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The Prominin-1 derived peptide improves cardiac function following ischemia

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Abstract:

Myocardial infarction (MI) remains the leading cause of death in the western world. Although medical advancements have been made in interventional revascularization technologies, a large percentage of patients are not candidates for them due to co-morbidities or lack of local resources. Thus, there remains a need for the development of novel non-invasive strategies to treat MI. Approaches to accelerate revascularization within ischemic tissues through angiogenesis by providing Vascular Endothelial Growth Factor (VEGF) in protein or gene form has been shown to be effective in animal models but not in humans likely due to its short half-life and systemic toxicity. We previously showed that a small peptide (PR1P) we developed stabilizes VEGF in its active dimer state, increases VEGF binding to its receptors and potentiates VEGF activity. Here we show that systemic PR1P treatment targeted, stabilized and upregulated endogenous VEGF within ischemic myocardium following left coronary artery surgery in mice and rats. Targeted VEGF upregulation led to augmentation of heart function at two weeks following surgery. We conclude that PR1P is a potential candidate therapeutic for MI.

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Introduction

Myocardial infarction (MI) is a leading cause of morbidity and mortality worldwide[1]. Rescuing cardiomyocytes (CM) from cell death in the ischemic region and restoring blood flow in obstructed arteries are two crucial strategies for treatment. Conventional invasive therapies to improve coronary artery blood flow and reduce myocardial damage following MI include coronary artery dilation or stenting, and coronary artery bypass surgery. However, these invasive procedures are often not available, require delay (often due to co-morbidities) or not entirely effective. Therapeutic induction of *angiogenesis would potentially be more easily available and less invasive as it could theoretically* only require the systemic administration of angiogenic factors to restore vascularization to damaged areas of the heart. The most promising and extensively studied pro-angiogenic therapeutic is vascular endothelial growth factor (VEGF)[2] which has been shown to function as both a survival and angiogenesis factor[3]. Specifically, VEGF facilitates CM regeneration and protects CMs from apoptosis in vitro by activation of downstream VEGF signaling pathways including the activation of AKT and the upregulation of Bcl-2 expression[4, 5]. However, despite success in animal ischemia models, VEGF therapies have failed in human clinical trials[6]. Disappointing clinical results can potentially be attributed to short plasma and tissue half-lives which limit the retention of VEGF within target (ischemic) zones. Also, VEGF's propensity to induce severe hypotension and tissue edema when delivered intravascularly limited dosing to levels far below those likely to show efficacy in under perfused areas[7, 8].

We have previously shown that PR1P, a small peptide derived from the extracellular domain of prominin-1 and which binds to VEGF and protects it from proteolytic degradation[9, 10], augmented angiogenesis in multiple in-vivo angiogenesis models, and improved perfusion of threatened limbs in a murine hind limb ischemia model[9]. Additionally PR1P has been shown to increase VEGF levels and signaling within lungs when delivered via inhalation[10] and to protect neurons[11] and lung cells[10] from apoptosis. We hypothesized here that PR1P would upregulate endogenous VEGF signaling within compromised heart tissue and improve outcome from myocardial infarction. Herein we provide evidence that PR1P improved CM survival following serum starvation in vitro, and when delivered systemically to mice and rats following left coronary artery ligation, PR1P targeted, stabilized and upregulated VEGF signaling within ischemic myocardium. This targeted upregulation of endogenous VEGF was associated with improved functional outcome measured using intravascular hemodynamic monitoring and echocardiography. Collectively, these data support using PR1P in a novel non-invasive approach to treat MI by targeting and upregulating endogenous VEGF within ischemic myocardium.

Material and Methods

Murine model of Myocardial Infarction

Ten-week-old female C57BL/6J mice (Jackson Laboratory (Bar Harbor, ME, and from Harlan, Rehovot, Israel) and 10-week-old female Sprague-Dawley rats (Harlan, Rehovot, Israel) were used for the MI experiments. Anesthesia was induced using ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5mg/kg). Upon adequate anesthesia, a left thoracotomy was performed, the heart was exposed, and the left anterior descending coronary artery ligated using a 6.0 silk suture. Following ligation, the thorax and skin were closed, and the animals allowed to recover at ambient room temperature. Animals were randomized in a blinded fashion to receive treatment consisting of intraperitoneal (IP) administration of PR1P or scrambled peptide (SP, 10µg in 100 ul saline/mouse and 200ug in 100ul saline/rat), starting on day 0 and administered every other day until day 12. A select group of animals underwent Sham surgery and did not receive treatment of any kind. Animals were euthanized on day 14. Protocols for these studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital and at the Beth Israel Deaconess Medical Center (BIDMC) (murine MI model) and Institutional Animal Care and Use Committee (IACUC) at the Hebrew University, Jerusalem, Israel (rat MI model).

Hemodynamic Measurements in mice

Hemodynamic pressure-volume parameters were measured in mice under inhaled isoflurane anesthesia using a 1.4-Fr microtip pressure-volume catheter (Scisense, Ontario, Canada) inserted into the right common carotid artery and advanced into the left ventricle. Data were recorded using a Powerlab system (ADInstruments, Colorado Springs, CO). Beat-by-beat pressure-volume parameters including stroke

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work (SW), cardiac output (CO), ejection fraction (EF) were measured and analyzed using CardioSoft Pro software as was done in the past (CardioSoft, Houston, TX)[12, 13].

Echocardiographic Studies in Mice and Rats

Echocardiography was performed in mice and rats by a blinded technician on anesthetized ((ketamine, 80 mg/kg, IP) and xylazine hydrochloride (5 mg/kg, IP)) animals at 3 or 14 days after coronary artery ligation surgery. Echocardiographic imaging was performed using a GE Vivid3 platform equipped with a 13-MHz linear epiaortic transducer (General Electric, Haifa, Israel). The probe was positioned in a left parasternal position, and two-dimensional imaging of the heart in the short axis was performed using a high frame rate. This image was used to guide an M-mode cursor down the medial axis of the LV. Measurements were performed in triplicate using the leading-edge convention for myocardial borders. MAX-LVP (maximum left ventricular pressure), MIN-LVP (minimum left ventricular pressure), EDM-LVP (minimum end diastolic left ventricular pressure), MAX-LV-V (maximum left ventricular volume), MIN-LV-V (minimum left ventricular volume).

Histology and Immunochemistry (Rat Heart Infarct Quantification)

Rat hearts were fixed in 10% formalin and paraffin-embedded using standard protocols. Heart sections (4 μm) were treated with Masson's trichrome (MTC, Thermo Scientific, Rockford, IL), which stains collagen blue and myocardial cells pink red. Photomicrographs of sections of the left ventricle were used to quantify myocardial infarction size as described by Grad et al.[14] Briefly, infarct size was calculated as a normalized ratio of the minimal width of the infarcted area compared to the area of the left ventricle (LV) muscle area ((min width * 100)/LV Area, i.e. blue stain)/total LV muscle area (cm^2), as well as a normalized ratio of the infarct area to the whole left ventricle area ((Infarct Area *100)/LV area) using ImageJ© software (National Institute of Health, Bethesda, MD).

Cell culture

Primary human cardiomyocytes (CM) were grown in Cellartis CM Culture base (Takara, Mountain View, CA USA) and used between passages 4–6.[15]

Apoptosis assay

Cardiomyocytes (CMs) were incubated in serum free medium without supplements in the presence or absence of PR1P (0.2 mg/ml) for 24 hours, removed from the plate by treatment with 0.25% trypsin, and then incubated with FITC-conjugated Annexin V to identify apoptotic cells (Ebioscience, San-Diego, CA) according to manufacturer's instructions. Cells were then analyzed on FACS Calibur flow cytometer (BD Biosciences, Pharmingen, San Diego, CA) using Flowjo software.

Western blot analysis

CMs were serum starved for 24 hours and incubated with 50μg/ml of PR1P for 15 minutes at 37°C. The cells were scraped and washed twice with 1xPBS. Cells were homogenized with RIPA buffer [50 mMTris-HCl (pH 7.4), 150 mMNaCl, 1% Nonidet P-40, 0.5% Na+ deoxycholate, and 0.1% SDS] (IPA-Boston Bioproducts, Worchester, MA), containing a protease inhibitor cocktail tablet (Sigma-Aldrich, St. Louis, MO) on ice. Blotting was done using standard methods and repeated three times to ensure reproducibility. Primary antibodies for western blot analysis in this study were used at 1:1,000 dilution and included anti-VEGF (Santa Cruz, Santa Cruz, CA), pAKT (Cell signaling, Danvers, MA) and β-actin (Sigma-Aldrich, St. Louis, MO).

Reagents and peptides

The 12-mer peptide PR1P (DRVQRQTTTVVA) and SP (QATVDTRQVTRV) were commercially synthesized by Biomatik (Wilmington, DE).

Statistical Analysis

All results are expressed as mean ±SD. Statistical comparisons between 2 groups were performed by Student's t test. P values <0.05 were considered statistically significant. All experiments were repeated in triplicate.

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Results

PR1P improves murine myocardial function post-infarction

	Heart rate (bpm)	MAX-LVP (mmHg)	MIN-LVP (mmHg)	EDM-LVP (mmHg)	MAX-LV_V (μl)	MIN-LV_V (μl)
Sham	452.8 ± 22.8	100.9 ± 4.9	-3.4 ± 2	1.9 ± 0.6	49.2 ± 11.2	15.8 ± 5.9
SP	468.9 ± 48.3	89.7 ± 6.3	7.9 ± 5.2	16 ± 6.4	53.2 ± 6.7	35.5 ± 6.5
PR1P	483.5 ± 66.8	94.5 ± 8.3	5 ± 6.6	10.7 ± 7.6	45.4 ± 7.3	22.8 ± 6.8
p value	0.4713	0.0629	0.1644	0.0351	0.003	0.0001
	Stroke work (mjoules)	dp/dt MAX (mmHg/sec)	dp/dt MIN (mmHg/sec)	Aortic pressure systolic (mmHg)	Aortic pressure diastolic (mmHg)	
Sham	2672 ± 719	8677 ± 751	-7876 ± 919	100.2 ± 5.1	70.8 ± 7.5	
SP	1066 ± 296	5999.7 ± 1031	-4942 ± 765	90.2 ± 5.6	68.6 ± 5.5	
PR1P	1493 ± 414	6817.7 ± 1518	5214.1 ± 1050	92.9 ± 7.9	71.9 ± 5.7	
p value	0.0017	0.0706	0.3821	0.2446	0.0935	

Table 1. PR1P enhances functional outcome of the heart following myocardial infarction in mice. Shown are hemodynamic parameters and indices of heart function (with p values comparing PR1P to SP) in mice obtained using vascular transducer catheters placed in the left ventricle of anesthetized animals 14 days following sham surgery (Sham) and no treatment, or left coronary artery ligation and every other day treatments with intraperitoneal injections of PR1P or scrambled peptide (SP). BPM (beats per minute), MAX-LVP (maximum left ventricular pressure), MIN-LVP (minimum left ventricular pressure), EDM-LVP (minimum end diastolic left ventricular pressure), MAX-LV-V (maximum left ventricular volume), MIN-LV-V (minimum left ventricular volume).

To determine whether systemic PR1P therapy could be used to improve the functional outcome of injured myocardial tissue in vivo, we used invasive and non-invasive tools to assess myocardial performance at 14 days post surgically induced MI in mouse and rat MI models. In these models, surgical ligation of the left anterior descending coronary artery leads to myocardial infarction characterized initially by increased levels of VEGF mRNA and protein expression in compromised myocardium[16-19]. There was no mortality from surgery, and no differences between groups in body weights before, during or after experiments in any of the animals (data not shown). As summarized in Table 1, PR1P treatment in mice led to significant reduction in the minimum end diastolic left ventricular pressure (EDM-LVP, 10.7 ± 7.6 in PR1P, 16 ± 6.4 in SP, Table 1), maximum and minimum left ventricular volumes (MAX-LV-V, 45.4 ± 7.3 in PR1P, 53.2 ± 6.7 in SP, MIN-LV-V, 22.8 ± 6.8 in PR1P, 35.5 ± 6.5 in SP, Table 1), and a significant increase in stroke work (1493 ± 414 in PR1P, 1066 ± 296 in SP, Table 1) at 14 days post coronary artery ligation as determined using left ventricular vascular catheter probes. Also, PR1P improved flow-based hemodynamic cardiac function parameters including ejection fraction (EF, Fig. 1A), stroke volume (SV, Fig. 1B) and cardiac output (CO, Fig. 1C). Echocardiography revealed that PR1P reduced left ventricular internal diameter at end systole (i.e. LVIDs, Fig.1D) and mitigated the reduction in left ventricular fractional shortening (FS, Fig. 1D) at day 14 following coronary artery ligation. Collectively, these data demonstrate that systemic PR1P therapy significantly improved outcome from coronary artery ligation in mice.

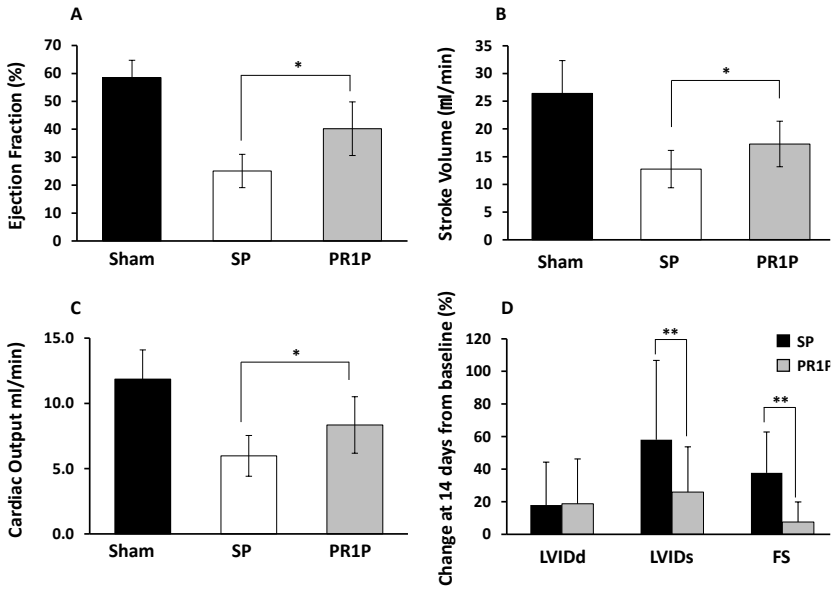

Figure 1. PR1P enhances the functional outcome of the heart following myocardial infarction in mice. **A-C)** Bar graphs showing left ventricular ejection fraction (EF, **A)** stroke volume (SV, **B)** and cardiac output (CO, **C)** measured using left ventricular vascular transducer catheters in anesthetized mice 14 days following sham surgery (Sham) and no treatment, or left coronary artery ligation and every other day intraperitoneal injections with scrambled peptide (SP) or PR1P. **D**) Bar graph showing average relative change from baseline of indicated echocardiographic measurements made on anesthetized mice described in Fig. 1A indicating improved outcome following PR1P treatment. LVIDd (left ventricular internal diameter at end diastole), LVIDs (left ventricular internal diameter at end systole), FS (Fractional shortening) calculated as FS=((LVIDd- LVIDs)/LVIDd). *p<0.001, **p<0.015, (n=6 Sham, 17 SP, 18 PR1P).

PR1P improves rat myocardial function post-infarction

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We next characterized the ability of systemic PR1P to mediate functional outcome from myocardial infarction in rats. In these studies, echocardiography at two weeks post coronaryartery ligation demonstrated decreased LV dilatation post-MI as evidenced by a reduction in the left ventricular cavity area during both systole and diastole (LV Cavity Area, Fig. 2A) and in the LVID at end of systole (LVIDs) and diastole (LVIDd, Fig. 2B). In addition, PR1P therapy led to improved cardiac contractility as indicated by an increase in the fractional change in area (Fig 2C) and increased left ventricular shortening fraction (Fig. 2D). Representative photomicrographs of Masson trichrome stained cross sections of myocardium (to identify collagen at sites of infarction[14]) from PR1P and SP treated rats 14 days after coronary artery ligation surgery are shown in Fig. 2E-F. Quantification of the infarct sizes from micrographs in similarly stained heart sections show decreased areas of infarction at both 3 and 14 days from surgery (Fig. 2G-H). Taken together, these data suggest that systemic delivery of PR1P enhances the functional outcome of murine and rat myocardium following acute ischemic injury.

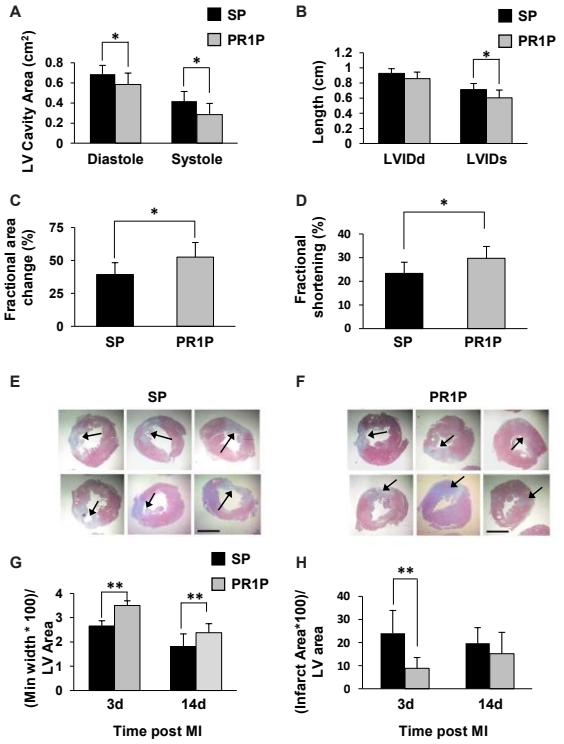

Figure 2: PR1P enhances the functional outcome of the heart following myocardial infarction in rats. A-D) Bar graphs showing echocardiographic assessments of rat heart function at 14 days following left anterior coronary artery ligation and every other day treatment with PR1P or scrambled peptide (SP) indicating improved outcome following PR1P treatment. LV Cavity Area (left ventricular cavity area). LVIDd (left ventricular internal diameter at end diastole), LVIDs (left ventricular internal diameter at end systole), FS (fractional shortening). (n= 9 PRIP, 7 SP) **p* < 0.04, **E-H**) **PR1P reduces infarct size following ischemia. E-F)** Representative photomicrographs of Masson trichrome stained sections of rat heart from animals described in Fig. 2A indicating infarcted regions (collagen, blue) and regions with viable muscle (pink red) at 14 days after surgery. Black arrows indicate areas of infarction. Scale bar, 100 mm. **G-H**) Bar graphs showing quantification of infarct size from photomicrographs described in E-F from hearts prepared 3 (N=5 PR1P, 5 SP) and 14 (N=6 PR1P, 5 SP) days after surgery. **p<0.05.

PR1P stabilizes VEGF, upregulates VEGF signaling and mitigates apoptosis

We next turned to molecular biology studies to determine the molecular mechanism by which PR1P augments recovery from myocardial ischemia caused by coronary artery ligation. We recently showed that PR1P bound VEGF, enhanced the prevalence of VEGF dimers (the active form of VEGF) and prevented VEGF degradation in vitro and in vivo in the lungs when delivered via inhalation[10]. We therefore investigated here whether systemically delivered PR1P similarly stabilized VEGF dimers within injured myocardium. Western blot analyses of tissue homogenates from ischemic and non-ischemic zones from rat heart biopsies 14 days after coronary artery ligation and every other day treatment with PR1P or SP showed increased expression of VEGF dimers in ischemic zones in the presence of PR1P (IZ, Fig. 3A). Interestingly, there was no effect of PR1P on the prevalence of VEGF dimers in non-ischemic zones (NZ, Fig. 3B) at this same time period.

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We previously showed that PR1P significantly reduced retinal ganglion cell apoptosis in an optic nerve crush injury model[11] and prevented respiratory epithelial cell apoptosis from cigarette smoke extract in-vitro and from LPS in-vivo in mice[10]. Downstream VEGF signaling including the phosphorylation and activation of the protein kinase AKT has been shown to inhibit CM apoptosis [20-22]. Akt also plays a key role in multiple cellular processes that mediate cell survival during stress including glucose metabolism, gene transcription, cell proliferation, and cell migration[23, 24]. To determine whether PR1P mediates AKT phosphorylation and apoptosis in injured myocardial cells, we first incubated CMs *in vitro* in the presence of increasing concentrations of PR1P and assessed levels of phosphorylated AKT in cell homogenates using Western blotting. Figure 3C shows that PR1P increased AKT phosphorylation in these cells at 15 minutes in a dose dependent manner. To determine whether PR1P similarly augments AKT phosphorylation *in vivo* following tissue injury, we biopsied cardiac tissue from ischemic zone (IZ) and normal zone (NZ) of rat heart at 3 and 14 days following coronary artery ligation, and analyzed levels of phosphorylated AKT relative to total AKT in tissue homogenates (Fig. 3D-E). Quantification of Western blots in these experiments revealed that PR1P increased AKT phosphorylation at 14 days following MI in the ischemic zones only (Fig. 3E). Together with our findings that PR1P augmented levels of VEGF dimers in ischemic zones only, these results suggest that PR1P targets and stabilizes endogenous VEGF, and augments VEGF signaling only within sites of ischemic myocardium. To determine whether PR1P has the potential to mediate apoptosis in CMs, we cultured CMs in serum free medium for 48 hours in the presence and absence of PR1P, and then fixed and stained the cells with annexin V (to identify cell apoptosis). FACS analysis of these cells revealed that PR1P significantly reduced serum starvation-induced CM apoptosis (Fig. 3F). Together, these data strongly suggest that systemic PR1P therapy augmented the functional outcome from coronary artery ligation by targeting and upregulating endogenous VEGF signaling within ischemic myocardium.

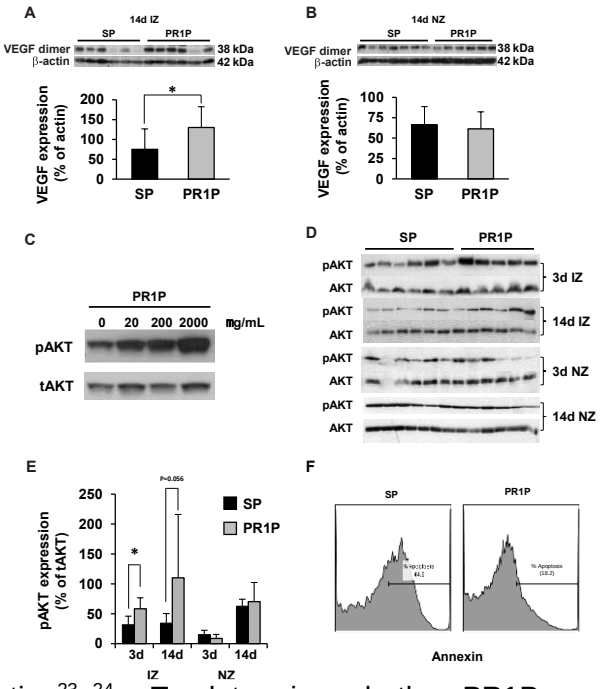

Figure 3: PR1P targets, stabilizes and upregulates endogenous VEGF within ischemic myocardium. A-B) Representative Western blot analyses (at top) and corresponding Bar Graphs showing quantification of blots of rat heart tissue homogenates from ischemic zone (IZ, **A**) or normal zone (NZ, **B**) at 14 days following left coronary artery ligation and every other day intraperitoneal treatment with PR1P or scrambled peptide (SP). Bar graphs show the corresponding quantification of VEGF expression as a percentage of β-actin expression for indicated groups of myocardial tissue homogenates and indicate that PR1P treatment augments the expression of VEGF in the IZ. *p<0.05. (**C**) Representative Western blot analysis of protein from human cardiomyocyte cell homogenates following 15-minute incubation in vitro at 37 °C in serum free cell culture media in the presence of increasing concentrations of PR1P showing dose dependent increase in phosphorylated AKT (pAKT) relative to total AKT (tAKT). (**D**) Representative Western blot analyses (D) and corresponding Bar Graphs (E) showing quantification of phosphorylated AKT (p-AKT) relative to AKT (total, t-AKT) of rat heart tissue homogenates from ischemic or normal zones (IZ or NZ, respectively) at 3 or 14 days following left coronary artery ligation and treatments described in Fig. 3A. Membranes were immunoblotted with antibodies to phosphorylated AKT (p-AKT) and then AKT (total, t-AKT) in order to quantify the relative expression of pAKT as a percentage of tAKT. (**E**) Bar graphs showing corresponding quantifications of pAKT expression as a percentage of tAKT in IZ and NZ myocardium at indicated time points showing increased pAKT in IZ following PR1P therapy. *p<0.01. **F)** Representative FACS analysis of cardiomyocytes following 48-hour serum starvation in the presence or absence of PR1P (0.2mg/mL) and stained with FITC-annexin V showing that PR1P reduces serum starvation-induced apoptosis.

Discussion

Here we report on studies conducted in mice and rats supporting that PR1P is a novel candidate therapeutic to treat MI. We used established rodent cardiac ischemia models that have been shown to induce a short transient upregulation of VEGF expression and signaling[17-19]. We found that systemic delivery of PR1P, a proprietary peptide that binds and stabilizes endogenous VEGF, resulted in improved cardiac function two weeks from coronary artery ligation as measured by echocardiography and invasive intravascular monitoring. Mechanistic studies suggested that systemically delivered PR1P targeted cardiac tissue within ischemic zones leading to sustained upregulation of endogenous VEGF signaling beyond that which is normally found during natural recovery from MI in rodents.

Myocardial infarction affects 7.9 million US adults annually[25]. As populations age, an increasing proportion of patients who might benefit from standard revascularization procedures will be rejected as

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candidates due to significant co-morbidites[26]. Thus, there has been an interest to develop an effective and easily accessible non-invasive treatment capable of reducing myocardial injury following ischemia. Gene, protein, and cellular therapies have been considered as potential strategies to treat coronary artery and peripheral vascular occlusive diseases that do not respond to conventional treatment[27]. VEGF remains the leading candidate target to stimulate both angiogenesis and prevent apoptosis, the two major processes that mediate atherosclerosis as well as ischemic heart disease[28]. However, although different types of VEGF therapies, including treatment with genes and proteins, have been shown to be effective in animal myocardial infarction and ischemic limb models, clinical trials for these purposes have not been successful[29, 30]. Failure in these studies was multi-factorial. VEGF is rapidly cleared from the circulation [31, 32], and its tissue half-life is short[33], and so high VEGF doses required to prove therapeutic efficacy were used which resulted in significant toxicity including hypotension, localized edema, anemia, and thrombocytopenia[34, 35]. Myocardial VEGF production is significantly but only transiently upregulated under normal circumstances following ischemic injury[16]. It would therefore seem plausible that a therapeutic such as PR1P that can target, stabilize and upregulate endogenous VEGF signaling within tissue microenvironments for extended periods of time without disturbing pre-existing VEGF gradients may prove beneficial in mitigating tissue injury and may also limit toxicity. PR1P addresses the need for an alternative systemic therapy that targets and enhances the effect of endogenous VEGF only within sites of tissue injury (see Fig 3). Unlike with systemic VEGF therapy, the hypoxia induced VEGF gradient in the injured tissue microenvironment is targeted by PR1P and is maintained thereby preserving the mechanical and chemical cues necessary to stimulate appropriate blood vessel growth and myocardial repair[36, 37].

The role of VEGF signaling following ischemia in the rodent heart after coronary artery ligation has been extensively studied[28, 38]. Within hours of coronary artery ligation, there is a transient increase in the level of VEGF mRNA in zones bordering ischemic regions that returns to baseline within 24 hours[18]. In contrast, there is a gradual decline in VEGF mRNA in ischemic zones that begins hours after ischemia and that remains low 2 weeks thereafter[18]. VEGF levels similarly increase transiently for 24 hours in border zones, and similarly decline within hours in ischemic zones and remain low for up to one month.[18] Interestingly, there is inflammation within the injured myocardium that becomes apparent within hours to days: neutrophils infiltrate the ischemic tissue within the first few days, then macrophages appear (~4 days) followed by lymphocytes (7-14 days)[38]. There is proliferation of fibroblasts and collagen accumulation early beginning within the 7-14 days, and there is completion of scar formation by day 21[38]. Thus, ischemia induced VEGF signaling within the border and ischemic zones mediates angiogenesis and multiple cellular events including apoptosis and inflammation that ultimately determine the degree of functional recovery of the myocardium[16, 18]. We found that every other day treatment with systemic PR1P both targets and stabilizes endogenous VEGF within ischemic zones and alters the natural progression of endogenous VEGF signaling that was associated with improved functional outcome. Specifically, PR1P therapy led to increase in VEGF dimer levels and increased downstream VEGF signaling (AKT phosphorylation) within ischemic zones, but not within zones remote from injury (see Fig. 3). We recently showed that PR1P binding to VEGF prevented its degradation by proteases, including plasmin and elastase, naturally released by inflammatory cells during inflammation, and which compete with PR1P for binding sites within the VEGF heparin binding domain (HBD)[10]. PR1P binding to VEGF within ischemic zones of the myocardium likely similarly increases endogenous VEGF levels and signaling by preventing VEGF degradation by proteases released by inflammatory cells recruited into the heart following ischemia. Ohta et al found that increased elastase activity that correlated with inflammatory cell infiltration of the murine heart following MI increased as early as 6 h after left coronary artery ligation and persisted at 7 days[39]. Overexpression of an elastase inhibitor in mice in their model led to improvement in diastolic dysfunction at 4 days and improved cardiac performance at 28 days following coronary artery ligation suggesting that elastase inhibition suppresses inflammation associated cardiac dilatation and dysfunction after MI[39]. Importantly, PR1P does not inhibit elastase per se, but instead blocks its binding to VEGF[10]. As such, the effect of PR1P on the functional outcome in the ischemic myocardium may be due in part to its ability to prevent VEGF degradation by elastase and other proteases which might limit potential signaling effects of VEGF degradation products[40, 41] Kurtagic et al showed that VEGF degradation product generated by elastase is a macrophage chemoattract[41], and so reduced VEGF degradation product could in itself mitigate inflammation. Although the mechanism by which PR1P targets the injured myocardium is not clear, this novel property of targeting VEGF signaling where needed could

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potentially be used to target oxygen deprived but not yet infarcted regions in the heart during unstable angina or for peripheral vascular occlusive disease where inflammation is also noted[42, 43].

We found that PR1P stabilized VEGF in its dimeric (active) form within ischemic zones of rat myocardium at 2 weeks following coronary artery ligation leading to upregulation of endogenous VEGF signaling. Downstream VEGF signaling has been shown to play a key role in multiple cellular processes in CMs that mediate cell survival during stress including glucose metabolism, gene transcription, cell proliferation, and cell migration[23, 24]. In addition, VEGF mediated AKt activation has been shown to reduce apoptosis in CMs following ischemia-reperfusion injury[44, 45], pressure overload[46], and oxidative stress[47]. Thus, these multiple cellular processes collectively mediate cell survival, along with angiogenesis, which would serve to augment perfusion to deprived tissues, and ultimately determine the functional outcome of the heart following ischemic injury in vivo[48]. In fact, increased expression of anti-apoptotic proteins in cardiomyocytes, such as Bcl-2, and increased apoptosis in general is a consistent feature of end-stage heart failure[49] and correlates with the clinical severity of cardiomyopathy[5]. Interestingly, knockdown of Akt resulted in impaired endothelial progenitor cell function and neovascularization of hind-limb muscles following experimentally induced unilateral limb ischemia in mice[50]. PR1P upregulated angiogenesis in multiple VEGF dependent angiogenesis models in vitro and in vivo and in so doing increased blood flow to compromised hind limbs in a murine hind-limb ischemia model[9]. Our current study further supports that PR1P therapy enhances outcomes following tissue injury by targeting endogenous VEGF in the ischemic tissue where it facilitates AKt phosphorylation and the downregulation of apoptosis.

In summary, these studies revealed that systemically administered PR1P targeted and stabilized VEGF to augment VEGF levels and signaling in ischemic zones following coronary artery ligation in mouse and rat MI models. Sustained stabilization and upregulation of endogenous VEGF led to improved functional outcome of the heart in both animal models at 2 weeks from MI. Our findings have direct relevance to human diseases involving tissue injury from arterial insufficiency leading to tissue ischemia including coronary artery and peripheral vascular disease. Success of this novel VEGF targeting approach would enable patients to be treated earlier and without the need for invasive technologies.

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