Rennin–Angiotensin System

Angiotensin-Converting Enzyme 2 Metabolizes and Partially Inactivates Pyr-Apelin-13 and Apelin-17
Physiological Effects in the Cardiovascular System

Wang Wang, Shaun M.K. McKinnie, Maikel Farhan, Manish Paul, Tyler McDonald, Brent McLean, Catherine Llorens-Cortes, Saugata Hazra, Allan G. Murray, John C. Vederas, Gavin Y. Oudit

See Editorial Commentary, pp 307–309

Abstract—Apelin peptides mediate beneficial effects on the cardiovascular system and are being targeted as potential new drugs. However, apelin peptides have extremely short biological half-lives, and improved understanding of apelin peptide metabolism may lead to the discovery of biologically stable analogues with therapeutic potential. We examined the ability of angiotensin-converting enzyme 2 (ACE2) to cleave and inactivate pyr-apelin 13 and apelin 17, the dominant apelin peptides. Computer-assisted modeling shows a conserved binding of pyr-apelin 13 and apelin 17 to the ACE2 catalytic site. In ACE2 knockout mice, hypotensive action of pyr-apelin 13 and apelin 17 was potentiated, with a corresponding greater elevation in plasma apelin levels. Similarly, pharmacological inhibition of ACE2 potentiated the vasodepressor action of apelin peptides. Biochemical analysis confirmed that recombinant human ACE2 can cleave pyr-apelin 13 and apelin 17 efficiently, and apelin peptides are degraded slower in ACE2-deficient plasma. The biological relevance of ACE2-mediated proteolytic processing of apelin peptides was further supported by the reduced potency of pyr-apelin 12 and apelin 16 on the activation of signaling pathways and nitric oxide production from endothelial cells. Importantly, although pyr-apelin 13 and apelin 17 rescued contractile function in a myocardial ischemia–reperfusion model, ACE2 cleavage products, pyr-apelin 12 and 16, were devoid of these cardioprotective effects. We designed and synthesized active apelin analogues that were resistant to ACE2-mediated degradation, thereby confirming that stable apelin analogues can be designed as potential drugs. We conclude that ACE2 represents a major negative regulator of apelin action in the vasculature and heart.

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Online Data Supplement

Key Words: angiotensin-converting enzyme 2 ▪ apelin 17 ▪ blood pressure ▪ computer-based model ▪ enzyme kinetics ▪ ischemia reperfusion injury ▪ pyr-apelin 13

The apelin and angiotensin family of peptides have a wide range of related physiological and pathophysiological effects on the heart and vasculature.1–5 Apelin is synthesized as a precursor 77 amino acid pro-pro-peptide and is subsequently processed into a family of apelin peptides, with pyr-apelin 13 and apelin 17 being the dominant apelin peptides found in vivo.6,7 Apelin acts on the apelin receptor and regulates vascular homeostasis, angiogenesis, myocardial adaptation to stress, and fluid balance, thereby playing a key role in vascular diseases, such as systemic and pulmonary arterial hypertension, myocardial infarction, and heart failure.8–11 The C-terminal region of the apelin peptide is central for its overall biological activity. N-terminal deletions of apelin-17 reveal that the 12 C-terminal amino acids are core requirements for the internalization and biological potency of apelin action on the apelin receptor.12 Indeed, apelin-17-induced internalization of the apelin receptor decreases with every N-terminal deletion to apelin-12. Similarly, the N-terminal residues 2–5 of apelin 13 are critical for functional potency,13 and the C-terminal sequence consisting of residues 8–11 is important for binding activity and receptor internalization.12,14 The C-terminal residue of apelin, phenylalanine, is critical for binding of the apelin peptide to the apelin receptor.15 However, the functional relevance of the C-terminal phenylalanine

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From the Division of Cardiology, Department of Medicine (W.W., B.M., G.Y.O.), Mazankowski Alberta Heart Institute (W.W., B.M., G.Y.O.), Department of Chemistry, Faculty of Science (S.M.K.M., T.M., J.C.V.), and Division of Nephrology, Department of Medicine (M.F., A.G.M.), University of Alberta, Edmonton, AB, Canada; Department of Zoology, RBC College, West Bengal State University, West Bengal, India (M.P.); INSERM, Laboratory of Central Neuropeptides and Regulations of Water Balance and Cardiovascular Functions, Center for Interdisciplinary Research in Biology, College de France, Paris, France (C.L.-C.); Department of Biotechnology, Indian Institute of Technology, Roorkee, India (S.H.).
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Correspondence to Gavin Y. Oudit, Division of Cardiology, Department of Medicine, Mazankowski Alberta Heart Institute, University of Alberta, 2C2 Walter Mackenzie Health Sciences Centre, 8440-112 St, Edmonton, Alberta, Canada, T6G 2B7. E-mail gavin.oudit@ualberta.ca
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365
residue and the enzymatic processes involved in its removal from the native apelin peptide remains poorly defined. Using loss-of-function and gain-of-function strategies, we here define a critical role of angiotensin-converting enzyme 2 (ACE2) in the proteolytic cleavage of the C-terminal phenylalanine residue in pyr-apelin 13 and apelin 17. Importantly, this degradative site can be modified to produce relatively stable apelin analogues as potential therapeutic agents.

Methods

In Silico Modeling of Apelin Peptide Binding to ACE2

We selected the structure of human apo-ACE2 (PDB ID: 1R42) mainly because of its high resolution, appropriate R-factor, and errorless electron density map. This structure was equilibrated in constant pressure–temperature condition (NVT, NPT) in the Groningen Machine for Chemical Simulations (GROMACS).21 We performed knowledge-based docking using our understanding from ACE–substrate bound complex structure. For this purpose, we have used the Angiotensin II (Ang II)-bound ACE complex (PDB ID: 4APH)24 and MLN-4760 inhibitor-bound ACE2 complex (PDB ID: 1R4L).18,19,20 We modeled the ACE2–Ang II, ACE2–pyr-apelin 13, and ACE2–apelin 17 complexes using these 2 above mentioned reference structures as a template. We built the structure of peptide substrates, pyr-apelin 13 and apelin 17, using the modeling server Peptide Builder.

To model the ACE2–substrate complexes, we used the knowledge-based Autodock Vina docking method.21 The grid for docking was developed using structurally aligned ACE/ACE2 structures. Using the in-built Grid map option, we prepared the axes dimensions and center of Alberta.

Animal Care Guidelines, and animal protocols were reviewed and used at 12 weeks of age as previously described.5,26 All animal experiments were performed in accordance with the Canadian Council on Animal Care Guidelines, and animal protocols were reviewed and approved by the Animal Care and Use Committee at the University of Alberta.

Apelin Intravenous Injection, Plasma Collection, and Blood Pressure Measurement

Mice were anesthetized using 1.5% isoflurane/oxygen, and body temperature was monitored and maintained at 36°C. The aorta was cannulated via the right carotid artery using a PV loop catheter (Model 1.2F from Scisense, Transonic) to continuously record arterial blood pressure and heart rate (LabScribe 2.0, Scisense). Pyr-apelin 13 (1.4 μmol/kg body weight; Tocris Bioscience, Bristol, UK) or apelin 17 (1.4 μmol/kg body weight; Tocris Bioscience, Bristol, UK) or same volume of saline was injected via the right jugular vein, and blood was collected at 1 or 5 minutes posta post-apelin injection from the right carotid artery, and plasma was isolated. Samples were collected in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St Louis) to stabilize the apelin peptides in plasma during sample collection, which was stored at −80°C until analysis. In a separate series of experiments, pyr-apelin 13 (1.4 μmol/kg body weight), apelin 17 (1.4 μmol/kg body weight), or candesartan cilexetil (1 mg/kg body weight; Sigma-Aldrich, St Louis)27 was administered, and blood pressure was continuously measured for 1 hour postinjection.

LC-MS/MS-Based Quantification of Circulating Apelin Peptide Levels

Circulating levels of pyr-apelin 12, pyr-apelin 13, apelin 16, and apelin 17 were determined by mass spectrometry. Plasma samples were collected as described earlier. Samples were thawed and spiked with stable isotope-labeled internal standards at a concentration of 1 ng/mL. After acidification and C18-based solid-phase extraction, samples were subjected to LC-MS/MS analysis using a reversed-phase analytical column (Acquity UPLC C18, Waters) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters) in MRM mode.28 Apelin concentrations were calculated by relating endogenous peptide signals to internal individual standard apelin signals, provided that there is a minimal signal-to-noise ratio of >10. The quantification limits for plasma pyr-apelin 13 and apelin 17 were 48.4 and 12.7 pg/mL, respectively, with an intra-assay coefficient of variation of 7.41% (n=3) for pyr-apelin 13.

In Vitro ACE2 Hydrolysis of Pyr-1-Apelin-13 and Apelin-17

Pyr-apelin 13 and apelin 17 (Tocris Bioscience, Bristol, UK) were diluted in Milli-Q water. Recombinant human ACE2 (rhACE2; R&D Systems Inc, Minneapolis, MN) was dissolved in 2-(N-morpholino)ethanesulfonic acid (MES) buffer (50 mmol/L MES, 300 mmol/L NaCl, 10 μmol/L ZnCl2, 0.01% Brij L23, pH 6.5) at a concentration of 40 mmol/L. Five microliter of apelin peptide (400 μmol/L) was added to 45 μL of MES buffer and had 50 μL of rhACE2 added and incubated at 37°C for a defined period of time (final [apelin]=20 μmol/L, [rhACE2]=20 μmol/L). Experiments (n=3) were quenched by the addition of 100 μL EDTA (0.1 mol/L); 5 μL of 1 mmol/L Fmoc-Asp-OH was added as an internal standard and analyzed by C18 RP-high performance liquid chromatography (HPLC).

HPLC Analyses of In Vitro ACE2 Degradation Products

Apelin peptide products were resolved using a VyDAC C18 RPLC Protein-Peptide Column (300 Å, 5 μm, 4.6 mm×250 mm). Peptides were separated using the following method: 0 to 3 minutes 10% B, 3 to 23 minutes 10% to 45% B, 23 to 25 minutes 45% to 100% B, 25 to 26 minutes 100% B, 26 to 27.25 minutes 100% to 10% B, 27.25 to 30 minutes 10% B (A=0.1% aqueous trifluoroacetic acid [TFA]; B=0.1% TFA in acetonitrile). The extent of apelin peptide degradation was done by comparing the areas of substrate and product peaks (area of product peak/area of product peak+area of substrate peak).

Kinetic Determination of ACE2 Hydrolysis of Apelin Peptides

In vitro ACE2 assays were set up as previously described with 5, 20, 50, and 100 μmol/L apelin concentrations (n=2 at each concentration for kinetic determinations). Assays were quenched after 30, 60, and 120 seconds and analyzed by C18 RP-HPLC. The percent of apelin degradation products were converted to micromoles of products formed and used to calculate the initial velocities of apelin substrate degradation. These initial velocities were graphed against substrate concentration and analyzed using GraphPad PRISM version 4.0 software to determine kinetic parameters. A calculated ACE2 molecular mass of 85 KDa was used to determine the turnover numbers (kcat) analogous to that previously reported.29

Determination of Hydrolysis of Apelin Peptides in Plasma

Plasma (20 μL) was portioned into microfuge tubes and prewarmed to 37°C. Five microliter of apelin peptide (400 μmol/L) was added and incubated at 37°C for varying lengths of time (n=3 for each time point).
Experiments were quenched by the addition of 20 μL of 10% aqueous TFA, and 5 μL of internal standard (1 mmol/L Fmoc-Asp-OH, mouse plasma; 1 mmol/L dansyl-Tyr-Val-Gly-OH (Sigma-Aldrich, St Louis, MO; human plasma) was added. The experiments were diluted ≤100 μL with 0.1% aqueous TFA and loaded onto a pre-equilibrated Harvard Apparatus C18 spin column and centrifuged at 300g for 2 minutes and repeated twice. Samples were washed with ≥300 μL of 0.1% aqueous TFA and centrifuged at 300g for 2 minutes. Desired peptides were eluted with 300 μL of 75% acetonitrile (murine plasma) or 40% acetonitrile (human plasma) in 0.1% aqueous TFA, centrifuged at 300g for 2 minutes, and analyzed by C18 RP-HPLC. The remaining apelin peptides in plasma were quantified by comparing the apelin internal standard ratio of samples at 0 minutes to the ratio at other time points.

**Langendorff-Isolated Heart Perfusion and Ischemia-Reperfusion Injury**

Langendorff-isolated heart perfusion was used as a model of global ischemia-reperfusion (IR) injury as described before. WT male murine (C57Bl6) hearts were mounted on a Langendorff system and perfused at a constant pressure of 60 mm Hg with modified Krebs–Henseleit solution (116 mmol/L NaCl, 3.2 mmol/L KCl, 2.0 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 25 mmol/L NaHCO₃, 1.2 mmol/L KHPO₄, 11 mmol/L glucose, 0.5 mmol/L EDTA, and 2 mmol/L/L pyruvate) kept at 37°C and continuously oxygenated with 95% O₂ and 5% CO₂ to maintain a pH at 7.4. Left ventricular pressure was recorded continuously using a PowerLab system, ADInstruments, Australia; after a 10 minutes baseline recording, global ischemia was induced for 30 minutes followed by 40 minutes of reperfusion. A postconditioning protocol was used with pyr-apelin 13, pyr-apelin 12, apelin 17, or apelin 16 (1 μmol/L) given at the start of reperfusion. Hearts and coronary effluents were collected and flash-frozen in liquid nitrogen.

**ACE2 and Creatine Kinase Activity Assays**

ACE2 enzymatic activity in plasma and tissue extract was assayed using a fluorescence-based assay using 7-methoxy coumarin-Tyr-Val-Ala-Asp-Ala-Pro-Lys-(2,4-dinitrophenyl)-OH (R&D Systems) and a fluorescence plate reader (Spectramax M5 from Molecular Devices, LLC) as previously described. A specific ACE2 inhibitor, was used to eliminate nonspecific ACE2 activity. Creatine kinase activity was performed in coronary effluent from the Langendorff preparations at the indicated time points using a commercial kit (ECPK-100, BroAssay Systems, Hayward, CA).

**Western Blot Assay Using Langendorff Perfused Hearts**

Total protein extraction and immunoblotting were performed as previously described. Total ventricle tissue was homogenized using a TissueLyserII (Qiagen) with PhosSTOP and complete protease inhibitors (Roche) in CellLytic M Cell Lysis Reagent (Sigma-Aldrich), TissueLyserII (Qiagen) with PhosSTOP and complete protease inhibitors (Roche) in CellLytic M Cell Lysis Reagent (Sigma-Aldrich), separated on 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and subjected to immunoblotting of phospho-(Ser473)/total Akt, phospho-(Thr308)/total Akt, and phospho/total Akt (Cell Signaling Technology, Beverly, MA). Blots were visualized and quantified with ImageQuant LAS 4000 (GE Healthcare, Baie-d’Urfé, QC, Canada).

**Human Umbilical Vein Endothelial Cell Culture, Western Blot Analysis, and Nitric Oxide Oxidase Assay**

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously. HUVECs were serum starved in M199 culture medium containing 5 μM DAF-FM (D-23841; Molecular Probes, Inc, Eugene, OR) and cultured at 37°C for 60 minutes. Fluorescence from the harvested cells were measured in a fluorescence plate reader at excitation and emission of 495/515 nm. L-NMMA (Nω-N-monomethyl arginine; 10 μmol/L) was used as a specific nitric oxide synthase inhibitor to determine the actual NO production.

**Statistical Analysis**

Statistical analyses were performed using SPSS software (Chicago, IL; Version 19). Hypothesis testing methods included Student’s t test or 1-way analysis of variance followed by the Student–Neuman–Keuls test to compare the data between 22 experimental groups, respectively. Statistical significance is recognized at P<0.05. All the results were expressed as means±SEM.

**Results**

**Modeling of the ACE2–Apelin Complex**

We performed in silico modeling of ACE2 bound with Ang II, pyr-apelin 13, and apelin 17 and analyzed the molecular interactions between ACE2 and substrate, formation of the zinc-mediated tetrahedral intermediate, and the substrate cleavage site.

**ACE2 and Pyr-Apelin 13**

Analysis of the ACE2–Pyr-apelin13 model illustrated the electrostatic surface of the binding cleft in ACE2 with the substrate-binding site S1 and S1′ pockets binding with P1 (Pro) and P1′ (Phe) residues of pyr-apelin 13 (Figure 1A and 1B). The oxygen atom of the aromatic side chain of Tyr385 and the nitrogen atom of His401 in the S1 pocket of ACE2 interact with the carbonyl oxygen of proline (P1) of pyr-apelin 13 (Figure 1C). Importantly, His345 and Pro346 of the S1′ pocket of ACE2 interact with the amino terminal nitrogen atom of phenylalanine (P1′) of pyr-apelin 13; pyr-apelin 13 is cleaved at its C–N peptide bond between the P1–P1′ residues, resulting in a loss of the C-terminal phenylalanine residue (Figure 1D). These modeling features are consistent with the ACE2–Ang II interaction, which showed that the substrate binding pocket of the electrostatic surface of the complex model is constructed by positively charged basic and nonpolar residues (Figure S1A in the online-only Data Supplement), with well-delineated hydrogen bonds with Pro (P1) and Phe (P1′) residues of Ang II and with the zinc-bound tetrahedral intermediate (Figure S1B through S1D).

**ACE2 and Apelin 17**

The minimized model and electrostatic surface analysis of apelin-17-bound ACE2 showed that the peptide substrate fits within a positively charged cleft inside the ACE2 protein (Figure 1E). The P1 (proline) and P1′ (phenylalanine) residues in peptide substrate are intruded into a hydrophobic cleft (Figure 1F). Zinc forms a tetrahedral intermediate by making interactions with His374, His378, and Glu402 of ACE2, Pro (P1) of substrate and a water molecule. Glu375 of ACE2 deprotonates the zinc-bound water molecule (Figure 1G). His401 and Tyr385 are 2 residues forming the S1 pocket in ACE2. These 2 residues interact with carboxyl oxygen atom of Pro (P1) of the substrate apelin 17 (Figure 1H). His345 and Pro346 form the S1′ pocket in ACE2, which interacts with the nitrogen atom of the Phe (P1′) of substrate. The
Figure 1. Modeling of the interactions between angiotensin-converting enzyme 2 (ACE2) and pyr-apelin 13 and apelin 17. Pyr-apelin 13 and zinc bound with ACE2 (A) with the electrostatic surface of pyr-apelin 13 bound to ACE2 at a lower (left) and higher (right) magnification (B). Interaction between P1 site of pyr-apelin 13 and S1 pocket of ACE2 (C) and interaction between P1′ site of (Continued)
P1-P1′ cleavage site of the substrate is identified at the C–N bond between Pro (P1) and Phe (P1′), which results in the formation of apelin 16 (Figure 1I). Our in silico modeling data clearly demonstrates that ACE2 is predicted to interact with pyr-apelin 13 and apelin 17 in a manner compatible with proteolytic cleavage of the substrate.

Figure 1 Continued. pyr-apelin 13 and S1′ pocket of ACE2 (D). The minimized model (E) and the electrostatic surface analysis of apelin 17 bound to ACE2 at a lower (left) and higher (right) magnification (F). The interaction in the zinc-mediated tetrahedral intermediate (G), interaction between P1 site of apelin 17 and S1 pocket of ACE2 (H), and interaction between P1′ site of apelin 17 and S1′ pocket of ACE2 (I).

Figure 2. Genetic loss of angiotensin-converting enzyme 2 (ACE2) potentiates the hypotensive response to apelin peptides in association with elevated plasma apelin levels. Schematic of the parent apelin peptides, pyr-apelin 13 and apelin 17, and their C-terminal truncated peptides, pyr-apelin 12 and apelin 16 (A). Blood pressure in anesthetized mice showing systolic blood pressure (SBP; B), diastolic blood pressure (DBP; C), and mean arterial blood pressure (MABP; D) in response to pyr-apelin 13 (1.4 μmol/kg body weight IV). The SBP (E), DBP (F), and MABP (G) in response to apelin 17 (1.4 μmol/kg body weight IV) in anesthetized mice. Plasma levels of pyr-apelin 13 (H) and apelin 17 (I) in wild-type (ACE2+/y) and ACE2KO (ACE2−/y) mice at 1 minute and 5 minutes after intravenous administration. Plasma and tissue ACE2 activity in wild-type (ACE2−/y) and ACE2KO (ACE2−/y) mice (J). ND=not detected. Values are mean±SEM; n=8 for the vehicle group and n=10 for the apelin peptide group (A–F); n=12 for the ACE2+/y and ACE2−/y groups (G–I). *P<0.05 compared with the vehicle-injected group; #P<0.05 compared with the ACE2+/y group; $P<0.05 compared with the ACE2+/y group.
Figure 3. Pharmacological inhibition of angiotensin-converting enzyme 2 (ACE2) potentiates apelin peptides, and biochemical analysis shows a key role of ACE2 in the proteolytic degradation of apelin peptides. Plasma levels of pyr-apelin 13 (A) and apelin 17 (B) in anesthetized mice in response to pyr-apelin 13 and apelin 17 (1.4 μmol/kg body weight IV) in wild-type (ACE2+/y) mice pretreated with either placebo or MLN-4760 (10 mg/kg IP) for 1 hour. Plasma ACE2 activity in response to treatment with MLN-4760 (10 mg/kg IP) for 1 hour (C). Analysis of the degradation of pyr-apelin 13 (left) and apelin 17 (right) as illustrated by representative high performance liquid chromatography (HPLC) traces (D) and quantification (E) after incubation with plasma from wild-type (ACE2+/y) and ACE2 ko (ACE2−/y) mice (E). Hill plot (F) showing enzymatic degradation of apelin peptides when incubated with recombinant human ACE2 (rhACE2) and representative HPLC traces of the degradation of pyr-apelin 13 (G) and apelin 17 (H) in response to rhACE2, showing a complete loss of the native peptide within 120 and 240 s, respectively. Catalytic properties of the rhACE2-mediated proteolytic processing of pyr-apelin 13 and apelin 17 peptide substrates (I). Values are mean±SEM; n=8 for the vehicle group and n=10 for the MLN-4760 group (A and B); *P<0.05 compared with the vehicle-injected group; †P<0.05 compared with the ACE2+/y group.

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<td>19 ± 4</td>
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Inhibition of ACE2 Potentiates the Hypotensive Effect of Apelin Peptides in Association With Increased Apelin Peptide Levels

We next tested a critical in vivo role of ACE2 in modulating the depressor response mediated by apelin peptides (Figure 2A). In male WT mice, intravenous administration of pyr-apelin 13 and apelin 17 resulted in a predictable hypotensive response (Figure 2B through 2G). In comparison to AT1 receptor blockade with candesartan (Figure S2), the initial hypotensive action of pyr-apelin 13 was similar in magnitude but dissipated faster because of its shorter biological half-life. Interestingly, ACE2 knockout (KO) mice showed a marked increase in the hypotensive response which persisted over the 1 hour measurement period (Figure 2B through 2G). Indeed, the reduction in arterial blood pressure approached 50% of baseline values in the ACE2KO mice, demonstrating a potent vasodepressor action of...
apelin peptides in an ACE2-deficient state. The observed disparity in the hypotensive effects was concomitant with differences in steady-state plasma levels of apelin peptides in WT and ACE2KO mice. In WT mice, plasma levels of pyr-apelin 13 levels (Figure 2H) and apelin 17 (Figure 2I) were markedly increased at 1 minute and drastically lowered at 5 minutes after intravenous delivery. In contrast, plasma pyr-apelin 13 and apelin 17 levels were increased to a greater extent at 1 and 5 minutes after intravenous delivery in ACE2KO mice (Figure 2H through 2I). We confirmed that plasma and tissue ACE2 activity was markedly lowered in the ACE2KO mice (Figure 2J).

We also used a specific pharmacological inhibitor of ACE2, MLN-4760, to provide further evidence for a role of ACE2 in metabolizing apelin peptides. Pharmacological inhibition of ACE2 using in vivo administration of MLN-4760 (10 mg/kg) increased plasma levels of pyr-apelin 13 (Figure 3A) and apelin 17 (Figure 3B) in association with marked suppression of plasma ACE2 activity (Figure 3C). Incubation of pyr-apelin 13 and apelin 17 in plasma from WT and ACE2KO mice resulted in a greater loss of apelin isoforms in WT samples illustrated by the HPLC chromatograms (Figure 3D) and quantitative analysis (Figure 3E). We next performed in vitro assays to directly assess the ability of ACE2 to metabolize apelin peptides. Recombinant human ACE2 was used to generate enzyme kinetics of the degradation of pyr-apelin 13 and apelin 17. The Hill plot and Michaelis–Menten
analysis showed a classic concentration-dependent proteolytic degradation of pyr-apelin 13 and apelin 17 (Figure 3F through 3H). The catalytic efficiency determined by the ratio of $k_{\text{cat}}/K_m$ was 4× higher for pyr-apelin 13 compared with apelin 17 (Figure 3F through 3I). These data clearly demonstrate that genetic and pharmacological inhibition of ACE2 potentiates the hypotensive action of apelin peptides, and ACE2 is a key enzyme which degrades apelin peptides.
Loss of C-Terminal Phenylalanine Attenuates Apelin Peptide Physiological Effects

To elucidate the physiological relevance of C-terminal phenylalanine, we examined the effects of native and C-terminal truncated apelin peptides on blood pressure and endothelial cells and in postconditioning myocardial IR injury. The hypertensive effect of pyr-apelin 12 was clearly lowered compared with the hypotensive action of pyr-apelin 13 in WT mice (Figure 4A through 4C). Importantly, although pyr-apelin 12–mediated hypotension started to wane after 20 minutes, pyr-apelin 13–induced hypotension persisted for a longer period (Figure 4A through 4C). NO is a main final effector of the vasodilating effect of apelin in an endothelium-dependent manner.23,24 The ability of apelin to activate endothelial nitric oxide synthase (eNOS) leading to NO production in HUVECs was used as a bios assay to assess the potency of the apelin peptides. Pyr-apelin 13 and apelin 17 stimulated a robust increase in NO production (Figure 4D), which was concordant with the alterations in signaling pathways characterized by a marked increase in phosphorylation of Akt at the serine-473 residue (Figure 4E) and eNOS at the serine-1177 residue (Figure 4F) in a time-dependent manner. Equimolar doses of pyr-apelin 12 and apelin 16 resulted in lowered NO production (Figure 4D) and lowered phosphorylation of Akt and eNOS (Figure 4E and 4F).

We also used the ex vivo Langendorff heart IR preparation as a disease model to further determine the importance of the C-terminal phenylalanine residue. Using a postconditioning protocol, we showed that 1 μmol/L of pyr-apelin 13 and apelin 17 result in a predictable cardioprotection in response to global myocardial IR injury (Figure 5A and 5B). In contrast, pyr-apelin 12 and apelin 16 failed to mediate significant functional improvement after myocardial IR injury. Activation of the reperfusion injury salvage kinase signaling pathway is responsible for mediating beneficial effect in response to IR injury. Consistent with the differential response in functional recovery, Western blot analysis showed greater elevation in pAktThr308 and pAktSer473 (Figure 5C and 5D), resulting in lowered creatine kinase level in the coronary effluent in response to pyr-apelin 13 and apelin 17 (Figure 5E). In comparison, pyr-apelin 12 and apelin 16 resulted in lowered phosphorylation of the Akt pathway and failed to suppress creatine kinase release in the coronary effluent (Figure 5C through 5E). Collectively, these results illustrate a critical role of the C-terminal residue in mediating vascular, endothelial, and myocardial effects of the native pyr-apelin 13 and apelin 17 peptides.

Design and Synthesis of Apelin Analogues Resistant to ACE2 Degradative Action

The identification of a key degradative site in native apelin peptides allowed us to design synthetic apelin analogues which are potentially resistant to ACE2 action. We modified, synthesized, and purified 2 novel apelin analogues, NleAibBrF-pyr-apelin 13 and NleAibBrF-apelin 17, using a previously described method.8 Using structure–activity relationships conducted on pyr-apelin 13,8,34 we made multiple novel single amino acid substitutions combined into the same peptide with the aim of masking the susceptible C-terminal amide bond from proteolytic cleavage. These analogues were purified using HPLC, and high-resolution mass spectrometry and nuclear magnetic resonance were used to confirm the sequence of the synthesized analogues (Figure 6A and 6B). In contrast to native apelin peptides which were completely degraded within 4 minutes (Figure 3G and 3H), in vitro incubation of the apelin analogues with rhACE2 showed a marked resistance to proteolytic degradation with a minimal loss of apelin analogue over a 24-hour time period (Figure 6C). Similarly, incubation of the apelin analogues with human plasma further confirmed that NleAibBrF-pyr-apelin 13 is partially resistant to enzymatic degradation (Figure 6D; Figure S3). The therapeutic relevance of NleAibBrF-pyr-apelin 13 was shown by demonstrating that its in vivo hypotensive action was more pronounced and longer lasting compared with the native pyr-apelin 13 in WT mice (Figure 6E through 6G). These results highlight that apelin peptides can be manipulated to create apelin analogues resistant to ACE2 degradative action, supporting a potential therapeutic application of apelin analogues.

Discussion

Apelin peptides are known to mediate a range of beneficial effects in the cardiovascular system and on fluid homeostasis.1,3,31 Endogenous apelin maintains endothelium structure and function, promotes angiogenesis, counteracts the renin–angiotensin system, and regulates metabolism, myocardial hypertrophy and fibrosis, and inflammation.1,3,11 The 13 amino acid apelin peptide with a post-translational pyroglutamate substitution at the N terminus (pyr-apelin-13) has a high affinity for the apelin receptor and is the predominant isopeptide in the human myocardium and plasma.16 Importantly, pyr-apelin 13 is beneficial in patients with heart failure.18 However, apelin peptides have extremely short half-lives in vivo, suggesting the presence of a high-efficiency degradative system. Based on in vitro kinetic assays, ACE2 is also known to cleave several pep tidues essentially functioning as a monocarboxypeptidase.29 ACE2 has emerged as a dominant modulator of angiotensin metabolism and is one of the key enzymes that converts Ang II into Ang 1–7.2,5 We identified ACE2 as a major enzyme that determines the magnitude and duration of native apelin peptide action in the cardiovascular system. However, although in an ACE2-deficient state, the half-lives of the apelin peptides were prolonged, they were still metabolized at a relatively fast rate, clearly supporting a key role of other proteases. This concept is illustrated by the NleAibBrF-apelin 17 analogue which was initially more resistant than apelin-17 in human plasma, an effect lost after 60 minutes of incubation. Collectively, these results support a propensity of ACE2 to cleave the peptide amide bond characterized by proline–phenylalanine as the penultimate and C-terminal residues, respectively.

Importantly, in our current studies, we proved that the endogenous levels of ACE2 dramatically affect the action of exogenous apelin on the cardiovascular system. ACE2 is widely expressed, including expression in the heart, kidneys, vasculature, gut, and central nervous system, and is proteolytically shed by ADAM-17/TACE into the plasma.35,36 Therefore, ACE2 can be categorized into tissue ACE2 or soluble ACE2 existing in plasma. We showed
that soluble ACE2 is able to cleave apelin, as demonstrated by in vitro plasma incubation. However, the kinetics of the degradation of native apelin peptides was markedly slower compared with the in vivo responses, suggesting that tissue ACE2 is the dominant form of ACE2, which modulates native apelin peptide degradation in vivo. The use of rhACE2 in healthy human volunteers lowered plasma Ang II levels and increased plasma Ang 1–7 levels without altering systolic or diastolic blood pressure.37 The ability of rhACE2 to degrade pyr-apelin 13 and apelin 17 into product peptides with lowered vasodpressor action may explain the lack of rhACE2 depressor action in vivo in healthy human volunteers. Similarly, in Wistar–Kyoto rats, rhACE2 antagonism of Ang II–induced pressor effect was mild and transient.38 The ability of ACE2 to reduce the vasodpressor properties of apelin peptides may serve to negate the hypotensive action resulting from its role as a physiological negative regulator of the renin–angiotensin system. ACE2 is known to metabolize other vasoactive peptides, and ACE2 inhibition is expected to increase Ang II and lower Ang 1–7 plasma levels, leading to an increase in blood pressure, thereby counteracting the hypotensive action of apelin peptides. The persistent hypotensive effects of the apelin peptides is likely driven by the activation of signaling transduction pathways, such as phosphorylation of Akt/eNOS and Erk1/2.8,32,39,40 Based on blood pressure responses, loss of function experiments involving ACE2, and degradation assays, ACE2 has a greater efficacy for metabolizing pyr-apelin 13 compared with apelin 17.

Our results are consistent with several elegant biochemical studies showing that apelin-induced activation of intracellular signaling involves both G-protein and β-arrestin-dependent pathways and that the β-arrestin-dependent effects require the presence of the C-terminal phenylalanine residue.15,41 The loss of phenylalanine in pyr-apelin 13 and apelin 17 clearly lowered its ability to produce a hypotensive effect through systemic vasodilation. These results are consistent with previous studies using the apelin 17 peptide where the deletion of the phenylalanine reduced its hypotensive effect by 80%.12 We linked these differences in physiological response to the differential ability of the apelin peptides to activate and increase NO production. Given the beneficial effect of apelin peptides coupled with its short half-life in vivo, modification of apelin by protecting the C-terminal phenylalanine residue is a critical step in the generation of stable apelin analogues with therapeutic potential. Our future experimental work will include detailed pharmacokinetic analysis in a large animal model and pharmacodynamic studies with receptor binding and second messenger generation.

Perspectives

Apelin peptides mediate important physiological effects in the heart and vasculature and are important pharmacological targets. Decreased apelin action has been linked to systemic and pulmonary arterial hypertension and heart failure progression. However, therapeutic applications of apelin peptides are limited by their short biological half-lives. Genetic and pharmacological inhibition of ACE2 activity resulted in greater elevation in plasma levels of pyr-apelin 13 and apelin 17 and enhanced vasodepressor action. Importantly, ACE2 catalytic efficiency for apelin peptides is similar to that compared with its classic peptide substrate, angiotensin II. Pyr-apelin 12 and apelin 16 peptides were devoid of cardioprotective effects in the myocardial IR model, whereas they only partially lowered blood pressure and stimulated Akt signaling pathways and eNOS in HUVECs compared with their respective native apelin peptides. ACE2-mediated cleavage of the C-terminal phenylalanine residue from pyr-apelin 13 and apelin 17 resulted in loss of function, thereby functioning as a key enzyme which suppresses apelin peptide action. ACE2-resistant pyr-apelin 13 and apelin 17 analogues were designed, synthesized, and tested and shown to be resistant to ACE2 proteolytic properties. We have clearly demonstrated that ACE2 partially inactivates apelin peptides, and apelin analogues can be synthesized which are resistant to ACE2 inactivation. Because ACE2 negatively regulates the renin–angiotensin system by converting the pressor peptide, angiotensin 1–7, into angiotensin 1–7, the ability of ACE2 to negatively regulate apelin action may have a physiological adaptive role by limiting ACE2 vasodpressor action in vivo.

Acknowledgments

W. Wang and G.Y. Oudit designed the experiments and prepared the article and figures with contributions from all other authors; S.M.K. McKinnie and J.C. Vederas synthesized apelin analogues; S.M.K. McKinnie, T. McDonald, and J.C. Vederas performed all enzymatic kinetic studies; M. Farhan and A.G. Murray cultured human umbilical vein endothelial cells (HUVECs) and performed endothelial nitric oxide synthase (eNOS) Western blot assay; B. McLean guided the Western blot assay on Langendorff-perfused hearts and edited the article; C. Llorens-Cortes assisted with the experimental design and edited the article; M. Paul and S. Hazra modeled the apelin-ACE2 binding in silico; W. Wang performed blood pressure measurement in vivo, heart perfusion ex vivo; angiotensin-converting enzyme 2 (ACE2) and creatine kinase (CK) activities and nitric oxide (NO) level assays. We acknowledge technical support from Attioquant Diagnostics (Vienna, Austria).

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Disclosures

None.

References


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**What Is New?**

- Angiotensin-converting enzyme 2 is a major enzyme which degrades pyr-apelin 13 and apelin 17 in vivo.
- Angiotensin-converting enzyme 2–resistant analogues can be designed and synthesized.

**What Is Relevant?**

- Inhibition of angiotensin-converting enzyme 2 action potentiated apelin peptide levels and action.

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**Novelty and Significance**

- Stabilization of apelin peptides potentiates their therapeutic effects.

**Summary**

Angiotensin-converting enzyme 2 is a major enzyme involved in proteolytic degradation of pyr-apelin 13 and apelin 17 peptides, thereby negatively regulating their effects.
Angiotensin-Converting Enzyme 2 Metabolizes and Partially Inactivates Pyr-Apelin-13 and Apelin-17: Physiological Effects in the Cardiovascular System

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ANGIOTENSIN CONVERTING ENZYME 2 METABOLIZES AND PARTIALLY INACTIVATES PYR-APELIN-13 AND APELIN-17: PHYSIOLOGICAL EFFECTS IN THE CARDIOVASCULAR SYSTEM

by

Wang Wang¹,², Shaun M. K. McKinnie³, Maikel Farhan⁴, Manish Paul⁵, Tyler McDonald³, Brent McLean¹,², Catherine Llorens-Cortes⁶, Saugata Hazra⁷, Allan G. Murray⁴, John C. Vederas³ and Gavin Y. Oudit¹,²

¹Division of Cardiology, Department of Medicine, ²Mazankowski Alberta Heart Institute, ³Department of Chemistry, Faculty of Science, ⁴Division of Nephrology, Department of Medicine, University of Alberta, Edmonton, Canada, ⁵RBC College, West Bengal State University, India, INSERM, Laboratory of Central Neuropeptides and Regulations of Water Balance and Cardiovascular Functions, CIRB College de France, Paris, France, ⁷Department of Biotechnology, Indian Institute of Technology, Roorkee, India
Figure S1. Modeling of the interactions between angiotensin converting enzyme 2 (ACE2) and angiotensin II. A computer-simulated model of angiotensin II and zinc-bound ACE2 (left) and the electrostatic surface of angiotensin II and zinc-bound ACE2 (right) (A). Catalytic site of the ACE2-angiotensin II interaction (B), interaction between the P1 site of angiotensin II and S1 pocket of ACE2 (C) and the interaction between the P1' site of angiotensin II and S1' pocket of ACE2 (D).
Figure S2. Blood pressure in anaesthetized mice showing systolic blood pressure (SBP) (A), diastolic blood pressure (DBP) (B), and mean arterial blood pressure (MABP) (C) in response to AT1 receptor blockade using candesartan (1 mg/kg body weight i.v.) (n=6). Comparative hypotensive action of pyr-apelin-13 (1.4 µmol/kg body weight) and candesartan at 20 and 60 min following i.v. administration (D). *p<0.05 compared with the basal blood pressure; #p=0.05 compared with pyr-apelin-13.
Figure S3. Incubation of the apelin analogues with human plasma compared to native apelin peptides further confirming that these apelin analogues are resistant to enzymatic degradation as illustrated by representative HPLC traces.