0 fmol per tube.

**High-performance liquid chromatography.** The peptides were characterized by HPLC with an ERC HPLC equipment (Model ABE 88; ERC, Altdorf, FRG). All separations were carried out at room temperature with a 200 × 4 mm reversed-phase C18 column packed with 7-μm diameter particles (Macherey und Nagel, Düren, FRG) and an acetonitrile gradient elution system. This procedure provides baseline separation of angiotensin peptides in human plasma and sweat (9). The mobile phases were as follows: solution A, 1 mL of 1 mol/L morpholine solution adjusted to pH 6.5 with concentrated phosphoric acid in 100 mL of HPLC-grade water; solution B, 1 mL of 1 mol/L morpholine solution adjusted to pH 6.5 with concentrated phosphoric acid plus 25 mL of methanol in 100 mL of acetonitrile. A multiple step-gradient was performed (9). The column was calibrated with synthetic standard peptides, which were detected at 225 nm with a sensitivity of 0.1 absorbance unit full scale. Urine samples from control subjects were purified on Sep Pak C18 cartridges, pooled, and then dried in the vacuum centrifuge. The dried residues were dissolved in 1 mL of 0.01 mol/L HCl, centrifuged at 10,000 × g for 2 min at room temperature, after which 0.95 mL was injected for HPLC separation. The column was developed at a flow rate of 1.0 mL/min, and 1.0-mL fractions were collected. The eluent was removed from the eluted fractions by use of the vacuum centrifuge, and the dried samples were dissolved in 0.25 mL of RIA buffer. We measured Ang I and Ang II by RIA in 0.05-mL samples.

**Chemicals.** All chemicals used were of analytical grade. Tris (Trizma, pH 7.2) and bovine serum albumin were obtained from Sigma Chemie, Deisenhofen, FRG, and morpholine "Gold Markes" was purchased from Aldrich Chemie, Steinheim, FRG. All other chemicals, including HPLC solvents, were from Merck AG, Darmstadt, FRG. The synthetic peptides were purchased from Bachem Biochemica GmbH, Heidelberg, FRG.

**Results**

**Recovery.** The analytical recovery of the purification step with Sep Pak C18 cartridges was monitored by adding $^{125}$I-labeled Ang I or Ang II to the urine samples. In the purified urine samples, 99.09% (SE 0.56%) and 91.76% (SE 3.82%) of the respective radiolabeled peptides were recovered (n = 16).

**Angiotensin peptides in normal human urine.** Human urine samples contained immunoreactive Ang I and Ang II in the following concentrations (collected over 24 h from healthy volunteers): 189.00 (SE 38.36) and 17.54 (SE 3.07) pmol/24 h, respectively (n = 12). With regard to the urine volume excreted in 24 h, the mean (and SE) concentrations of Ang I or Ang II were 148.09 (32.22) and 12.82 (3.43) pmol/L, respectively (n = 12). The concentration of Ang II, the biologically inactive precursor of Ang II, was 11-fold that of Ang II.

The excretion profile of Ang I and Ang II over 24 h showed no circadian excretion pattern (Figure 1). The increase of the Ang I excretion at 1700 h was not significantly different from the concentrations at other times. Individual variation in the urine concentrations of both peptides was greater during the day, whereas little variation was seen at night. The Ang II concentrations tended to be less at night than during the day.

We investigated the effects of the presence of biologically active Ang II-degrading enzymes in urine. In vitro incubation of acidified urine with synthetic Ile$^8$-Ang II did not indicate any significant metabolism of Ang II. Even after 60 min, 94% (SE 2%); n = 6) of intact Ile$^8$-Ang II could be identified after HPLC separation (Figure 2). No degradation of $^{125}$I-labeled Ang I or $^{125}$I-labeled Ang II was noted after in vitro incubation with acidified urine. After 60 min, 97% (SE 3%) and 100% (SE 7%) of radiolabeled Ang I or Ang II could be recovered in the HPLC fractions (n = 6; Figure 3). However, when untreated urine was incubated with the radiolabeled peptides there was substantial degradation of both peptides. After incubation for 60 min, 76% (SE 9%) and 50% (SE 12%) of intact Ang I and Ang II, respectively, could be identified in the HPLC fractions (n = 3; Figure 3). This indicates the presence of peptidases in urine and their effective inhibition with the acid treatment.

HPLC characterization of pooled urine samples from control subjects displayed the presence of Ile$^8$-Ang I,
Ile<sup>5</sup>-Ang II, and Ang II metabolites such as Ile<sup>4</sup>-Ang III, Ile<sup>3</sup>-Ang II<sub>5-8</sub> hexapeptide, and Ile<sup>2</sup>-Ang II<sub>4-8</sub> pentapeptide (Figure 4). In three different pools, ~71% of the peptides that were detected with anti-Ang I antibody in the HPLC fractions corresponded to Ile<sup>5</sup>-Ang I. The other three substances that cross-reacted with the anti-Ang I antibody were immunoreactive material of unknown nature. Several unidentified substances that

Fig. 1. Daily excretion profile of Ile<sup>5</sup>-Ang I (upper panel) and Ile<sup>5</sup>-Ang II (lower panel)

No circadian rhythm for the excretion of angiotensin peptides was observed. Compared with the variation during the day, the concentrations of excreted Ang I and Ang II are less variable during the night. Results are expressed in pmol/h (mean ± SE; n = 12)

Fig. 2. In vitro degradation of Ile<sup>5</sup>-Ang II added to acidified human urine

Degradation of Ang II was insignificant even after 60 min. Results are expressed in percent of intact Ile<sup>5</sup>-Ang II after HPLC separation (mean ± SE; n = 6). The amount of added peptide exceeded the amount of endogenous peptide present

Fig. 3. In vitro degradation of <sup>125</sup>I-labeled Ang I (upper panel) and <sup>125</sup>I-labeled Ang II (lower panel) in human urine

In acidified urine, pH 2.5 (<sup>125</sup>I), the degradation of the radiolabeled peptides was not significant (n = 6). However, in untreated urine, pH 5.5 (C), both <sup>125</sup>I-labeled peptides were degraded (n = 3). Results are expressed as mean ± SE of intact <sup>125</sup>I-labeled Ang I or <sup>125</sup>I-labeled Ang II after HPLC separation

Fig. 4. HPLC characterization of angiotensin peptides in human urine with a reversed-phase C<sub>18</sub> column and an acetonitrile gradient elution

Besides Ile<sup>5</sup>-Ang I and Ile<sup>5</sup>-Ang II, Ang II metabolites such as Ile<sup>4</sup>-Ang III, Ile<sup>3</sup>-Ang II<sub>5-8</sub> hexapeptide, and Ile<sup>2</sup>-Ang II<sub>4-8</sub> pentapeptide were identified. Arrows indicate the retention times of the corresponding synthetic standard peptides. Chromatographic conditions given in the text. (...) gradient profile, (---) measurement with the anti-Ang I antibody, (- - -) measurement with the anti-Ang II antibody
Ang II hexa- and pentapeptides that were measurable with the anti-Ang II antiserum accounted for 24%, 18%, 15%, and 3%, respectively, of the total concentration of reactive peptides.

Angiotensin peptides in urine in patients. Ang I and Ang II were measured radioimmunologically in urine samples collected over a 24-h period from patients with AR to such drugs as cyanocobalamin, propyphenazono, and acetylsalicylic acid, and to food additives such as sodium benzoate. In all of the patients, Ang I and Ang II were markedly increased in the urinary fractions taken after the patients reported AR symptoms (Figure 5). Ang I increased by a factor of 3.81 (SE 0.54) and Ang II by a factor of 5.54 (SE 2.18) (n = 5). In the urine specimens collected after the symptoms had diminished or vanished, the concentrations of the immunoreactive peptides returned to normal.

Discussion

We report for the first time the presence of immunoreactive Ang I and Ang II in human urine. Ang II has been described in dog urine (11). The origin of Ang I and Ang II in human urine is not clear. The most likely source is plasma, but we cannot exclude the possibility that endogenous angiotensin peptides derived from the kidney are excreted into the urine. Infusion studies with [3H]Ang II in anesthetized, uninephrectomized dogs showed that, under physiological conditions, urinary Ang II was derived from Ang II generated by the kidney; at higher infusion rates, Ang II underwent glomerular filtration (11).

Because of the low concentrations of angiotensin peptides in urine, we used a procedure to enrich these concentrations. Octadecasyl-silica cartridges, widely used for purifying and enriching the content of peptides from various biological sources, were the method of choice (9, 12–15). This solid-phase extraction procedure allows the specific enrichment of Ang I and Ang II, with recovery values exceeding 90% and with no incompatibilities with the RIA measurement of both peptides. The Sep Pak purification procedure we used does not separate Ang I from Ang II and Ang II metabolites. Because of the negligible cross-reactivity of the anti-Ang I antiserum with Ang II and of the anti-Ang II antiserum with Ang I, both peptides can be determined by RIA of the eluate from the Sep Pak column as Ang I- and Ang II-immunoreactive material. To further characterize the peptides, one needs a combination of HPLC and RIA.

Based on the calculation of the urine concentrations per volume excreted, the concentration of Ang II in urine was in the same range as in plasma. However, the concentration of Ang I in urine was three- to fourfold higher than in plasma.

The excretion profile of Ang I and Ang II during 24 h did not show a circadian rhythm, but a high individual variation in their excretion was evident. The reduced physical activity and reduced environmental stress inputs during the night may account for the lesser variation during the night.

Because of possible in vitro degradation of urine Ang I and Ang II by peptidases after urine collection, we acidified samples with hydrochloric acid to inactivate peptidases and to prevent the degradation of angiotensins. Only intact synthetic Ang II, 125I-labeled Ang I, or 125I-labeled Ang II could be identified on HPLC after in vitro incubation with acidified urine for 60 min at 37 °C. However, we saw a substantial degradation of 125I-labeled Ang I or 125I-labeled Ang II when untreated urine was incubated for 60 min at 37 °C. We conclude that the acid treatment effectively inhibited angiotensin degradation.

HPLC characterization of Ang I- and Ang II-like immunoreactive material in partially purified urine extracts showed Ang I, Ang II, and Ang II metabolites. The decapeptide Ang I made up 71% of the total Ang I concentration in the urine extracts without HPLC separation. Similar results have been reported for human plasma, where 65% of the Ang I-like material represented true endogenous plasma Ang I (9). In human plasma, 62% to 64% of the Ang II-like material represented true endogenous Ang II (9, 16). Only 24% of the Ang II-like material in urine was true Ang II.

Patients with AR to drugs, food, or food additives often show cardiovascular symptoms such as hypotension, arrhythmia, or shock after exposure to these compounds. This prompted us to study whether the urinary excretion rate of Ang I and Ang II might be altered in these patients after oral provocation. In all five patients undergoing the oral test, the urine concentrations of
both Ang I and Ang II were markedly increased in the urine fractions after clinical symptoms were reported. The identification of altered concentrations of Ang I and Ang II in urine from these patients suggests that both peptides may play a role in AR.

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References