Published in final edited form as:

J Mol Cell Cardiol. 2018 May ; 118: 1–12. doi:10.1016/j.yjmcc.2018.03.005.

Overexpression of Prostaglandin E2 EP4 Receptor Improves Cardiac Function after Myocardial Infarction

Timothy D. Bryson^{1,2}, Xiaosong Gu^{1,4}, Remonda M. Khalil^{1,2}, Safa Khan¹, Liping Zhu¹, Jiang Xu¹, Edward Peterson³, Xiao-Ping Yang¹, and Pamela Harding^{1,2}

¹Hypertension & Vascular Research Division, Dept. of Internal Medicine, Henry Ford Hospital, Detroit, Michigan, USA

²Dept. of Physiology, Wayne State University School of Medicine, Henry Ford Hospital, Detroit, Michigan, USA

³Dept. of Public Health Sciences, Henry Ford Hospital, Detroit, Michigan, USA

⁴Department of Cardiology, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu, 215004, China

Abstract

Background—Prostaglandin E2 (PGE₂) signals through 4 separate G-protein coupled receptor sub-types to elicit a variety of physiologic and pathophysiological effects. We recently reported that PGE₂ via its EP3 receptor could reduce cardiac contractility of isolated myocytes and the working heart preparation. We thus hypothesized that there is an imbalance in the EP3/EP4 ratio towards EP3 in the failing heart and that overexpression of EP4 in a mouse model of heart failure would improve cardiac function.

Methods and Results—Our hypothesis was tested in a mouse model of myocardial infarction (MI) with the use of AAV9-EP4 driven by the myosin heavy chain promoter to overexpress EP4 in the cardiac myocytes. Echocardiography was performed to assess cardiac function. We found that overexpression of EP4 improved shortening fraction (p=0.0025), ejection fraction (p=0.0003), and reduced left ventricular dimension at systole (p=0.0013). Overexpression of EP4 also significantly reduced indices of cardiac hypertrophy and interstitial collagen fraction. Animals treated with

Address for Correspondence: Pamela Harding PhD, Senior Bioscientific Staff, Hypertension & Vascular Research Division, Henry Ford Hospital, E& R Building room 7097, 2799 West Grand Blvd., Detroit, MI 48202, Tel: (313) 916 2549, Fax: (313) 916 1479, phardin1@hfhs.org.

Author contributions: TB; manuscript preparation, experimental design, real time RT-PCR. XG; surgery/intracardiac injections, echocardiography, myocyte cross sectional area, and interstitial collagen fraction analysis. RK; real time RT-PCR, myocyte cross sectional area analysis. LZ; Macrophage migration analysis. SK; CD3 staining and analysis, manuscript preparation, manuscript review. JX; Surgery/intracardiac injections, echocardiography. EP; statistical analysis. X-P Y; surgery/intracardiac injections, echocardiography, manuscript revision. PH; principal investigator, manuscript preparation, manuscript review, experimental design and compilation of data.

Disclosures

None of the authors have anything to disclose

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

AAV9-EP4 also had a significant decrease in TNF α mRNA expression and in the number of macrophages and T cells migrated post MI coupled with a reduction in the expression of iNOS.

Conclusion—Overexpression of EP4 improves cardiac function post MI. This may be mediated through reductions in adverse cardiac remodeling or via inhibition of cytokine/chemokine production.

Keywords

myocardial infarction; heart contractility; inflammation; chemokines; cytokines; prostaglandin E2; EP4 receptor; heart; cardiac remodeling

1. INTRODUCTION

Prostaglandin E2 (PGE₂) signals through 4 separate G-protein coupled receptor sub-types (EP1, EP2, EP3 and EP4) to elicit a variety of physiological and pathophysiologic effects. EP2 and EP4 increase cAMP levels in the cell via adenylate cyclase activation, whereas EP3 inhibits cAMP production, and EP1 increases Ca²⁺ levels in the cell, although the cardiac effects of the receptors have not been studied in depth. Our laboratory has studied the role of PGE₂ via its EP4 receptor over the last decade and reported that the EP4 receptor causes hypertrophy of cardiac myocytes in vitro; that deletion of the EP4 receptor only in the cardiac myocyte worsens cardiac function after myocardial infarction (MI) and that male mice with cardiomyocyte deletion of the EP4 receptor develop a dilated cardiomyopathy with age that is characterized by reduced ejection fraction, left ventricle dilation, thinning of the posterior wall, and an increased interstitial cellular infiltrate [1–3]. Moreover, we recently reported that PGE₂ via its EP3 receptor could reduce contractility of preparations ranging from isolated myocytes to the whole heart by mechanisms that appeared to involve decreased phosphorylation of phospholamban (PLN) [4]. Presumably, this would lead to an inhibitory effect on SERCA, thereby decreasing contraction. Additionally, we reported that the EP3 receptor is upregulated after MI and although the EP4 receptor is also upregulated, it does not appear to be to a similar extent. We thus hypothesized that an imbalance in the EP3/EP4 ratio favoring expression of EP3 would explain some of the reduced contractility observed after MI and that over-expression of EP4 in the same model would improve cardiac function. This was tested in the present study using a mouse model of MI with the use of AAV9-EP4 driven by the myosin heavy chain promoter to overexpress EP4 in the left ventricle.

2. METHODS

2.1. Animal Use

10–12 week old male C57Bl/6 mice used for the MI studies and in vitro studies were from Jackson labs. The mouse model of MI using permanent ligation of the left anterior descending coronary artery was previously described by us [2]. All studies involving the use of animals were approved by the animal care and use committee (IACUC) at Henry Ford Hospital, in accordance with federal guidelines.

2.2. AAV-9 EP4 Transduction of the Mouse Heart

Our viral constructs consisted of adeno associated virus serotype 2/9 with EP4 (hereafter termed AAV9-EP4) and the control virus AAV2/9-CMV luciferase (hereafter termed AAV9-luc). AAV9-EP4 driven by the myosin heavy chain promoter and AAV9-CMV luciferase were obtained from Vector BioLabs (Malvern, PA) and both were administered at the same viral load. To first determine the localization of AAV9-EP4, we performed intracardiac injections of AAV9-EP4 containing a hemagglutinin (HA) tag on the N-terminus (hereafter termed AAV9-EP4-HA; AAV9-aMHCp-HA-mPTGER4-WPRE; Vector BioLabs), with the same viral titer as AAV9-EP4/AAV9-luc. In the other experiments, we performed either sham operation or ligation of the coronary artery and just before the ligation we administered 30 μ L (3 separate 10 μ L injections) of either AAV9-EP4 or AAV9-luc for a total of 1×10^{12} viral particles. These injections were given into three different areas of the left ventricle free wall, but not in the predicted infarct zone. Two weeks after injection we performed immunohistochemistry with antibodies against the HA tag to visualize expression of the construct. We also performed confocal microscopy to examine localization of the construct in animals that received AAV9-luc or AAV9-EP4 virus.

2.3 Echocardiography

Echocardiography was performed on conscious animals at 2 weeks after sham or MI surgery. The cardiac function of all mice was assessed by echocardiography using an Acuson 256 system (Mountain View, CA) with a 15-MHz linear transducer, as reported previously [5]. Diastolic measurements were made at the maximum left ventricle cavity dimension, whereas systolic parameters were measured during maximum anterior motion of the posterior wall. All echocardiography was performed by the same investigator who was blinded to the experimental groups.

2.4. Histology: Measurement of Myocyte Cross Sectional Area (MCSA), Picrosirius Red Staining, and Infarct Size

Histological assessment of myocyte cross sectional area (MCSA) was performed as previously described [6]. Briefly, mouse hearts were harvested and sectioned transversely into three slices from apex to base (sections A–C). The sections were frozen in OCT media pre-chilled in isopentane and stored at -80°C for determination of MCSA. All three sections of the heart were stained with fluorescein-labeled peanut agglutinin to delineate the myocytes. Four radially oriented microscope fields were selected from each section and photographed under the 20x objective. However, we did not analyze the scar itself as there are no visible cardiomyocytes in that region. MCSA was measured by computer-based planimetry (NIH Image J) and averaged across all 4 fields of the sections. The mean cardiomyocyte area was then calculated for each animal. All assessments were performed by blinded observers. Infarct size was determined using the method described by Takagawa et al for the mouse [7]. Briefly, using Metamorph software, infarct area was digitally traced and calculated as $[(\text{epicardial infarct ratio} + \text{endocardial infarct ratio})/2] \times 100$ for all sections and the average of the three sections was taken. Picrosirius red staining was performed using frozen sections of left ventricles and a method previously published [8]. Briefly, sections were postfixed in Bouin's fluid and they were then stained with 0.1% picrosirius red for 1

hour and washed twice with 0.5% acetic acid. Images of collagen staining were obtained under 2X objective and analyzed using Nikon Elements software.

2.5. Immunohistochemistry for Macrophages, T cells, and HA Tag-EP4

The number of macrophages was assessed using CD68⁺ staining (rat anti-CD68⁺ antibody, BioRad) on frozen heart sections as we previously described [6]. T cells were visualized using CD3⁺ staining (rabbit-anti CD3⁺ antibody, Abcam). EP4 containing the HA tag was visualized using anti-HA Tag staining (mouse anti-HA, Cell Signaling). Briefly, frozen sections were fixed in acetone for 10 minutes, followed by incubation with fresh 0.3% hydrogen peroxide for 30 minutes. Sections were then blocked in 5% centrifuged milk in TBS (Tris- buffered saline) blocking buffer and incubated with either anti-CD68⁺ (1:200 in blocking buffer), anti-CD3⁺ (1:100 in blocking buffer), or anti-HA tag (1:100 in blocking buffer) overnight at 4°C. Biotinylated secondary antibodies (1:200 dilution of anti-mouse IgG for CD68⁺ and HA Tag staining, 1:200 dilution of goat anti-rabbit IgG for CD3⁺ staining) were placed onto sections for 1 hour at room temperature. Sections were then incubated with horseradish peroxidase reagent for 40 minutes at room temperature and then visualized by AEC single solution (Vector laboratories; Burlingame, CA). After a rinse in tap water, slides were counterstained with Harris' hematoxylin solution for 1 min, then rinsed again in tap water and mounted onto slides using Aquamount (Lerner laboratories). Negative controls consisted of sections incubated in the absence of primary antibody. Photographs of five randomly-chosen fields per CD68⁺ stained section were taken under the 20X objective and the number of positively—stained cells were counted by a blinded observer. For CD3⁺ staining, four randomly-chosen fields were taken under the 20X, 40X, and 100X oil-immersion objective and the number of cells per mm² was measured by a blinded observer using Image J software. For HA tag staining, photographs of the negative control were taken under 20X and positive slides were taken under the 20X, 40X, and 100X oil-immersion objective.

2.6. Co-localization of HA-EP4 Using Double Immunofluorescence

Frozen left ventricle sections were allowed to warm to room temperature followed by fixation with ice-cold acetone for 15 minutes. Slides were rinsed in ice-cold TBS, twice, followed by permeabilization with TritonX-100 (0.1 % in TBS; pH 7.6) for 5 minutes at room temperature. Slides were then blocked for 1 hr. at room temperature in a humidified chamber in 2% bovine serum albumin (BSA; pH 7.6). Slides were incubated with primary antibodies sequentially at room temperature in a humidified chamber. First, slides were incubated with biotinylated anti-HA tag (BAM0601; R&D Systems) for 2 hours, followed by three washes with TBS. These slides were then incubated with Streptavidin conjugated to Northern LightsTM 557 (NL999; R&D Systems) for 1 hour in the dark. Slides were then washed 5 times with TBS, 5 minutes each, followed by incubation with either rat anti-CD31 (557355; BD BioSciences) or rabbit anti- α -Sarcomeric Actinin (ab137346; Abcam) for 2 hours. Slides were washed as before followed by secondary antibody detection with either goat anti-rat FITC or goat anti-rabbit FITC for 1 hour in the dark. Slides were washed 5 times with TBS, followed by incubation with DAPI nuclear stain (1: 18,000 in TBS) for 10 minutes. Slides were then mounted using AquaMount (Lerner laboratories). Control sections

were cryosections from animals that received AAV9-Luc virus and thus do not express HA-EP4. Slides were imaged using confocal microscopy.

2.7. Confocal Microscopy

Fixed and labeled left ventricle tissue slices were imaged by confocal microscopy using an Olympus FV1200 confocal microscope. Images were acquired using a 40X objective and an optical zoom function was used to acquire images at 200X. DAPI was excited with a 405 nm diode laser and fluorescence acquired between 420 and 490 nm. FITC labeled CD31 or α sarcomeric actinin was excited at 489 nm and fluorescence acquired between 495 and 555 nm. Northern LightsTM was excited at 557 nm and fluorescence acquired between 570 and 700 nm. No cross-bleed between channels was observed under these conditions. Images on the three channels were acquired simultaneously.

2.8. Western Blot

Phospholamban (PLN), phosphorylated PLN (p-PLN), and HA-EP4 expression were measured by Western Blot using homogenized left ventricles from sham and MI mice who received either AAV9-EP4 or AAV9-Luc, as we have previously described [4]. The p-PLN antibody is from Cell Signaling (Danvers, MA) and recognizes phosphorylation at Ser16/Thr17. After detection of p-PLN, the blot was stripped and a total phospholamban antibody (Danvers, MA) was applied. The p-PLN signal was corrected to total PLN. Expression of HA-EP4 was measured using antibodies raised against the HA tag (mouse anti-HA; Cell signaling, Cat No.2367) at a 1:1000 dilution. GAPDH was used to control for the amount of protein loaded.

2.9. In Situ Zymography

To assess MMP activity, we performed In situ zymography on frozen sections as previously described [9]. Fluorescein conjugated, dye-quenched gelatin from pig skin (DQTM-gelatin) was obtained from Molecular Probes (Invitrogen, Basel, Switzerland). A 1 mg/ml stock solution of DQ-gelatin was prepared in gelatinase reaction buffer (150 mM NaCl, 5 mM CaCl₂, 0.2 mM NaN₃, 50 mM Tris-HCl, pH 7.6) and stored at 4°C. The working solution for in situ zymography was made by directly diluting DQ-gelatin stock solution in reaction buffer to a final concentration of 20 μ g/ml. Unfixed cryosections were thawed, rounded with a wax pen, overlaid with 25 μ l DQ-gelatin working solution and incubated at 37°C in a humidified dark chamber for 5 hours and pictures were immediately taken under the microscope. Control sections were incubated in the presence of 20 mM EDTA which inhibits gelatinase activity. Photographs of four randomly-selected fields were taken under the 20X objective and a threshold was set using Nikon elements that encompassed only the bright green fluorescence indicative of gelatinase activity. The percent area of gelatinase activity was calculated for each animal per field and the mean calculated for each group.

2.10. Real Time RT-PCR

Measurement of β -myosin heavy chain (β -MHC), brain natriuretic peptide (BNP), tumor necrosis factor α (TNF α), inducible nitric oxide synthase (iNOS), Secreted protein, acidic and rich in cysteines (SPARC), matrix metalloproteinase 2 (MMP2), EP1, EP2, EP3, and

EP4 mRNA expression was performed by quantitative real-time RT-PCR using a SYBR green method. Real time RT-PCR was performed as follows: 1 µg of DNase-treated total RNA sample was reverse transcribed using random primers and Omniscript reverse transcriptase (Qiagen, Valencia, CA) in a total volume of 20 µl for 1 hr. at 37 °C followed by an inactivation step of 95 °C for 5 min. 2 µl of the reverse transcription reaction was then amplified in a Roche version 2.0 lightcycler PCR instrument (Roche, Indianapolis, IN) using SYBR green dye (SA Biosciences, Frederick, MD) and specific primers. Reactions were set up in a final volume of 20 µl, which contained 2 µl of sample, 1 µM each of both the primers and 10 µl of 2x SYBR green PCR mix. After an initial “hot start” at 95°C for 10 min, amplification occurred by denaturation at 95°C for 15s, then annealing at 58–60°C for 40 seconds for a total of 30–40 cycles, followed by extension at 72°C for 40 seconds. At the end of PCR cycling, melting curve analyses were performed. For all Qiagen primers, extension and annealing were performed together in one step at 60°C for 1 minute. A relative quantitation method (Ct) [10] was used to evaluate expression of each gene relative to control. Real time RT-PCR of GAPDH was used for normalization of all data. Primer sequences: TNFα (RefSeq No. NM_013693.3), EP1 (RefSeq No. NM_013641.2), EP2 (RefSeq No. NM_008964), EP3 primers (NM_011196), and SPARC (RefSeq No. NM_009242) were pre-designed from SA Biosciences (Qiagen; Frederick, MD). All other primers were designed by TIB MolBiol (Adelphia, NJ) β-MHC (MYH7) sense: 5′-ACAGGAAGAACCTACTGCGRC-3′; β-MHC antisense: 5′-AGCTTGTGACCTGGGACT-3′; BNP (Nppb) sense: 5′-CCCAAAAAGAGTCCTTCGGT-3′; BNP antisense: 5′-CCTACAACAACCTCAGTGCGTT-3′; iNOS sense: 5′-CCCTTCAATGGTTGGTACATGG-3′; iNOS antisense: 5′-ACATTGATCTCCGTGACAGCC-3′; MMP2 sense: 5′-ATACCCCAAGCCACTGACCA-3′; MMP2 antisense: 5′-CCAGGAGTCTGCGATGAGCT-3′; GAPDH sense: 5′-CAAGGTCATCCCAGAGCTG-3′; GAPDH antisense: 5′-TGTCATCATACTTGGCAGGTT-3′.

2.11. Cytokine/Chemokine Multiplex ELISArray

To identify changes in expression of major cytokines/chemokines after MI with injection of either control AAV9-Luc virus or AAV9-EP4 virus, we performed a multiplex Enzyme-Linked ImmunoSorbent Assay (ELISA) using a commercially available kit from Qiagen (Cat No. MEM-005A). Following the manufacturer’s instructions, we homogenized 20 mg of left ventricle tissue in sample buffer provided in the kit with the addition of a protease inhibitor (Roche). The 96-well plate was analyzed at 450 nm and 570 nm using a plate reader. The absorbance at 570 was to account for autofluorescence of the plate and was subtracted from final readings. After being corrected, absorbance values were corrected for amount of protein in each well.

2.12. Statistical Analysis

All statistics were performed by a statistician in the Department of Public Health Sciences of Henry Ford Hospital using the statistical package SAS Version 9.4. All data are shown as mean ± standard error of the mean (SEM). Data was analyzed using multiple two-sample

Wilcoxon tests for unequal variances along with a Hochberg's correction for multiple comparisons. This procedure provides a series of adjusted cutoff values for significance.

3. RESULTS

3.1. Effect of AAV9-EP4 Injection on EP4 mRNA Expression

To determine the extent of EP4 expression after viral injection, we performed real time RT-PCR for EP4. After 14 days, sham-operated animals receiving AAV9-EP4 had a 2.86-fold increase in EP4 mRNA expression compared with control (1.08 ± 0.25 vs. 3.09 ± 0.73 in Sham + AAV9-EP4; $p=0.05$). The expression levels of EP1 (1.25 ± 0.10 vs. 1.11 ± 0.11 in Sham + AAV9-EP4), EP2 (1.02 ± 0.15 vs. 0.71 ± 0.16 in Sham + AAV9-EP4), and EP3 (1.13 ± 0.33 vs. 2.23 ± 1.01 in Sham + AAV9-EP4), were not significantly changed following administration of AAV9-EP4.

3.2. AAV9-EP4-HA Overexpression Level and Localization

To assess the level of AAV9-EP4 expression and localization after injection, we performed intracardiac injections as described in the methods in sham animals with an AAV9-EP4-HA construct. After two weeks, we performed immunohistochemistry with antibodies against the HA tag. Figure 1A shows representative images of positive staining under different magnifications and of the negative control (bottom right panel). We observed intense positive staining throughout the left ventricle with staining around cardiomyocyte nuclei and on the cell membrane. This expression was confirmed by western blot. Figure 1B shows that HA-EP4 expression in the left ventricles of mice injected with AAV9-EP4 is highly abundant two weeks post MI, and is negative in animals that received AAV9-Luc.

3.3. Localization of HA-EP4 in the Left Ventricle

To determine localization of overexpressed EP4 within the heart, we performed double labeled immunofluorescence using the HA tag as a surrogate of EP4 expression. As a control we used cryosections from animals that received AAV9-Luc virus and therefore do not express the HA tag. Figure 1C clearly shows that the HA tag is expressed only in those animals that received AAV9-EP4 virus, and not in animals that received the control virus AAV9-Luc. We next used confocal microscopy to determine whether the HA tag co-localized with either the endothelial cells (CD31) or within the myocytes (α -Sarcomeric Actinin) (figure 1D). The top panel clearly shows no association between the HA tag (red) and the endothelial cells (green). However, the HA-tag does co-localize with the α -Sarcomeric Actinin (green; bottom panel) within the cardiomyocytes.

3.4. Effect of EP4 Overexpression on Cardiac Function

Representative echocardiography images (figure 2A) and data (figure 2B and table 1) are presented. As expected, animals with MI exhibited a lower ejection fraction (EF) than sham operated animals (37.8 ± 2.2 % for MI+AAV9-luc vs. 76.9 ± 2.1 % for sham + AAV9-luc, $p=0.0001$). The administration of AAV9-EP4 to MI animals resulted in a significant improvement in EF (to 47.7 ± 2.4 %, $p=0.0025$ compared to animals receiving AAV9-luc) whereas it had no effect in sham-operated mice. In addition to increased EF, our data shows an improvement in shortening fraction (SF; $p=0.0003$) coupled with less ventricle dilatation

at systole (LVD_s; $p=0.0013$) and a tendency for reduced chamber dimension at diastole (LVD_d) also ($p = 0.028$ NS). Altogether the data suggest that expression of EP4 improves cardiac function and alters ventricular remodeling. Furthermore, the effects on cardiac function appear to be independent of infarct size since there was no difference between MI + AAV9-luc vs. MI + AAV9-EP4 ($33.53 \pm 4.39\%$ versus $31.29 \pm 3.32\%$, respectively).

With respect to survival, mice typically die of cardiac rupture in the first week following MI. However, we did not observe any differences in mortality between mice receiving control virus (17.6%) and those receiving AAV9-EP4 (19.0%).

3.5. Effect of EP4 Overexpression on Collagen Fraction (Picrosirius Red Staining)

As expected, MI increased the collagen content in all three regions of the left ventricle examined (infarct, peri-infarct, and remote; Figure 3B left, middle, and right panels respectively) by picrosirius red staining (PSR). In the infarcted region, animals that received MI and control AAV9-Luc virus showed an increase in collagen to $18.78 \pm 2.58\%$ vs. $8.62 \pm 1.13\%$ in sham operated animals. In animals that received MI and AAV9-EP4 virus, positive PSR staining was increased even further to $29.96 \pm 2.94\%$. In the peri-infarct region, animals given MI and AAV9-Luc virus had a similar significant increase in collagen content ($25.10 \pm 2.00\%$ vs. $7.13 \pm 0.91\%$ in sham operated animals). In contrast to the infarcted region, collagen content in the peri-infarct was reduced after MI with injection of AAV9-EP4 to $17.12 \pm 2.24\%$. As anticipated, there was a small, but significant increase in collagen in the remote region of the heart ($6.72 \pm 1.14\%$ vs. $2.68 \pm 0.38\%$ in sham operated animals). This increase was also reduced to $3.62 \pm 0.91\%$ in animals that received MI with AAV9-EP4 injection. Representative images of the staining from the peri-infarct zone are shown in figure 3A.

3.6. Effect of EP4 Overexpression on Myocyte Cross-Sectional Area (MCSA)

As anticipated, MI increased MCSA but this increase was smaller in mice receiving AAV9-EP4 (Figure 3C: $259.4 \pm 4.0 \mu\text{m}^2$ vs. $296.6 \pm 6.0 \mu\text{m}^2$, $p < 0.01$). The administration of AAV9-EP4 did not affect MCSA in sham-operated animals ($202.3 \pm 5.2 \mu\text{m}^2$ for sham-operated receiving AAV9-luc vs. $231.4 \pm 18.5 \mu\text{m}^2$ for mice receiving AAV9-EP4, NS).

3.7. Effect of EP4 Overexpression on Gene Markers of Cardiac Hypertrophy

Since treatment with AAV9-EP4 reduced MCSA in response to MI, we also measured expression of β -myosin heavy chain (β -MHC) and BNP; gene markers of hypertrophy using real time RT-PCR, corrected to GAPDH. As shown in figure 3D, both β -MHC and BNP were increased after MI and these increases were attenuated after treatment with AAV9-EP4; consistent with the MCSA data. After MI, β -MHC mRNA expression (Figure 3D, left panel) was significantly increased 5-fold compared with sham animals (6.64 ± 1.9 vs. 1.31 ± 0.43 , $p < 0.001$). Animals treated with AAV9-EP4 virus had a significant increase in β -MHC mRNA expression after MI, although this increase was only 2.2-fold that of sham animals (3.76 ± 1.2 vs. 1.75 ± 0.87 , $p < 0.05$). Similarly, BNP mRNA expression (Figure 3D, right panel) significantly increased after MI (3.46 ± 1.0 vs. 1.11 ± 0.17 , $p < 0.001$). However, there was no difference in BNP mRNA expression after MI in animals who first received AAV9-EP4 treatment (1.65 ± 0.34 vs. 1.03 ± 0.11).

3.8. Effect of EP4 Overexpression on MMP-2 mRNA

Matrix metalloproteinases (MMPs) play an important role in extracellular matrix remodeling in various pathological conditions, including myocardial infarction [11–13]. Specifically, MMP-2 has been shown to play an important role in LV remodeling [12, 14]. For this reason, we determined the effect of EP4 overexpression on MMP-2 mRNA expression (Figure 4, left panel). There was a significant increase in MMP-2 expression in MI animals receiving control AAV9-Luc virus (2.91 ± 0.62 vs. 1.00 ± 0.06 in Sham + AAV9-Luc, $p < 0.05$). However, in animals receiving AAV9-EP4, there was no change in expression of MMP-2 after MI (1.68 ± 0.65 vs. 1.47 ± 0.31 in Sham + AAV9-EP4, $p = 0.85$). Since mRNA levels do not always reflect activity, we also performed in situ zymography to assess gelatinase activity (MMP-2 and MMP-9), shown in figure 4, right panel. As expected, gelatinase activity was increased in the infarct zone after MI in animals that received control virus (10.47 ± 3.71 RFU vs. 2.35 ± 0.60 RFU in sham animals that received the same virus; $p = 0.07$). Surprisingly, treatment of sham-operated mice with AAV9-EP4 increased MMP activity (2.35 ± 0.60 RFU for sham-operated AAV9-luc vs 7.23 ± 1.75 RFU, $p < 0.05$ for sham animals receiving AAV9-EP4). There was no further increase in MMP activity after MI in animals that received AAV9-EP4 virus (7.15 ± 1.60 RFU vs. 7.23 ± 1.75 RFU in sham + AAV9-EP4 group).

In the remote region of the heart, a similar increase in MMP activity was noted after MI in mice receiving control virus (0.42 ± 0.16 RFU in sham + AAV9-Luc vs. 5.67 ± 1.59 RFU in MI + AAV9-Luc, $p < 0.05$). Similar to results noted in the infarct zone, administration of AAV9-EP4 to sham-operated animals tended to increase MMP activity although the change failed to achieve statistical significance (0.42 ± 0.16 RFU in sham + AAV9-Luc vs 2.75 ± 1.20 RFU in sham + AAV9-EP4, $p = 0.104$). After MI, there was no statistically significant change in MMP activity for animals that received AAV9-EP4 (2.75 ± 1.20 RFU for sham + AAV9-EP4 vs 5.31 ± 1.51 RFU for MI + AAV9-EP4). Secreted protein, acidic and rich in Cysteines (SPARC) is a glycoprotein that plays an important role in activation and production of MMPs. We measured the expression of SPARC mRNA by real time RT-PCR and we found that SPARC was significantly increased after MI in animals that received control virus, compared to sham animals (3.57 ± 0.69 vs. 1.02 ± 0.14 in sham animals; $p = 0.001$). SPARC expression was not changed in sham-operated animals overexpressing EP4 (1.02 ± 0.14 for AAV9-luc vs 0.83 ± 0.25 for AAV9-EP4) and neither was it altered in mice subject to MI (3.37 ± 0.63 in MI+AAV9-EP4; $p = 0.787$).

3.9. Effect of EP4 Overexpression on Macrophage Migration and Polarization

A representative image of macrophage migration in all four groups is presented in figure 5A. Figure 5B shows a representative image of the CD68⁺ staining observed in the infarct/peri-infarct zone after 2 weeks of MI under different magnifications. As expected, after 2 weeks of MI, the number of macrophages in the infarct/peri-infarct region was increased 2.2-fold (figure 5C, left panel), from a value of 181.79 ± 10.2 cells/mm² in sham-operated animals receiving AAV9-luc to 401.65 ± 58.6 cells/mm² in MI + AAV9-luc injected hearts ($p < 0.001$). Treatment with AAV9-EP4 substantially reduced the number of infiltrating macrophages in the MI heart to a value of 281.77 ± 28.2 cells/mm² ($p = 0.09$). In the remote zone of the heart (figure 5C, right panel), the number of CD68⁺ cells was unchanged after

MI (102.02 ± 22.04 cells/mm² vs. 98.10 ± 15.51 cells/mm² in sham-operated animals), however, injection of AAV9-EP4 resulted in a significant increase in CD68⁺ cells in sham animals (176.30 ± 13.41 cells/mm² vs. 98.10 ± 15.51 cells/mm²) and this was further increased after MI to 194.80 ± 27.48 cells/mm². We also performed real time RT-PCR for inducible nitric oxide synthase (iNOS), one established marker of the M1 macrophage phenotype. As shown in figure 5D, the expression of iNOS (corrected to GAPDH) in the left ventricles was significantly increased in MI mice receiving control virus (2.04 ± 0.31 ; $p < 0.01$). However, there was no increase in iNOS expression after MI in mice that received AAV9-EP4.

3.10. Effect of EP4 Overexpression on T cells

Figure 5E displays representative images of CD3⁺ staining for each of the four groups and figure 5F shows a representative image of the CD3⁺ staining observed in the infarct/peri-infarct zone after 2 weeks of MI under different magnifications. Figure 5G (left panel) shows that two weeks post MI there was a 2.26-fold increase in positive T cell staining in the infarct region of mice that received AAV9-luc compared to sham-operated mice that received the same control virus (504.39 ± 46.80 cells/mm² vs. 222.61 ± 18.27 cells/mm² respectively, $p < 0.001$). Furthermore, the increase in T cells was prevented when MI mice were treated with AAV9-EP4 (329.71 ± 33.53 cells/mm² vs. 504.39 ± 46.80 cells/mm² MI + AAV9-Luc mice). CD3⁺ cell staining was not different between groups when the remote zone was analyzed (figure 5G, right panel).

3.11. Effect of EP4 Overexpression on Cytokines

It is well known that cytokines like TNF α play an important role in the pathophysiology of heart failure. Since increased expression of TNF α has been correlated with an increased severity of heart failure, we determined if treatment with AAV9-EP4 would prevent its expression. Figure 6A shows that TNF α mRNA expression (corrected to GAPDH) significantly increased after MI in mice receiving control virus (2.65 ± 0.63 vs. 1.05 ± 0.23 in sham animals, $p = 0.002$). However, TNF α mRNA did not increase after MI in mice receiving AAV9-EP4 (1.74 ± 0.24 vs. 1.17 ± 0.39 ; NS). We next performed a multiplex ELISA on left ventricle homogenates to identify key cytokines/chemokines that might be changing in MI with overexpression of EP4 (Figure 6B). Using the array kit, we identified a significant reduction in MCP-1 expression (left panel) in mice receiving AAV9-EP4 (0.254 ± 0.0146 vs. 0.167 ± 0.0187 in MI + AAV9-EP4; $p < 0.01$) and an increase in IL-10 (0.025 ± 0.0061 vs. 0.044 ± 0.0121 in MI + AAV9-EP4; $p = 0.172$), although this did not reach statistical significance (right panel). Unfortunately, levels of these cytokines/chemokines were below the levels of detection in isolated cardiomyocytes obtained from MI mice receiving either control virus or AAV9-EP4. However, these data indicate that overexpression of EP4 may reduce the expression of pro-inflammatory chemokines/cytokines (MCP-1) while increasing expression of anti-inflammatory cytokines (IL-10).

3.12. Effect of EP4 Overexpression on phosphorylation of Phospholamban

Since phosphorylation of phospholamban is important in regulating the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) and therefore contractility, we performed western blot analysis of p-PLN and total PLN expression (Figure 7). There was a significant increase in

expression of p-PLN, corrected to total PLN, in sham animals who received AAV9-EP4 compared with sham animals that received the control AAV9-Luc virus (1.01 ± 0.16 vs. 0.35 ± 0.09 ; $p < 0.05$). In animals subject to MI with injection of AAV9-EP4, the expression of p-PLN was already elevated and thus there was no further increase.

4. DISCUSSION

The results of this study show, for the first time, that administration of AAV9-EP4 into the left ventricle of mice with MI is beneficial in improving cardiac function. We have previously reported that male mice lacking the EP4 receptor in cardiomyocytes (EP4-KO) have worsened cardiac function after MI and develop dilated cardiomyopathy with age [2, 3]. More recently we also reported that PGE₂ via its EP3 receptor reduced contractility whereas stimulation of EP4 had the opposite effect. These effects appeared to involve changes in the phosphorylation of phospholamban. Moreover, we also reported that after MI, expression of the EP3 receptor subtype in the left ventricle was increased to a greater extent than EP4 [4]. We therefore hypothesized that overexpression of EP4 in the heart would improve cardiac function after MI. Thus, in our experiments described here, we used an adeno-associated viral vector driven by the alpha myosin heavy chain promoter to increase the expression of the EP4 receptor in the cardiomyocytes rather than increase the activation of receptors already present. The results support our hypothesis, with significant improvements in ejection fraction and shortening fraction noted, coupled with reductions in left ventricular dimension after MI without effecting infarct size. Overexpression of EP4 also significantly reduced hypertrophy and fibrosis after MI as evidenced by a smaller MCSA and reduced picrosirius red staining in the peri-infarct and remote zone, and the former was confirmed by reductions in β -MHC and BNP mRNA expression. Previously, other studies have shown a protective role for the EP4 receptor in ischemia-reperfusion injury. Xiao et al [15] reported that global EP4 KO mice had larger infarcts after reperfusion injury while administration of an EP4 agonist had the opposite effect. A study by Hishikari et al [16], using a rat model described similar findings but also indicated that treatment with an EP4 agonist could reduce cytokine secretion stimulated by the ischemia/reperfusion. Our data using the permanent coronary ligation model with EP4 overexpression agrees with the aforementioned findings except that we did not observe a change in infarct size at the end of the study. Whether this relates to the different models or different time points remains unknown. Since we also recently reported that PGE₂ via its EP3 receptor reduced contractility of the heart via mechanism(s) that appeared to involve decreased phospholamban phosphorylation; we examined phosphorylation of phospholamban in these studies. Sham-operated mice who received AAV9-EP4 exhibited a significant increase in phosphorylated phospholamban, consistent with our in vitro data using the EP4 agonist in isolated cardiomyocytes [4]. A similar trend was observed in animals with MI, although the data did not achieve statistical significance. We therefore speculate that the increased EP3 receptor abundance after MI could account, in part, for the decreased contractility observed. The role of the EP3 receptor in cardiac function is controversial. Martin et al [17] reported that cardiac overexpression of the EP3 receptor attenuated ischemia-induced myocardial injury. However, that paper used an isolated heart perfusion method and curiously, EP3 transgenic animals exhibited a reduced ejection fraction as measured in vivo using MRI. In

subsequent studies, we think it will be necessary to identify the impact of EP3 receptor inhibition with simultaneous EP4 overexpression on cardiac function.

Treatment with AAV9-EP4 also improved aspects of adverse cardiac remodeling. The increased collagen deposition noted in the peri infarct and remote zone after MI was substantially attenuated by use of AAV9-EP4 and there were significant reductions in the number of macrophages and T cells infiltrating the left ventricle. Although the beneficial role of the EP4 receptor in heart failure has not been fully explored, our data agrees with several other studies suggesting an anti-inflammatory role for the PGE₂ EP4 receptor subtype. Our data agrees with the beneficial role of the EP4 receptor reported in other cell types. In a rat model of autoimmune myocarditis, Ngoc et al. [18] reported that treatment with an EP4 agonist reduced immune cell infiltration, heart size, and area of fibrosis. More recently, a publication by Wang et al. [19] demonstrated that an EP4 receptor agonist could prevent cardiac dysfunction and cardiac fibrosis in a pressure overload, transaortic constriction mouse model.

In human samples, Haywood et al. [20] showed that iNOS was undetectable in control heart samples but could be detected in 36 of 51 patients with heart failure. Our data show that iNOS expression in the left ventricle is increased after MI and can be attenuated by treatment with AAV9-EP4, consistent with a cardioprotective effect. iNOS is also one marker for the M1 macrophage phenotype but it is unclear from our data obtained from left ventricle samples whether the iNOS originates from resident/infiltrating macrophages or another cell type such as cardiomyocytes. If the former, then the reduction noted in iNOS may relate simply to a reduction in the number of infiltrating macrophages observed after AAV9-EP4 treatment. Alternatively, PGE₂ has been shown to have anti-inflammatory effects in macrophages by reducing TNF α expression and increasing Interleukin 10 expression via the EP4/cAMP/protein kinase A (PKA) pathway, which promotes macrophage M2 polarization [21–23]. It remains to be elucidated however, if overexpression of EP4 in the left ventricle mediates macrophage polarization towards an anti-inflammatory M2 phenotype and our data using a limited number of markers does not allow us to draw any conclusions.

Another aspect of cardiac remodeling involves the matrix metalloproteinases (MMPs) family of proteins and other molecules that play a role in collagen deposition. Specifically, MMP-2 has been implicated in LV remodeling and furthermore, MMP-2 KO mice are reported to have lower incidences of cardiac rupture after MI [24, 25], although the mechanism of how MMP-2 contributes to cardiac rupture is not well understood. Also, reports show that broad inhibition of MMPs contributes to attenuation of LV dilatation [26, 27]. Several MMPs are involved in this process, although MMP-2 may play a key role. Matsumura et al. [13] reported that targeted deletion of MMP-2 in mice profoundly improved survival rates after MI. Moreover, they reported that deletion of MMP-2 completely reduced macrophage migration after MI. Our data using in situ zymography which measures gelatinase activity (MMP-2 and MMP-9) is interesting but rather perplexing in that we observe increased MMP activity in sham-operated animals treated with AAV9-EP4 that is not significantly increased further after MI. Overall, our data indicates that treatment with AAV9-EP4 alters the cardiac remodeling process after MI and changes in MMP activity may be an important player in the mechanism of reduced macrophage migration and the subsequent prevention of LV

dilatation we observed with overexpression of EP4. The secreted protein, acidic and rich in cysteines (SPARC) was also shown to affect remodeling after MI. Schellings et al [28] reported that deletion of SPARC increased rupture following MI and a role for this molecule in macrophage clearance has also been postulated [29]. Our data shows no effect of EP4 overexpression on this molecule, consistent with its high expression in fibroblasts and endothelial cells but low expression in myocytes [30].

Previously, we have described increased expression of both cytokine and chemokine mRNA (e.g. MCP-5 and fractalkine) in the left ventricle of male EP4-KO mice with dilated cardiomyopathy [2]. Takayama et al. [31] have shown that PGE₂ potently inhibits cytokine/chemokine secretion (MCP-1, interleukin 8, macrophage inflammatory protein, and CxCL10) in human macrophages. Thus, inhibition of chemokine or cytokine release may be a significant mechanism to explain how treatment with AAV9-EP4 reduces macrophage infiltration. Treatment with AAV9-EP4 also significantly prevented increases in TNF α mRNA after MI. TNF α is a major cytokine implicated in heart failure and increases in TNF α have been correlated with the severity of cardiac disease although anti-TNF α therapies have met with little clinical success [32, 33]. In addition to alterations in TNF α , our results also show that overexpression of EP4 affects other chemokines/cytokines. Using a mouse cytokine/chemokine array we detected a significant reduction in MCP-1 protein levels in the left ventricle and increases in IL-10, an anti-inflammatory cytokine, after treatment of MI hearts with AAV9-EP4. Unfortunately, protein levels of these cytokines/chemokines were below the limits of detection in isolated mouse cardiomyocytes treated with AAV9-EP4 or control virus, even after stimulation with LPS. Thus, we cannot be certain if overexpression of EP4 affects production of these factors from cardiomyocytes or if it has paracrine effects. However, our data shows that reduction of pro-inflammatory cytokines/chemokines may be one mechanism by which over-expression of the EP4 prevents the deleterious effects of MI.

5. CONCLUSION

We have demonstrated for the first time that overexpression of the EP4 receptor is protective in a mouse MI model. We have also shown that overexpression of the EP4 receptor may prevent inflammation involved in cardiac remodeling post MI, thus contributing to the preserved cardiac function. If our results can be applied to the human condition, our data support therapeutic interventions that increase EP4 expression.

Acknowledgments

The authors would like to thank David Taube for excellent technical assistance.

Sources of Funding

These studies were funded by a National Institutes of Health grant [5P01HL028982] (sub-project 2) to PH. Timothy Bryson was supported by the predoctoral NIH T32 Detroit Cardiovascular Training Grant (5T32HL12082205).

References

1. Harding P, et al. Lack of microsomal prostaglandin E synthase-1 reduces cardiac function following angiotensin II infusion. *Am J Physiol Heart Circ Physiol*. 2011; 300(3):H1053–61. [PubMed: 21193590]
2. Harding P, et al. Gene expression profiling of dilated cardiomyopathy in older male EP4 knockout mice. *Am J Physiol Heart Circ Physiol*. 2010; 298(2):H623–32. [PubMed: 20008274]
3. Qian JY, et al. Reduced cardiac remodeling and function in cardiac-specific EP4 receptor knockout mice with myocardial infarction. *Hypertension*. 2008; 51(2):560–6. [PubMed: 18180401]
4. Gu X, et al. Prostaglandin E2 Reduces Cardiac Contractility via EP3 Receptor. *Circ Heart Fail*. 2016; 9(8)
5. LaPointe MC, et al. Inhibition of cyclooxygenase-2 improves cardiac function after myocardial infarction in the mouse. *Am J Physiol Heart Circ Physiol*. 2004; 286(4):H1416–24. [PubMed: 14670812]
6. Gu X, et al. Fractalkine neutralization improves cardiac function after myocardial infarction. *Exp Physiol*. 2015; 100(7):805–17. [PubMed: 25943588]
7. Takagawa J, et al. Myocardial infarct size measurement in the mouse chronic infarction model: comparison of area- and length-based approaches. *J Appl Physiol* (1985). 2007; 102(6):2104–11. [PubMed: 17347379]
8. Sharma U, et al. Novel anti-inflammatory mechanisms of N-Acetyl-Ser-Asp-Lys-Pro in hypertension-induced target organ damage. *Am J Physiol Heart Circ Physiol*. 2008; 294(3):H1226–H1232. [PubMed: 18178715]
9. Tsujita K, et al. Targeted deletion of class A macrophage scavenger receptor increases the risk of cardiac rupture after experimental myocardial infarction. *Circulation*. 2007; 115(14):1904–1911. [PubMed: 17389263]
10. Winer J, et al. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem*. 1999; 270(1):41–9. [PubMed: 10328763]
11. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol*. 2001; 17:463–516. [PubMed: 11687497]
12. Creemers EE, et al. Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res*. 2001; 89(3):201–10. [PubMed: 11485970]
13. Matsumura S, et al. Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. *J Clin Invest*. 2005; 115(3):599–609. [PubMed: 15711638]
14. Spinale FG, et al. Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: relation to ventricular and myocyte function. *Circ Res*. 1998; 82(4):482–95. [PubMed: 9506709]
15. Xiao CY, et al. Prostaglandin E2 protects the heart from ischemia-reperfusion injury via its receptor subtype EP4. *Circulation*. 2004; 109(20):2462–2468. [PubMed: 15123528]
16. Hishikari K, et al. Pharmacological activation of the prostaglandin E2 receptor EP4 improves cardiac function after myocardial ischaemia/reperfusion injury. *Cardiovasc Res*. 2009; 81(1):123–132. [PubMed: 18805784]
17. Martin M, et al. Cardiospecific overexpression of the prostaglandin EP3 receptor attenuates ischemia-induced myocardial injury. *Circulation*. 2005; 112(3):400–406. [PubMed: 16009796]
18. Ngoc PB, et al. The anti-inflammatory mechanism of prostaglandin e2 receptor 4 activation in rat experimental autoimmune myocarditis. *J Cardiovasc Pharmacol*. 2011; 57(3):365–72. [PubMed: 21383594]
19. Wang Q, et al. An EP4 Receptor Agonist Inhibits Cardiac Fibrosis Through Activation of PKA Signaling in Hypertrophied Heart. *Int Heart J*. 2016
20. Haywood GA, et al. Expression of inducible nitric oxide synthase in human heart failure. *Circulation*. 1996; 93(6):1087–94. [PubMed: 8653828]

21. Kim SH, et al. Distinct protein kinase A anchoring proteins direct prostaglandin E2 modulation of Toll-like receptor signaling in alveolar macrophages. *J Biol Chem.* 2011; 286(11):8875–83. [PubMed: 21247892]
22. Morato-Marques M, et al. Leukotrienes target F-actin/cofilin-1 to enhance alveolar macrophage anti-fungal activity. *J Biol Chem.* 2011; 286(33):28902–13. [PubMed: 21715328]
23. Domingo-Gonzalez R, et al. Prostaglandin E2-induced changes in alveolar macrophage scavenger receptor profiles differentially alter phagocytosis of *Pseudomonas aeruginosa* and *Staphylococcus aureus* post-bone marrow transplant. *J Immunol.* 2013; 190(11):5809–17. [PubMed: 23630358]
24. Hayashidani S, et al. Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling after experimental myocardial infarction. *Am J Physiol Heart Circ Physiol.* 2003; 285(3):H1229–35. [PubMed: 12775562]
25. Heymans S, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med.* 1999; 5(10):1135–42. [PubMed: 10502816]
26. Lindsey ML, et al. Selective matrix metalloproteinase inhibition reduces left ventricular remodeling but does not inhibit angiogenesis after myocardial infarction. *Circulation.* 2002; 105(6):753–8. [PubMed: 11839633]
27. Mukherjee R, et al. Myocardial infarct expansion and matrix metalloproteinase inhibition. *Circulation.* 2003; 107(4):618–25. [PubMed: 12566376]
28. Schellings MW, et al. Absence of SPARC results in increased cardiac rupture and dysfunction after acute myocardial infarction. *J Exp Med.* 2009; 206(1):113–23. [PubMed: 19103879]
29. Barrans JD, Stamatou D, Liew C. Construction of a human cardiovascular cDNA microarray: portrait of the failing heart. *Biochem Biophys Res Commun.* 2001; 280(4):964–969. [PubMed: 11162619]
30. Harris BS, et al. SPARC regulates collagen interaction with cardiac fibroblast cell surfaces. *Am J Physiol Heart Circ Physiol.* 2011; 301(3):H841–7. [PubMed: 21666116]
31. Takayama K, et al. Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. *J Biol Chem.* 2002; 277(46):44147–54. [PubMed: 12215436]
32. Feldman AM, et al. The role of tumor necrosis factor in the pathophysiology of heart failure. *J Am Coll Cardiol.* 2000; 35(3):537–44. [PubMed: 10716453]
33. Khanna D, McMahon M, Furst DE. Anti-tumor necrosis factor alpha therapy and heart failure: what have we learned and where do we go from here? *Arthritis Rheum.* 2004; 50(4):1040–50. [PubMed: 15077286]

New and Noteworthy

This manuscript is the first to show that viral overexpression of the Prostaglandin E₂ EP4 receptor in cardiomyocytes improves cardiac function in a mouse myocardial infarction model. This project could translate to therapeutic interventions that increase EP4 expression and/or activation.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HIGHLIGHTS

- Overexpression of the PGE2 E2 EP4 receptor improves cardiac function post MI
- Reduced inflammation may account for the beneficial effects of EP4
- Overexpression of EP4 increases phospholamban phosphorylation

Figure 1D

Figure 1.

Figure 1A: Representative immunohistochemistry images showing anti-HA staining using the 20X objective (top left), a portion of this staining at 40X (top right), and 100X (bottom left). Bottom right panel shows a section incubated in the absence of primary antibody for a negative control. Positive staining is indicated by the black arrows. Also note that the interstitial region, indicated by the white arrow is devoid of staining.

Panel B shows western blot for HA expression in homogenized left ventricles from MI animals that received injections of either CMV-Luciferase or AAV9-EP4.

Figure 1C: Representative immunofluorescence image showing expression of the HA tag in the left ventricle of a mouse that received AAV9-Luc virus (left panel) vs. one that received AAV9-EP4 (right panel).

Figure 1D: Confocal imaging showing double immunofluorescence labelling of the the HA tag with the endothelial cell marker CD31 (Figure 1D, top panel; 40X) and the cardiomyocyte marker α -Sarcomeric Actinin (bottom panel; 200X). Note that the HA tag does not co-localize with the positive green CD31 signal outlining capillaries and vessels.

Figure 2A

Figure 2B

Figure 2.
Figure 2A: Representative M-mode images of echocardiography from each of the four groups used for analysis.

Figure 2B: Quantitative analysis of echocardiography data. EF-ejection fraction, SF-shortening fraction, LVD_s – left ventricle dimension at systole, LVD_d - left ventricle dimension at diastole. N = 9 for Sham + AAV9-luc, N=10 for Sham + AAV9-EP4, N=16 for MI + AAV9-luc, N=17 for MI + AAV9-EP4. * denotes statistical significance vs. respective sham group, + denotes statistical significance vs. MI+AAV9-Luc.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Figure 3A: Representative images captured under 2X objective from each treatment group taken from the peri-infarct zone.

Figure 3B: Quantification of Picrosirius red staining performed from the infarcted region (left panel), peri-infarct region (middle panel), and remote region (right panel) of frozen left ventricle sections. Data is presented as mean $\pm$ SEM. *** $p < 0.001$ compared to respective

sham group. + $p < 0.05$, +++ $p < 0.005$ compared to MI + AAV9-Luc. (Left panel) N=6 for Sham + AAV9-Luc, Sham + AAV9-EP4, and MI + AAV9-Luc, N=14 for MI + AAV9-EP4. For middle and right panels N=6/group.

Figure 3C: MCSA-Myocyte cross sectional area analysis analyzed using NIH Image J software. MCSA is presented as mean $\pm$ SEM. *** $p < 0.005$ vs. Sham + AAV9-Luc, ++ $p < 0.01$ vs. MI + AAV9-Luc. N=5 for Sham + AAV9-Luc, N=5 for Sham + AAV9-EP4, N=7 for MI + AAV9-Luc, N=11 for MI + AAV9-EP4.

Figure 3D: β -myosin heavy chain (β -MHC) mRNA expression and Brain natriuretic peptide (BNP) mRNA expression. For both β -MHC and BNP data was corrected to GAPDH and presented as fold of Sham + AAV9-Luc control group. N = 10 for Sham + AAV9-Luc, N=9 for Sham + AAV9-EP4, N=14 for MI + AAV9-Luc, N=12 for MI + AAV9-EP4. *** $p < 0.005$ vs. Sham + AAV9-Luc, + $p < 0.05$ vs. Sham + AAV9-EP4.

Figure 4.

Figure 4 (left panel): MMP-2 mRNA expression from left ventricles. Data is presented as mean $\pm$ SEM. N=3 for Sham + AAV9-Luc, N=4 for Sham + AAV9-EP4, N=8 for MI + AAV9-Luc, and N=6 for MI + AAV9-EP4. * $p < 0.05$ vs. Sham + AAV9-Luc. Figure 4 (right panel): In situ zymography to assess gelatinase activity in infarct zone. Data is presented as mean % area positive. N=3/group, + $p < 0.05$ vs. Sham + AAV9-Luc.

Figure 5.

Figure 5A: Representative images from all four groups showing CD68⁺ staining for macrophages in the infarct/peri-infarct region of frozen left ventricle sections.

Figure 5B: Representative images of CD68⁺ staining from the infarct zone under 2X (left panel), 10X (middle panel) and 20X (right panel).

Figure 5C: Quantification of CD68⁺ staining in the infarct/peri-infarct region (left panel) and in the remote region (right panel) of the left ventricle. Data are presented as means $\pm$ SEM. N = 12 per group. ** p < 0.01, *** p < 0.005 vs. respective sham group, ++ p < 0.01 vs. MI + AAV9-Luc.

Figure 5D: Inducible nitric oxide synthase (iNOS) mRNA expression. Data are corrected to GAPDH control and are presented as mean $\pm$ SEM fold of Sham + AAV9-Luc. ** p < 0.05 vs. Sham + AAV9-Luc. N = 3 for Sham + AAV9-Luc, N=4 for Sham + AAV9-EP4, N=8 for MI + AAV9-Luc, N=6 for MI + AAV9-EP4.

Figure 5E: Representative images showing CD3⁺ staining for T lymphocytes in the peri-infarct zone in frozen left ventricle sections taken under 20X objective.

Figure 5F: Representative images of CD3⁺ staining taken under 40X (left panel) and 100X (right panel). White arrows depict positive staining.

Figure 5G: Quantification of CD3⁺ staining in the infarct + peri-infarct zones (left panel), and in the remote zone of the left ventricle (right panel). Data are presented as means $\pm$

SEM. N = 12/group for Sham + AAV9-Luc, Sham + AAV9-EP4, and MI + AAV9-Luc.
N=16 for MI + AAV9-EP4. * $p < 0.05$, *** $p < 0.005$ vs. respective sham group. ++ $p < 0.01$
vs. MI + AAV-Luc.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6.

Figure 6A: Tumor necrosis factor α (TNF α) mRNA expression. Data was corrected to GAPDH and all data are presented as mean $\pm$ SEM as fold of Sham + AAV9-Luc. N = 3 for Sham + AAV9-Luc, N=4 for Sham + AAV9-EP4, N=8 for MI + AAV9-Luc, N=6 for MI + AAV9-EP4. *** $p < 0.001$ vs. Sham + AAV9-Luc.

Figure 6B: Cytokine/chemokine multiplex ELISA data. Left panel shows protein expression of the chemokine MCP-1 and the right panel shows protein expression of the cytokine IL-10. Data presented is optical density (O.D.) corrected for μ g of protein in each well x 1000. Data are presented as mean $\pm$ SEM. Panel A: N = 4 for MI + AAV9-Luc, N=5 for AAV9-EP4.

Panel B: N = 6 for MI + AAV9-Luc, N=5 for AAV9-EP4. ** $p < 0.01$ vs. MI + AAV9-Luc.

Figure 7.

Western blot for the expression of phosphorylated phospholamban (p-PLN) in left ventricles of mice that received either sham or MI operation with injection of either AAV9-Luc or AAV9-EP4. Total phospholamban (t-PLN) served as a loading control. Top panel shows a representative blot, while the bottom panel shows the quantification. Data is presented as mean $\pm$ SEM. *p < 0.05 compared to Sham+AAV9-Luc. N=5 per group

Table 1

Echocardiography Data

Parameters	Sham + AAV9-Luc	Sham + AAV9-EP4	MI + AAV9-Luc	MI + AAV9 EP4
BW (g)	25.62 ± 0.62	26.73 ± 0.48	26.35 ± 0.38	27.46 ± 0.36
LV (mg)/10 g BW	32.88 ± 0.65	31.88 ± 0.54	37.55 ± 1.53 *	36.15 ± 1.02 ‡
HR (bpm)	670.22 ± 14.07	680.97 ± 11.27	648.03 ± 14.60	655.21 ± 78.03
CO (mL/min/10 g BW)	12.71 ± 0.67	10.75 ± 0.45	8.92 ± 0.63 *	9.65 ± 1.22
EF (%)	76.92 ± 2.14	75.47 ± 1.11	37.84 ± 2.21 *	47.69 ± 2.39 + ‡
SF (%)	53.90 ± 0.66	53.60 ± 0.97	28.20 ± 2.88 *	40.25 ± 2.46 + ‡
LVD _d (mm)	2.59 ± 0.07	2.48 ± 0.07	4.05 ± 0.21 *	3.49 ± 0.34 ‡
LVD _s (mm)	1.20 ± 0.03	1.15 ± 0.04	2.98 ± 0.26 *	2.13 ± 0.16 + ‡

BW-body weight, LV- left ventricle, HR- heart rate, CO- cardiac output, EF- ejection fraction, SF- shortening fraction, LVD –left ventricle dimension. Data presented as mean ± SEM.

* represents a significant difference vs. Sham + AAV9-Luc, + represents significant difference vs. MI + AAV9-Luc, and ‡ represents a significant difference vs. Sham + AAV9-EP4.

N = 9 for Sham + AAV9-Luc, N=10 for Sham + AAV9-EP4, N=16 for MI + AAV9-Luc, N=17 for MI + AAV9-EP4.