Reperfusion therapy with recombinant human relaxin-2 (Serelaxin) attenuates myocardial infarct size and NLRP3 inflammasome following ischemia/reperfusion injury via eNOS-dependent mechanism

Juan Valle Raleigh  
Virginia Commonwealth University

Adolfo G. Mauro  
Virginia Commonwealth University

Teja Devarakonda  
Virginia Commonwealth University

See next page for additional authors

Follow this and additional works at: http://scholarscompass.vcu.edu/intmed_pubs

Part of the Medicine and Health Sciences Commons

Published on behalf of the European Society of Cardiology. All rights reserved. VC The Author 2017.

Downloaded from  
http://scholarscompass.vcu.edu/intmed_pubs/129

This Article is brought to you for free and open access by the Dept. of Internal Medicine at VCU Scholars Compass. It has been accepted for inclusion in Internal Medicine Publications by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
Authors
Juan Valle Raleigh, Adolfo G. Mauro, Teja Devarakonda, Carlo Marchetti, Jun He, Erica Kim, Scott Filippone, Anindita Das, Stefano Toldo, Antonio Abbate, and Fadi N. Salloum

This article is available at VCU Scholars Compass: http://scholarscompass.vcu.edu/intmed_pubs/129
Reperfusion therapy with recombinant human relaxin-2 (Serelaxin) attenuates myocardial infarct size and NLRP3 inflammasome following ischemia/reperfusion injury via eNOS-dependent mechanism

Juan Valle Raleigh, Adolfo G. Mauro, Teja Devarakonda, Carlo Marchetti, Jun He, Erica Kim, Scott Filippone, Anindita Das, Stefano Toldo, Antonio Abbate, and Fadi N. Salloum*

Pauley Heart Center, Division of Cardiology, Department of Internal Medicine, Virginia Commonwealth University, 1101 East Marshall Street, Room 7-070, Richmond, VA 23298-0204, USA

Received 25 May 2016; revised 9 November 2016; editorial decision 18 November 2016; accepted 1 December 2016; online publish-ahead-of-print 10 January 2017

Time for primary review: 47 days

Aims
The preconditioning-like infarct-sparing and anti-inflammatory effects of the peptide hormone relaxin following ischemic injury have been studied in the heart. Whether reperfusion therapy with recombinant human relaxin-2, serelaxin, reduces myocardial infarct size and attenuates the subsequent NLRP3 inflammasome activation leading to further loss of functional myocardium following ischemia/reperfusion (I/R) injury is unknown.

Methods and results
After baseline echocardiography, adult male wild-type C57BL or eNOS knockout mice underwent myocardial infarction (MI) by coronary artery ligation for 30 min followed by 24 h reperfusion. Mice were treated with either serelaxin (10 μg/kg; sc) or saline 1 h prior to ischemia or 5 min before reperfusion. In both pre-treatment and reperfusion therapy arms, serelaxin improved survival at 24 h post MI in wild-type mice (79% and 82%) as compared with controls (46% and 50%, \( P < 0.05 \)), whereas there was no difference in survival between serelaxin- and saline-treated eNOS knockout mice. Moreover, serelaxin significantly reduced infarct size (64% and 67% reduction, \( P < 0.05 \)), measured with TTC staining, and preserved LV fractional shortening (FS) and end-systolic diameter (LVESD) in wild-type mice as compared with controls (\( P < 0.05 \)). Interestingly, caspase-1 activity in the heart tissue, a measure of inflammasome formation, was markedly reduced in serelaxin-treated wild-type mice compared with controls at 24 h post-MI in both treatment modalities (\( P < 0.05 \)). Genetic deletion of eNOS abolished the infarct-sparing and anti-inflammatory effects of serelaxin as well as functional preservation. Serelaxin plasma levels assessed at 5 min and 1 h after treatment, using ELISA, approximated physiologic relaxin levels during pregnancy in mice and parallels that in humans.

Conclusion
Serelaxin attenuates myocardial I/R injury and the subsequent caspase-1 activation via eNOS-dependent mechanism.

Keywords
Serelaxin • Ischemia-reperfusion injury • eNOS • Caspase-1 • LV function

* Corresponding author. Tel: +804 827 2340, fax: +804 828 8700, E-mail: fadi.salloum@vcuhealth.org
Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2017. For permissions, please email: journals.permissions@oup.com.
1. Introduction

Acute myocardial infarction (AMI) is a major cause of death and disability worldwide. In patients suffering ST-elevation myocardial infarction (STEMI), minimizing ischemic time by urgent revascularization, either by way of thrombolytic therapy or primary percutaneous coronary intervention, has been a successful strategy in reducing morbidity and mortality. Restoration of coronary flow, despite re-introducing oxygen, and nutrients to the ischemic environment, may however lead to further myocardial injury through a process known as reperfusion injury. Numerous experimental interventions during myocardial reperfusion in animal models have been shown to significantly reduce infarct size. However, most attempts for clinical translation of promising experimental findings in the context of STEMI patients have been disappointing.

Serelaxin, a human recombinant form of relaxin-2, has been recently shown in RELAX-AHF to improve symptoms and reduce cardiac damage and cardiovascular death in patients with acute heart failure. The hormone relaxin is a 6 kDa polypeptide that belongs to the insulin superfamily. Relaxin is mostly produced in the corpus luteum and placenta in mammals, however, it can also be produced by other tissues including brain, heart, and kidney. The relaxin receptor 1 (RXFP1), one of four members of the relaxin family peptide receptors, is a G-coupled cognate ligand receptor for relaxin. RXFP1 expression is not restricted to reproductive tissues, as it is also present in non-reproductive tissues including endothelial and smooth muscle cells of arteries and veins, kidney tubules, cardiomyocytes and heart tissue, lung, liver, blood cells, and brain. In experimental animal models of ischemia/reperfusion (I/R) injury, the benefits of relaxin, after coupling with its cognate receptor RXFP1, have been linked to nitric oxide (NO) pathway, particularly by blocking the beneficial effects of relaxin with non-specific inhibition of NO synthase (NOS). Suggested mechanisms of cardioprotection include reduction of oxidative stress, calcium overload, neutrophil activity, endothelial adhesive protein expression, mast cell degranulation, apoptosis, platelet adhesion, as well as improving coronary blood flow.

Based on this background information, we tested whether pre-treatment (prior to ischemia) or reperfusion therapy with serelaxin will improve outcomes at 24 h in a murine model of MI. Additionally, we evaluated the role of endothelial NOS in mediating the infarct-sparing and anti-inflammatory effects [specifically the Nod like receptor containing a pyrin domain-3 (NLRP3) inflammasome via caspase 1 activation] by taking advantage of eNOS knockout mice, since most previous studies relied on pharmacologic NOS inhibition.

2. Methods

2.1 Animals

C57BL and eNOS knockout (stock # 002684) 8-week-old male mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed under temperature-controlled conditions with free access to food and water. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (8th edition revised in 2011) and all procedures were approved by Virginia Commonwealth University IACUC.

2.2 Drugs and chemicals

Triphenyltetrazolium chloride (TTC) and Zymosan A from Saccharomyces cerevisiae were purchased from Sigma-Aldrich (St. Louis, MO). Phthalo blue dye was purchased from Quantum Ink Company (Louisville, KY). Serelaxin was provided by Novartis Pharmaceuticals.

2.3 Experimental Protocol

2.3.1 Prior to ischemia

Adult male C57BL or eNOS knockout mice were pretreated with either serelaxin (10 μg/kg, sc) or volume-matched saline 1 h prior to regional ischemia. This dose was based on previous studies in the literature that showed that a dose of 5 μg/kg was protective in a swine model of I/R injury. We initially tested both doses of 5 μg/kg and 10 μg/kg and found that the higher dose offers better protection in terms of infarct size reduction compared to the lower dose (not shown).

2.3.2 Prior to reperfusion

A bolus dose of 10 μg/kg serelaxin (sc) or volume-matched saline was given 5 min prior to initiation of reperfusion following 25 min of ischemia.

2.4 MI protocol

The methodology of myocardial infarction was described previously. In brief, mice were anesthetized with 70 mg/kg pentobarbital (i.p.) and the left descending coronary artery was identified and occluded for 30 min by a 7.0 silk ligature that was placed around it and a small piece of polyethylene tubing (PE10) that was positioned on top of it. After coronary artery occlusion for 30 min, reperfusion was established by removing the PE10 tube that was compressing the coronary artery. After reperfusion, the air was expelled from the chest and the animals were extubated and then received analgesia (buprenorphine SR LAB, 0.5 mg/kg; s.c., which lasts up to 72 h) and intramuscular antibiotic (Gentamicin, 0.7 mg/kg).

2.5 Experimental groups

Two treatment arms were used in wild-type and eNOS knockout mice: (i) Pretreatment (serelaxin was administered 1 h prior to I/R) and (ii) At Reperfusion (serelaxin was administered 5 min prior to reperfusion).

Eight total treatment groups were used: (i) Wild-Type Control, pretreatment: C57BL wild-type mice received 0.1 ml subcutaneous (s.c.) normal saline 1 h before I/R; (ii) Wild-Type Serelaxin, pretreatment: C57BL wild-type mice received serelaxin (10 μg/kg, sc.) 1 h before I/R; (iii) eNOS knockout Control, pretreatment: eNOS knockout mice were treated as in group 1; (iv) eNOS knockout Serelaxin, pretreatment: eNOS knockout mice were treated as in group 2; (v) Wild-Type Control, reperfusion therapy: C57BL wild-type mice received 0.1 ml (s.c.) normal saline 5 min prior to the onset of reperfusion; (vi) Wild-Type Serelaxin, reperfusion therapy: C57BL wild-type mice received serelaxin (10 μg/kg, sc.) 5 min prior to the onset of reperfusion; (vii) eNOS knockout Control, reperfusion therapy: eNOS knockout mice received were treated as in group 5; and (viii) eNOS knockout Serelaxin, reperfusion therapy: eNOS knockout mice were treated as in group 6. Sham mice underwent the same surgical protocol without coronary artery occlusion.

In all groups, LV function was assessed using echocardiography and infarct size was measured at 24 h after I/R. The detailed experimental protocol is illustrated in Figure 1.

2.6 Survival

Survival rate was determined based on the animals that survived the experimental protocol starting at recovery following surgery until 24 h after infarction.
2.7 Infarct size assessment
After 24 h of reperfusion, the heart was quickly removed under deep anaesthesia with 100 mg/kg pentobarbital (i.p.) and mounted on a Langendorff apparatus as described previously. The coronary arteries were perfused with 37°C Krebs–Henseleit buffer. After the blood was washed out, 3 ml of 10% TTC in isotonic phosphate buffer (pH 7.4) at 37°C was infused over several minutes before the ligature was tightened and ~1 ml of 5% Phthalo blue dye was injected as a bolus into the aorta until most of the heart turned blue. The heart was perfused with saline to wash out the excess Phthalo blue. Finally, the heart was removed, frozen, and cut into 8–10 transverse slices from apex to base of equal thickness (~1 mm). The slices were then fixed in 10% neutral formaldehyde for 4–24 h with a weight on top to keep the heart slices flat for the initial 30 min. The areas of infarcted tissue, the risk zone, and the whole left ventricle were determined by computer morphometry using ImageJ imaging software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011).  

2.8 Echocardiography
Echocardiography was performed using the Vevo770™ imaging system (VisualSonics Inc., Toronto, Canada) prior to surgery (baseline) and 24 h after surgery prior to sacrificing the animal. Isoflurane (2.5%) was used for anaesthesia and the procedure was carried out as previously described to measure LV end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD). LV fractional shortening (FS) was calculated as (LVEDD-LVESD)/LVEDD×100.

2.9 Determination of plasma H2-relaxin
Plasma serelaxin levels were measured in un-infarcted mice at 5 min, 1-, 2-, and 3-h time points after treatment to determine how rapidly subcutaneous administration of serelaxin achieved physiologically relevant blood concentrations and to establish a pharmacokinetic profile. This information is especially important in the reperfusion therapy arm, which received serelaxin only 5 min prior to reperfusion. Blood was obtained during aortotomy at the specified time points. The collected blood was immediately transferred into pre-heparinized tubes placed on ice. Samples were centrifuged at 8000 rpm for 5 min, and the supernatant was decanted into fresh Eppendorf tubes—which were stored in -20°C until usage. H2-relaxin was determined by the use of a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions. The kit had a detection limit of 4.57 pg/ml. The intra- and inter-assay coefficients of variation are lower than 4.6 and 10.2, respectively.

2.10 Determination of plasma nitrite levels
Plasma nitrite concentrations were quantified by an automated ion chromatography system (ENO30 Analyzer, Eicom). Nitrite was separated by a column (NO-PAK with polystyrene polymer, Eicom). The mobile phase, delivered at a pump rate of 0.33 ml/min, was 10% methanol containing 0.15 mol/L NaCl–NH4Cl and 0.5 g/L of tetrasodium ethylenediaminetetraacetic acid (EDTA). The Griess reagent, which was 1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L N-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min.

2.11 Haemodynamics
Healthy mice were deeply sedated (pentobarbital 70 mg/kg), and a pressure probe catheter (AD Instruments, Colorado Springs, CO) was retrogradely positioned in the aortic arch from the right carotid artery. Aortic systolic and diastolic pressures were measured and recorded using LabChart Pro 5 (ADInstruments). After acquisition of baseline values, serelaxin (10 µg/kg, s.c.) was administered to the mice and pressure values were recorded at 5-min intervals to detect changes in the aortic pressures.

2.12 Adult primary cardiomyocyte preparation
Adult rat ventricular cardiomyocytes were isolated using an enzymatic technique as previously reported and has been extensively employed in our laboratory.

2.13 Western blotting
The general methodology for Western blot has been previously described. Isolated LV samples (n = 3/group) or adult primary cardiomyocytes were homogenized and centrifuged at 12 000 g for 10 min at 4°C. Total proteins (75 µg) for each sample were separated by SDS-PAGE on 4–20% gradient acrylamide gels, transferred onto a nitrocellulose membrane, and blocked with 5% non-fat dry milk in Tris-buffered saline. Membranes were incubated overnight with rabbit monoclonal antibody (1:1000 dilution, Cat# PA1-41532; Thermo Fisher Scientific) for relaxin receptor 1 (RXFP1), mouse monoclonal antibody (dilution 1:1000, Cat# 4691 or 1060; Cell Signaling, MA) specific for total eNOS (goat polyclonal, 1:1000 dilution, Cat# PA1-41532; Thermo Fisher Scientific) or phospho-eNOS (Ser1177 and Thr495), respectively, and GAPDH antibody (1:1000 dilution, Cat# 4691 or 1060; Cell Signaling, MA) for the corresponding phospho-eNOS (Ser1177 and Thr495), respectively, and GAPDH antibody (1:1000 dilution, Cat# 4691 or 1060; Cell Signaling, MA) specific for total eNOS (goat polyclonal, 1:1000 dilution, Cat# PA1-41532; Thermo Fisher Scientific). The blot was then incubated for 1 h with the corresponding secondary peroxidase-conjugated antibody and developed using Western Lightning Plus-Ecl substrate (Perkin Elmer, MA, USA). The densitometric analysis for the corresponding phospho- and total eNOS and beta actin band was done using ImageJ software.

2.14 Caspase-1 activity in the heart
An additional subset of mice was sacrificed 24 h after surgery in the reperfusion therapy arm (n = 6 per treatment group). The heart was removed, rinsed in phosphate-buffered saline (PBS) and the free left ventricular free wall distal to left anterior descending (LAD) occlusion was collected and snap frozen in liquid nitrogen. Caspase-1 activity was measured in the tissue using a fluorogenic substrate (Ac-YVAD-AMC) specific for caspase-1 (Enzo Life Sciences, Farmingdale, NY, USA). After homogenization using radioimmunoprecipitation assay buffer (RIPA) buffer...
containing a cocktail of protease inhibitors and centrifugation at 16,000 rpm for 20 min, 50 µg of protein from each sample were used for the assay as previously described. Fluorescence was measured after 60 min and was expressed as arbitrary fluorescence units produced by one microgram of sample per minute (fluorescence/µg/min) and calculated as fold change compared to the caspase-1 activity in homogenates of the hearts of sham-operated mice, whereby oral intubation and a left thoracotomy were performed and a 7.0 silk suture was placed around the left coronary artery but not tightened.

2.15 Inflammasome-dependent Peritonitis Model
To determine the effects of serelaxin on the inflammasome in vivo independent of its effects on ischemia or infarction, we used a model of peritonitis induced by the intra-peritoneal injection of zymosan A (1 mg/mouse), which induces the activation of the cryopyrin inflammasome. Six hours after zymosan A injection, peritoneal lavage was performed with sterile NaCl 0.9% (7 ml), and the fluid was assessed for leukocyte content using the Thoma chamber. The total amount of leukocytes was determined in the presence or absence of serelaxin (10 µg/kg).

2.16 Statistics
All measurements of infarct size and risk areas are expressed as group means ± SE. Changes in echocardiography parameters and infarct size were analysed using one-way ANOVA to determine the main effect, and the post-hoc two-sided Dunnett’s test or Tukey’s test to compare two groups at a time. Statistical differences were considered significant if the P-value was <0.05. Discrete variables were presented as absolute and percent value. The Chi-square test (or the Fisher’s exact test when appropriate) was used to compare discrete variable in different groups. The Bonferroni correction for post-hoc analysis was used when comparing two groups from three or more groups.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

3. Results

3.1 Mouse heart and cardiomyocytes express RXFP1
Western blot analysis demonstrated that RXFP1 is expressed in the mouse heart as well as adult primary murine ventricular cardiomyocytes (n = 3/group). (B) Survival of mice following I/R in the various treatment groups at 24 h post-MI. Note that serelaxin-treated wild-type mice exhibited a significant increase in survival compared with animals treated with saline or eNOS knockout mice in both treatment arms. The Chi-square test was used to determine statistical significance.

Figure 2 RXFP1 is expressed in mouse cardiomyocytes and heart and serelaxin improves survival following myocardial infarction. (A) Western blot analysis demonstrating expression of RXFP1 in the mouse heart and isolated primary adult mouse ventricular cardiomyocytes (n = 3/group). (B) Survival of mice following I/R in the various treatment groups at 24 h post-MI. Note that serelaxin-treated wild-type mice exhibited a significant increase in survival compared with animals treated with saline or eNOS knockout mice in both treatment arms. The Chi-square test was used to determine statistical significance.
3.3 Serelaxin reduces infarct size following MI

3.3.1 Pre-treatment arm
Infarct size (% of risk area) was reduced from 47.2 ± 4.0 with saline to 17.6 ± 2.8 with serelaxin 24 h after infarction (P < 0.05, Figure 3A). Genetic deletion of eNOS abrogated the infarct-sparing effect of serelaxin (39.4 ± 3.1%). The risk area (% of left ventricle) was similar between the groups (P = NS) and was 54.9 ± 1.3% in the saline I/R C57BL group, 54.7 ± 1.2% in the serelaxin C57BL group, 54.8 ± 1.4% in the serelaxin eNOS knockout group and 53.5 ± 1.0% in the saline eNOS knockout group.

3.3.2 Reperfusion therapy arm
Similar to the pre-treatment arm, infarct size was reduced from 46.5 ± 3.5 with saline to 15.2 ± 2.8 with serelaxin 24 h after infarction (P < 0.05, Figure 3B). Genetic deletion of eNOS abrogated the preservation of LV function as demonstrated by marked increase in LVESD to 3.3 ± 0.2mm and a decline in FS to 19 ± 1%, P < 0.05 vs. serelaxin in wild-type mice.

3.4 Serelaxin preserves LV function following MI

3.4.1 Pre-treatment arm
None of the groups presented with significant LV dilatation at 24 h post infarction (not shown), however, serelaxin decreased LVESD (2.6 ± 0.1 mm) and preserved FS (27 ± 2%) as compared to saline in wild-type mice (LVESD: 3.4 ± 0.3mm and FS: 17 ± 2%, respectively; P < 0.05, Figure 4A). Genetic deletion of eNOS abrogated the preservation of LV function as demonstrated by marked increase in LVESD to 2.9 ± 0.2mm and a decline in FS to 17 ± 2%, P < 0.05 vs. serelaxin in wild-type mice.

3.4.2 Reperfusion therapy arm
Serelaxin decreased LVESD (2.6 ± 0.1 mm) and preserved FS (28 ± 2%) as compared to saline in wild-type mice (LVESD: 3.5 ± 0.2mm and FS: 18 ± 2%, respectively; P < 0.05, Figure 4B). Genetic deletion of eNOS abrogated the preservation of LV function as demonstrated by marked increase in LVESD to 2.9 ± 0.2mm and a decline in FS to 17 ± 2%, P < 0.05 vs. serelaxin in wild-type mice.

3.5 Serelaxin approximates pregnancy relaxin levels 5 min after subcutaneous administration and levels are maintained 1 h later
Following subcutaneous administration of serelaxin (10 µg/kg) in mice, H2-relaxin blood levels were significantly increased at 5 min (779 ± 61 pg/mL), and remained elevated after 1 h (865 ± 35 pg/mL) as shown in Figure 5A. Serum levels declined by the 2-h time point

Figure 3 Serelaxin reduces myocardial infarct size when administered prior to ischemia or at the time of reperfusion. Top Panel: Representative heart sections showing TTC-stained viable and infarcted tissue in the (A) pre-treatment arm and (B) reperfusion therapy arm. Bottom Panel: (A) Myocardial infarct size (% of RA, n = 8/group) measured 24 h post-MI in the pre-treatment arm. Note that infarct size was significantly reduced with serelaxin in wild-type mice, but not in eNOS knockout mice. (B) Myocardial infarct size measured 24 h post-MI in the reperfusion therapy arm (n = 9/group). Similar to pretreatment, infarct size was significantly reduced with serelaxin in wild-type mice, but not in eNOS knockout mice. One-way ANOVA was used to determine the main effect and Tukey’s post-hoc multiple comparisons test was used to compare the means of the various groups.
(170.19 ± 36.07 pg/ml) and were negligible at 3 h post injection time (4.89 ± 1.78 pg/ml). Saline-treated mice did not express H2-relaxin in their plasma.

3.6 Subcutaneous Serelaxin increases plasma nitrite levels
Plasma nitrite levels increased at all time points, but only reached statistical significance at 6 h after injection when compared to control, 1-h and 3-h groups (p = 0.0109, 0.0011, and 0.0053, respectively) (Figure 5B).

3.7 Haemodynamic parameters show no change with 10 µg/kg serelaxin
The systolic and diastolic blood pressure recordings indicate no change in pressure up to 30 min following serelaxin administration in sham mice (Figure 5C). This suggests that the cardioprotective effects of serelaxin against I/R injury at a dose of 10 µg/kg are not secondary to haemodynamic changes caused by the drug.

3.8 Reperfusion treatment with serelaxin attenuates caspase-1 activity in the heart post-MI
Serelaxin given at reperfusion attenuated activation of the inflammasome in the heart in WT mice when compared with saline in wild-type mice in (A) pretreatment arm (n = 8/group) and (B) reperfusion therapy arm (n = 9/group). Genetic deletion of eNOS abolished this preservation in LV function. Bottom Panel: LV FS measured in the various treatment groups. One-way ANOVA was used to determine the main effect and Tukey’s post-hoc multiple comparisons test was used to compare the means of the various groups.

3.9 Serelaxin does not attenuate inflammasome-mediated peritonitis in the mouse
To further evaluate the direct capacity of serelaxin to attenuate NLRP3 inflammasome activation in vivo and independent of its infarct-sparing effects, we used the zymosan A-induced peritonitis model, an inflammatory model in which leukocyte migration into the peritoneal cavity is mediated by the inflammasome without induction of myocardial ischaemia. In this model, pretreatment with serelaxin did not reduce the leukocyte infiltrate in the peritoneal cavity following challenge with zymosan A (p = NS vs. control, Figure 6B). This suggests that the effects of serelaxin on caspase-1 activity are mediated by its powerful infarct-sparing capacity.

4. Discussion
In the current study, we show that serelaxin, recombinant H2-relaxin, attenuates myocardial I/R injury by reducing infarct size and left ventricular dysfunction after 24 h when given as a preconditioning mimetic or as reperfusion therapy in mice. Several studies have demonstrated the protective effect of recombinant human (rh)-relaxin against I/R injury in cultured cardiomyocytes or in vivo models of myocardial infarction, recently reviewed by Valle Raleigh et al. Most of the in vivo studies, however, were limited by the short duration of reperfusion (3 h), which precluded better assessment of clinical outcomes. Moreover, the mechanistic findings that implicated NO in mediating the cardioprotective effects of rh-relaxin relied on pharmacoologic non-specific NOS inhibition. Our study is the first to take advantage of mice with genetic deletion of eNOS.
eNOS and characterize the infarct-sparing and anti-inflammatory effects of serelaxin in the absence of eNOS-derived NO. Our study design was also clinically relevant since we administered serelaxin as a pre-conditioning mimetic 1 h prior to experimental ischemia and also tested its effects when given at the onset of reperfusion. Both approaches are important since the pre-treatment strategy may be beneficial in scenarios where ischemia is predicted, such as cardiac surgery or transplantation, and reperfusion therapy is applicable in the catheterization lab setting. Lethal reperfusion injury is known to cause cardiomyocyte death from oxidative stress, calcium overload, rapid restoration of physiological pH, and inflammation. Interestingly, relaxin seems to target many of these mechanisms of cell injury as shown in previous studies. 

Serelaxin (10 μg/kg, s.c.) given prior to ischemia or at the onset of reperfusion was very effective in reducing infarct size and improving LV function and survival at 24 h after treatment. In both treatment arms, genetic deletion of eNOS blunted the cardioprotective effects of serelaxin and attenuated the overall survival benefit seen in wild-type mice. This finding is particularly important since it demonstrates, for the first time, the significance of eNOS in mediating the protective effects of rh-relaxin and also sheds new light on potential future implications for serelaxin in other diseases that involve endothelial dysfunction or reduced NO bioavailability. The dose of serelaxin used in our studies resulted in plasma concentrations (~0.8 ng/ml) that approximated relaxin levels in pregnant mice (~5–7 ng/ml). Moreover, these levels were achieved as early as 5 min after subcutaneous injection of serelaxin in mice, which clearly supports the role of serelaxin in conferring the cardioprotection observed when administered at the time of reperfusion. Despite the route of administration, the significant rise in plasma levels within a short duration can be explained by referring to the absorption profiles of other peptides injected in murine subcutaneous sites. A study performed by Wu et al. to quantify the rate of loss of peptides within subcutaneous injection sites in mice revealed that half-life of removal is inversely correlated with the molecular weight of proteins. VEGF-C156S, with the mass of 23 KDa, had a half-life of removal of 31 min. Therefore, it is reasonable to expect that serelaxin, a peptide weighing 6 KDa, would be found at much shorter times within the plasma. However, the study focused on lymphatic absorption specifically since peptides weighing greater than 16 KDa are predominantly absorbed via the lymphatic system. Smaller
peptides (<16 KDa) can directly diffuse into capillaries and be expected to have shorter absorption times than can be achieved via the lymphatic route. The ELISA kit was also utilized to quantify protein levels at 2-h and 3-h time points. Serelaxin levels declined to negligible values within 3 h and were not detected in saline-treated mice. The recombinant peptide has a terminal half-life of 1–4 h, varying across species. Since pharmacokinetic parameters for serelaxin can be scaled allometrically, the rapid clearance as observed in our mouse model is consistent with observations made in current literature.

The effect of rh-relaxin on myocardial I/R injury was first evaluated in a model of isolated-perfused guinea pig hearts. In this study, relaxin increased coronary flow when administered in the perfusion buffer at the time of coronary occlusion. Nitric oxide production was increased with relaxin and, intriguingly, these effects were blunted with non-specific NOS inhibition. Additionally, relaxin significantly reduced calcium overload and malonyldialdehyde, markers of myocardial injury and mast cell degradation, as well as mitochondrial swelling and hyper-contraction of myofibrils. In a rat model of I/R injury, pretreatment with relaxin 30 min before ischemia reduced myocardial injury, ventricular arrhythmias and mortality. Furthermore, morphological studies with electron microscopy revealed that relaxin reduced endothelial and cardiomyocyte swelling and conserved normal ultrastructure of myofilaments, which were also accompanied by reduction in neutrophil accumulation in capillary vessels. These results correlated with a decline in myeloperoxidase activity, a marker of neutrophil accumulation in tissue, and myocardial calcium content. Although most I/R models that tested relaxin were ex vivo and the drug was given prior to ischemia, reperfusion therapy with rh-relaxin following 30 min of ischemia in pigs

Figure 6 Serelaxin attenuate NLRP3 inflammasome activation following myocardial infarction. (A) Caspase-1 activity was markedly increased at 24 h following I/R injury in the hearts of wild-type mice and eNOS knockout mice (n = 9/group). The increase in caspase-1 activity; however, was attenuated with serelaxin treatment only in wild-type mice but not in eNOS knockout mice. (B) In the inflammasome-dependent peritonitis model, serelaxin did not attenuate leukocyte infiltration in wild-type mice as compared to saline controls after zymosan injection (n = 5/group). One-way ANOVA was used to determine the main effect and Tukey’s post-hoc multiple comparisons test was used to compare the means of the various groups.
was shown to cause dose-dependent reduction in serum biomarkers of myocardial damage.18,27 These findings correlated with increased myocardial viability measured with cardiac single-photon emission computed tomography with Thallium chloride. Malondialdehyde, calcium overload and myeloperoxidase were also reduced with rh-relaxin in this study. Unfortunately, the study design prevented further assessment of more clinical outcomes (including infarct size at 24 h, survival and cardiac function with longer follow-up) since the animals were sacrificed after 180 min of reperfusion.

The role of NO in mediating the protective effects of relaxin has been examined not only in the heart, but also in the lung15 and kidney.29 Alexiou et al.15 showed that relaxin-2 attenuated I/R-induced lung injury via NO-dependent pathway. Although the protective effect of relaxin was abolished in the presence of various NOs inhibitors, including LN3-Nitroarginine methyl ester (L-NAME), 1400W and S-methyl-L-thiocitulline (SMTc), relaxin was shown to promote moderate inducible NOS stimulation. Similarly, Collino et al.34 reported that rh-relaxin attenuated functional renal injury following I/R by reducing oxidative stress and neutrophil activation in a NO-dependent mechanism by enhancing eNOS activation and up-regulation of inducible NOS. Another study tested the anti-inflammatory properties of relaxin in lipopolysaccharide (LPS)-primed rat coronary endothelial (RCE) cells.29 RCE cells and neutrophils were co-cultured and their adhesion was studied in the presence or absence of relaxin. The NOS inhibitor, L-N(G)-monomethyl arginine citrate (L-NMMA) was utilized to test the possible involvement of NO in mediating the down-regulation of endothelial adhesiveness to neutrophils. Incubation of LPS-primed RCE cells with relaxin for 24 h resulted in significant reduction in adherent neutrophils as well as endothelial expression of adhesion molecules P-selectin and vascular cell adhesion molecule (VCAM)-1 protein and mRNA. These effects were abolished in the presence of L-NMMA, advocating a key role of NO in NOs-derivatives NO. Our results demonstrating an essential role of eNOS in mediating the cardioprotective role of relaxin against I/R injury fully support the previous findings. To study the immediate impact of relaxin treatment on eNOS activity, we assessed the phosphorylation status of cardiac eNOS at 1, 3, and 6 h after treatment. Surprisingly, our results indicate no difference between serelaxin treatment and control saline at these time points (see Supplementary material online, Figure S1 showing no change at 1 h). However, plasma nitrite levels were increased at the specified time points and reached statistical significance at 6 h after treatment, suggesting that global eNOS activation and thus NO generation mediated the cardioprotective effect of relaxin against I/R injury. Indeed, these protective effects were absent in eNOS knockout mice treated with serelaxin.

Recombinant relaxin mediates its influence on signalling by acting on its cognate receptor RXFP1. Despite its structural homology with other GPCRs, RXFP1 belongs to the unique LGR family as it possesses the N terminal leucine rich repeat (LRR) region and an LDLa module attached to the LRR domain.35 These additional moieties confer unique agonist binding sites within RXFP1. Specifically, the R00xR00xV region within the B chain of relaxin binds to LRR4-8, which leads to the interaction of attached LDLa module with Extracellular loops 1 and 2 of the transmembrane region. Therefore, the LDLa module serves as a true ligand to the receptor and relaxin is needed to initiate this process to result in downstream signalling.35

The canonical signalling pathway invoked after the activation of RXFP1 leads to a Gαs-mediated initial surge in cAMP that is inhibited by GoRx. A later cAMP response is mediated by the βγ subunits.35 These subunits also activate PI3K/Akt. PI3K activity has previously been shown to regulate eNOS phosphorylation and hence alter its functional significance at the level of post-translational modifications.36 As such, treatment of rat aortic rings with H2-relaxin in a study performed by Dschietzig et al.36 led to increased eNOS phosphorylation at Ser1177 and Ser633, which enhance the enzymatic activity and Ser1177 phosphorylation was abolished upon incubation of rings with Wortmannin, a PI3K inhibitor.

Our results also demonstrate, for the first time, that reperfusion therapy with serelaxin attenuates the activation of the NLRP3 inflammasome as shown by significant reduction in caspase-1 activity. The role of sterile inflammation that occurs following MI in promoting further injury leading to heart failure has been in the spotlight of recent investigations.21,23,37,38 The NLRP3 inflammasome is a macromolecular structure that functions as a platform for the production of pro-inflammatory cytokines of the Interleukin-1 family (i.e. IL-1β and IL-18) and is involved in the progression of several diseases.39 In the cardiovascular system, it is activated in cardiomyocytes, fibroblasts, leukocytes, and endothelial cells.38 There is enough evidence in animal models incriminating NLRP3 activation after myocardial injury18,21,23 in related impairment of heart function and remodelling by producing IL-1β and IL-18.40,41 Although there is no direct proof supporting a modulatory role of relaxin on NLRP3, an inhibitory effect of NO on NLRP3 has been reported. NO was shown to inhibit caspase-1, IL-1β, and IL-18 release from stimulated peritoneal macrophages in vitro and in vivo in mice.42 In a mouse model of renal I/R, relaxin was able to blunt the inflammatory response by reducing IL-1 and IL-18 levels, among other inflammatory markers.43 Interestingly, our results also indicate that the inhibitory effects of serelaxin on caspase-1 following I/R injury were lost in eNOS knockout mice. This further supports an obligatory role of eNOS in mediating both the infarct-sparing and anti-inflammatory effects of serelaxin. While serelaxin attenuated caspase-1 activity following MI, our results also show that serelaxin did not interfere with inflammasome activity following canonical inducers of the NLRP3 inflammasome in a peritonitis model in vivo (Zymosan A). These data suggest that the inhibitory effect of serelaxin on cardiac caspase-1 activity is likely dependent upon its infarct-sparing properties. Nonetheless, the overall anti-inflammatory effects of relaxin have been shown to occur in models of LPS-mediated inflammation29 and therefore future long-term studies
with follow-up at 1 or more years are needed to shed more light on the anti-inflammatory effect of serelaxin in preventing adverse remodelling post-MI.

In conclusion, the present study has provided evidence for a novel mechanism by which serelaxin exerts cardioprotective effects against I/R injury in mice. In particular, the treatment of reperfusion injury with serelaxin is especially attractive, considering its profound effect on myocardial infarct size and LV function, which are strong predictors of adverse outcomes following MI. Interestingly, relaxin has been shown to attenuate the occurrence of severe ventricular arrhythmias following I/R injury in animal models of MI, which may explain the improvement in survival in our study. Although our infarction model utilizes healthy mice, which may represent a departure from human coronary disease due to differences in coronary circulation and confounding factors, it still serves as proof-of-concept for the potential infarct-sparing effects of serelaxin post-MI. Given the favourable safety profile of serelaxin and the wealth of data suggesting its protective properties, serelaxin may be considered a promising candidate for clinical trials to study its infarct-sparing effects when given at reperfusion in patients with acute heart failure.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Funding

This study was supported by an Investigator-Initiated Trial from Novartis Pharmaceuticals (CRLX0304USNC02T) to F.N.S.

Conflict of interest: F.N.S. and A.A. received research grants from Novartis Pharmaceuticals. The other authors have no conflict of interest to disclose.

References


