A Novel 4-Oxo-2(E)-nonenal-Derived Modification to Angiotensin II: Oxidative Decarboxylation of N-Terminal Aspartic Acid

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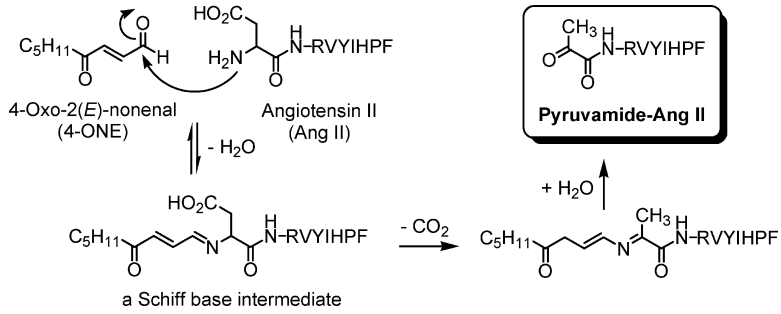

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A Novel 4-Oxo-2(*E*)-nonenal-Derived Modification to Angiotensin II: Oxidative Decarboxylation of N-Terminal Aspartic Acid

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4-Oxo-2(*E*)-nonenal (4-ONE) is a major bifunctional electrophile derived from lipid hydroperoxides. A substantial amount of past research on 4-ONE-derived modifications to cellular macromolecules has led to the conclusion that 4-ONE is more reactive toward DNA and protein than 4-hydroxy-2(*E*)-nonenal (4-HNE). In this study, a novel discovery was made that 4-ONE mediates not only adduct formation but also oxidative decarboxylation of N-terminal aspartic acid on angiotensin (Ang) II and des-Ile^5, His^6, Pro^7, and Phe^8-Ang II [Ang II (1−4)]. This reaction was not mediated by other lipid peroxidation-derived aldehydes such as 4-HNE and *trans*-4,5-epoxy-2(*E*)-decenal (4,5-EDE). The initial reaction of 4-ONE with an N-terminal α-amino group of Ang II or Ang II (1−4) resulted in the formation of a Schiff base intermediate. The resulting intermediate underwent tautomerization and decarboxylation followed by hydrolysis to provide an α-keto amide (pyruvamide) moiety at the N terminus of Ang II and Ang II (1−4). The structures of 4-ONE-derived pyruvamide-Ang II and -Ang II (1−4) were confirmed by matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) and postsource decay (PSD)-TOF/MS analyses and by comparisons to their authentic standards. The presence of a ketone group on the N terminus was confirmed by reduction with sodium borohydride, which resulted in the addition of two hydrogen atoms. Reactivity of 4-ONE toward N-terminal aspartic acid on Ang II was then compared with that of pyridoxal 5′-phosphate (PLP), a well-known aldehyde that efficiently converts N-terminal aspartic acid residue to pyruvate residue. The results indicated a rapid formation of 4-ONE-derived pyruvamide-Ang II and a higher reactivity of 4-ONE at its physiological concentration. This suggests that peptides or proteins containing N-terminal aspartic acid can readily react with lipid hydroperoxide-derived 4-ONE to form pyruvamides, which could modulate their biological functions.

Introduction

Oxidative stress has been implicated in the degenerative diseases of aging; these include cancer, cardiovascular diseases, immune system decline, and brain dysfunctions (*1*). The increased production of reactive oxygen species (ROS)[1] occurs in settings of oxidative stress and results in damage to lipids and cellular macromolecules, such as DNA and proteins. Among them, the polyunsaturated fatty acids (PUFAs) found in membranes and lipoproteins exhibit particularly high susceptibility to ROS-induced damage, leading to the formation of lipid hydroperoxides. Enzymes such as lipoxygenases (LOXs) and cyclooxygenases (COXs) can also oxidize PUFAs to form lipid hydroperoxides, which undergo transition metal ion- and vitamin C-dependent decomposition to form α,β-unsaturated aldehyde genotoxins, 4-oxo-2(*E*)-nonenal (4-ONE), *trans*-4,5-epoxy-2(*E*)-decenal (4,5-EDE), and 4-hydroxy-2(*E*)-nonenal (4-HNE) (*2*). 4-HNE has received the most attention since it was identified as a cytotoxic molecule during lipid peroxidation by Benedetti et al. in 1980 (*3*). It has been detected from various biological samples in the range of nM to lower μM (*4*−*6*), and the level was increased significantly in oxidized samples (*4, 5*). The rich chemistry and biochemistry of lipid hydroperoxide-derived 4-HNE have resulted in an intensive study of its cytotoxic properties (*7*) and its ability to induce various signal transduction pathways (*8*). There is also a substantial amount of literature describing the modifications induced by 4-HNE to DNA bases, peptides, and proteins (*9, 10*). However, it has been recently demonstrated that significant quantities of 4-oxo-2(*E*)-nonenol (4-ONO), an isomer of 4-HNE, were formed in endothelial cells subjected to oxidative stress. 4-ONO was shown to react with glutathione (GSH) to yield GSH adducts similar to those formed by 4-HNE. This suggests that peptide and protein adducts ascribed to arise solely from endogenous 4-HNE needs to be reappraised (*11*).

Recently, 4-ONE was identified as a major product from the homolytic decomposition of ω-6 PUFA hydroperoxides (*2, 12*).

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[1] Abbreviations: Ang, angiotensin; AT1 receptor, angiotensin II type 1 receptor; CHCA, α-cyano-4-hydroxycinamic acid; COX, cyclooxygenase; des-Ile^5, His^6, Pro^7, Phe^8-Ang II, Ang II (1−4); 4,5-EDE, *trans*-4,5-epoxy-2(*E*)-decenal; GSH, glutathione; 4-HNE, 4-hydroxy-2(*E*)-nonenal; LC/UV, liquid chromatography/ultraviolet; LOX, lipoxygenases; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time-of-flight/mass spectrometry; MH^+, protonated molecule; 4-ONE, 4-oxo-2(*E*)-nonenal; 4-ONO, 4-oxo-2(*E*)-nonenol; PLP, pyridoxal 5′-phosphate; PSD, postsource decay; PUFA, polyunsaturated fatty acid; RAS, renin/angiotensin system; ROS, reactive oxygen species; SEM, standard error of the mean.

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4-ONE is much more efficient than 4-HNE at modifying DNA bases through the formation of heptanone-etheno adducts (*13*−*15*). 4-ONE has also been recognized to be a more reactive protein modification and cross-linking agent than 4-HNE (*16, 17*). In previous studies, we have demonstrated the formation of dihydrofuran and dihydropyrrole derivatives from the reaction of 4-ONE with lysine and the formation of a substituted imidazole derivative with arginine (*18*). The reaction of histidine and lysine residues with 4-ONE resulted in the formation of a novel cyclic structure within bovine histone H4 (*19*). 4-ONE was also shown to form stable furan derivatives with histidines of α- and β-hemoglobin chains (*20*). A recent study demonstrated that the 4-ONE-Lys adduct that corresponds to the expected mass of the Michael adduct is actually an isomeric 4-ketoamide (*21*).

Angiotensin (Ang) II is the major bioactive peptide involved in the renin/angiotensin system (RAS), a crucial part of the physiological and pathological responses of the cardiovascular system. Ang II has been implicated in the development of various cardiovascular diseases, such as hypertension, atherosclerosis, and heart failure. Although the mechanisms by which Ang II contributes to each of these diseases remain unclear, oxidative stress is considered a central mechanism based on studies demonstrating the involvement of ROS in numerous signaling pathways of Ang II (*22*). NADPH oxidase is known to be the major source of vascular ROS. Ang II stimulates ROS generation via activation of NADPH oxidase, mainly through its G protein-coupled Ang II type 1 (AT1) receptor (*23*). ROS induced by Ang II activates downstream ROS-sensitive kinases that are critical to mediating cardiovascular remodeling (*24*). Another major consequence of ROS-derived damage to cardiovascular system is lipid peroxidation with production of the genotoxic aldehydes. However, there has been little attention given to the potential for the formation of lipid hydroperoxide-derived modifications that can modulate the biological activity of Ang II.

Recently, Gilmore et al. reported that an unusual decarboxylation of N-terminal aspartic acid residue on Ang I occurred via formation of a Schiff base intermediate stabilized by the aromatic pyridyl group of pyridoxal 5′-phosphate (PLP) (*25*). We reasoned that the conjugation through α,β-unsaturated ketone moiety on 4-ONE should lead to the formation of a stable Schiff base in a reaction with Ang II (DRVYIHPF). Therefore, 4-ONE can mediate not only adduct formation but also oxidative decarboxylation of the N-terminal aspartic acid on Ang II. This paper rigorously characterizes this modification and shows its rapid formation under the physiological conditions.

Materials and Methods

Materials. 4-ONE was purchased from Cayman Chemical Co. (Ann Arbor, MI). Human Ang II was obtained from Calbiochem (a brand of EMD, San Diego, CA), and human des-Ile^5, His^6, Pro^7, Phe^8-Ang II [Ang II (1−4)] was obtained from American Peptide Co. Inc. (Sunnyvale, CA). Ang I, α-cyano-4-hydroxycinamic acid (CHCA), sodium hydroxide, and trifluoroacetic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). PLP monohydrate was purchased from Fluka Analytical (Buchs, Switzerland). Sodium borohydride was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Chelex-100 chelating ion-exchange resin (100−200 mesh size) was purchased from Bio-Rad Laboratories (Hercules, CA). LC grade acetonitrile and ethanol were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Purified water was purchased from Daiwa-Yakuhin Co. Ltd. (Sendai, Japan) and further filtered through Ultrapure Water System, CPW-100 (Advantec Toyo Kaisha, Ltd. Tokyo, Japan). Oasis HLB cartridge was obtained from Waters Corp. (Milford, MA). ZipTipC_{18} cartridges were obtained from Millipore Co. (Bedford, MA).

Liquid Chromatography. Chromatography was carried out using a Nanospace SI-1 semimicrocolumn LC system (Shiseido Co. Ltd., Tokyo, Japan) equipped with a UV detector. System 1 employed a Jupiter C18 column (150 mm × 2.0 mm i.d., 5 μm, 300 Å; Phenomenex, Torrance, CA). System 2 employed a Zorbax Extend-C18 column (150 mm × 2.1 mm i.d., 5 μm; Agilent Technologies, Inc., Santa Clara, CA). For system 1, solvent A was water:acetonitrile (98:2, v/v) containing 0.08% trifluoroacetic acid, and solvent B was acetonitrile:water (98:2, v/v) containing 0.08% trifluoroacetic acid. The linear gradient was as follows: 20% B at 0 min, 27.5% B at 4 min, 52.5% B at 30 min, 90% B at 31 min, 90% B at 36 min, and 20% B at 37 min with a flow rate of 0.2 mL/min. For system 2, solvent A was water:acetonitrile (98:2, v/v) containing 0.08% trifluoroacetic acid, and solvent B was acetonitrile:water (70:30, v/v) containing 0.08% trifluoroacetic acid. The linear gradient was as follows: 20% B at 0 min, 27.5% B at 4 min, 52.5% B at 30 min, 90% B at 31 min, 90% B at 36 min, and 20% B at 37 min with a flow rate of 0.2 mL/min. All separations were performed at ambient temperatures.

Matrix-Assisted Laser Desorption Ionization-Time-of-Flight/Mass Spectrometry (MALDI-TOF/MS). MALDI-TOF/MS and MALDI-postsource decay (PSD)-TOF/MS experiments were carried out using an AXIMA-CFR Plus MALDI-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan) located in the Department of Instrumental Analysis (Technical Division, School of Engineering, Tohoku University). The mass spectrometer was equipped with a nitrogen laser (337 nm) and had a flight path of 120 cm for linear mode and 225 cm for reflectron mode. All spectra presented were acquired in the positive ion mode with an accelerating voltage of 20 kV and in the reflectron mode with an average of 100−150 laser shots. TOF/MS experiments were performed in the mass range of *m/z* 100−3000. For PSD-TOF/MS experiments, the mass window for the precursor ion selection was ca. ±10 Da. Calibration was made by using four or five internal calibrants from the following: monoisotopic masses of matrix monomer at *m/z* 190.0504, matrix dimer at *m/z* 379.0930, Ang II (1−4) at *m/z* 552.2776, matrix trimer at *m/z* 568.1400, Ang II at *m/z* 1046.5418, and Ang I at *m/z* 1296.6800. For MALDI-PSD-TOF/MS analyses, PSD fragments were calibrated using monoisotopic masses of fragment ions generated from Ang II (MH^+ at *m/z* 1046.5418, $MH^+ - NH_3$ at *m/z* 1029.5152, y_7 at *m/z* 931.5148, $b_7 + H_2O$ at *m/z* 899.4734, b_6 at *m/z* 784.4100, a_6 at *m/z* 756.4151, b_5 at *m/z* 647.3511, a_5 at *m/z* 619.3562, $b_4 - NH_3$ at *m/z* 517.2405, y_3 at *m/z* 400.1979, $b_3 - NH_3$ at *m/z* 354.1772, and y_2 at *m/z* 263.1390). The samples were desalted and eluted with ZipTipC_{18} cartridges. In brief, the ZipTips were conditioned with acetonitrile (10 μL × 3) and then equilibrated with 0.1% aqueous trifluoroacetic acid (10 μL × 3). The peptide solutions in 5% aqueous acetonitrile containing 0.1% trifluoroacetic acid were subsequently loaded onto the ZipTip and washed with 0.1% aqueous trifluoroacetic acid (10 μL × 3). Finally, the samples were eluted with 75% aqueous acetonitrile containing 0.1% trifluoroacetic acid (10 μL × 5) and evaporated to dryness under nitrogen. The samples were redissolved in 50% aqueous acetonitrile (20 μL). Aliquots (0.5 μL) were loaded onto MALDI sample plates followed by 0.5 μL of matrix solution (saturated CHCA in 50% aqueous acetonitrile containing 0.1% trifluoro-

acetic acid and 1−2 μM of internal calibrants) and allowed to dry at room temperature.

Reaction of 4-ONE with Ang II or Ang II (1−4). A solution of 4-ONE (92.4 μg, 0.6 μmol) in 20 μL of methylacetate:ethanol (1:1, v/v) was added to Ang II (209 μg, 0.2 μmol) or Ang II (1−4) (110 μg, 0.2 μmol) in Chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 180 μL). The reaction mixture was incubated for 24 h at 37 °C. After incubation, excess 4-ONE was removed by extraction using hexane (200 μL × 2). A portion of the sample (10 μL) was analyzed by liquid chromatography/ultraviolet (LC/UV) (235 nm) using LC system 1 for Ang II reaction mixture and LC system 2 for Ang II (1−4) reaction mixture.

Reaction of Ang II with 4-HNE or 4,5-EDE. A solution of Ang II (209 μg, 0.2 μmol) in Chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 180 μL) was added to 4-HNE (93.7 μg, 0.6 μmol) in 20 μL of ethanol or 4,5-EDE (100.9 μg, 0.6 μmol) in 20 μL of methylacetate:ethanol (1:1, v/v). The reaction mixture was incubated for 24 h at 37 °C. After incubation, excess aldehyde was removed by extraction using hexane (200 μL × 2). A portion of the sample (10 μL) was analyzed by LC/UV (235 nm) using LC system 1.

Preparation of Authentic Pyruvamide-Ang II and -Ang II (1−4) Using PLP. PLP monohydrate (159.1 μg, 0.6 μmol) was added to Ang II (209 μg, 0.2 μmol) or Ang II (1−4) (110 μg, 0.2 μmol) in Chelex-treated 50 mM sodium phosphate buffer (pH 7.4, 200 μL). The reaction mixture was incubated for 24 h at 37 °C. After incubation, pyruvamide-Ang II and -Ang II (1−4) were isolated using LC systems 1 and 2, respectively, by monitoring the UV absorbance at 235 nm. The retention time of pyruvamide-Ang II on LC system 1 was 8.1 min, and the retention time of pyruvamide-Ang II (1−4) on LC system 2 was 5.5 min.

Sodium Borohydride Reduction of Pyruvamide-Ang II or -Ang II (1−4). PLP- or 4-ONE-derived pyruvamide-Ang II and -Ang II (1−4) were purified using LC systems 1 and 2, respectively. Solutions of pyruvamide-Ang II or -Ang II (1−4) in 50 mM sodium phosphate buffer (pH 7.4, 100 μL) were treated with 100 μL of 0.1 M sodium borohydride in 0.1 M sodium hydroxide. The reaction mixtures were incubated for 1 h at 37 °C. After they were cooled down at room temperature, the resulting modified peptides were purified and desalted chromatographically on Oasis HLB (1 cc, 10 mg) cartridges. The cartridges were conditioned with acetonitrile (1 mL) followed by 0.1% aqueous trifluoroacetic acid (1 mL). The reaction solutions (200 μL) were loaded on the cartridges and washed with 0.1% aqueous trifluoroacetic acid (200 μL × 3). The peptides were recovered by elution with 60% aqueous acetonitrile containing 0.1% trifluoroacetic acid (200 μL × 2) and evaporated to dryness under nitrogen. The samples were redissolved in 50% aqueous acetonitrile (20 μL). Aliquots (0.5 μL) were loaded onto MALDI sample plates followed by 0.5 μL of matrix solution (saturated CHCA in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid and 1−2 μM of internal calibrants) and allowed to dry at room temperature for MALDI-TOF/MS analysis.

Sodium Borohydride Reduction of Ang II or Ang II (1−4). Solutions of Ang II or Ang II (1−4) in 50 mM sodium phosphate buffer (pH 7.4, 100 μL) were treated with 100 μL of 0.1 M sodium borohydride in 0.1 M sodium hydroxide. After incubation for 1 h at 37 °C, the reaction mixtures were purified and desalted as described above. Aliquots (0.5 μL) were loaded onto MALDI sample plates followed by 0.5 μL of matrix solution (saturated CHCA in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid and 1−2 μM of internal calibrants) and allowed to dry at room temperature for MALDI-TOF/MS analysis.

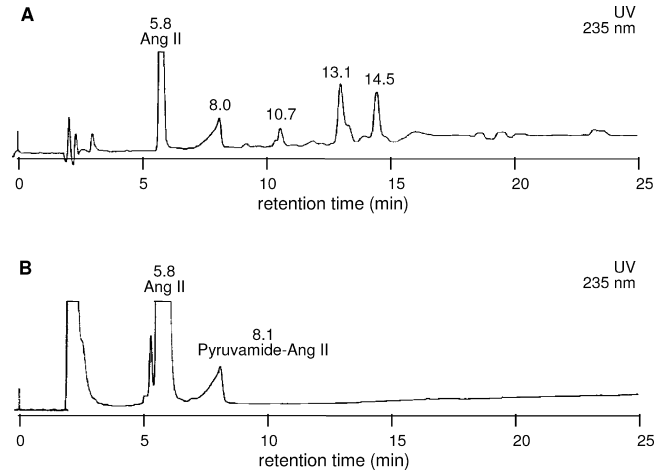


Figure 1. (A) LC/UV (235 nm) analysis of products from the reaction of Ang II with 4-ONE at 37 °C for 24 h. (B) LC/UV (235 nm) analysis of pyruvamide-Ang II (retention time of 8.1 min) from the reaction of Ang II with PLP at 37 °C for 2 h.

Reaction of Ang II with Decreasing Concentrations of 4-ONE or PLP. A solution of Ang II (209 μg, 1.0 mM final concentration) in Chelex-treated 50 mM sodium phosphate buffer (pH 7.4) was treated with decreasing concentrations of 4-ONE or PLP (3.0, 1.0, 0.5, 0.1, and 0.05 mM final concentration). Each reaction mixture was incubated for 2 and 24 h at 37 °C. A portion of the sample (10 μL) was analyzed by LC/UV (235 nm) using LC system 1.

Results

Analysis of Reaction between Ang II and 4-ONE. LC/UV (235 nm) analysis of the products from the reaction between 4-ONE and Ang II at 37 °C for 24 h revealed the presence of four major products together with residual Ang II (Figure 1A). Each product peak was isolated using LC system 1 and further desalted as described above for MALDI-TOF/MS analysis. The MALDI-TOF/MS spectrum of the most polar modified peptide eluting at 8.0 min revealed an MH^+ at *m/z* 1001.49 corresponding to a loss of 45 Da from Ang II (MH^+; *m/z* 1046.51). The 4-ONE-modified peptides that eluted at 10.7 and 13.1 min had an identical MH^+ at *m/z* 1200.70, which corresponded to an increase in mass of 154 Da (+[4-ONE]). They were tentatively identified as Michael addition products formed at the N-terminal amino group of Ang II after MALDI-PSD-TOF/MS analysis. The last eluting 4-ONE-modified peptide had a retention time of 14.5 min. It showed an MH^+ at *m/z* 1182.64 corresponding to an increase in mass of 136 Da (+[4-ONE − H_2O]). The MALDI-PSD-TOF/MS analysis of *m/z* 1182.64 revealed the modification occurred on Arg^2.

Preparation of Authentic Pyruvamide-Ang II Using the Reaction of PLP with Ang II. The LC/UV (235 nm) chromatogram of the reaction mixture after 2 h at 37 °C showed the formation of pyruvamide-Ang II with a retention time of 8.1 min on LC system 1 (Figure 1B). Its peak area on the LC/UV chromatogram was increased approximately four times after 24 h of incubation. Pyruvamide-Ang II was then isolated using LC system 1 and further desalted as described above. MALDI-TOF/MS analysis of pyruvamide-Ang II revealed an MH^+ at *m/z* 1001.49 corresponding to a loss of 45 Da from Ang II. These LC/UV and MS characteristics were identical to those for the first-eluting 4-ONE-modified peptide (Figure 1A).

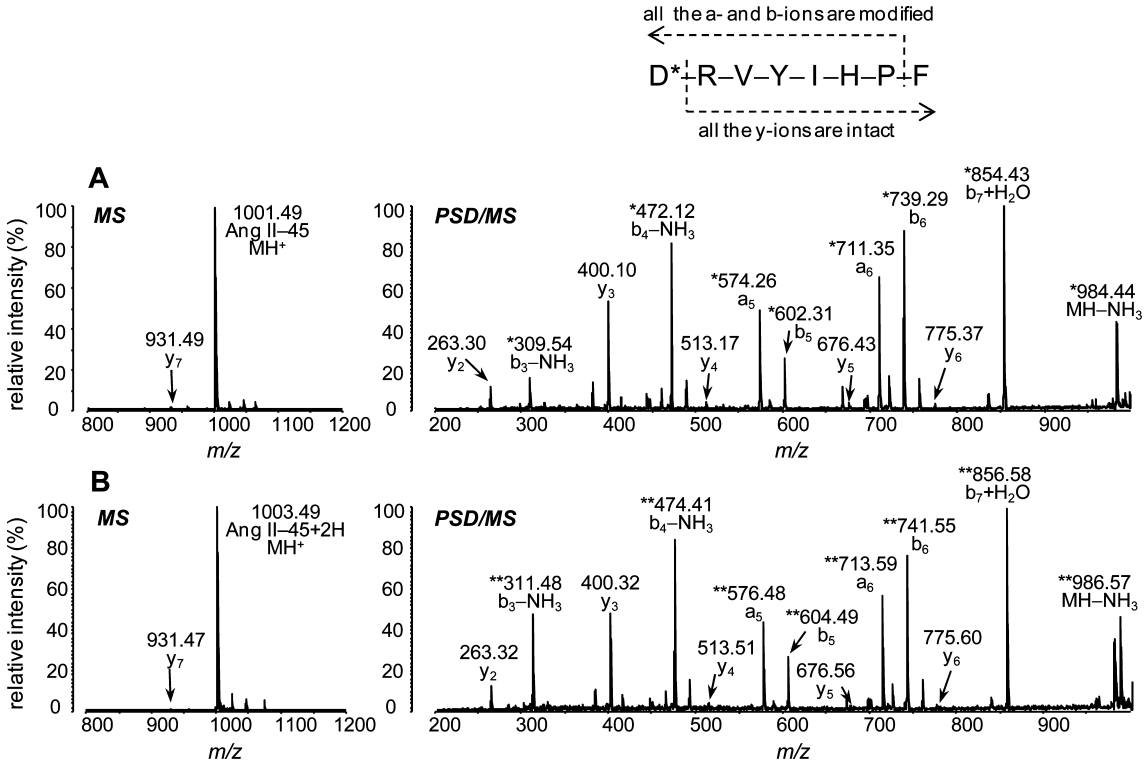


Figure 2. (A) MALDI-TOF/MS and -PSD-TOF/MS analyses of the most polar product from the reaction of Ang II with 4-ONE. Left, MS spectrum; right, PSD/MS spectrum of MH^+ (*m/z* 1001.49). * represents a modified ion (−45 Da). (B) After sodium borohydride reduction. Left, MS spectrum; right, PSD/MS spectrum of MH^+ (*m/z* 1003.49). ** represents a modified ion (−45 Da + 2H). The *m/z* value on each peak indicates the monoisotopic mass observed.

MALDI-TOF/MS and -PSD-TOF/MS Analysis of Ang II. The theoretical monoisotopic mass of protonated Ang II (DRVYIHPF) is 1046.5418. The MALDI-TOF/MS spectrum exhibited an MH^+ of Ang II at *m/z* 1046.51 after calibration. The MALDI-PSD-TOF/MS analysis of *m/z* 1046.51 resulted in the generation of a good series of fragment ions. An almost complete series of a- and b-ions, including either a loss of NH_3 or an addition of H_2O (a_3 to a_7 and b_2 to b_7), were observed with relatively higher intensities as follows: $b_7 + H_2O$ at *m/z* 899.43, b_6 at *m/z* 784.41, a_6 at *m/z* 756.38, b_5 at *m/z* 647.38, a_5 at *m/z* 619.39, b_4 at *m/z* 534.32, $b_4 - NH_3$ at *m/z* 517.27, $b_3 - NH_3$ at *m/z* 354.02, and $b_2 - NH_3$ at *m/z* 255.24. All of the y ions (y_2−y_7) were also observed as follows: y_7 at *m/z* 931.47, y_6 at *m/z* 775.41, y_5 at *m/z* 676.37, y_4 at *m/z* 513.33, y_3 at *m/z* 400.20, and y_2 at *m/z* 263.14. However, the relative intensities for some of y ions (y_4−y_6) were less than 10%. As described in the Materials and Methods section, PSD fragments were calibrated using theoretical monoisotopic masses of fragment ions of Ang II.

MALDI-TOF/MS and -PSD-TOF/MS Analysis of Pyruvamide-Ang II Derived from 4-ONE Before and After Sodium Borohydride Reduction. MALDI-TOF/MS analysis of the most polar modified peptide (retention time = 8.0 min) isolated from the reaction between 4-ONE and Ang II revealed an MH^+ at *m/z* 1001.49, together with a small y_7 ion at *m/z* 931.49 (Figure 2A, left). The MALDI-PSD-TOF/MS analysis of *m/z* 1001.49 revealed that a modification occurred at the N-terminal aspartic acid (Figure 2A, right). Thus, all of the a and b ions that were detected (a_3−a_7 and b_3−b_7) appeared with a loss of 45 Da. On the other hand, all of the y ions (y_2−y_7) remained unmodified. The 4-ONE-modified peptide (retention time = 8.0 min) was then subjected to sodium borohydride reduction. After the reaction, the resulting product was further purified and desalted by solid-phase extraction as described in the Materials and Methods section. MALDI-TOF/MS analysis of the product revealed that no starting material was left. One major product was formed with an MH^+ at *m/z* 1003.49 (Figure 2B, left) that corresponded to the addition of two hydrogen atoms. MALDI-PSD-TOF/MS analysis of *m/z* 1003.49 resulted in the generation of all modified a and b ions (a_3−a_7 and b_3−b_7) and all intact y ions (y_2−y_7), which is consistent with a modification to N-terminal aspartic acid (Figure 2B, right). All of the modified a and b ions showed an increase in mass units of 2 Da when compared with those in the PSD spectrum of pyruvamide-Ang II (Figure 2A, right). This confirmed that two hydrogen atoms were added to the ketone group of N-terminal α-keto amide (pyruvamide) moiety. These MALDI-TOF/MS and -PSD-TOF/MS characteristics were identical to those of authentic pyruvamide-Ang II. On the basis of these results, the most polar modified peptide formed in the reaction between 4-ONE and Ang II was identified as pyruvamide-Ang II.

Analysis of Reactions between Ang II and 4-HNE or 4,5-EDE. LC/UV (235 nm) analysis after 24 h at 37 °C using LC system 1 revealed that pyruvamide-Ang II was not formed from both reactions of Ang II with 4-HNE or 4,5-EDE.

MALDI-TOF/MS and PSD/MS Analysis of Pyruvamide-Ang II (1−4) Derived from 4-ONE Before and After $NaBH_4$ Reduction. The authentic pyruvamide-Ang II (1−4) was prepared from the reaction between PLP and Ang II (1−4) and was purified using LC system 2. The retention time of pyruvamide-Ang II (1−4) on LC system 2 was 5.5 min. LC/UV (235 nm) analysis of the reaction between 4-ONE and Ang II (1−4) at 37 °C for 24 h revealed the presence of pyruvamide-Ang II (1−4). It was isolated using LC system 2 and further desalted as described above for MALDI-TOF/MS analysis. The protonated Ang II (1−4) (DRVY) has a theoretical monoisotopic *m/z* of 552.2776. The MALDI-TOF/MS spectrum exhibited an MH^+ of Ang II (1−4) at *m/z* 552.28 after

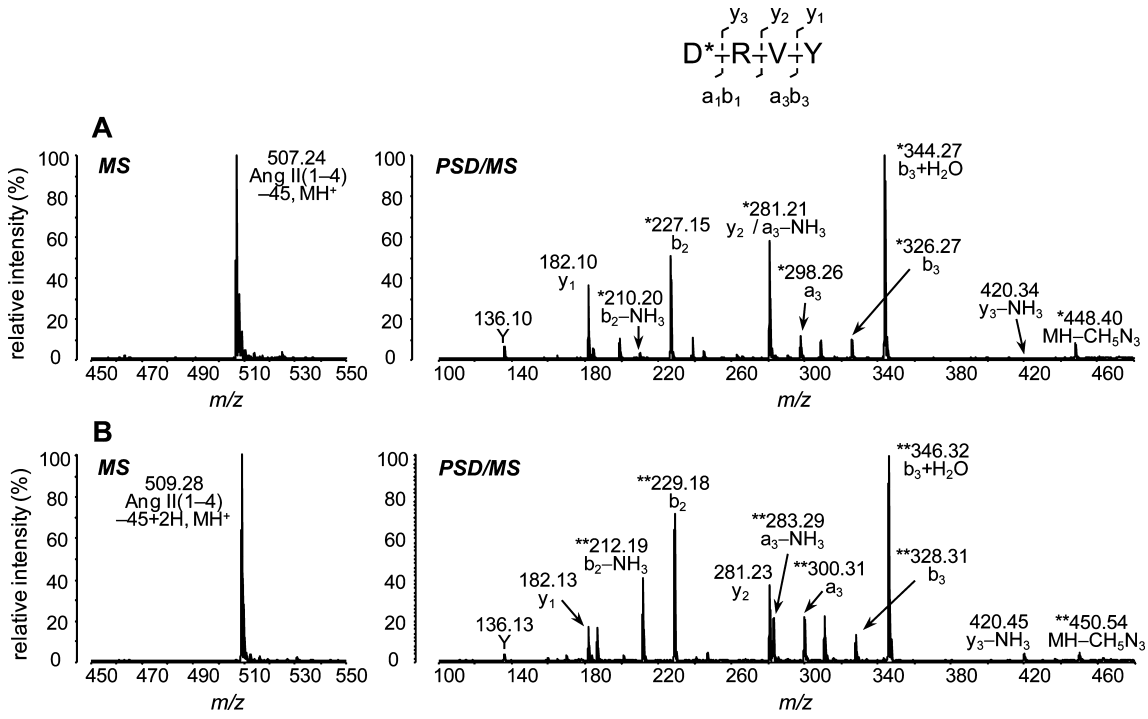


Figure 3. (A) MALDI-TOF/MS and -PSD-TOF/MS analyses of pyruvamide-Ang II (1−4) from the reaction of Ang II (1−4) with 4-ONE. Left, MS spectrum; right, PSD/MS spectrum of MH^+ (*m/z* 507.24). * represents a modified ion (−45 Da). (B) After sodium borohydride reduction. Left, MS spectrum; right, PSD/MS spectrum of MH^+ (*m/z* 509.28). ** represents a modified ion (−45 Da + 2H). The *m/z* value on each peak indicates the monoisotopic mass observed.

calibration. MALDI-TOF/MS analysis of pyruvamide-Ang II (1−4) isolated from the reaction between 4-ONE and Ang II (1−4) revealed an MH^+ at *m/z* 507.24 (Figure 3A, left), corresponding to a loss of 45 Da from Ang II (1–4). The MALDI-PSD-TOF/MS spectrum of *m/z* 507.24 was consistent with a modification to N-terminal aspartic acid (Figure 3A, right). All of the a and b ions (a_2, a_3 and b_2, b_3) were modified (−45 Da), and all of the y ions (y_1-y_3) were intact. The isolated pyruvamide-Ang II (1−4) was then subjected to sodium borohydride reduction. After the reaction, the resulting product was further purified and desalted. MALDI-TOF/MS analysis of the product revealed that no starting material was left. One major product was formed with an MH^+ at *m/z* 509.28 (Figure 3B, left) that corresponded to the addition of two hydrogen atoms. MALDI-PSD-TOF/MS analysis of *m/z* 509.28 resulted in the formation of intact y ions (y_1-y_3) and reduced (−45 Da + 2H) a and b ions (a_2, a_3 and b_2, b_3) (Figure 3B, right), suggesting that the reduction occurred on the ketone group of N-terminal pyruvamide moiety. These LC/UV, MALDI-TOF/MS, and -PSD-TOF/MS characteristics were identical with those of authentic pyruvamide-Ang II (1−4).

Sodium Borohydride Reduction of Ang II or Ang II (1−4). MALDI-TOF/MS analysis revealed that Ang II and Ang II (1−4) were recovered intact after the reaction with sodium borohydride.

Formation of Pyruvamide-Ang II from Decreasing Concentrations of PLP or 4-ONE. Ang II (1.0 mM final concentration) in Chelex-treated phosphate buffer (pH 7.4) was reacted with decreasing concentrations of PLP or 4-ONE (3.0, 1.0, 0.5, 0.1, and 0.05 mM, final concentration) at 37 °C to compare the reactivity, especially when the concentrations are close to physiological conditions. The formation of pyruvamide-Ang II was monitored by LC system 1 after 2 and 24 h of incubation. Results are presented as mean ± standard error of the mean (SEM) from duplicate samples (Figure 4). In the reaction of PLP with Ang II, the formation of pyruvamide-Ang II after 2 h at all PLP concentrations was a mean of 13.7 ± 7.4% over the formation after 24 h of incubation (Figure 4A). In contrast, the mean formation of 4-ONE-derived pyruvamide-Ang II was 56.8 ± 4.9% at 2 h over 24 h of incubation (Figure 4B). The formations of pyruvamide-Ang II from PLP and 4-ONE were then compared at 2 h (Figure 4C). When the concentration of aldehydes was 3.0 mM, the PLP-derived formation was approximately 2.0 times higher than the 4-ONE-derived formation. There was then a rapid decrease in the PLP-derived formation as the aldehyde concentration decreased. In contrast, 4-ONE-derived formation decreased gradually. Thus, the 4-ONE-derived pyruvamide-Ang II formation was 1.6 times and 3.0 times higher than the PLP-derived formation at the aldehyde concentrations of 0.5 and 0.05 mM, respectively. At 24 h (Figure 4D), the PLP-derived formation of pyruvamide-Ang II was approximately 5.3 times higher than the 4-ONE-derived formation when the aldehyde concentration was 3 mM. However, it was only 2.6 times higher than 4-ONE-derived formation at the 0.05 mM concentration point.

Discussion

The oxidative decarboxylation of N-terminal aspartic acid was identified very recently during studies on peptide-cleaving catalysts selective for target proteins as drug candidates. In these studies, human Ang I and Ang II were chosen as target peptides based on their physiological importance. Two derivatives of Co(III)cyclen were selected from chemical libraries as the most active catalysts. Upon incubation with Co(III)cyclen compounds, Ang I and Ang II were cleaved by oxidative decarboxylation instead of peptide hydrolysis; the N-terminal aspartic acid residues of Ang I and Ang II were converted to pyruvate residues (*26*). In a separate study on N-terminal protein modification, unexpected decarboxylation on N-terminal aspartic acid residue of Ang I was observed upon treatment with glyoxylic acid. The product of the reaction was identified as

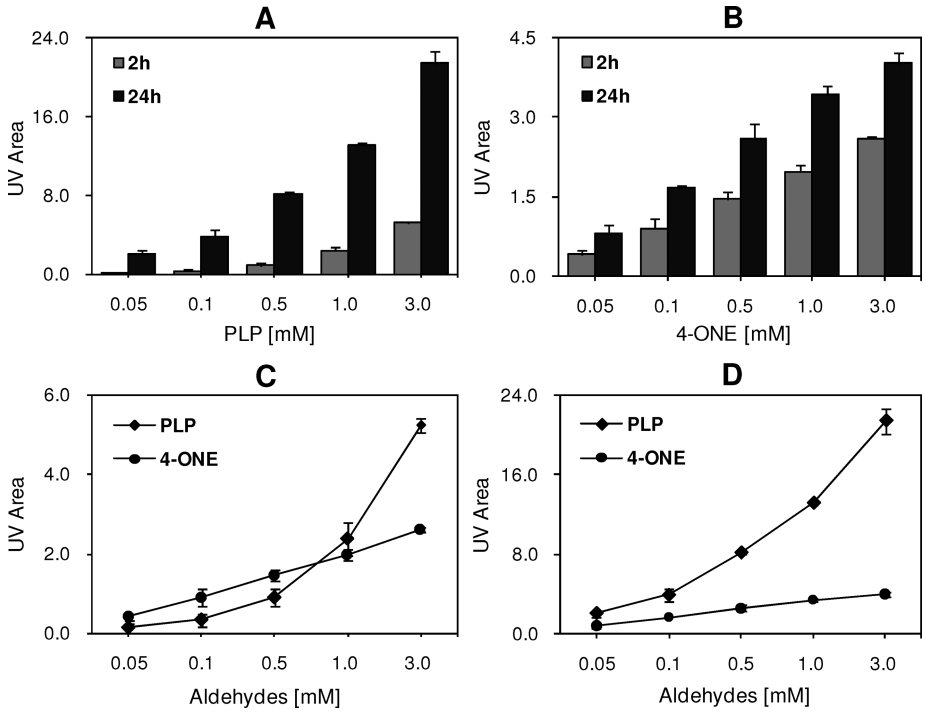


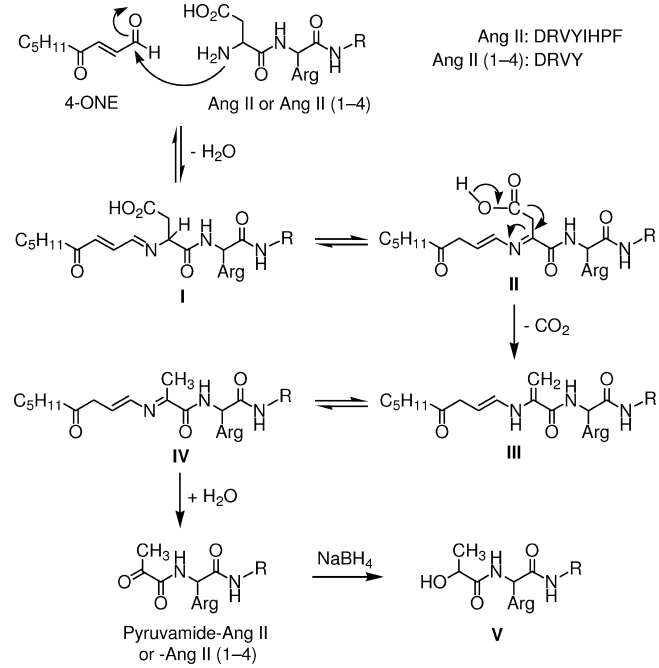
Figure 4. Formation of pyruvamide-Ang II from the reaction of Ang II with decreasing concentrations of (A) PLP at 2 (gray bar) and 24 h (black bar). (B) 4-ONE at 2 (gray bar) and 24 h (black bar). (C) PLP (closed diamond) or 4-ONE (closed circle) at 2 h. (D) PLP (closed diamond) or 4-ONE (closed circle) at 24 h. Data are presented as means ± SEM (error bars or within symbols) from duplicate samples.

pyruvamide, and further experiments found that PLP was the most efficient at converting Ang I to pyruvamide-Ang I (*25*).

The authentic standards for pyruvamide-Ang II and -Ang II (1−4) were prepared using PLP. Chelex-treated buffer was used for the reactions between 4-ONE and Ang II or Ang II (1−4) to ensure that no transition metal ions in the reaction solution were involved in the 4-ONE-derived formation of pyruvamides (*2*). The reaction of Ang II with 4-ONE resulted in the formation of pyruvamide-Ang II with an MH^+ at *m/z* 1001.49, corresponding to a loss of 45 Da from Ang II (MH^+; *m/z* 1046.51) and a retention time on LC/UV analysis identical to that of the authentic standard (Figure 1). In contrast, the formation of pyruvamide-Ang II was not observed in the reaction of Ang II with 4-HNE or 4,5-EDE. The structure of pyruvamide-Ang II was confirmed by MALDI-TOF/MS and -PSD-TOF/MS analyses (Figure 2) and by comparison with the authentic standard. The modification site at N-terminal aspartic acid was revealed by MALDI-PSD-TOF/MS analysis and showed all modified a and b ions (− 45 Da) and all intact y ions. The presence of a ketone group on the N terminus was confirmed by reduction with sodium borohydride, which resulted in the addition of two hydrogen atoms. The possibility was considered that one of the imine groups on Ang II (Arg^2 and His^6) may have been reduced upon sodium borohydride reaction. Thus, intact Ang II was subjected to the sodium borohydride reaction and was recovered unmodified after the reaction. This provided unequivocal evidence for the presence of pyruvamide moiety on the N terminus of Ang II. The formation of pyruvamide-Ang II (1−4) from the reaction of 4-ONE with Ang II (1−4) further confirmed the oxidative decarboxylation of N-terminal aspartic acid derived from 4-ONE (Figure 3).

The previous studies with 2′-deoxyguanosine, 2′-deoxyadenosine, 2′-deoxycytidine, arginine, and GSH showed that reactions between 4-ONE and the primary amine take place at the C-1 of aldehyde (*11, 13−15, 18*). Therefore, we propose that the initial reaction of the primary amino group on the N terminus of Ang II occurs at C-1 of 4-ONE, yielding a

Scheme 1. Proposed Mechanism for the Formation of Pyruvamide-Ang II from 4-ONE



carbinolamine that is dehydrated and the Schiff base intermediate I is formed (Scheme 1). The formation of stable Schiff base intermediate through the longer conjugation system with the α,β-unsaturated ketone moiety on 4-ONE was further supported by the reactions of Ang II with 4-HNE or 4,5-EDE that did not result in the formation of pyruvamide-Ang II. The intermediate I has an α-proton with a lower pK_a value, which allows tautomerization to occur uniquely at this site (II). The imine group on intermediate II facilitates the decarboxylation reaction leading to enamine (III), which undergoes tautomerization to form imine (IV). The subsequent hydrolysis of the resulting

imine (IV) yields the ketone at the N terminus of Ang II (pyruvamide-Ang II). Upon sodium borohydride reduction, two hydrogen atoms were added to the N-terminal ketone group (V).

Time- and concentration-dependent reactivity of 4-ONE for the oxidative decarboxylation of N-terminal aspartic acid was compared to that of PLP at pH 7.4 and 37 °C in Chelex-treated buffer. It has been reported that PLP afforded a 65% conversion of Ang I into pyruvamide-Ang I in 2 h at pH 6.5 and 37 °C (*25*). Current data demonstrated a rapid formation of 4-ONE-derived pyruvamide-Ang II and a higher reactivity of 4-ONE at its lower concentration (less than 0.5 mM), which is close to its physiological concentration (Figure 4). 4-ONE can modify other amino acids such as Arg^2 and His^6 of Ang II, as shown in Figure 1. UV intensities of the peaks eluting at 13.1 and 14.5 min were increased approximately 5.0 and 4.0 times, respectively, after 24 h of incubation, as compared to those after 2 h of incubation. There are also possibilities of intra- or intermolecular cross-linking of Ang II induced by 4-ONE. This could explain the lesser formation of 4-ONE-derived pyruvamide-Ang II at higher concentrations of 4-ONE or the longer incubation time when compared to PLP-derived formation. Although the basal physiological concentration of lipid hydroperoxide-derived bifunctional electrophiles such as 4-HNE (nM to lower μM range) (*4*−*6*) is similar to that of PLP (nM range) (*27*), it is noteworthy that it further increases in response to an oxidative stress (*4, 5*).

In addition to Ang II, contributions of other Ang peptides to vascular regulation have also been demonstrated, including Ang (1–7), Ang III, and Ang IV (*28*). Among them, Ang III was recently proposed as the physiologically relevant peptide that regulates blood pressure rather than Ang II. This was based on studies showing an inhibition of increase in blood pressure by a selective inhibitor of aminopeptidase A, which mediates the generation of Ang III by the cleavage of the N-terminal aspartate residue of Ang II (*28, 29*). More recently, a new Ang peptide, Ang A, has been identified from human plasma (*30*). It shows a strong vasoconstrictive effect and a higher concentration in end stage renal failure patients. Interestingly, it differs from Ang II in Ala^1 instead of Asp^1 and was proposed to be formed enzymatically through the action of a yet unknown decarboxlyase in human mononuclear leukocytes on Asp^1 of Ang II. The structural similarity of 4-ONE-derived pyruvamide-Ang II to Ang III and Ang A warrants further investigation on its biological activity.

In summary, we have provided unequivocal evidence that 4-ONE can generate pyruvamide-Ang II by oxidative decarboxylation of N-terminal aspartic acid residue. The plasma concentration of Ang II in normal human subject is reported as 32 ± 6 pM (*31*). There are also the local RASs separate from the peripheral RAS such as intrarenal and brain RAS that produce Ang II independently. It has been demonstrated that the Ang II concentrations in interstitial fluid are much higher than the plasma concentrations, with values in the range of 3−5 nM in rat model system (*32*). As for the chemical modifications on Ang II, the nitration and nitrosylation of tyrosine (*33, 34*), the nitrosation of arginine (*33*), and the metal-catalyzed oxidation of histidine (*35*) have been reported. In vivo study using the nitrated Ang II showed that nitration of the tyrosine residue inhibits vasoconstrictive properties of Ang II (*33*). However, these modifications have not been detected in physiological systems. That could be mainly because the conventional enzyme immunoassay for Ang II quantitation does not distinguish small modifications on Ang II. Therefore, our current studies are now focused on the development of analytical methodology for the quantitation of 4-ONE-derived pyruvamide-Ang II in biological fluids. The ability to quantify 4-ONE-derived pyruvamide-Ang II will also help to further improve understanding of the role that lipid hydroperoxide-derived bifunctional electrophile, 4-ONE, plays in cardiovascular diseases.

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