Loss of Apelin Exacerbates Myocardial Infarction Adverse Remodeling and Ischemia-reperfusion Injury: Therapeutic Potential of Synthetic Apelin Analogues

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Background—Coronary artery disease leading to myocardial ischemia is the most common cause of heart failure. Apelin (APLN), the endogenous peptide ligand of the APJ receptor, has emerged as a novel regulator of the cardiovascular system.

Methods and Results—Here we show a critical role of APLN in myocardial infarction (MI) and ischemia-reperfusion (IR) injury in patients and animal models. Myocardial APLN levels were reduced in patients with ischemic heart failure. Loss of APLN increased MI-related mortality, infarct size, and inflammation with drastic reductions in prosurvival pathways resulting in greater systolic dysfunction and heart failure. APLN deficiency decreased vascular sprouting, impaired sprouting of human endothelial progenitor cells, and compromised in vivo myocardial angiogenesis. Lack of APLN enhanced susceptibility to ischemic injury and compromised functional recovery following ex vivo and in vivo IR injury. We designed and synthesized two novel APLN analogues resistant to angiotensin converting enzyme 2 cleavage and identified one analogue, which mimicked the function of APLN, to be markedly protective against ex vivo and in vivo myocardial IR injury linked to greater activation of survival pathways and promotion of angiogenesis.

Conclusions—APLN is a critical regulator of the myocardial response to infarction and ischemia and pharmacologically targeting this pathway is feasible and represents a new class of potential therapeutic agents. (J Am Heart Assoc. 2013;2: e000249 doi: 10.1161/JAHA.113.000249)

Key Words: angiogenesis • cardiomyopathy • heart failure • ischemia-reperfusion injury • myocardial infarction

Apelin (APLN) is the endogenous ligand for the apelin receptor (APJ receptor) and is synthesized as a 77-amino acid prepropeptide which is processed into C-terminal fragments denoted by their lengths as Apelin-36, Apelin-19, Apelin-17 and Apelin-13. APLN is predominantly expressed in the endocardial and vascular endothelial cells while the APJ receptor is localized to endothelial and smooth muscle cells as well as cardiomyocytes, allowing for autocrine and paracrine effects of APLN in the heart. APLN mediates positive inotropic effect on isolated cardiomyocytes, isolated perfused rat heart and in vivo and mediates endothelium-dependent vasodilation. Genetic variation in the APJ receptor modifies the progression of heart failure in patients with dilated cardiomyopathy and the APLN /APJ system is compromised in human heart failure. In patients with chronic heart failure, APLN administration increased cardiac index and lowered peripheral vascular resistance in the absence of hypotension providing a promising new drug target for heart failure.

Coronary artery disease is now the most common cause of heart failure. APLN promotes the phosphorylation of Akt and increases the proliferation of endothelial cells in vitro suggesting an important proangiogenic role.
plethora of biochemical and cellular effects of APLN, we hypothesized that loss of APLN may enhance the suscepti-
bility to myocardial ischemic injury. We used APLN knockout
(APLN<sup>−/−</sup>) and wildtype (APLN<sup>+/+</sup>) mice and showed that loss of APLN impaired the functional recovery, post-MI remodeling
and angiogenesis and exacerbate myocardial ische-
mia-reperfusion (IR) injury. We used novel synthetic APLN
analogues, which were markedly more resistant to proteolytic
clavage by angiotensin converting enzyme 2 (ACE2) than
native pyr-1-apelin-13, and identified one analogue that
mimicked the function of APLN and resulted in marked
protection against ex vivo and in vivo myocardial IR injury. We
conclude that APLN critical regulates the myocardial response
to ischemia and pharmacologically targeting this pathway is
feasible and represents a new class of potential therapeutic
agents.

Methods

Experimental Animals

APLN -deficient (APLN<sup>−/−</sup>) and littermate wildtype (APLN<sup>+/+</sup>)
mice were generated and bred in a C57BL/6 background as
previously described. All animal experiments were carried
out 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.

Human Explanted Hearts

Cardiac tissues from patients with subacute MI or idiopathic
dilated cardiomyopathy and with advanced heart failure
were collected from the explanted hearts at the time of
cardiac transplantation as part of the Human Explanted
Heart Program (HELP) at the Mazankowski Alberta Heart
Institute. Nonfailing control hearts were obtained by the
Human Organ Procurement and Exchange (HOPE) program.
All experiments were performed in accordance with the
institutional guidelines and were approved by Institutional
Ethics Committee.

Myocardial Infarction

Ten-week-old male mice of both genotypes were subjected to
MI by permanent ligation of the proximal left anterior
descending (LAD) coronary artery in a manner blinded to
the genotype as previously described. Anaesthetized
mice underwent left thoracotomy in the fourth intercostal
space. The pericardium was opened to expose the left
ventricle (LV) and the LAD was encircled and ligated with a
6-0 silk suture; the muscle and skin were closed in layers. In
sham-operated mice, LAD was encircled but not ligated.
Animals were inspected at least 2 times daily. Following
1 day, 3 days, and 7 days after LAD ligation or sham-oper-
ation, mice were anaesthetized and sacrificed. Hearts were
quickly excised, dissected into infarct, peri-infarct, and
noninfarct regions, and then flash-frozen separately for further
protein and RNA analyses. For immunohistochemical analysis,
whole hearts were arrested in diastole with 1 mol/L KCl and
then fixed in 10% formalin or embedded in optimal cutting
temperature compound (OCT) and flash frozen.

Infarct Size Measurement and Neutrophil and
Macrophage Staining

For infarct size measurement, hearts were quickly excised and
sectioned in 0.5-mm slices from apex to the point of ligation,
then incubating with 1% Triphenyl Tetrazolium Chloride at
37°C for 10 minutes. The brick red represents viable tissue,
while pale indicates necrosis. Image Proplus software was
used for image analysis. Infarct size was reported as a
percentage of the total LV size. Neutrophils and macrophages
were stained in sham, 1-day and 3-days post-MI LVs using rat
antimouse neutrophil (AbD Serotec) and rat antimouse Mac-3
(BD Biosciences) primary antibodies and Cy3 conjugated goat
antirat and Alexa Fluor 488 conjugated goat antirabbit
secondary antibodies (Invitrogen), respectively. The sections
were visualized and imaged under fluorescence microscope
(Olympus IX81) and images were analyzed using Metamorph
software (Version 7.7.0.0).

APLN and CD31 Immunofluorescence

APLN and CD31 double immunofluorescence staining was
carried out in human heart and coronary arteries as well as in
murine heart tissues. Briefly, 5 μm thick OCT embedded
cryosections were fixed with 4% paraformaldehyde followed
by rehydration, permeabilization and blocking with PBS, 0.1%
triton X-100 and 4% BSA, respectively. The sections were then
incubated with primary antibodies, goat anti-APLN (Santa Cruz
Biotechnology Inc.) and mouse antihuman CD31 (BD Biosciences)
for human LV and goat anti- APLN (Santa Cruz
Biotechnology Inc.) and rat antimeouse CD31 (BD Biosciences),
at 4°C for overnight. The sections were then washed with PBS
and incubated with secondary antibodies, Alexa Fluor 488
conjugated donkey antigoat and Alexa Fluor 594 conjugated
donkey antigoat for mouse LV and Alexa Fluor 488 conjugated
donkey antigoat and Alexa Fluor 594 conjugated
donkey antigoat and Alexa Fluor 488 conjugated
donkey antigoat for mouse LV and Alexa Fluor 488 conjugated
donkey antigoat and Alexa Fluor 594 conjugated
donkey antigoat and Alexa Fluor 488 conjugated
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Echocardiography Measurements and Invasive Pressure-Volume Analysis

Transthoracic echocardiography was performed noninvasively as described previously using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics). The temporal resolution for M-mode imaging in this system is a pulse repetition frequency of 8 kHz with an axial resolution of 55 μm, lateral resolution of 115 μm, focal length of 12.7 mm, and depth of field of 2.2 mm. Mice were anesthetized with 0.8% isoflurane for the duration of the recordings. LV ejection fraction (EF) was calculated as a measure of systolic function using the following equation: EF (%) = ([LV end-diastolic volume – LV end-systolic volume] / LV end-diastolic volume) / 100. The maximal anteroposterior left atrial (LA) diameter was measured by M-mode in the parasternal long-axis view and used as LA size. Qualitative and quantitative measurements were made offline using analytic software (VisualSonics). Electrocardiogram kilohertz-based visualization (EKV™) software analysis produced offline reconstruction for simulated 250 to 1000 Hz static and cine loop images. Modified parasternal long axis EKV loops were also used to measure EF via Simpson’s method. M-mode images were used to measure LV chamber sizes and wall thicknesses. The wall motion score index (WMSI) was calculated based on the American Society of Echocardiography recommended assessment of wall motion function of the 17-segment LV model. In the murine model, use of WMSI and analysis of segmental wall motion abnormalities in the post-MI hearts has been validated. A flame dried 3-necked round bottom flask equipped with a stirring bar was flushed with Ar gas. Fmoc-4-bromophenylalanine (0.933 g, 2.0 mmol) was dissolved in dried DCM and cooled to 0°C. Diisopropylcarbodiimide (0.155 mL, 1.0 mmol) was added to the mixture and stirred at 0°C for 20 minutes. The reaction mixture was concentrated in vacuo and redissolved in a DMF:DCM solution (3:1). Wang resin (2.00 g) was added to a solid phase peptide synthesis vessel and washed with dry DCM (2 x 10 mL) and DMF (2 x 10 mL). The resin was preswollen by bubbling with Ar gas in DMF (10 mL) for 1 hour and filtered. The activated Fmoc-4-bromophenylalanine anhydride was added to the resin followed by catalytic 4-dimethylaminopyridine (DMAP) and bubbled under Ar gas for 1.5 hours. The solution was drained and the resin was washed with DMF (3 x 10 mL). To cap additional reactive sites on the resin, 20% acetic anhydride in DCM (15 mL, 15 minutes) was used and followed by washing with DMF (3 x 10 mL) and DCM (3 x 10 mL), yielding Fmoc-4-bromophenylalanine on Wang resin in 0.5 mmol/g loading.

Synthesis and Characterization of Novel APLN Analogues

Reagents and solvents

All commercially available reagents and protected amino acids were purchased and used without further purification. Dichloromethane (DCM) used for anhydrous reaction was distilled over calcium hydride prior to use. High-performance liquid chromatography (HPLC) grade dimethylformamide (DMF) and methanol were used without further purification.

Loading of C-terminal 4-bromophenylalanine onto Wang resin

N-methylmorpholine (NMM) (6 equiv) was added to a solution of Fmoc protected amino acid (5.0 equiv to resin loading), 1-hydroxybenzotriazole hydrate (HOBT) (5.0 equiv) and benzo-triazol-1-yloxypyrrolidinophosphonium hexafluorophosphate (PyBOP) (4.9 equiv) in DMF (10 mL). The solution was allowed to preactivate for 5 minutes. The solution was transferred to the reaction vessel containing preswelled resin and was bubbled with argon for 2 hours. A small sample of the peptide was cleaved from the resin (by treatment with 95% trifluoroacetic acid (TFA)/2.5% triisopropylsilane (TIPS)/2.5% water (H2O) for 2 hours) and the completion of the reaction...
was determined by matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) analysis using 4-hydroxy-α-cyanocinnamic acid (HCCA) as a matrix. Resin was washed with DMF (3 x 10 mL), then 20% Ac₂O in DMF (10 mL) was added to the resin for 10 minutes to affect end capping. Resin was again washed with DMF (3 x 10 mL). Then 20% piperidine in DMF (3 x 10 mL) was added to remove the N-terminal Fmoc protecting group, this reaction was monitored by ultraviolet-visible (UV-Vis) spectroscopy, observing the dibenzofulvene-piperidine adduct at λ = 301 nm.

General procedure for peptide synthesis using automated Fmoc Solid Phase Peptide Synthesis

All peptides were synthesized on a CEM Liberty 1 Microwave Peptide Synthesizer. Solid phase synthesis was carried out on a 0.1 mmol scale using Fmoc chemistry on Wang resin (0.65 mmol/g loading). Commercially available protected amino acids and were loaded on the peptide synthesizer as 0.2 mol/L solutions in DMF. All amino acid subunits were coupled using O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as the activating agent and heated at 70°C for a 5 minutes coupling time. Fmoc residues were deprotected using a 20% solution of piperidine in DMF using UV-Vis spectroscopy to observe the dibenzofulvene-piperidine adduct absorption monitored at λ = 301 nm.

Syntheses of NleInpBrF pyr-1-apelin-13 and NleAibBrF pyr-1-apelin-13 analogues

For both analogues, 4-bromophenylalanine-loaded Wang resin was subjected to Fmoc deprotection as described above, and either Fmoc-Inp-OH or Fmoc-Aib-OH were coupled onto the free amine to initiate the synthesis of NleInpBrF or NleAibBrF pyr-1-apelin-13, respectively. The following amino acids were coupled as previously described in the following order: Fmoc-Nle-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-His(Trt)-OH, Fmoc-Ser(O₂Bu)-OH, Fmoc-C-Leu-OH, Fmoc-Arg(Pmc)-OH, Fmoc-Pro-OH, Fmoc-Arg(Pmc)-OH, and pyroglutamic acid.

General procedure for cleavage and purification of pyr-1-apelin-13 analogues

To simultaneously cleave the peptide from Wang resin and remove side chain protecting groups, a solution of 95:2.5:2.5 TFA:anisole:H₂O was added to the resin-bound peptide for 2 hours. The resin beads were removed via filtration through glass wool and the filtrate was concentrated in vacuo. The crude peptide was obtained by precipitation with cold Et₂O. The crude peptide was redissolved in a 9:1 H₂O:methanol (0.1% TFA) solution and purified by HPLC, which was performed on a Varian Prostar chromatograph equipped with a model 325 variable wavelength UV detector and a Rheodyne 7725i injector fitted with a 1000 μL sample loop. The column used for semipreparative purification was a Phenomenex Luna C18(2) column (5 μm, 100 x 250 mm), and for analytical purification was a C18 reverse-phase peptide/protein HPLC column (Vydac, 4.6 x 250 mm, 5 μm) using H₂O (0.1% TFA) and acetonitrile (0.1% TFA) as eluents. All HPLC solvents were filtered with a Millipore filtration system under vacuum before use.

The HPLC method followed was: gradient beginning at 2% acetonitrile, climb to 50% acetonitrile over 10 minutes, then climb to 100% acetonitrile over 4 minutes, remain at 100% for 4 minutes, return to 2% over 4 minutes, hold at 2% over 3 minutes (flow rate 1 mL/min [analytical] or 2.5 mL/min [semipreparative] with UV detection at 220 nm). The peptide was collected as a sharp peak at 13.5 minutes and solvent was removed in vacuo. The residue was then resuspended in 0.1% TFA in H₂O and lyophilized to give the final product. NleInpBrF pyr-1-apelin-13 (apelin analogue I) was isolated as a white solid powder after lyophilization in 25.6% yield (10.5 mg purified). NleAibBrF pyr-1-apelin-13 (apelin analogue II) was isolated as a white solid powder after lyophilization in 36.4% yield (17.9 mg purified).

Angiotensin-converting Enzyme 2 Proteolysis Assay of pyr-1-apelin-13 and APLN Analogues

Recombinant angiotensin converting enzyme 2 (rACE2) was suspended in Tris buffer (25 mmol/L Tris, 200 mmol/L NaCl, 5 μmol/L ZnCl₂, pH 8.0) to a final concentration of 1 μmol/L. 1 mmol/L solutions of APLN peptides (pyr-1-apelin-12 [65 to 76] [New England Peptide Inc] and pyr-1-apelin-13 [65 to 77] [Tocris Bioscience]) and APLN analogues were prepared in Milli-Q water. A modified procedure was adapted to determine the extent of susceptibility of APLN peptides and APLN analogues to ACE2 proteolysis.24 5 μL of 1 μmol/L recombinant ACE2 was added to 90 μL of buffer (25 mmol/L Tris, 200 mmol/L NaCl, 5 μmol/L ZnCl₂, pH 8.0) and 5 μL of aqueous 1 mmol/L APLN peptide or APLN analogue were added to the reaction mixture for varying quantities of time at room temperature or 37°C to achieve final concentrations of 5 mmol/L rACE2 and 5 μmol/L APLN peptide/analgoue, respectively. Reactions were quenched upon addition of 100 μL of 100 mmol/L ethylenediaminetetraacetic acid (EDTA) pH 7.0, and products were monitored by both HPLC and MALDI-TOF analyses. Analytical HPLC separation of peptides was accomplished using a C18 reverse-phase peptide/protein HPLC column (Vydac, 4.6 x 250 mm, 5 μm) using a gradient of 10% to 45% B (A: H₂O [0.1% TFA]; B: acetonitrile [0.1% TFA]), a flow rate of 1 mL/min was used and peptides were detected by absorbance at 220 nm. The
amount of hydrolyzed product was determined by comparing the areas under the substrate and product peaks after integration as previously reported.\textsuperscript{24} Hydrolysis products and initial substrates were analyzed on a Perspective Biosystems Voyager\textsuperscript{TM} Elite MALDI-TOF MS using 4-hydroxy-\textalpha{-}cyanocinnamic acid (HCCA) as a matrix to confirm masses.

Isolated Langendorff Heart Perfusion and Ischemia-Reperfusion Protocol

Langendorff heart perfusion was used to study the contractile function. Mice were heparinized and anaesthetized with 1.5\% to 2\% isoflurane inhalation. Heart was excised and mounted on Langendorff system and perfused at a constant pressure of 80 mm Hg with modified Krebs-Henseleit solution (116 mmol/L NaCl, 3.2 mmol/L KCl, 2.0 mmol/L CaCl\textsubscript{2}, 1.2 mmol/L MgSO\textsubscript{4}, 25 mmol/L NaHCO\textsubscript{3}, 1.2 mmol/L KH\textsubscript{2}PO\textsubscript{4}, 11 mmol/L glucose, 0.5 mmol/L EDTA and 2 mmol/L pyruvate), which was kept at 37\degree C and continuously oxygenated with 95\% O\textsubscript{2} and 5\% CO\textsubscript{2} to maintain a pH at 7.4. By inserting a water-filled balloon into the LV chamber, which on the other side connected to a pressure transducer, the pressure changes were recorded by PowerLab system (ADInstruments). After stabilization and 10 minutes baseline recording, global ischemia was induced for 30 minutes followed by 40 minutes of reperfusion and hearts were flash frozen in liquid nitrogen. The coronary effluents were collected at baseline, at the start of reperfusion, and following 10 minutes of reperfusion, for the determination of CK activity. A postconditioning protocol with wildtype (APLN\textsuperscript{+/y}) hearts was used with APLN analogue I and II (1.5 \mu g/mL for 20 minutes) given at the start of reperfusion.

Myocardial Ischemia-Reperfusion Injury In-Vivo

Ten-week-old male mice of both genotypes were subjected to ischemia by LAD ligation followed by reperfusion. Reliability of IR was confirmed by Evan’s blue staining. Anaesthetized mice underwent left thoracotomy in the fourth intercostal space. The pericardium was opened to expose the LV and the LAD coronary artery was encircled and ligated with a 6-0 silk suture for 30 minutes; the reperfusion was established by releasing the ligation. In sham-operated mice, the LAD was encircled but not ligated. Following 3 hours of reperfusion or sham-operation, mice were reanaesthetized and sacrificed. The non-IR parts of the hearts were distinguished with Evan’s Blue by injection into the coronary circulation after the LAD was ligated again at the same location. Hearts were quickly excised and sectioned in 1-mm slices from apex to the point of ligation, then incubating with 1\% triphenyl tetrazolium chloride (TTC) (Sigma) at 37\degree C for 10 minutes. The blue stained area represents the non-IR myocardium while the rest was termed area at risk (AAR) in which the brick red represents viable tissue, while the white/yellowish region indicates nonviable tissue. In a separate group of wildtype mice, APLN analogue II (60 \mu g/kg/min for 10 minutes) was administered via the right internal jugular vein at the moment of reperfusion.

CK Activity Assay

Perfusates from the Langendorff preparations and plasma from 1-day post-MI mice were collected and preserved at \textminus 80\degree C for creatine kinase (CK) activity assay using a commercial kit (BioAssay).

Aortic Ring Angiogenesis Assay

The ex vivo mouse ring angiogenesis assay was carried as previously described.\textsuperscript{25} Thoracic aorta excised from 10-week old APLN\textsuperscript{+/y} and APLN\textsuperscript{+/y} mice under sterile conditions were cut into 1 mm-thick rings under a stereomicroscope. The aortic rings were placed between two layers of Matrigel matrix (BD Biosciences; 354234) in a 48-well culture plate. EBM-2 media (Lonza; CC-3156) with 2\% FBS was added containing PBS, rhVEGF (20 ng/mL; Lonza; CC-4114A), APLN analogue I and II (100 ng/mL). Rings were cultured at 37\degree C and 5\% CO\textsubscript{2} for 12 days. Phase contrast images were taken using an Olympus IX81 microscope.

Fibrin Gel Bead Angiogenesis Assay

Human endothelial progenitor cells (hEPCs) were isolated from peripheral blood as previously described.\textsuperscript{26} Briefly, mononuclear blood cells were isolated from leukopheresis products under a protocol approved by the Health Ethics Review Board of the University of Alberta. After density separation over a ficoll gradient, CD34\textsuperscript{+} cells were isolated using antibody-coated magnetic beads (StemCell Technologies). The late outgrowth endothelial colony-forming cells (ECFC) clones were expanded in EBM-2 medium with 10\% FBS, and routinely monitored for expression of endothelial marker gene products using quantitative RT-PCR, flow cytometry, or Western blot. Where indicated, hEPCs were transfected with 50 mmol/L non-silencing or siRNA directed to APLN (Qiagen) and in vitro angiogenesis of hEPCs was evaluated as described previously.\textsuperscript{27,28} EPCs transfected with si Nonsense (siNS) or siAPLN were loaded onto Cytoex (Sigma) beads (~400 cells/bead) and cultured for 2 hours, then the beads were suspended in fibrinogen/fibronectin solution (2 mg/mL) containing aprotinin (0.15 U/mL) and 0.625 U/mL thrombin was added. Images of the beads after 16 hours culture were captured using a 20\times objective and a charge-coupled device (CCD) camera-equipped inverted microscope (Leica).
number and length of sprouts were analyzed using image analysis software (OpenLab) of 30 beads/experiment. Sprout length was grouped into tertiles established from mock-transfected hEPC (<75, 76 to 125, >126 μm).

Isolated Cardiomyocyte Contractility
Adult murine LV cardiomyocytes were isolated and cultured as previously described except that the 2,3-butanedione monoxime was omitted to preserve contractile function. Briefly, mice were injected with 0.05 mL of 1000 USP/mL heparin for 15 minutes and then anesthetised using 2% isoflurane (1 L/min oxygen flow rate) provided through a nose cone. After opening the chest cavity, the heart was quickly excised and perfused using a Langendorff system within 45 s. Following 3-minute perfusion, the heart was then digested with 2.4 mg/mL collagenase type 2 (Worthington) for 7 to 8 minutes. After sufficient digestion, the ventricles were removed, dissociated using forceps and transfer pipettes, and resuspended in stopping buffer (10% FBS perfusion buffer). The isolated cardiomyocytes were then exposed to increasing Ca²⁺ concentrations (100 μmol/L, 400 μmol/L, and 900 μmol/L) for 15 minutes each and were kept in perfusion buffer solution (pH 7.4). An aliquot of isolated cardiac myocytes were transferred in a glass-bottomed recording chamber on top of inverted microscope (Olympus IX71) and allowed to settle for 5 to 6 minutes. Cells were superfused at a rate of 1.5 to 2 mL/min with modified Tyrode’s solution (containing in mmol/L: 135 NaCl, 5.4 KCl, 1.2 CaCl₂, 1 MgCl₂, 1 NaH₂PO₄, 10 taurine, 10 HEPES, 10 glucose; pH 7.4 with NaOH). The perfusion solutions were heated to an in-bath temperature of 35 to 36°C using in-line heater (SH-27B, Harvard Apparatus) controlled by automatic temperature controller (TC-324B, Harvard Apparatus). Quiescent rod-shaped myocytes with clear striations were selected for study. Platinum-wire electrodes were placed near the cell just outside of the microscope view at 400 x magnification. Cardiomyocytes were paced at 1 Hz with voltage of 3 to 4 V (50% above threshold) and pulse duration of 2.5 ms using S48 stimulator (Grass Technology). Sarcomere length was estimated in real time from images captured at a rate 200 frame/sec via 40 x objective (UAPO 40 x 3/40, Olympus) using high-speed camera (IMPERX IPX- VGA-210, Aurora Scientific). Sarcomere length was calculated by HVSL software v. 1.75 (Aurora Scientific) using auto-correlation function (ACF/Sine-fit) algorithm. Myocytes were paced for at least 2 minutes. Only recordings of myocytes that produced stable contractions at a steady-state were selected for analysis. At about 2 minutes of stimulation time, 10 consecutive contractions were selected and averaged to reduce noise and make calculations of derivatives more precise. Averaged contraction was used to calculate fractional shortening (FS), relaxation times (t50 and t90), and ±dL/dt. Calculations were performed in Origin 8.5 (OriginLab) using custom-made script of built-in LabTalk language.

TUNEL, CD31, Lectin and HIF-1α Immunofluorescence
In situ DNA fragmentation was detected by TUNEL assay kit (Invitrogen) according to manufacturer’s instructions. Briefly, 5 μm thick LV cryosections were fixed with 4% paraformaldehyde and washed in Dulbecco’s PBS. The sections were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate and washed with wash buffer. After one hour incubation with DNA labeling solution (terminal deoxynucleotidyl transferase and BrdUTP in reaction buffer) the sections were treated with Alexa Fluor 488 conjugated mouse anti-BrDU and counterstained with propidium iodide. The sections were mounted using Prolong Gold antifade mounting medium (Olympus IX81). Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) positive cells were counted using 20× magnification images with MetaMorph (Basic version 7.7.0.0) software and magnitude of apoptosis was expressed as a percentage of apoptotic cells.

Vascular endothelial cells were stained in sham and 7 days post-MI LVs by immunohistochemistry using rat anti-CD31 (BD Pharmingen) primary antibody and Cy3 conjugated goat antirat (Invitrogen) secondary antibody. Briefly, 5-μm thick OCT-embedded cryosections were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X100, and blocked with 4% bovine serum albumin. The sections were then incubated with primary antibody (1:10, clone MEC 13.3, BD Pharmingen) overnight at 4°C in a humidified chamber. After several washings the sections were incubated with Cy3 conjugated goat antirat (Invitrogen) secondary antibody for 1 hour at 37°C. The sections were visualized and imaged under fluorescence microscope (Olympus IX81) after mounting with Prolong Gold antifade mounting medium (Invitrogen). The images were analyzed using Metamorph software (Version 7.7.0.0) and the magnitude of vascularization was represented as CD31+ area/mm² of the myocardium.

Lectin immunofluorescence assay using fluorescein conjugated Ricinus communis agglutinin I (RCA; lectin; Vectorlabs) perfusion method was used to assess blood flow and new vessel formation in peri-infarct area after 7 days of MI or sham surgery in APLN+/y and APLN-/y mice. Briefly, heparinized mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and right jugular vein was cannulated using PE10 tubing. 0.2 mg of lectin in 100 μL saline was injected into the jugular vein; 50 μL of saline was injected immediately to expel lectin from cannula. After 18 minutes of lectin circulation, 0.2 mg of papaverine HCl (Sigma Aldrich) in
50 µL saline was injected through the same cannula to promote maximal dilation of blood vessels and circulated for 2 minutes. After 20 minutes of lectin administration, hearts were collected in OCT in cold conditions. Five-µm thick cryosections were cut, fixed with 4% paraformaldehyde for 20 minutes followed by rehydration using PBS for 30 minutes. The sections were mounted in Prolong Gold antifade mounting medium with DAPI (Invitrogen), visualized, and imaged under fluorescence microscope (Olympus IX81). Images were analyzed using Metamorph (Version 7.7.0.0) software and magnitude of vascularization in peri-infarct area was represented as lectin perfused area/mm² of the myocardium.

To assess the nuclear translocation of hypoxia inducible factor-1α (HIF-1α), a key mediator of cellular adaptation to hypoxia, immunofluorescence was performed on 5-µm thick formalin fixed-paraffin embedded sections. Briefly, after deparaffinization sections were heated to 95 to 100°C in citrate buffer pH 6.0 for the antigen retrieval. The sections were then immune-stained with anti-HIF-1α primary antibody (Novus Biologicals) and TRITC conjugated antirabbit secondary antibody (Invitrogen) and counterstained DAPI. The section were mounted and visualized under fluorescence microscope (Olympus IX81) and nuclear HIF-1α expression in the peri-infarct region was morphometrically measured using Metamorph (Version 7.7.0.0) software.

**TaqMan Real-time PCR**

RNA expression levels of various genes were determined by TaqMan real-time PCR as previously described (see Table S1 for list of primers and probes). Total RNA was extracted from flash-frozen tissues using TRIzol, and cDNA was synthesized from 1 µg RNA by using a random hexamer. For each sample, a standard curve was generated using known concentrations of cDNA (0.625, 1.25, 2.5, 5, 10, and 20 µg) as a function of cycle threshold (CT). Expression analysis of the reported genes was performed by TaqMan real-time-PCR using ABI 7900 Sequence Detection System. The SDS2.2 software (integral to ABI7900 real-time machine) fits the CT values for the experimental samples and generates values for cDNA levels. All samples were run in triplicates in 384 well plates. 18S rRNA was used as an endogenous control.

**Western Blot Analysis and Nuclear Fractionation**

Total protein extraction and immunoblotting (IB) were performed as previously. Typically, 150 µg of total protein isolated from LV was separated on 8% or 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subject to IB of phospho-(Ser473)/total Akt and phospho/-total Erk1/2 (Cell Signaling Technology); and of anti APLN (ab59469) and anti-APJ receptor (ab84296) from Abcam Inc. The anti-APLN antibody (ab59469) was raised against the conserved C-terminus of APLN. VEGF (147) and α-Tubulin (DM1A) antibodies were purchased from Santa Cruz Biotechnology Inc. Blots were visualized and quantified with ImageQuant LAS 4000 (GE Healthcare). Western blot for phospho-(serine-473)/total Akt was also performed on hEPCs. Human EPCs were serum-starved overnight in incomplete EBM-2 supplemented with 2% FBS. The next day EPCs were stimulated with placebo (PBS), APLN analogue I and II at 30 and 100 ng/mL for 10 minutes and cells were collected for analysis of phospho-Akt (Ser473)/total Akt levels.

Nuclear fractionation was performed as previously described with modifications. Briefly, LV tissues were homogenized in hypotonic lysis buffer (10 mmol/L K-HEPES pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L DTT, 0.2 mmol/L Na3VO4, 1× protease inhibitor cocktail (Calbiochem), 1× phosphatase inhibitors (Sigma and Calbiochem). The total homogenate was centrifuged at 100g for 5 minutes to collect unbroken tissues. The supernatant was then centrifuged at 2000g for 10 minutes to precipitate crude nuclei from cell membrane and cytosolic proteins (second supernatant). The second supernatant was further centrifuged at 100 000g for 90 minutes to separate soluble cytosolic proteins (third supernatant) from membrane pellet. The crude nuclear fraction was resuspended in hypotonic lysis buffer supplemented with 2.4 mmol/L sucrose, and then layered on top of a 2.4 mmol/L sucrose cushion and purified by centrifugation at 100 000g for 90 minutes. Following ultracentrifugation, the purified nuclear pellet was resuspended in storage buffer (20 mmol/L Na-HEPES pH 7.9), 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 0.2 mmol/L EGTA, 0.5 mmol/L PMSF, 0.5 mmol/L DTT, 25% Glycerol, 1× protease and phosphatase inhibitors). 30 µg of nuclear protein from LV was subject to HIF1α blotting (Novus). The purity of nuclear and cytosolic fractions was verified by using Histone H-3 (Cell Signaling; nuclear marker) and Caspase-3 (Cell Signaling; cytosolic marker).

**Statistical Analysis**

Statistical analyses were performed using the SPSS Statistics 19 software and statistical significance was recognized at P<0.05. Student t-test or one-way ANOVA, followed by multiple-comparison Student Neuman-Keuls testing was performed to compare the data between two or three experimental groups, respectively. Two-way ANOVA using the MI and APLN status as the two independent variables (factors) was performed to compare the data between four experimental groups (WT, APLNKO, Sham and MI) (Figures 2E, 2F, 3, 4B through 4E, 4G through 4L, 7A, and 7C through 7F). We first confirmed that our data were normally distributed.
(Shapiro-Wilk Statistic; \( P<0.05 \)), and then performed the statistical analyses as noted above. Survival data were analyzed using the Kaplan-Meier method and the log-rank test was used to test for statistical significance (Figure 2B).

Results

Downregulation of APLN in Diseased Murine and Human Hearts

Acute MI, by ligation of the LAD artery, resulted in a drastic reduction in APLN levels in the infarct and peri-infarct regions at 1 day post-MI which persisted at 7 days post-MI. In contrast, APJ levels showed a bimodal change in the post-MI setting with an early increase followed by downregulation at 7 days post-MI (Figure 1A). In failing human hearts explanted following subacute MI, APLN levels showed a marked decrease with a corresponding increase in APJ levels (Figure 1B). Immunofluorescence staining confirmed a downregulation of APLN in the endothelial compartment of the infarcted murine and human hearts and in the myocardial interstitium with a concordant loss of APLN in epicardial coronary arteries in patients with coronary artery disease (Figures 1A, 1B, and S1).

Loss of APLN Enhances the Susceptibility to Myocardial Infarction

To ascertain whether APLN is a critical determinant of the cardiac response to ischemic injury, we subjected APLN knockout (APLN\(^{-/}\)) and littermate wild-type (APLN\(^{+/}\)) mice to MI. Western blot analysis confirmed loss of APLN in the absence of an upregulation of APJ levels in APLN\(^{-/}\) hearts (Figure 2A). Acute MI resulted in increased mortality in APLN\(^{-/}\) compared to APLN\(^{+/}\) mice based on Kaplan-Meier survival analysis with a 16% (8/50) and 32% (16/50) mortality, respectively (Figure 2B). The greater mortality in APLN\(^{-/}\) mice correlated with larger infarct size as demonstrated by TTC staining and greater elevation in plasma creatine kinase activity at 1 day post-MI (Figure 2C) leading to greater ventricular dilation and fibrosis (Figure 2D). Modulation of prosurvival pathways and angiogenesis are critical determinants of post-MI adverse myocardial remodeling.\(^{34}\) APLN is an agonist of the G-protein coupled receptor, APJ, and can activate the well-known prosurvival kinase, PI3K/Akt. The TUNEL assay revealed a greater degree of apoptosis in the infarct-related region (Figure 2E) in 1-day post-MI APLN\(^{-/}\) hearts. Western blot analysis showed a consistent increase in phospho-Akt and Erk1/2 in the infarct, peri-infarct and

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**Figure 1.** Myocardial apelin is downregulated in diseased murine and human hearts. Western blot analysis and immunofluorescence staining for apelin (green) and CD31 (red) illustrating marked loss of myocardial apelin in infarcted murine (A) and human hearts (B) with coronary artery disease (CAD) associated with a drastic loss of apelin in human epicardial coronary arteries (B). Immunofluorescence staining for apelin (green) and CD31 (red) with the individual brightfield images shown in the lower lane illustrating a marked loss of apelin in infarcted human myocardium (C) and in the left anterior descending coronary arteries from patients with CAD compared to healthy controls (HC) (D). The arrow indicates the human myocardial interstitium while the arrowheads illustrate the diseased human coronary endothelium. Sh indicates sham-operated; MI, myocardial infarction; Inf, infarct region; Peri, peri-infarct region; Non, non-infarct region; LAD, left anterior descending artery; R.R., relative ratio; LV, left ventricle; n=3; \( ^*P<0.05 \) compared to the corresponding sham group; \( ^\$P<0.05 \) compared with HCs and noninfarcted LV.
Loss of apelin enhances susceptibility to myocardial infarction. Western blot analysis of APLN and APJ levels showing a complete loss of APLN in APLN knockout (APLN-/y) hearts without compensatory changes in APJ level (A). LAD-ligation resulting in increased mortality in APLN-/y (B), larger infarct size shown as representative images of triphenyl tetrazolium chloride (TTC)-stained heart sections and plasma creatine kinase (CK) activity at 1 day post-MI (C) and greater ventricular dilation and fibrosis at 7 day post-MI (D). TUNEL staining and quantification of apoptotic nuclei (E) revealed a greater increase in apoptosis in APLN-deficient hearts with Western blot analysis of serine-473 Akt and Erk1/2 phosphorylation showing increased levels at 1 day post-MI in APLN+/y hearts but with a dramatic loss of Akt and Erk1/2 phosphorylation in APLN-/y hearts (F). Sh indicates sham-operated; MI, myocardial infarction; Inf, infarct region; Peri, peri-infarct region; Non, noninfarct region; p, phospho; t, total; R.R., relative ratio; LAD, left anterior descending artery; APLN, Apelin. Values are mean±SEM; n=5 for each group except for B where n=50 (P<0.01 based on Kaplan–Meier survival analysis and log-rank test) and C where n=8. P<0.05 compared to the corresponding sham group.*P<0.05 for the main effects and #P<0.05 for the interaction using two-way ANOVA.

Figure 2. Loss of apelin enhances susceptibility to myocardial infarction. Western blot analysis of APLN and APJ levels showing a complete loss of APLN in APLN knockout (APLN-/y) hearts without compensatory changes in APJ level (A). LAD-ligation resulting in increased mortality in APLN-/y mice based on Kaplan–Meier survival analysis (B), larger infarct size shown as representative images of triphenyl tetrazolium chloride (TTC)-stained heart sections and plasma creatine kinase (CK) activity at 1 day post-MI (C) and greater ventricular dilation and fibrosis at 7 day post-MI (D). TUNEL staining and quantification of apoptotic nuclei (E) revealed a greater increase in apoptosis in APLN-deficient hearts with Western blot analysis of serine-473 Akt and Erk1/2 phosphorylation showing increased levels at 1 day post-MI in APLN+/y hearts but with a dramatic loss of Akt and Erk1/2 phosphorylation in APLN-/y hearts (F). Sh indicates sham-operated; MI, myocardial infarction; Inf, infarct region; Peri, peri-infarct region; Non, noninfarct region; p, phospho; t, total; R.R., relative ratio; LAD, left anterior descending artery; APLN, Apelin. Values are mean±SEM; n=5 for each group except for B where n=50 (P<0.01 based on Kaplan–Meier survival analysis and log-rank test) and C where n=8. P<0.05 compared to the corresponding sham group.*P<0.05 for the main effects and #P<0.05 for the interaction using two-way ANOVA.

noninfarct regions in APLN+/y hearts (Figure 2F). In contrast, loss of APLN resulted in a drastic lowering of phospho-Akt and Erk1/2 levels in the infarct and peri-infarct regions in APLN-/y hearts (Figure 2F). Increased cell death in APLN-/y hearts was also associated with greater neutrophil and macrophage infiltration (Figure 3A and 3B) associated with increased expression of proinflammatory cytokines (Figure 3C), and matrix metalloproteinases (MMPs), MMP8, MMP9 and MMP12 (Figure 3D) which will likely lead to degradation of the myocardial extracellular matrix.

The greater infarct size and increased myocardial inflammation coupled with compromised prosurvival signaling pathways likely contributes to worsened systolic dysfunction in APLN knockout mice. While baseline systolic function was not significantly different between APLN+/y and APLN-/y mice (Figure 4), which was confirmed at the single cardiomyocyte level (Figure S2), echocardiographic M-mode, parasternal long axis, and left atrial (Figure 4A) views showed worsening of systolic dysfunction, greater LV and left atrial dilation at 1 week post-MI in APLN-/y hearts. Quantitative assessment of systolic function showed increased left ventricular end-systolic volume (LVESV) (Figure 4B) and left atrial size (Figure 4C) with greater reduction in EF (Figure 4D) and worsening WMSI (Figure 4E) in APLN-deficient hearts. Invasive LV pressure-volume loop analysis confirmed equivalent basal systolic function and that APLN-/y mice showed a marked exacerbation of systolic dysfunction in response to MI independent of alterations in preload and afterload (Figure 4F through 4L).

Beneficial Effects of APLN Analogue in Myocardial Ischemic Injury

Exacerbation of post-MI dysfunction in APLN-deficient hearts suggests that enhancing APLN action can have salutary beneficial effects in ischemic heart disease. Given the short
half-life of native APLN peptides,\textsuperscript{11,24,35} we modified, synthesized and purified two novel APLN analogues with the aim to enhance their therapeutic effects: NleInpBrF pyr-1-apelin-13 (APLN analogue I) and NleAibBrF pyr-1-apelin-13 (APLN analogue II) (Figure S3; Tables S2 and S3). Using structure-activity relationships conducted on pyr-1-apelin,\textsuperscript{36} 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 (APLN analogue I) or combined two potent APJ binding substitutions into the same peptide analogue while maintaining comparable stability to the native peptide (APLN analogue II). These analogues were purified using high-performance liquid chromatography (HPLC) (Figures S4 and S5), and high-resolution mass spectrometry and nuclear magnetic resonance (NMR) were used to confirm the sequence of the synthesized analogues (Figures S6 and S7). Proteolysis analysis using HPLC coupled with MALDI-TOF mass spectrometry confirmed efficient ACE2-mediated cleavage of pyr-1-apelin-13 (but not pyr-1-apelin-12) with 79.5±2.2%, 54.6±4.2% and 18.3±3.8% (n=3) of pyr-1-apelin-13 remaining at 30 s, 1 and 2 minutes following incubation with ACE2 (Figure S8) while APLN analogue I and analogue II were markedly resistant to ACE2-mediated proteolysis (Figure S9). We confirmed that APLN analogue I and II were not inhibitors of ACE2 activity (Figure S10) and as such the lack of formation of degradation products is due to the intrinsic resistance of the APLN analogues to ACE2 action.

We used the ex vivo Langendorff heart preparation to further determine the role of APLN in myocardial ischemic injury and to evaluate the therapeutic effects of the synthetic APLN analogues. Notably, in response to myocardial IR injury, APLN\textsuperscript{+/y} hearts exhibited suppressed functional recovery compared to APLN\textsuperscript{-/-} hearts as illustrated by representative hemodynamic responses (Figure 5A), LV developed pressure (Figure 5B), rate-pressure product (Figure 5C) and measures of myocardial contractility, dP/dt\textsubscript{max} and dP/dt\textsubscript{min} (Figure 5D). The enhanced susceptibility to IR injury is consistent with the reduced viability of adult cardiomyocytes isolated from APLN-deficient hearts (Figure 6A and 6B).
A postconditioning protocol was used in which APLN analogues were applied following the ischemic period in APLN+/y (wildtype) hearts in order to simulate a potential clinical application. While APLN analogue I (1 μmol/L) failed to prevent the IR injury (Figure 6C through 6E), APLN analogue II (1 μmol/L) resulted in a greater recovery of function as illustrated by representative hemodynamic responses (Figure 5A) and the quantitative measure of myocardial performance (Figure 5B through 5D). Analysis of creatine kinase activity in the coronary perfusate corroborated the greater myocardial damage in the reperfused APLN−/y hearts with a marked protection seen in response to APLN analogue II (Figure 5E). The increased phosphorylation of Akt and Erk1/2, two critical pathways in mediating cardioprotection against myocardial IR injury,37 were reduced in the absence of APLN at 10 minutes (Figure 5F) and at 40 minutes postreperfusion (Figure S11) with APLN analogue II leading to greater activation of these protective signaling pathways (Figure 5F). Next, we extrapolated our findings to an in vivo model of IR injury in which the proximal LAD coronary...
Apelin Controls Myocardial Ischemic Injury

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Critical Role of APLN in Myocardial Angiogenesis: Stimulation by APLN Analogue

APLN stimulates the angiogenic response, a key adaptive mechanism in ischemic heart disease and a determinant of infarct expansion.34,38,39 We next examined the role of APLN in the adaptive angiogenesis response. Nuclear translocation of the key transcription factor, HIF-1α, was reduced in the infarct region as delineated by immunofluorescence staining (Figure 7A) and Western blot analysis (Figure 7B) resulting in a lack of upregulation of VEGF in the infarcted APLN-deficient myocardium (Figure 7C). While the regional expression of angiopoietin-1 (Ang-1) was not differentially affected, angiopoietin-2 (Ang-2) levels were decreased in infarcted APLN−/− hearts at 3 days post-MI (Figure 7D). Collectively, these data show a clear and important role of angiogenesis and as such

Figure 5. Myocardial ischemia-reperfusion injury using the ex vivo Langendorff system is exacerbated by the absence of apelin and is rescued by apelin analogue. (A) Representative hemodynamic tracings of APLN+/y and APLN−/− hearts showing a marked reduction in post-ischemic functional recovery of APLN+/y hearts with a dramatic protection in response to apelin analogue II (1.5 µg/mL for 20 minutes) at the start of reperfusion. Functional assessment of the LV developed pressure (LVDP) (B), rate-pressure product (RPP) (C), maximum and minimum rate of change in LV pressure (±dP/dt) (D) showing marked suppression of post-ischemic functional recovery in apelin-deficient hearts and a dramatic protection in response to apelin analogue II. (E) Creatine kinase activity in the coronary perfusate showing a marked increase in APLN−/− hearts at the start and after 10 minutes of reperfusion with reduced damage in response to apelin analogue II. (F) Western blot analysis of serine-473 Akt and Erk 1/2 phosphorylation showing increased phosphorylation in response to ischemia-reperfusion (IR) injury in APLN+/y hearts which was markedly suppressed by loss of APLN and further stimulated by apelin analogue II. (G) In vivo IR due to 30 minutes ischemia in the LAD territory followed by 3 hours reperfusion with Evan's blue and TTC staining showing greater infarct size in APLN−/− hearts and a lack of upregulation of VEGF in the infarcted APLN-deleted myocardium (Figure 7C). While the regional expression of angiopoietin-1 (Ang-1) was not differentially affected, angiopoietin-2 (Ang-2) levels were decreased in infarcted APLN−/− hearts at 3 days post-MI (Figure 7D). Collectively, these data show a clear and important role of angiogenesis and as such

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we examined the in situ angiogenic response in the post-MI heart using CD31 immunofluorescence (Figure 7E) and lectin staining (Figure 7F) of the coronary endothelial cells and microvasculature, respectively. Our results show a marked reduction in capillary density and vessel integrity in the post-MI APLN−/− hearts compared to APLN+/+ hearts (Figure 7E and 7F).

We used the aortic ring culture method and showed that vessel sprouting was impaired in APLN-deficient vessels, which was rescued by supplementation with APLN analogue II and recombinant human VEGF (Figure 8A). To further substantiate the role of APLN in angiogenesis, we isolated and purified hEPCs and confirmed the endothelial lineage by flow-cytometry for the endothelial-specific marker, VE-cadherin and expression of Von Willebrand factor and eNOS (Figure 8B). siRNA knockdown of APLN in hEPCs reduced APLN expression by ~80% (Figure 8C) and using an in vitro angiogenesis culture, expression of characteristic tip cell genes revealed markedly decreased expression of DLL4 (Figure 8D), a gene implicated in directing the tip versus stalk cell phenotype without affecting Flt1 and KDR expression (Figure S12). Silencing of APLN function disrupted endothelial sprouting with increased short/long vessel ratio and reduced endothelial sprout density correlating with a marked suppression of DLL4 (Figure 8E). Importantly, APLN analogue II stimulated the endothelial sprouting resulting in longer capillary tube formation (Figure 8E) in association with increased phospho-Akt in hEPCs (Figure 8F). In contrast, APLN analogue I failed to alter the angiogenic response and Akt phosphorylation of hEPCs (Figure 6F).

Figure 6. Reduced viability of cardiomyocytes isolated from APLN-deficient hearts and lack of biological effects of Apelin analogue I. Reduced viability of isolated adult cardiomyocytes from APLN+/− hearts compared with wildtype APLN+/+ as illustrated by morphological assessment (A, B). Apelin analogue I (NleInpBrF pyr-1-apelin-13) given at 1.5 μg/mL for 20 minutes into the coronary circulation of WT mice at the time of reperfusion failed to protect the ex vivo wildtype hearts from ischemia-reperfusion injury (C, E) and was unable to stimulate angiogenesis (F, G) or Akt phosphorylation in human EPCs at 100 ng/mL (H). LVDP indicates left ventricle developed pressure; APLN, Apelin; RPP, rate-pressure product; dP/dt, rate of change in LV pressure; p, phospho; t, total; R.R., relative ratio. Values are mean ±SEM; n=8 per groups except for H where n=5. *P<0.05 compared with the APLN+/+ group.

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through 6H). These findings confirmed that APLN deficiency inhibits the angiogenic response in the post-MI heart and in hEPCs.

**Discussion**

Coronary artery disease characterized by adverse post-MI remodeling and IR injury is now the most common cause of heart failure.\(^{12,37}\) We showed the APLN/APJ pathway is drastically altered in post-MI myocardial tissue characterized by a marked reduction in APLN levels in murine hearts. Importantly, in explanted human hearts with primary ischemic injury, there was a marked loss of APLN in various compartments confirming a critical role of APLN in human heart failure secondary to ischemic heart disease. Using a genetic model we showed that loss of APLN impaired post-MI remodeling, angiogenesis and functional recovery, and exacerbated myocardial IR injury ex vivo and in vivo demonstrating a critical causal role of APLN in myocardial ischemic injury. The loss of APLN clearly compromises the activation of the protective Akt/PI3K\(^{43}\) and Erk 1/2 signaling pathways,\(^{44}\) both in vivo and ex vivo, resulting in increased myocardial damage and worsening heart function. Our synthetic apelin analogue provided salutary beneficial effects against ex vivo and in vivo myocardial injury. The C-terminal phenylalanine residue of apelin-13 is essential for activity and its removal leads to a 10-fold decrease in binding and functional efficacy.\(^{36,45}\) We have shown that in contrast to pyr-1-apelin-13, APLN analogue I and II were extremely resistant to proteolytic cleavage of the C-terminal phenylalanine by ACE2. The ability of APLN analogue II to improve both systolic and diastolic function is particularly useful in the setting of myocardial ischemia. In addition to the prosurvival effects of APLN and APLN analogues, the ability of these agonists to activate Akt and eNOS\(^{15,46}\) can directly improve the contractility and relaxation of the cardiomyocytes.\(^{47,48}\)

Genetic variation in the APJ receptor modifies the progression of heart failure in patients with dilated cardiomyopathy.\(^{9}\)
In Dahl salt-sensitive hypertensive (DS) rats, cardiac APLN / APJ pathway is markedly downregulated with the onset of heart failure. APLN mediates positive inotropic effect in vitro and in vivo and minimizes increases in systemic arterial and venous tone with corresponding reductions in LV afterload and preload. The integrative physiological role of the APLN system strongly suggest that enhancing APLN action may serve to minimize myocardial ischemic damage and the progression to advanced heart failure. The APLN/APJ signaling pathway is downstream of Cripto, a member of the EGF-CFC family of signaling molecules, promotes cardiomyocyte differentiation and enhanced cardiac differentiation of embryonic stem cells, processes which may be recruited in the post-MI setting. Using in vitro and in vivo assessment of angiogenesis, we showed that APLN is required for normal angiogenesis, a key adaptive mechanism in ischemic and pressure-overload–induced heart failure. We observed a marked deficiency in angiogenic sprout formation in a robust assay of 3-dimensional angiogenesis using primary hEPCs when APLN was silenced. APLN induces phosphorylation of eNOS and NO release from endothelial cells thereby stimulating angiogenesis while loss of APLN may sensitize endothelial cells to apoptosis. As such, the APLN/APJ system has emerged as a critical mediator of the spatial and temporal control of angiogenesis in heart disease. Our study has several limitations. We used a germ-line knockout of APLN and therefore we cannot distinguish cell-specific effects of APLN action versus the systemic changes associated with a whole-body knockout of apelin. In addition, a detailed molecular pharmacological characterization of the APLN analogues including in vivo pharmacokinetics and their binding properties to their cognate receptors is needed and we cannot exclude off-target effects of the APLN analogues given the concentrations used in this study.

Collectively, our results and previous studies have delineated a critical role of the APLN/APJ axis in the regulation of cardiovascular functions and fluid homeostasis. APLN increases cardiac contractility in vitro and in vivo and minimizes increases in systemic arterial and venous tone with corresponding reductions in LV afterload and preload. In addition, APLN receptor agonism mediates central effect and suppresses arginine vasopressin thereby promoting renal fluid loss which may be particularly attractive in patients.
with HF. Since the APLN/APJ system is compromised in human heart failure,3,10 the integrative physiological role of the APLN/APJ system strongly suggest that enhancing APLN action may serve to minimize myocardial ischemic damage and the progression to advanced heart failure. Enhancing APLN action may serve to minimize myocardial ischemic damage in patients with chronic heart failure. Eur J Heart Fail. 2006;8:355–360.


DATA SUPPLEMENT

Loss of Apelin exacerbates myocardial infarction adverse remodeling and ischemia-reperfusion injury: therapeutic potential of synthetic Apelin analogues

by


Department of Physiology, Mazankowski Alberta Heart Institute, Department of Chemistry, Division of Cardiology, Department of Medicine, Division of Nephrology, Department of Medicine, University of Alberta, Edmonton, Canada, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria
Supplemental Figure 1. Immunofluorescence staining for apelin (green) and CD31 (red) with the individual brightfield images shown on the right illustrating a marked loss of apelin in infarcted (Inf) murine hearts compared to sham (Sh) hearts. The specificity of the apelin antibody was confirmed in the APLN^{+/y} hearts which did not show any evidence of non-specific staining.
Supplemental Figure 2. Preserved basal cardiomyocyte length (A), contractility (B) and relaxation (C-D) in isolated APLN^{-/y} cardiomyocytes compared to APLN^{+/y} cardiomyocytes (n=8 cardiomyocytes in each group).
Supplemental Figure 3, Wang W et al.

**Analogue I**
(NleInpBrF pyr-1-apelin-13)

**Analogue II**
(NleAibBrF pyr-1-apelin-13)

**Supplemental Figure 3.** Schematic representation of the synthesized NleInpBrF pyr-1-apelin-13 (apelin analogue I) and NleAibBrF pyr-1-apelin-13 (apelin analogue II) analogues. Regions indicated in red represent synthetic alterations from the native apelin-13.
Supplemental Figure 4, Wang W et al.

**NleInpBrF pyr-1-apelin-13 analogue**

Semi-preparative purification

![Semi-preparative purification graph](image1)

Analytical reinjection

![Analytical reinjection graph](image2)

**Supplemental Figure 4.** High performance liquid chromatography (HPLC) trace of NleInpBrF pyr-1-apelin-13 analogue (analogue I) showing semi-preparative purification and analytical reinjection of isolated peptide peak.
Supplemental Figure 5, Wang W et al.

**NleAibBrF pyr-1-apelin-13 analogue**

Semi-preparative purification

Analytical reinjection

**Supplemental Figure 5.** High performance liquid chromatography (HPLC) trace of NleAibBrF pyr-1-apelin-13 analogue (analogue II) showing semi-preparative purification and analytical reinjection of isolated peptide peak.
Supplemental Figure 6. High Resolution Mass Spectrometry of NleNpBrF pyr-1-apelin-13 analogue (analogue I). Mass spectra (MS) were recorded on a Kratos AEIMS-50, Bruker 9.4T Apex-Qe FTICR (high resolution, HRMS) using either 4-hydroxy-α-cyanocinnamic acid (HCCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as matrices. MS/MS was performed on a Bruker Ultraflextreme MALDI/TOF/TOF to successfully confirm the peptides’ sequences.
Supplemental Figure 7, Wang W et al.

**Supplemental Figure 7.** High Resolution Mass Spectrometry of NleAibBrF pyr-1-apelin-13 analogue (analogue II). Mass spectra (MS) were recorded on a Kratos AEIMS-50, Bruker 9.4T Apex-Qe FTICR (high resolution, HRMS) using either 4-hydroxy-α-cyanocinnamic acid (HCCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as matrices. MS/MS was performed on a Bruker Ultraflextreme MALDI/TOF/TOF to successfully confirm the peptides’ sequences.
Supplemental Figure 8. Analytical high performance liquid chromatography (HPLC) analysis showing efficient degradation of pyr-1-apelin-13 (A), but not pyr1-apelin-12 (B), by ACE2. **A. Pyr-1-apelin-13 incubation with ACE2;** black – pyr-1-apelin-12 and pyr-1-apelin-13 co-injection; red – 30 s pyr-1-apelin-13 incubation; blue – 1 min pyr-1-apelin-13 incubation; green – 2 min pyr-1-apelin-13 incubation. **B. Pyr-1-apelin-12 incubation with ACE2;** black – pyr-1-apelin-12 standard; red – 1 h pyr-1-apelin-12 incubation; blue – 48 h pyr-1-apelin-12 incubation.
Supplemental Figure 9, Wang W et al.

**A. Apelin Analogue I incubation with ACE2;** black – analogue I standard; red – 1 h incubation; blue – 24 h incubation; green – 48 h incubation (at 37 °C).

**B. Apelin Analogue II incubation with ACE2;** black – analogue II standard; red – 1 h analogue II incubation; blue – 48 h analogue II incubation; green – 48 h analogue II incubation (at 37 °C).
Supplemental Figure 10. Analytical high performance liquid chromatography (HPLC) analysis showing inability of apelin analogue I (A) and apelin analogue II (B) to inhibit the ability of ACE2 to cleave pyr-1-apelin-13. **Apelin Analogue I (A):** black – analogue I standard; red – 1 min of pyr-1-apelin-13 incubation with ACE2; blue – 1 min of pyr-1-apelin-13 incubation with 1:1 (pyr-1-apelin-13:analogue I) preincubated with ACE2 for 1 h. **Apelin Analogue II (B):** black – analogue II standard; red – 1 min of pyr-1-apelin-13 incubation with ACE2; blue – 1 min of pyr-1-apelin-13 incubation with 1:1 (pyr-1-apelin-13:analogue II) with analogue II preincubated with ACE2 for 1 h.
Supplemental Figure 11, Wang W et al.

Supplemental Figure 11. Western blot analysis of phosho Akt (serine-473) signaling pathway in the ex vivo hearts following ischemia-reperfusion injury at 40 mins of reperfusion. *p<0.05 compared with corresponding aerobic group; #p<0.05 compared with the APLN+/y group.
Supplemental Figure 12. Apelin knockdown using siRNA did not affect Flt1 and KDR expression using qRT-PCR and mRNA isolated from angiogenic sprout cultures of hEPCs.
### Supplemental Table 1. Taqman Primers and Probes.

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Ang-1=angiopoietin-1; Ang-2=angiopoietin-2, TNFα=tumor necrosis factor-alpha, IL-1β=Interleukin-1-beta; IL-6=interleuking-6; MMP=matrix metalloproteinase, HPRT= hypoxanthine phosphoribosyltransferase. Assay ID refers to the commercial assay available from Applied Biosystems.
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<td>1.81, 1.74</td>
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*2 sets of signals were observed for Nle11 due to rotamers*
Supplemental Table 3. NMR proton chemical shifts of Apelin Analogue II (NleAibBrF pyr-1-apelin-13).

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Loss of Apelin Exacerbates Myocardial Infarction Adverse Remodeling and Ischemia-reperfusion Injury: Therapeutic Potential of Synthetic Apelin Analogues


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DATA SUPPLEMENT

Loss of Apelin exacerbates myocardial infarction adverse remodeling and ischemia-reperfusion injury: therapeutic potential of synthetic Apelin analogues

by


Department of Physiology, Mazankowski Alberta Heart Institute, Department of Chemistry, Division of Cardiology, Department of Medicine, Division of Nephrology, Department of Medicine, University of Alberta, Edmonton, Canada, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria
Supplemental Figure 1, Wang W et al.

**Supplemental Figure 1.** Immunofluorescence staining for apelin (green) and CD31 (red) with the individual brightfield images shown on the right illustrating a marked loss of apelin in infarcted (Inf) murine hearts compared to sham (Sh) hearts. The specificity of the apelin antibody was confirmed in the APLN+/y hearts which did not show any evidence of non-specific staining.
Supplemental Figure 2. Preserved basal cardiomyocyte length (A), contractility (B) and relaxation (C-D) in isolated APLN⁻/⁻ cardiomyocytes compared to APLN⁺/⁺ cardiomyocytes (n=8 cardiomyocytes in each group).
Supplemental Figure 3, Wang W et al.

Analogue I
(NleInpBrF pyr-1-apelin-13)

Analogue II
(NleAibBrF pyr-1-apelin-13)

Supplemental Figure 3. Schematic representation of the synthesized NleInpBrF pyr-1-apelin-13 (apelin analogue I) and NleAibBrF pyr-1-apelin-13 (apelin analogue II) analogues. Regions indicated in red represent synthetic alterations from the native apelin-13.
Supplemental Figure 4, Wang W et al.

**NleInpBrF pyr-1-apelin-13 analogue**

Semi-preparative purification

![Semi-preparative purification graph](image)

Analytical reinjection

![Analytical reinjection graph](image)

**Supplemental Figure 4.** High performance liquid chromatography (HPLC) trace of NleInpBrF pyr-1-apelin-13 analogue (analogue I) showing semi-preparative purification and analytical reinjection of isolated peptide peak.
Supplemental Figure 5, Wang W et al.

**NleAibBrF pyr-1-apelin-13 analogue**

Semi-preparative purification

Analytical reinjection

**Supplemental Figure 5.** High performance liquid chromatography (HPLC) trace of NleAibBrF pyr-1-apelin-13 analogue (analogue II) showing semi-preparative purification and analytical reinjection of isolated peptide peak.
Supplemental Figure 6. High Resolution Mass Spectrometry of NleNpBrF pyr-1-apelin-13 analogue (analogue I). Mass spectra (MS) were recorded on a Kratos AEIMS-50, Bruker 9.4T Apex-Qe FTICR (high resolution, HRMS) using either 4-hydroxy-α-cyanocinnamic acid (HCCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as matrices. MS/MS was performed on a Bruker Ultraflextreme MALDI/TOF/TOF to successfully confirm the peptides’ sequences.
Supplemental Figure 7, Wang W et al.

**Supplemental Figure 7.** High Resolution Mass Spectrometry of NleAibBrF pyr-1-apelin-13 analogue (analogue II). Mass spectra (MS) were recorded on a Kratos AEIMS-50, Bruker 9.4T Apex-Qe FTICR (high resolution, HRMS) using either 4-hydroxy-α-cyanocinnamic acid (HCCA) or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) as matrices. MS/MS was performed on a Bruker Ultraflexextreme MALDI/TOF/TOF to successfully confirm the peptides’ sequences.
Supplemental Figure 8, Wang W et al.

Supplemental Figure 8. Analytical high performance liquid chromatography (HPLC) analysis showing efficient degradation of pyr-1-apelin-13 (A), but not pyr-1-apelin-12 (B), by ACE2. **A. Pyr-1-apelin-13 incubation with ACE2;** black – pyr-1-apelin-12 and pyr-1-apelin-13 co-injection; red – 30 s pyr-1-apelin-13 incubation; blue – 1 min pyr-1-apelin-13 incubation; green – 2 min pyr-1-apelin-13 incubation. **B. Pyr-1-apelin-12 incubation with ACE2;** black – pyr-1-apelin-12 standard; red – 1 h pyr-1-apelin-12 incubation; blue – 48 h pyr-1-apelin-12 incubation.
Supplemental Figure 9, Wang W et al.

Supplemental Figure 9. Analytical high performance liquid chromatography (HPLC) analysis showing a complete lack of degradation of apelin analogue I (A) and apelin analogue II (B) by ACE2. **A. Apelin Analogue I incubation with ACE2;** black – analogue I standard; red – 1 h incubation; blue – 24 h incubation; green – 48 h incubation (at 37 °C). **B. Apelin Analogue II incubation with ACE2;** black – analogue II standard; red – 1 h analogue II incubation; blue – 48 h analogue II incubation; green – 48 h analogue II incubation (at 37 °C).
Supplemental Figure 10. Analytical high performance liquid chromatography (HPLC) analysis showing inability of apelin analogue I (A) and apelin analogue II (B) to inhibit the ability of ACE2 to cleave pyr-1-apelin-13. **Apelin Analogue I (A):** black – analogue I standard; red – 1 min of pyr-1-apelin-13 incubation with ACE2; blue – 1 min of pyr-1-apelin-13 incubation with 1:1 (pyr-1-apelin-13:analogue I) preincubated with ACE2 for 1 h. **Apelin Analogue II (B):** black – analogue II standard; red – 1 min of pyr-1-apelin-13 incubation with ACE2; blue – 1 min of pyr-1-apelin-13 incubation with 1:1 (pyr-1-apelin-13:analogue II) with analogue II preincubated with ACE2 for 1 h.
Supplemental Figure 11, Wang W et al.

Supplemental Figure 11. Western blot analysis of phosho Akt (serine-473) signaling pathway in the ex vivo hearts following ischemia-reperfusion injury at 40 mins of reperfusion. *p<0.05 compared with corresponding aerobic group; #p<0.05 compared with the APLN+/y group.
Supplemental Figure 12. Apelin knockdown using siRNA did not affect Flt1 and KDR expression using qRT-PCR and mRNA isolated from angiogenic sprout cultures of hEPCs.
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*2 sets of signals were observed for Nle11 due to rotamers
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<td>3.13, 2.99</td>
<td>Ar-H 7.49, 7.16</td>
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