Plasma Concentrations of Angiotensin Metabolites in Young Male Normotensive and Mild Hypertensive Subjects

Toshiro Matsui, Kei Tamaya, Kiyoshi Matsumoto, Yutaka Osajima, Keiko Uezono* and Terukazu Kawasaki*

The plasma concentrations of angiotensin (Ang) I, Ang II, and their metabolites (Ang (3-8), (4-8), (5-8), and (3-4)) following in vitro ACE inhibitory activity were examined in young male normotensive (NT) (n = 7), and mild hypertensive (HT) volunteers (n = 6). There were no differences in supine plasma levels of Ang I, Ang II, and Ang (5-8) between the NT and HT groups: Ang I, 304 ± 43 fmol/ml vs. 293 ± 15 fmol/ml; Ang II, 32 ± 6 fmol/ml vs. 43 ± 10 fmol/ml; Ang (5-8), 176 ± 22 fmol/ml vs. 133 ± 32 fmol/ml. In addition, there were no significant differences between groups in any of these Ang levels when measured after standing for 60 min. However, the HT group showed significantly reduced supine and upright plasma Ang (3-8) and Ang (3-4) levels as compared to the NT group. In particular, the supine plasma level of Ang (3-4) (71 ± 13 fmol/ml-plasma) in the HT group was significantly (1/3-fold) lower than that in the NT group (197 ± 35 fmol/ml-plasma). An inverse correlation between the plasma level of Ang (3-4) and the upright systolic blood pressure (r = -0.627, p < 0.02, n = 13) was observed, indicating that the metabolism of Ang (3-4) might have been associated with the change in blood pressure. (Hypertens Res 1999; 22: 273-277)

Key Words: angiotensin II, angiotensin (3-4), metabolism, hypertension, normotension

In the renin-angiotensin-aldosterone (RAA) system, angiotensin (Ang) II, as well as Ang III and Ang (1-7), is known to be a pressor metabolite, increasing blood pressure (BP) through the activation of vasoconstriction or retention of Na+ in the kidneys via promotion of aldosterone release (1). However, other Angs are considered to have no bioactivity in the RAA system.

In our previous series of studies, we isolated 20 angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides from the the Bacillus licheniformis alkaline protease hydrolyzate of sardine muscle (2-4). Among these identified peptides, the sequence of the tripeptide, Arg-Val-Tyr, corresponded to the sequence of Ang (2-4) (3). Our subsequent in vitro ACE inhibitory studies on thirteen synthetic Ang II fragments (5) revealed the following: 1) 7 Angs showed potent ACE inhibitory activities (IC50 < 200 μM); 2) 4 ACE inhibitory Angs, i.e., Ang (3-8), Ang (4-8), Ang (5-8) and Ang (3-4) (Val-Tyr), occurred in human plasma (6); and 3) oral administration of Val-Tyr with the dose of 6 mg per d in mild hypertensive human subjects resulted in a significant decrease in BP during a 4-wk protocol (7). We have also reported that activation of the RAA system influences the metabolism of Angs in normotensive human subjects (8). We found that activation of the RAA system accelerated the production of smaller Angs, such as Ang (3-8), Ang (5-8) and Ang (3-4), in plasma. In particular, an approximately 4-fold increase in the production of Ang (3-4) was observed after subjects maintained an upright posture for 60 min. This suggested that the production of smaller Angs, particularly Ang (3-4), which are assumed to be biologically inactive, might be closely combined with the RAA system. To obtain more useful information on the metabolism of these Angs, we have examined the metabolic behavior of smaller Angs in young male normotensive and mild hypertensive subjects.

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Subjects and Methods

Human Plasma

The study group consisted of 13 male students of Kyushu University, 7 normotensives and 6 mild hypertensives aged 22 to 26 yr (normotensive mean age, 24.4 ± 0.4; hypertensive mean age, 23.3 ± 1.3). All subjects gave their informed written consent prior to the study. The subjects were instructed not to consume any beverages and not to smoke over the 12 h prior to the start of the study. After overnight fasting, venous blood samples (20 ml) were taken after subjects had fasted overnight and assumed a supine posture (SuP) for 30 min and an upright posture (UpP) for 60 min. Just prior to blood sampling, systolic and diastolic BP (SBP/DBP) were measured 3 times in succession. Each blood sample was drawn into a chilled vacutainer tube containing EDTA-2Na (TERUMO, Ltd., Tokyo, Japan), and immediately centrifuged at 1,500 × g for 15 min (4°C). To avoid the loss or degradation of Angs of interest by any enzyme in the tube, shortly after concentration, the obtained plasma was ultracentrifuged with a Molucut L (< M.W. 5,000; Nihon Millipore, Ltd., Yonezawa, Japan) at 4°C. As will be seen later, the 93.0 ± 3.3% recovery of Ang I from spiked (100 fmol/ml in plasma) plasma sample indicated that the degradation would be minimized under these experimental conditions. The plasma was separated and frozen at −30°C until the determination was performed.

Materials

Ang I, Ang II, and Ang (3-4) (Val-Tyr) were purchased from Sigma Chemical Co. (St. Louis, MO). Naphthalene-2,3-dialdehyde (NDA) as a fluorogenic reagent was obtained from Fluka (Tokyo). All other chemicals were of analytical-reagent grade and used without further purification.

Determination of Endogenous Angs in Plasma

Ten ml of the plasma sample was ultrafiltered, and concentrated to 0.5 ml by evaporation under reduced pressure. Then, the sample was directly applied to a reversed-phase column (Cosmosil 5C18-AR, 4.6 × 250 mm, Nacalai Tesque, Ltd.). The following linear gradient system was applied to a clean-up column: 20% to 40% CH3CN within 40 min for Ang I and Ang II assays, and 40% to 60% CH3CN within 60 min for the other Ang assays. After the zone of the retention of a desired NDA-Ang was column-switched, the heart-cutting fraction was separated on an analytical column (Cosmosil 5C18-ARII). The mobile phase was the CH3CN in 0.1% TFA containing 5 mM sodium octyl sulfonate as an ion-paired reagent. The following system was applied to an analytical column to determine each Ang: 60% CH3CN for Ang (3-4) assay, 55% CH3CN for Ang (5-8) assay, and 30% to 40% CH3CN within 25 min for the other Ang assays. The flow rate was 0.4 ml/min for both columns. The fluorescence detection (excitation and emission wavelengths: 420 nm and 490 nm, respectively) was done with a fluorescence detector (FP-9205, Nippon Bunko, Tokyo). Under the above column-switching HPLC conditions, complete separation and specific detection of the desired Angs was achieved without overlapping with other contaminating endogenous compounds, using a detection limit of >1.5 fmol/ml in plasma for all Angs (signal-to-noise ratio, >2). The method also showed a high reproducibility with less than 4.0% of C.V. value from 3 runs in each of three separate days. The recoveries of Angs from spiked (100 fmol/ml) plasma samples were more than 92%, e.g., 95.0 ± 5.6% for Ang II, and 92.4 ± 2.4% for Ang (3-4). The reliability of this method was confirmed by the agreement of the range of Ang II plasma level by the present (25-47 fmol/ml in plasma) with that by the radioimmunoassay method (28-52 fmol/ml in plasma) (11).

Data Analysis

Data are expressed as means ± SEM. One-way analysis by ANOVA was used to compare variables between 2 groups. Correlations and differences were considered significant when the p value was less than 0.05. The correlation coefficient between 2 variables was determined by Pearson's simple regression analysis.

Results

Table 1 summarizes the baseline characteristics of normotensive and mild hypertensive subjects (denoted as NT and HT groups, respectively) in the SuP and UpP groups. There was no difference in age between these groups. An apparent BP increase was observed in both groups under condition of 60-min UpP.

Figures 1 and 2 show the SuP and UpP plasma concentrations of endogenous Angs for the NT and
Matsui et al: Plasma Levels Angiotensins 275

As shown in Fig 1, there were no differences in the SuP plasma levels of Ang I, Ang II, or Ang (5-8) between the NT and HT groups: Ang I, 304 ± 43 fmol/ml vs. 294 ± 15 fmol/ml; Ang II, 32 ± 6 fmol/ml vs. 43 ± 10 fmol/ml; Ang (5-8), 176 ± 22 fmol/ml vs. 133 ± 32 fmol/ml, respectively. In addition, no differences in the levels of these Angs were found between groups after 60-min UpP (Fig. 2). However, the HT group showed significantly lower SuP and UpP plasma Ang (3-8) and Ang (3-4) levels than the NT group. In particular, the SuP plasma level of Ang (3-4) (71 ± 13 fmol/ml-plasma) in the HT group was significantly (1/3-fold) lower than that in the NT group (197 ± 35 fmol/ml-plasma). The productions of all Angs investigated in this study were accelerated by UpP in both the HT and NT groups. There was thus an inverse correlation between the plasma level of Ang (3-4) and the SBP of UpP ($r = -0.627, p < 0.02, n = 13$), as shown in Fig. 3, indicating that the metabolism of Ang (3-4) might be associated with the BP change.

**Discussion**

To our knowledge, the predominant angiotensins showing physiological effects on the RAA system are Ang II and Ang III, although other Ang metabolites, such as Ang (4-8), Ang (5-8), and Ang (6-8) also show slight dipogenic activity (12). Recently, it has been established that Ang (1-7) also displays some pressor actions in the RAA system, such as coronary constriction, neuronal excitation, and prostaglandin release (13-15). In addition, Ang IV (Des-Asp$^1$-Arg$^2$-Ang II; Ang (3-8)) has been shown to be a specific AT$_4$ receptor agonist, and to play a potential role in neuronal develop-

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**Table 1. Baseline Characteristics of the Volunteer Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Mild hypertensive</th>
<th>Normotensive</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>23.3 ± 1.3</td>
<td>24.4 ± 0.4</td>
</tr>
<tr>
<td>Men/Women</td>
<td>6/0</td>
<td>7/0</td>
</tr>
<tr>
<td>Body height (cm)</td>
<td>169 ± 1.2</td>
<td>176 ± 2.8</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>72.3 ± 6.9</td>
<td>73.3 ± 6.4</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)</td>
<td>25.3 ± 2.6</td>
<td>23.7 ± 2.3</td>
</tr>
<tr>
<td>Supine systolic BP*   (mmHg)</td>
<td>131 ± 3.9</td>
<td>111 ± 3.2</td>
</tr>
<tr>
<td>Supine diastolic BP*  (mmHg)</td>
<td>75 ± 2.1</td>
<td>61 ± 3.4</td>
</tr>
<tr>
<td>Upright systolic BP** (mmHg)</td>
<td>140 ± 1.8</td>
<td>120 ± 4.0</td>
</tr>
<tr>
<td>Upright diastolic BP** (mmHg)</td>
<td>84 ± 2.4</td>
<td>74 ± 2.6</td>
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Values are means ± SEM. BP, blood pressure. *Subjects kept in the supine position for 30 min, followed by **the upright position for 60 min.

**Fig. 1.** Comparison of plasma angiotensin levels between young male normotensive (n=7) and hypertensive (n=6) subjects in the supine position. Columns and bars show mean ± SE. Statistically significant difference by ANOVA: *p<0.05.

**Fig. 2.** Comparison of plasma angiotensin levels between young male normotensive (n=7) and hypertensive (n=6) subjects in the upright position. Columns and bars show mean ± SE. Statistically significant difference by ANOVA: *p<0.05.
At the present time, the other Angs are considered to be biologically inactive and of little physiological importance in the RAA system. On the other hand, our previous in vitro study with respect to Ang inhibition of lung ACE revealed that two of the smaller Angs, Ang (5-8) and Ang (3-4), retarded the action of ACE with the IC50 value of 11.6 and 26.0 \( \mu \)M, respectively (5). Apart from the neuronal effect, Ang (3-8) elicited a potent in vitro ACE inhibitory activity (IC50 = 6.6 \( \mu \)M) as well. Also, oral administration of exogenous Ang (3-4) derived from sardine muscle hydrolyzate showed a marked BP reduction in mild hypertensive subjects during a 4-wk protocol (7). These findings led us to examine the relationship between BP and the metabolic behavior of smaller Angs. In our previous report (8), the SuP plasma levels of Ang (3-8), Ang (5-8), and Ang (3-4) in normotensives were >30 fmol/ml, with that of Ang (3-4) (197 ± 35 fmol/ml; \( n = 7 \)) being predominant. In the present study, Angs with in vitro ACE inhibitory activity were also detectable in the HT group, as shown in Fig. 1, whereas the HT group showed significantly lower (1/2- and 113-fold) SuP plasma Ang (3-8) and Ang (3-4) levels, respectively, and higher (3-fold) plasma Ang (4-8) levels than the NT group. A marked reduction in Ang (3-8) (29 ± 6 fmol/ml) and Ang (3-4) (71 ± 13 fmol/ml) levels in the HT group indicated the poor activity or secretion of angiotensinases involved in their productions. Allard et al. (17) suggested that, in the metabolic process of \(^{[3]}\)ANG II on cultured mouse spinal cord cells, dipeptidyl aminopeptidase III might be responsible for the Arg2-Val3 as well as the Tyr4-Ile bond. Ang (3-8) was found to be produced from Ang III by the action of Aminopeptidase M (18, 19). Therefore, a low activity of these angiotensinases in the HT group might be responsible for the poor metabolism of Ang (3-4) and Ang (3-8), respectively. According to the report by Herrmann et al. (20), patients with a history of hymenoptera venom anaphylaxis showed significantly reduced Ang I and Ang II plasma levels as compared to healthy volunteers. In the present study, however, no significant difference in Ang I and Ang II plasma levels was observed between the HT and NT groups (Fig. 1).

Similar metabolic behavior was observed under condition of UpP (Fig. 2), although the productions of all Angs were accelerated by maintaining the UpP for 60 min. In general, the upright position may lead to RAA activation or an increase in plasma renin activity (21). Based on our previous finding (5) of an approximately 4-fold increase in plasma renin activity in normotensive subjects after 60-min UpP, we speculated that the plasma renin activity would be increased in the UpP-HT groups (although we did not assay for this increase in this study). Thus, it was considered that the metabolic process of Angs would remain constant irrespective of the RAA activation.

Our results confirmed the existence of a relationship between the SBP and the plasma level of Ang (3-4) (Fig. 3). Contrary to this finding, the lack of a significant difference in the plasma levels of Ang I and Ang II (Figs. 1 and 2) led to the assumption that plasma ACE activity would not differ between the two groups. Further studies will thus be needed to clarify the physiological action of Ang (3-4) not only in the circulatory RAA system, but also in localized systems.

In summary, we concluded that the metabolism of smaller Angs, i.e., Ang (3-8) and Ang (3-4), was significantly depressed in the HT group as compared with NT group.

References

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Matsui et al: Plasma Levels Angiotensins


