Novel Mass Spectrometric Methods for Evaluation of Plasma Angiotensin Converting Enzyme 1 and Renin Activity

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Abstract—This article demonstrates the applicability of quantitative proteomics to assays of proteolytic enzyme activity. A novel assay was developed for measurement of renin and angiotensin-converting enzyme (ACE) activity in plasma. The method was validated in animal models associated with alterations of the renin angiotensin system (RAS). Using surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) with a ProteinChip Array technology, plasma renin and ACE1 could be measured in <0.5 μL of plasma. Plasma is incubated with peptide substrates for renin and ACE, tetradecapeptide (TDP), and angiotensin I (Ang I), respectively. The reactions mixtures are spotted onto the ProteinChip WCX2 and detected using SELDI-TOF-MS. Peak height or area under curve for TDP, Ang I, and angiotensin II (Ang II) peaks are measured. There was a linear relationship between disappearance of substrate and appearance of products for both renin and ACE ($R^2$=0.95 to 0.98). ACE1 activity was blocked with chelating agents (EDTA and 1,10 phenanthroline), indicating action of a metalloprotease. The ACE1 inhibitor, captopril, selectively blocked ACE1. Renin activity was specifically blocked with renin inhibitor and was not affected by phenanthroline or captopril. Animal models tested were Ang AT1a receptor-deficient and streptozotocin (STZ) diabetic mice. Plasma renin activity was increased >2-fold in AT1a−/− as compared with AT1a+/+. In STZ diabetic mice, ACE1 was increased 2-fold as compared with controls. The advantage of the method is that it is tagless, does not require additional purification steps, and is extremely sensitive. The approach can be multiplexed and used for identification of novel substrates/inhibitors of the RAS. (Hypertension. 2005;46 [part 2]:953-959.)

Key Words: angiotensin ■ angiotensin-converting enzyme ■ captopril ■ hypertension ■ renin

The renin angiotensin system (RAS) has long been recognized to play a crucial role in the regulation of blood pressure and electrolyte balance.1 The processing scheme begins with the conversion of angiotensinogen (AGT) to angiotensin I (Ang I) via renin (EC 3.4.23.15).2 This is followed by the action of angiotensin-converting enzyme (ACE) (EC 3.4.15.1), a peptidyl dipeptidase that belongs to the glu zincin family of metalloproteases.3 ACE catalyzes the cleavage of the C-terminal dipeptide (L-histidyl-L-Leucine) from the inactive decapeptide, Ang I, to produce the potent vasoconstrictor, angiotensin II (Ang II). In addition to the circulating RAS, there is also tissue expression of the RAS in brain, kidney, pancreas, adipocytes, and other organs.4-6 Ang II acts through 2 receptor types, AT1 and AT2 receptors.1 Molecular genetics studies demonstrated that AT1 receptors exist as AT1a and AT1b forms that are homologous in gene and protein structure.7,8 Ang AT1 receptors mediate virtually all of the known physiological actions of Ang II in renal, cardiovascular, and neuronal systems. Ang AT1a receptors are critical in the control of blood pressure, autonomic function, and fluid/electrolyte balance, as seen in studies of AT1a gene deletion models.9,10 Ang AT2 receptors are more prevalent during development and are thought to mediate vasodilation and cell growth.11,12 There is also evidence for interaction with ACE expression, with suggestions that AT2 activation decreases ACE activity.13 Chronic hypotension and dramatic increase in renin mRNA has been shown in mice deficient in angiotensinogen11 and Ang AT1a receptors.15

Until recently, quantitative analysis of proteolytic enzyme activities required the use of specially designed chromatographic substrates whose kinetic behavior may not parallel the natural substrates. An alternative is to use synthetic peptides identical to the endogenous substrates and directly analyze the enzymatic peptide products. For this we have taken advantage of the sensitivity and accuracy of a mass spectrometric (MS)-based method, SELDI-TOF-MS ProteinChip technology (Ciphergen Biosystems, Fremont, Calif). It is particularly useful for quantification of low-molecular-weight peptides and has been used for on-chip enzymatic peptide sequencing.16,17 However, it has not yet been applied to a great extent to measurement of enzyme activity.
The focus is on the development of MS enzyme assays for ACE1 and renin, representative proteolytic enzymes, with defined substrates and peptide products. SELDI-TOF-MS provided the key for developing a new method for measurement of proteolytic enzyme activities, specifically targeting the RAS. The methodological approach uses peptide substrates, TDP and Ang I, which release endogenous angiotensin peptide that can be analyzed with SELDI-TOF-MS. It is anticipated that these MS enzyme assays may have clinical applications because renin and ACE activity are associated with disease states, such as hypertension, diabetes, and renal dysfunction. One advantage of ProteinChip Arrays is that a simple washing step removes unbound peptides, residual salts, and detergents that are present in crude biological extracts or buffers. These substances can interfere with MS analysis and reduce assay sensitivity.\textsuperscript{16} ProteinChip arrays are derivatized with affinity matrices, which mirror the properties of conventional chromatographic media to capture peptides and proteins.\textsuperscript{16} Finally, because of the low femtomole sensitivity of SELDI-TOF, measurements can be made with small sample volumes and substrate concentrations. A previous study showed that MS coupled with high-performance liquid chromatography could be used for kinetic analysis of galactosidase activity.\textsuperscript{18}

In the present study, sample preparation methods and data acquisition protocols were optimized for the application of SELDI-TOF-MS for quantitative analysis of products of plasma-based proteolytic enzyme-catalyzed reactions. The study characterizes plasma renin and ACE1 reactions including testes of specificity, kinetics, and physiological stimulation.

Materials and Methods

Water was purified by an Elga LabWater purification system (Lowell, Mass). Trifluoroacetic acid was purchased from Pierce Biotechnology, Inc (Rockford, Ill). Streptozotocin (STZ), phenyl-methane-sulfonfyl fluoride, and 1,10 phenanthroline were purchased from Sigma Aldrich Co (St. Louis, Mo). Organic solvents were high-performance liquid chromatography grade. Ang I (molecular weight, 1296.7), Ang II (molecular weight, 1046.3), and the specific renin inhibitor [Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe] and the selective ACE1 inhibitor, captopril, were purchased from Bachem Bioscience Inc (King of Prussia, Pa). SELDI-TOF-MS ProteinChip and the calibration standards were purchased from Ciphergen Biosystems, Inc (Fremont, Calif).

Animals and Plasma Samples

Adult male AT1a gene deletion (AT1a\textsuperscript{−/−}) and control (AT1a\textsuperscript{+/+}) mice (4 months of age) were obtained from a breeding colony at Wright State University. The strain was shown to have low blood pressure and high levels of plasma renin activity.\textsuperscript{15,19} The original breeding pairs were obtained from Dr Thomas Coffman (Duke University, Durham, NC). Genotypes were determined by polymerase chain reaction of DNA isolated from tail biopsy specimens as previously described.\textsuperscript{19} C57BL/6 male mice were purchased from a commercial source (Harlan Inc, Indianapolis, Ind). Animals are housed at 22°C under a 12-hour light/12-hour dark cycle with ad libitum access to water and standard mouse chow. For the collection of blood samples, mice were decapitated and trunk blood was collected in ice-chilled heparinized tubes. The plasma was immediately separated and stored frozen at −80°C. All experimental protocols were approved by the Wright State University Animal Care and Use Committee.

Streptozotocin-Induced Diabetes

Diabetes was induced by a single intraperitoneal injection of 150 mg/kg STZ freshly dissolved in 0.01 N sodium citrate (pH 4.5) after a 6-hour fast. Controls were injected with buffer. The syndrome induced in rodents by STZ injection closely resembles type I diabetes in humans.\textsuperscript{20} A drop of tail blood was used for measurement of blood glucose using Accu-Chek Advantage glucose meter and test strips (Roche Diagnostics, Indianapolis, Ind). Results were expressed in mg/dL. After 2 weeks, blood glucose in the STZ-treated mice was 21.3 ± 2.0 mmol/L, as compared with 6.85 ± 0.4 mmol/L in controls.

SELDI-TOF-MS

The weak cation exchange (WCX2) ProteinChip was used for the retention and analysis of substrate and peptide enzyme products. WCX2 ProteinChip spots were first outlined with a hydrophobic pap-pen and air-dried. Spots were activated with 1 μL 0.01 N HCL and washed with deionized water (5 μL, 3 times). For measurement of enzyme activities, substrates (Ang II or TDP) were incubated with plasma and peptide products were measured by SELDI-TOF MS. For ACE1 activity, plasma (0.5 to 2.5 μL) was incubated for 1 to 3 hours in 50 μL MES buffer (50 mmol/L, pH 6.75) containing 2 mmol/L phenyl-methane-sulfonfyl fluoride and varying excess concentrations of Ang I (10 to 0.1 μmol/L) at 37°C. The reaction mixture (1 μL) was then spotted onto ProteinChip Arrays and analyzed as previously described.\textsuperscript{17} Briefly, after the incubation period, 1 μL of the reaction mixtures were spotted onto ProteinChip WCX2 and incubated for an extra 15 minutes in a humidified chamber at 37°C. ProteinChip were washed with deionized water (5 μL, 3 times) to remove nonbound proteins, salts, and other contaminants. Enzymatic reactions were terminated by addition of 1 μL of freshly prepared saturated matrix (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile [v/v], containing 0.1% trifluoroacetic acid) and chips were dried at room temperature. The peptides retained by the ProteinChip were directly read in a ProteinChip reader system, PBS II (Ciphergen Biosystems). Peptides were analyzed with a spot protocol that analyzes 13 different areas in each spot. The source and detector voltages were 20,000 and 1800 V, respectively. The ionized peptides were detected and their molecular masses determined according to their time of flight. The Spectral analysis was performed with proprietary software (version 3.1; Ciphergen Biosoftware), which integrate the area under each peak for use in quantitative analysis.

For renin activity, the plasma samples were thawed quickly in a water bath to minimize cryoactivation of prorennin and the reaction mixture was spiked with renin substrate (TDP, m/z 1759); 1 mmol/L EDTA was included to block the activity of ACE1/ACE2. In separate experiments, 1.10 phenanthroline, specific renin inhibitor [Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe] and the selective ACE1 inhibitor, captopril, were pre-incubated for 15 minutes at room temperature before the addition of the substrates. The K\textsubscript{m} values were calculated by the Lineweaver–Burk method using 0.5 μmol/L plasma and variable concentration of TDP (0.5 to 30 μmol/L).

Statistics

Values of peptide spectra relative intensity or area under the curve (AUC) were expressed as means ± SEM. Student t test was used for comparison between groups. Differences were considered to be statistically significant at P<0.05.

Results

A typical example of the SELDI-TOF-MS profile for peptide subsets and peptide products resulting after incubation of Ang I or TDP with mouse plasma (0.5 μL) in the absence or presence of inhibitors. ACE1 converted Ang I (Ang 1 to 10: 1296.5 m/z) to Ang II (Ang 1 to 8: 1046.3 m/z) as expected by cleavage of the C-terminal His-Leu dipeptide.
Figure 1A and 1B). ACE activity was blocked by the ACE1 inhibitor, captopril (10 μmol/L) (Figure 1C), and by the chelating agents 10 mmol/L EDTA and 0.44 mmol/L 1,10 phenanthroline (data not shown). The reaction blockade by captopril, EDTA, and 1,10 phenanthroline indicates that the cleavage products were not produced by nonspecific degradation of Ang I, but rather via the zinc metalloproteases ACE1.21

A similar MS protocol was applied to measurement of renin activity. In this case, renin substrate, TDP (Ang 1 to 14, 1759 m/z) (0.1 to 5 μmol/L) was used as the substrate. Plasma incubated with TDP (1759, m/z) in absence of EDTA resulted in the production of peptides with m/z that matched Ang I and Ang II (Figure 1E). This is related to sequential proteolytic actions of renin and ACE1. Captopril had no effect on renin activity, but as expected blocked ACE1 and increased Ang I levels (Figure 1F). This demonstrates one of the advantages of the assay, which is the ability to multiplex, eg, to screen for ACE1, rennin, and ACE2 in the same sample. It is well documented that ACE2 cleaves one amino acid from Ang I and Ang II to produce Ang 1 to 7 and Ang 1 to 9, respectively.21,22 This was verified when kidney extracts (a known source of ACE221,22) were incubated with Ang II. A peak with m/z of 899 was generated, providing evidence of renal ACE2 activity (data not shown). The presence of ACE1 and absence of ACE2 in plasma was verified using this method (Figure 1B) because there was no evidence of peaks corresponding to Ang 1 to 7 (899 m/z) or Ang 1 to 9 (1184 m/z). The specific renin inhibitor [Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe] (0.1 to 10 μmol/L) blocked the activity of plasma renin with >90% inhibition with 10 μmol/L (IC50 = 1 μmol/L) (Figure 2).

Figure 3 shows monitoring of plasma ACE1 reaction by SELDI-TOF-MS, in which both substrate and reaction product were quantified simultaneously. For assay evaluation, we determined the relationship between disappearance of substrate, Ang I, generation of product, Ang II, and plasma volume (Figure 3A). There was a linear relationship with a correlation coefficient of 0.92. Figure 3B shows a linear correlation between substrate depletion (Ang I) and generation of Ang II (R² = 0.97). As expected, there was a relationship between peptide peak height and laser intensity. When

Figure 1. SELDI-TOF-MS analysis of ACE and renin activity in normal mouse plasma. Plasma (0.5 μL) was incubated with Ang I (1 μmol/L) or TDP (4 μmol/L) for 2 hours in absence or presence of captopril (10 μmol/L), and generated peptides were analyzed with SELDI-TOF-MS. Conditions: A, Ang I; B, Ang I+plasma; C, Ang I+plasma+captopril; D, TDP; E, TDP+plasma; F, TDP+plasma+captopril.

Figure 2. Inhibition of plasma renin with renin inhibitor (RI). Top, SELDI-TOF-MS analysis of renin activity in normal plasma (0.5 μL). Conditions: A, TDP+plasma; B, TDP+plasma+RI (0.1 μmol/L); C, TDP+plasma+RI (10 μmol/L). Bottom, Percent inhibition of renin activity with variable concentrations of RI (0.1 to 20 μmol/L).
laser intensities were compared, there was a higher Ang II peak intensity at 150 than at 140 (66/5.5 versus 29/5.5; n = 7). When the ratio of the peptide peaks (Ang I/Ang II) was used as the experimental index, there was no differences between the low and high laser intensity (0.71/0.02 versus 0.70/0.03, low versus high laser intensity; n = 7). There was a linear relationship between Ang I/Ang II ratio and plasma concentrations (Figure 3C) (R^2 = 0.98). The data verify the usefulness and the reproducibility of the method and demonstrate that the peptide ratio provides a better index of activity. It is not dependent on the exact laser setting or on the substrate/enzyme concentrations.

To validate the MS assay for renin activity, the kinetics of normal plasma renin activity was studied with variable concentration of TDP. Figure 4 shows the construction of Lineweaver–Burk plot (1/V versus 1/S) for determination of the Michaelis constant (K_m) for TDP.

To physiologically validate the assays, we tested plasma samples from animals with alterations in the RAS system, STZ diabetic, and AT1a receptor-deficient mice. Plasma ACE1 in control and STZ-diabetic mice was measured using the MS enzyme assay (Figure 5). Results showed that plasma ACE activity was increased in STZ diabetes. Ang II (produced from Ang I in vitro after 2 hours of incubation) was increased when measured as peak intensity (P < 0.05), AUC (P < 0.01), or substrate/peptide ratio (P < 0.05). This is consistent with published results showing increased plasma ACE activity in diabetes.

Plasma renin activity in AT1a−/− and AT1+/+ mice was monitored by the MS enzyme assay. Renin substrate (TDP) and Ang I formed were expressed as peak intensity and AUC (Figure 6). Results showed that plasma renin activity was increased in AT1a−/− (Figure 6A). Ang I (produced from renin substrate TDP in vitro after 2 hours of incubation) was increased when measured as peak intensity (P < 0.01), AUC (P < 0.05), or substrate/peptide ratio (P < 0.05) (Figure 6B). As expected, the increase in renin activity was associated with a significant decrease in renin substrate (P < 0.05) (Figure 6A). These data are consistent with published results showing increased plasma renin activity in AT1a−/− mice.

Discussion

The RAS is a major target for cardiovascular drugs. Because this system is essentially a peptide enzymatic cascade, characterization of current and prospective drugs requires data on peptide targets. For this reason we decided to take advantage of the power of MS coupled with new matrix systems to develop novel enzyme assay for renin and ACE. Using a SELDI-TOF MS approach with endogenous peptide substrates, the new renin and ACE assays show: (1) high sensitivity, allowing measurement in < 1 μL plasma; (2) applicability for time course studies in animals and humans; (3) ability to measure RAS changes under pathological conditions, STZ diabetes, and genetic deletion of the Ang AT1a receptor; (4) usefulness as a prototype for other enzyme assays; and (5) promise in the development of high-throughput testing systems for drugs targeting the RAS.

MS is a powerful tool with the potential to replace fluorometric, radioactive, and photometric monitoring of certain enzyme assays. It has the advantage of specificity,
speed, and reproducibility. An important strength is the use of computerized laser scanning that ionizes different areas in a sample, providing an integrated measurement. This reduces problems associated with cell surface differences and sampling error. One restriction of MALDI-TOF-MS for quantitative analysis is the possible interference of matrix with peptides of interest in the mass range between 0 and 500 (m/z). However, this is not applicable in the present study because the Ang peptides analyzed have mass >500 m/z.

For the study of ACE activity, a widely used spectrophotometric assay method was developed by Cushman and Cheung in the 1970s and is still in use today. In this assay, hippuric acid release from an artificial ACE substrate, hippuryl-L-histidine-L-leucine, is directly related to ACE activity. One drawback of this assay is that it requires extraction of hippuric acid. The method of Holmquist et al uses furanacryoyl tripeptide as an ACE substrate. The hydrolysis produces a shift of absorption spectrum that is related to ACE activity. Plasma renin activity is determined by adding exogenous AGT or TDP to a plasma sample and measuring Ang I formation by radioimmunoassay. Sensitivity is a requirement of the renin assay because normal plasma renin activity concentrations are low and subnormal concentrations have diagnostic relevance.

Because of volume requirements of the classical methods and because of increasing use of mice in experimental studies, there was a need for more sensitive and specific ACE and renin assays. MS was chosen to play key role in the method because of the sensitivity, the ability to use endogenous peptides as substrates, and the capacity for direct analysis of the enzymatic peptide products. It should be noted that there has been a widespread expansion in the use of MS for identification of peptides and proteins. Today’s technological improvements are considered breakthroughs as compared with previous methods that required large amounts of protein and are difficult to implement. A recent modification of MS methodology is the ProteinChip technology (Ciphergen Biosystems, Inc), which facilitates protein/peptide profiling of complex biological mixtures. It provides a powerful tool for determination of biomarkers of physiological/pathological states. We used SELDI-TOF-MS to develop sensitive and specific assays for ACE and renin in tissue and plasma. The proteolytic Ang peptide products are identified by m/z and quantified by peak intensity and AUC. Results show that the enzymatic reactions produce the predicted Ang peptides with inhibition by specific inhibitors. For example, Ang I incubation with plasma resulted in the formation of Ang II with a linear relationship between substrate depletion and product formation. There was also a

Figure 5. Plasma ACE activity in STZ diabetic mice. Plasma (0.5 μL) from diabetic and control mice was incubated with Ang I (1 μmol/L) for 2 hours and formed Ang II was expressed as intensity (%), AUC (BA), or as ratio of Ang I/AngII (3B). *P<0.05, **P<0.01 vs control, n=6 to 7 per group.

Figure 6. Plasma renin activity was monitored by SELDI-TOF-MS in normal and AT1a receptor–deficient mice. Plasma (0.5 μL) from AT1a−/− and AT1a+/+ mice was incubated with TDP (4 mmol/L) for 2 hours and Ang I product was measured using SELDI-TOF-MS. Data are expressed as intensity %, AUC (A), or as ratio of Ang I/TDP (B). *P<0.05, **P<0.01 vs AT1a+/+, n=6 to 7 per group.
correlation between incubation time and product formation. Evidence that this reaction was mediated by ACE1 was shown by the inhibition with captopril and metal chelators. There was no evidence for the formation of other Ang peptides, Ang 1 to 7 and Ang 1 to 9, which indicates a lack of ACE2 in plasma as would be predicted. The MS renin assay was also specific and sensitive. When TDP was incubated with plasma, there was evidence for formation of Ang I and Ang II, related to the sequential action of renin and ACE. Captopril had no effect on renin, whereas a renin inhibitor blocked the reaction, showing specificity of the method.

For ACE it is difficult to directly compare this new MS enzyme method with traditional assays. This is the first method to use the endogenous peptide substrate, Ang I, as compared with the small synthetic artificial ACE substrate, hippuryl-L-histidine-L-leucine. Characterization experiments demonstrate specificity, linearity of reaction, and responses to physiological conditions. Comparison of the MS renin assay with traditional methods was performed by varying TDP and plotting 1/substrate versus 1/velocity (Lineweaver–Burke plot). The results showed a linear reaction with a regression coefficient of 0.97 and calculated $K_m$ of 6.3 $\mu$mol/L. This experimental value of $K_m$ for AGT during the renin reaction was similar to that measured with traditional methods.32

The emergence of SELDI-TOF-MS allows for the dissection of the RAS with more specificity and ease than previously possible. Complete or partial peptide sequences are the most discriminating criteria for the identification of proteins. Such structural information can be obtained from peptide fragmentation data by tandem MS and subsequent quantification of target peptides and proteins. MS enzyme assays.

A major hurdle in comparative proteomics is identification and subsequent quantification of target peptides and proteins. The traditional method for MS quantification is to compare the unlabeled peptide to an internal standard that is chemically identical, except for inclusion of stable heavy isotopes. New approaches for MS quantification have been developed33 and include the use of relative intensity of peak height, which is specific for each peptide. Mass spectrometric data give more confidence about the identity of the reaction products than any of the other enzyme assays and therefore avoids false-positive results.

Perspectives
Most of our understanding of RAS has come from classical pharmacological studies. However, the emergence of SELDI-TOF-MS allows for the dissection of this peptidergic system with more specificity and ease than previously possible. In conclusion, our results document the development of novel mass spectrometric enzyme assays for monitoring renin and ACE activity in plasma. SELDI-TOF-MS provides a viable alternative to existing analytical techniques with the advantage of the use of endogenous synthetic substrates for ACE1 and ACE2 and the ability to directly identify and measure enzyme peptide products with no ambiguity. The method may be useful as a tool for monitoring disease states, a screening mechanism for drug development, and a prototype for other MS enzyme assays.

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