New Mass Spectrometric Assay for Angiotensin-Converting Enzyme 2 Activity

Khalid M. Elased, Tatiana S. Cunha, Susan B. Gurley, Thomas M. Coffman, Mariana Morris

Abstract—A novel assay was developed for evaluation of mouse angiotensin-converting enzyme (ACE) 2 and recombinant human ACE2 (rACE2) activity. Using surface-enhanced laser desorption/ionization time of flight mass spectrometry (MS) with ProteinChip Array technology, ACE1 and ACE2 activity could be measured using natural peptide substrates. Plasma from C57BL/6 mice, kidney from wild-type and ACE2 knockout mice, and rACE2 were used for assay validation. Plasma or tissue extracts were incubated with angiotensin I (Ang I; 1296 m/z) or angiotensin II (Ang II; 1045 m/z). Reaction mixtures were spotted onto the ProteinChips WCX2 and peptides detected using surface-enhanced laser desorption/ionization time of flight MS. MS peaks for the substrates, Ang I and Ang II, and the generated peptides, Ang (1-7) and Ang (1-9), were monitored. The ACE2 inhibitor MLN 4760 (0.01 to 100 μmol/L) significantly inhibited rACE2 activity (IC_{50}=3 nmol/L). Ang II was preferably cleaved by rACE2 (k_m=5 μmol/L), whereas Ang I was not a good substrate for rACE2. There was no detectable ACE2 activity in plasma. Assay specificity was validated in a model of ACE2 gene deletion. In kidney extract from ACE2-deficient mice, there was no generation of Ang (1-7) from Ang II. However, Ang (1-7) was produced when Ang I was used as a substrate. In conclusion, we developed a specific and sensitive assay for ACE2 activity, which used the endogenous peptide substrate Ang II. This approach allows for the rapid screening for ACE2, which has applications in drug testing, high-throughput enzymatic assays, and identification of novel substrates/inhibitors of the renin-angiotensin system. (Hypertension. 2006;47:1010-1017.)

Key Words: angiotensin ■ renin-angiotensin system ■ mice ■ angiotensin converting enzyme

The renin–angiotensin system (RAS) is a key regulator of cardiovascular and renal function, both under physiological and pathological conditions. It consists of a cascade of enzymatic reactions, and in the classical system, the processing scheme begins with the conversion of angiotensinogen to angiotensin I (Ang I) via renin (EC 3.4.23.15). This step is followed by the action of angiotensin-converting enzyme (ACE, EC 3.4.15.1) 1, a peptidyl dipetidase, which belongs to the gluzincin family of metalloproteases. ACE1 cleaves the C-terminal dipeptide (L-histidyl-L-Leucine) of Ang I, generating the physiologically active vasoconstrictor peptide angiotensin II (Ang II). Ang II is the key mediator of the RAS, and its biological actions are produced through the selective binding of this peptide to 2 different types of receptors, Ang II type 1 and Ang II type 2. In addition to the initially described circulating RAS, the components are also expressed locally in brain, kidney, pancreas, adipocytes, and other organs.

New findings suggest that the RAS is a far more complex and dynamic system than suggested by previous research. In 2000, a homologue of ACE1, known as ACE2, was cloned by 2 independent groups. This newly discovered member of the RAS is an 805 amino acid protein that shares approximately 42% sequence identity to the N- and C-domains of somatic ACE1. Unlike ACE1, ACE2 functions predominantly as a carboxypeptidase with a substrate preference for hydrolysis between proline and a hydrophobic or basic C-terminal residue. Recombinant expressed ACE2 was first reported to cleave a single amino acid from Ang I to generate Ang (1-9), which is then converted by ACE1 to the potent vasodilator peptide Ang (1-7). Subsequent to this initial characterization, it was found that ACE2 has a preference for Ang II as a substrate, which it rapidly hydrolyzes to Ang (1-7). Thus, in vitro biochemical data suggest that ACE2 negatively regulates Ang II production and function, not only because it decreases local production of Ang II, but also because the cardiovascular effects of its product, Ang (1-7), oppose those of Ang II. In addition, there is evidence that other non-ACE enzyme systems may be active in tissue, adding to the local production of Ang II and Ang (1-7).

Initially, ACE2 appeared to be more limited in its tissue distribution than ACE1, with significant levels detected only in heart, kidney, and testis. However, a recent study detected ACE2 expression in a variety of other tissues. The exact in vivo role of ACE2 remains to be determined, but it is clearly multifunctional. Studies of gene deletion mice and of the effects of heart failure on ACE1 and ACE2 expression suggest that...
ACE2 is involved in the control of cardiovascular function.\textsuperscript{15–18} Moreover, ACE2 has been identified as a functional receptor for the coronavirus, which causes the severe acute respiratory syndrome.\textsuperscript{19} Recently, Santos et al\textsuperscript{20} identified the orphan G protein–coupled receptor mas as an Ang (1–7) receptor.

One issue, which is important for the investigation of the ACE2 system and its pathophysiological role, is the availability of selective, sensitive, and rapid assays. This is critical in view of accumulating evidence of the role of ACE2 both in cardiovascular homeostasis and as a cellular receptor for the severe acute respiratory syndrome virus.\textsuperscript{19} A classical method for measurement of ACE2 tissue activity is based on the use of fluorogenic peptide substrates.\textsuperscript{3,9,21,22} Another method that is accurate, but time consuming, is the use of radiolabeled peptide precursors followed by high-performance liquid chromatography (HPLC) separation of the peptide products.\textsuperscript{23}

Our recent studies have focused on the development of new proteolytic assay technologies. Using endogenous peptide substrates with surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) mass spectrometry (MS) measurements, we were able to show that ACE1 (conversion of Ang I to Ang II) or renin (conversion of tetradecapeptide to Ang I) activity could be measured in small sample volumes (<1 μL plasma).\textsuperscript{24} Studies of physiological conditions revealed that there were increases in plasma ACE1 activity in diabetic mice and plasma renin activity in the Ang AT1a receptor deletion model. The key issue behind the methodology is the ability to distinguish and quantify small molecular weight peptides.\textsuperscript{25,26} This means that it can be applied to peptide sequencing, as well as quantification of enzymatic reactions. This is different from spectroscopic assays in which the index of activity is the change in color or fluorescence with no information regarding the identity of the specific peptides formed in the proteolytic reactions.\textsuperscript{27}

The objective of the present study was to develop a sensitive and specific assay for ACE2 activity. The method was based on our previous work, which established MS as a useful tool for measuring proteolytic enzyme activity, specifically as related to the RAS.\textsuperscript{24} The approach uses the natural endogenous substrates for ACE2, Ang I, and Ang II, which are proteolytically cleaved to yield Ang (1–9) and Ang (1–7) respectively. It is anticipated that this MS enzyme assay will have applications in drug screening, antagonist development, and clinical investigations.

**Methods**

Recombinant HUMAN ACE2 was purchased from R&D System. Ang I and Ang II were obtained from Bachem Bioscience Inc. Bestatin was obtained from ALPCO Diagnostics. SELDI-TOF-MS ProteinChip and the calibration standard molecules were purchased from Ciphergen Biosystems, Inc. ACE2 inhibitor MLN 4760 was a gift from Dr Natalie Dales (Millennium Pharmaceuticals, Cambridge, MA). Trifluoroacetic acid was purchased from Pierce Bio-technology, Inc. PMSF, α-hydroxy-4-cinnamic acid, captopril, EDTA and I, and 10 phenanthroline were purchased from Sigma Aldrich Co. Organic solvents were HPLC grade.

**Animals and Plasma Samples**

C57BL/6 male mice were purchased from a commercial source (Harlan Inc, Indianapolis, IN). Male mice with targeted disruption of the ACE2 gene (Ace2\textsuperscript{−/−}) and their littermate controls (Ace2\textsuperscript{+/+}) were generated in the laboratory of Dr Thomas Coffman.\textsuperscript{18} ACE2-deficient mice (Ace2\textsuperscript{−/−}) showed no gross abnormalities, were capable of efficient reproduction, and showed normal cardiac function.\textsuperscript{18} Animals were 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. Plasma was immediately separated and stored frozen at −80°C. Tissues were removed, immediately frozen in liquid nitrogen, and stored at −80°C. All of the experimental protocols were approved by the Wright State University Animal Care and Use Committee.

ACE1 and ACE2 activity were measured in plasma and kidney extracts. The SELDI-TOF-MS method takes advantage of the ability to precisely quantify the peptide products of the ACE1 and ACE2 reactions, Ang II, Ang (1–9), and Ang (1–7). The methodology for tissue measurements is similar to that described previously.\textsuperscript{24} The tissues are homogenized on ice in 1:9 (weight/vol) of Tris HCl (50 mmol/L; pH 7.4) containing 2 mmol/L PMSF. The homogenate is centrifuged at 9000g for 10 minutes to remove cellular debris. Total protein content is determined in the supernatant using the Bradford protein assay with BSA as a standard (BioRad Protein Assay Reagent). The tissue extract is adjusted with Tris HCl (50 mmol/L; pH 7.4) to a concentration of 1 µg total protein per microliter. The components of the reaction mixture are the following: 25 μL MES buffer (50 mmol/L; pH 6.75); 2 mmol/L PMSF; 10 μmol/L bestatin, source of ACE [kidney extract, 0.5 to 2.5 µg protein in 0.5 to 2.5 µL recombinant human (r)ACE2 or plasma]; and Ang I or Ang II (10 μmol/L). The incubations time was from 30 to 120 minutes at 37°C. The contribution of endogenous angiotensinogen and Ang I and Ang II peptides in the reaction was negligible, because excess amounts of Ang I and Ang II were used.

**SELDI-TOF-MS**

Weak cation exchange (WCX2) ProteinChips were used for the retention and analysis of substrate and peptide enzyme products. WCX2 ProteinChip spots were first outlined with a hydrophobic mark (pap-pen; RPI Corp) and air dried. The chip surface was activated with 1 mg/L of 0.01 N HCl and washed with deionized water (5 μL, 3 times). After the incubation period, 1 μL of the reaction mixture was spotted onto ProteinChip WCX2 and analyzed as described previously.\textsuperscript{24}

**Statistics**

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

**Results**

**Measurement of rACE2 Activity**

An example of the SELDI-TOF-MS profile for peptides retained on ProteinChip WCX2 during rACE2 proteolytic assays is shown in Figure 1. The chromatograms show the substrates and peptide products formed after 30 minutes of incubation of Ang I or Ang II (10 μmol/L) with rACE2 (2 and 10 ng), rACE2 (10 ng) converted Ang I (1296.5 mU) to Ang (1–9) (1183.45 mU), by cleavage of the C-terminal leucine (Figure 1A and 1B). The Ang (1–9) peak was almost undetectable when using the lower concentration of rACE2 (2 ng; Figure 1A). The conversion from Ang I to Ang (1–9) was minimal, indicating that Ang I was not a good substrate for rACE2. Incubation of Ang II with rACE2 (2 ng) resulted in production of peptides with m/z that matched Ang (1–7) (898.8; Figure 1C and 1D). Ang II was a good substrate for rACE2, as seen by the formation of Ang (1–7) and by complete conversion of Ang II to Ang (1–7) when a higher concentration of rACE2 (10 ng) was used (Figure 1D).
Recombinant ACE2 activity was evaluated in the presence of the metallic chelators (EDTA and 1,10 phenanthroline) and the specific ACE2 inhibitor, MLN 4760. Like ACE1, ACE2 activity was blocked by the chelating agents (data not shown).

Figure 2 shows the dose-related inhibitory effect of MLN 4760 on rACE2 activity (2 ng). Ang II (10 μmol/L) was incubated with rACE2 (2 ng) in 25 μL MES buffer (pH 6.75) for 30 minutes in the absence and presence of variable concentrations of MLN-4760. SELDI-TOF-MS analysis of substrate and generated Ang (1-7) was monitored. (A) control Ang II; (B) Ang II+rACE2; (C) Ang II+rACE2+MLN 4760 (3 nmol/L); (D) Ang II+rACE2+MLN 4760 (30 nmol/L); (E) Ang II+rACE2+MLN 4760 (300 nmol/L).

Recombinant ACE2 activity was evaluated in the presence of the metallic chelators (EDTA and 1,10 phenanthroline) and the specific ACE2 inhibitor, MLN 4760. Like ACE1, ACE2 activity was blocked by the chelating agents (data not shown). Figure 2 shows the dose-related inhibitory effect of MLN 4760 on rACE2 activity (2 ng). Ang II (10 μmol/L) was incubated with rACE2 (2 ng) in the absence and presence of various concentrations of MLN 4760 (3 to 300 nmol/L; Figure 2B through 2E). The low dose (3 nmol/L) significantly inhibited rACE2 activity (Figure 2C), and the highest dose (300 nmol/L) produced >95% inhibition of rACE2 activity (Figure 2E). A test of 0.3 nmol/L MLN 4760 also showed inhibition of ~20% (data not shown). The results show the specificity of the reaction, indicating that peptide cleavage was not produced by nonspecific degradation of Ang II but rather via the zinc metalloproteases of ACE2.7,8

The reproducibility of the SELDI-TOF-MS assay for rACE2 activity was quantified using variable concentration of Ang II as substrate (0.5 to 50 μmol/L). The peptide was added to 25 μL of MES buffer containing rACE2 (2 ng/μL), and generated Ang (1-7) was monitored and expressed as...
AUC (data not shown). There was a good correlation between the concentrations of Ang II substrate and the quantity of generated Ang (1-7) \( (R^2 = 0.95) \). For assay evaluation, we determined the relationship between disappearance of substrate, Ang II, generation of product, Ang (1-7), and enzyme concentration \( (R^2 = 0.97 \text{ and } 0.98, \text{ respectively; Figure 3a}) \). When the ratio of the peptide peaks (Ang 1-7/Ang II) was used as the experimental index, there was a linear relationship between Ang (1-7)/Ang II ratio and rACE2 concentration \( (R^2 = 0.99) \) (Figure 3b). In addition, the generation of Ang (1-7) increased by time (data not shown). The data verify the use and the reproducibility of the method and demonstrate that the peptide ratio provides a good index of activity. Similarly, when different amounts of kidney extracts were added to a standard amount of Ang II, there was a linear relationship between kidney protein content and generated Ang (1-7) \( (R^2 = 0.96) \). The data demonstrate that SELDI-TOF-MS may be used as a biochemical for enzyme assay and for quantitative evaluation of peptide levels in the reaction mixture.

To further validate the MS enzyme assay, the kinetics of rACE2 activity were studied by constructing a Lineweaver-Burk plot \( (1/\text{substrate versus } 1/\text{velocity; data not shown}) \). Calculated Michaelis constant \( (K_m) \) for rACE2 using Ang II as a substrate was found to be 5.1 \( \mu \text{mol/L} \), which is in agreement with published data using another assay system.\(^{21}\)

### Measurement of ACE2 Activity in Plasma and Kidney

Further characterization studies were conducted using the specific ACE2 antagonist MLN 4760 with measurement of ACE1 and ACE2 activity in plasma and kidney extracts. Figure 4 shows the results of an experiment in which normal mouse plasma (source of ACE1) was incubated with Ang I (10 \( \mu \text{mol/L} \)) for 2 hours. Ang I was processed by ACE1 to yield Ang II (1046, \( m/z \)), a reaction that was blocked by the ACE1 inhibitor captopril (data not shown). There was no evidence for the
production of Ang (1-7) (898.8), and ACE1 activity was not affected by preincubation with MLN 4760 (0.01 and 100 μmol/L; Figure 4C and 4D). The composite data suggest that ACE2 is not active in plasma. To exclude the possibility that endogenous ACE2 inhibitors might be present in plasma and influence the enzyme reaction, rACE2 activity (2 ng) was measured in the presence of mouse plasma (0.5 to 2.5 μL). Plasma had no effect on rACE2 activity (data not shown), suggesting that circulating ACE2 inhibitors are not present.

ACE2-deficient (Ace2+/−) and wild-type ACE2 (Ace2+/+) mice were used for further characterization and validation of the ACE2 assay. Kidney extract was used as a source of ACE2. Figure 5 shows the results of a representative experiment in which kidney extract from wild-type ACE2 (Ace2+/+) and ACE2-deficient (Ace2+/−) mice were incubated with Ang II (10 μmol/L). In the wild-type (Ace2+/+), Ang II was metabolized to Ang (1-7) (899 m/z), providing direct evidence of renal ACE2 activity as expected (Figure 5B). In contrast, Ang (1-7) (899 m/z) was not formed in kidney extracts from ACE2-deficient (Ace2+/−) mice (Figure 5C), providing physiological evidence for an absence of ACE2.

Figure 6 compares Ang (1-7) generation in renal extracts from ACE2-deficient (Ace2+/−) and wild-type (Ace2+/+) mice using Ang II or Ang I as substrates. For quantitative evaluation, the ratio of peptide peaks [Ang (1-7)/Ang II] and [Ang (1-7)/Ang I] was used as the experimental index. As expected, there was marked decrease in the ratio of Ang (1-7)/Ang II in ACE2-deficient mice compared with the wild-type (P<0.001; Figure 6). However, when Ang I was used as the substrate, there was no difference in the ratio of Ang (1-7)/Ang I between the groups, indicating that other enzyme systems apart from ACE2 may be involved in the generation of Ang (1-7) from Ang I.

**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. The traditional view that Ang II is the major product of the RAS has been questioned with the recent discovery of a novel carboxypeptidase, ACE2, and growing evidence for a physiological role for its peptide product, Ang (1-7). Like ACE1, the substrate affinity of ACE2 is not confined to the RAS. ACE2 efficiently cleaves apelin-13, dynorphin A (1-13), and des-Arg9 bradykinin. However,
the ability of ACE2 to inactivate the vasoconstrictor Ang II and generate the putative cardioprotective metabolite Ang (1-7) implicates ACE2 as a potential regulator of the RAS. Further clarification of the role of ACE2 in disease states will be assisted by the development of sensitive and rapid methods for measurement of ACE2 activity. There is also a need for miniaturized, reliable assays because of increasing use of mice as experimental models and the volume requirements of classical enzyme methods.

The introduction of 2 ionization techniques, electrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization, in the late 1980s provided major advances in analytical techniques in biomedical research. MS is a powerful tool with the potential to replace fluorometric, radioactive, and photometric monitoring of certain enzyme assays. The method makes it possible to plot appearances and disappearance of products, reactants, and even intermediates over short reaction times. It has the advantage of specificity, speed, and reproducibility. We chose to develop an MS-based assay system because of the ability of the use of endogenous peptides as substrates and the capacity for direct analysis of the enzymatic peptide products. New approaches for MS quantification have been developed and include the use of relative intensity of peak height and ratio of products to substrate. We used both peak height intensity and AUC as parameters for the quantification of substrate and enzyme products. One restriction of MALDI-TOF-MS for quantitative analysis is possible interference of the matrix with peptides in the mass range between 0 and 500 m/z. However, this is not applicable in the present study, because the Ang I and II peptides analyzed have mass >700 m/z.

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 tool for the determination of biomarkers of physiological/pathological states. Recently we used this emerging technology for the evaluation of ACE1 and renin activity in mice, using plasma sample sizes of <1 μL. The method does not require fluorogenic substrates, radioactivity, or laborious purifications steps. The objective of the present study was to determine whether this technology could be extended to the measurement of ACE2 activity.

To initiate the project, we took advantage of the availability of recombinant human ACE2 to establish and optimize an MS-based assay for ACE2. A unique characteristic of this assay methodology is the ability to use the endogenous peptide substrates Ang I and Ang II. Our results showed that ACE2 activity could be measured using an MS assay similar to that developed for ACE1. We demonstrated that ACE2 preferentially hydrolyzes Ang II over Ang I and that the assay was specific, sensitive, and showed linearity of reaction. It is difficult to directly compare this new MS-enzyme method with traditional assays, which use fluorogenic substrates. However, we measured the $K_m$ for the ACE2 reaction and compared it with traditional methods. rACE2 was incubated with different concentrations of Ang II with measurement of peptide hydrolysis products [formation of Ang (1-7)] followed by construction of a Lineweaver-Burk plot (1/substrate versus 1/velocity). Results showed a linear reaction with a regression coefficient of 0.97 and a calculated $K_m$ of 5 μmol/L. This experimental $K_m$ value for Ang II in the ACE2 reaction was similar to that reported. In other studies, which used natural peptides as substrates, peptide hydrolysis products were separated using reverse-phase HPLC and measured with UV detection. The peaks corresponding to the peptide products were integrated to calculate product formation and compared with standard curves. This type of method is time consuming and subject to problems because of the requirement of multiple steps. The MS enzyme method described here is relatively simple, rapid (could be completed in 1 hour), and does not require desalting or further purification steps. This more direct approach takes advantage of MALDI-TOF-MS, which allows for real-time detection of m/z. Another advantage is that the matrix is tolerant of buffers and salts, which makes MALDI-TOF-MS suited for direct analysis of complex samples, such as enzymatic digests, without further purification. In addition, ProteinChip Arrays are preferable over other MALDI-TOF-MS methods, because they allow for a simple washing step, which 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.

Results show that the enzymatic reactions produce the predicted Ang I and Ang II peptides with inhibition by specific inhibitors. For example, initial studies of ACE1 revealed that incubation of Ang I with plasma resulted in the formation of Ang II with a linear relationship between substrate depletion and product formation. In the present study, ACE1 activity was measured in plasma using Ang I as a substrate with no evidence of production of Ang (1-7). There is a report of a secreted form of human ACE2 in culture medium from transfected endothelial cells and cardiomyocytes and in plasma of neonatal rats injected with lentivector encoding the secreted form of human ACE2. Recently we readdressed the idea of the presence of circulating ACE2 using the new assay technology. When plasma from normal mice was incubated with Ang II, Ang (1-7) could not be detected. This suggests that ACE2 activity is not present in normal plasma but does not rule out the possibility that ACE2 may be active in the circulation under pathological states.

To physiologically validate the method, we used a new genetic mouse model, which lacks the ACE2 enzyme. Renal ACE2 activity was measured in wild-type ACE2 (Ace2+/+) and ACE2-deficient (Ace2−/−) mice using Ang II as the substrate. There was a marked decrease in the formation of Ang (1-7) in kidney extracts from ACE2-deficient mice. The results verify the assay specificity and confirm the absence of renal ACE2 activity in ACE2-deficient (Ace2−/−) mice, consistent with the characterized model. Interesting results were obtained when Ang I was used as the enzyme substrate. If ACE2 is critical in the metabolism of Ang I, one would predict similar results with the Ang I and II substrates. However, Ang (1-7) generation was not different in the knockout and wild-type strains. This is likely explained by the reaction of Ang I with other proteolytic enzymes, such as the neutral endopeptidase neprilysin (E.C. 3.4.24.11), which also generates Ang (1-7).

One of the issues of concern with the classical enzyme assays is their reliance on fluorogenic, artificial substrates.
For example, 1 ACE2 assay uses Mca-YVAPK(Dnp)-OH, which was originally developed to measure caspase-1 activity.\textsuperscript{9,39,40} The current method of choice for ACE2 measurement uses the fluorogenic peptide substrates Mca-YVAPK(Dnp) and Mca-APK(Dnp).\textsuperscript{3,9,21,22} In this assay, the substrate peptide contains a fluorescent 7-methoxycoumarin group (Mca), which is quenched by energy transfer to a 2, 4-dinitrophenyl group (Dnp). It can be used to measure ACE2 activity, as well as activity of other peptidases. It is based on cleavage of an amide bond between the fluorescent and quencher groups, resulting in an increase in fluorescence. However, there is no information as to whether this substrate is specific for ACE2 over ACE1. In fact, in 1 study, captopril (ACE1 inhibitor) was included in the ACE2 assay system\textsuperscript{38} with no explanation as to its purpose.\textsuperscript{38} Furthermore, in a recent study using this fluorogenic substrate, there was a discrepancy between ACE2 activity and protein content (Western blot and immunohistochemistry).\textsuperscript{41} The reason could be because of the use of polyclonal antibodies or the use of the fluorogenic substrate for measuring enzyme activity. Using the MS approach described in this article, it is possible to determine whether a given substrate can act as a substrate for >1 enzyme and to determine the site(s) of proteolytic cleavage.

Use of artificial synthetic substrates can also generate conflicting data, and an example is provided by the effect of NaCl on ACE1\textsuperscript{13} and ACE2\textsuperscript{3,9,21,22} activity. When the synthetic substrate Mca-APK (Dnp) is used, there is either inhibition\textsuperscript{3,9} or activation of ACE2 by NaCl.\textsuperscript{13} However, when the natural substrate Ang II is used, NaCl inhibits ACE2 activity.\textsuperscript{3} These studies highlight the problems with the use of artificial substrates to study enzyme activity and suggest that observed differences in the activation of ACE1 and ACE2 cannot be directly attributed to differences in the enzymes, because other conditions, such as substrates, or ionic conditions could affect the outcome of the study.

In conclusion, our results document the development of a novel MS-enzyme assay for monitoring tissue ACE2 activity. Results document the absence of a circulating form of ACE2, the specificity of the method, and the alterations associated with specific gene deletion. SELDI-TOF-MS provides a viable alternative to existing analytical techniques with the advantage of the use of endogenous synthetic substrates and the ability to directly measure enzymatic peptide products. 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. The method provides a rapid, sensitive, and reproducible alternative to existing analytical techniques for detection of RAS enzymatic activities.

**Perspectives**

The focus in the present study was on the development of an MS-based enzyme assay for ACE2 using endogenous peptide substrates. Evaluation of enzyme activity by SELDI-TOF-MS is specific where substrates and products are unambiguously identified by their mass/charge ratio (m/z) and quantified as peptide peak intensity or AUC. The technology has been successfully used to monitor rACE2 and kidney ACE2 activity, and calculated kinetic parameters were shown to parallel those obtained by conventional techniques. MS data give more confidence about the identity of the reaction products than other enzyme assays and, therefore, avoid false-positive results. The short time, small sample size, and minimal handling requirements along with potential for high throughput represent an additional significant advantage in the application of the method in drug development of potential ACE2 inhibitors. The method could also be applied to studies of the functional role of ACE2 and screening for circulating ACE2 under pathological conditions.

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