Protease Activated Receptor-2 Contributes to Heart Failure

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Abstract

Heart failure is a major clinical problem worldwide. Previous studies have demonstrated an important role for G protein-coupled receptors, including protease-activated receptors (PARs), in the pathology of heart hypertrophy and failure. Activation of PAR-2 on cardiomyocytes has been shown to induce hypertrophic growth in vitro. PAR-2 also contributes to myocardial infarction and heart remodeling after ischemia/reperfusion injury. In this study, we found that PAR-2 induced hypertrophic growth of cultured rat neonatal cardiomyocytes in a MEK1/2 and p38 dependent manner. Furthermore, PAR-2 activation on mouse cardiomyocytes increased expression of the pro-fibrotic chemokine MCP-1. Cardiomyocyte-specific overexpression of PAR-2 in mice induced heart hypertrophy, cardiac fibrosis, inflammation and heart failure. Finally, in a mouse model of myocardial infarction induced by permanent ligation of the left anterior descending coronary artery, PAR-2 deficiency attenuated heart remodeling and improved heart function independently of its contribution to the size of the initial infarct. Taken together, our data indicate that PAR-2 signaling contributes to the pathogenesis of hypertrophy and heart failure.

Introduction

Heart failure (HF) is defined as the failure of the heart to provide the metabolic needs of tissues [1]. It is a major clinical problem of the Western world [2]. In the United States alone, HF results in more than 500,000 deaths per year [2]. HF reflects the end point of both acute and chronic insults, including coronary artery disease, myocardial infarction, hypertension, valve abnormalities and inherited mutations in sarcomere and cytoskeletal proteins [3–5].

The major process that contributes to HF is pathologic remodeling of the heart caused by cardiomyocyte hypertrophy, proliferation of cardiac fibroblasts and cardiac inflammation [3,5]. Cardiomyocytes are generally thought not to proliferate after birth, but can increase in size via hypertrophic growth [4]. Further, cardiac fibroblasts proliferate and synthesize extracellular matrix that contributes to cardiac fibrosis [3]. Depending on the heart disease etiology, different forms of fibrosis can be observed, including perivascular and interstitial fibrosis, as well as deposition of collagen-rich scar tissue at sites of myocardial infarction [3]. Aside from collagen deposition, dysregulated extracellular matrix turnover, orchestrated by the matrix metalloproteinase (MMP)/tissue inhibitor of MMPs (TIMP) system, mediates cardiac fibrosis leading to HF [6]. Cardiac fibrosis and HF severity was further connected to increased levels of inflammatory cytokines and chemokines, such as interleukin (IL)-6 and monocyte chemotactic protein-1 (MCP-1, CCL2) [7–10].

Protease activated receptors (PARs) are a family of seven transmembrane domain G protein-coupled receptors activated by proteolytic cleavage [11]. After their activation, a new amino terminus peptide is exposed that functions as a tethered ligand [11]. The PAR family consists of four members: PAR-1, PAR-2, PAR-3, and PAR-4. The coagulation protease thrombin is the main physiological activator of PAR-1, PAR-3 and PAR-4 [11]. PAR-2 is activated by various proteases, including trypsin, mast cells tryptase, and the coagulation proteases FVIIa and FXa [11–13]. PARs can also be activated by synthetic agonist peptides corresponding to the tethered ligand sequence [11]. PARs are widely expressed by cells within the cardiovascular system. Both PAR-1 and PAR-2 are expressed on vascular endothelium, smooth muscle cells, and cardiomyocytes [14]. It was reported that PAR-1 but not PAR-2 is expressed by rat cardiac fibroblasts [14,15]. However, more recent publications indicate that PAR-2 is expressed on cardiac fibroblasts of rats and mice [16,17]. In vivo studies demonstrated that activation of PAR-1 or PAR-2 on rat neonatal cardiomyocytes results in a series of molecular and morphological changes that lead to hypertrophic growth of these cells [15,18]. We have previously shown that PAR-1 contributes to cardiac remodeling after myocardial infarction by inducing eccentric hypertrophy of cardiomyocytes [19]. Recently, we demonstrated that inflammation and infarct size were reduced in PAR-2 deficient mice in an acute model of ischemia/reperfusion injury; this resulted in long-term beneficial effects reflected by a
better preservation of heart function [20]. In this model, we observed reduced levels of IL-6 in the heart after injury in PAR-2 deficient mice [20]. In addition, PAR-2 stimulation leads to MCP-1 expression in endothelial and epithelial cells [21,22].

In the current study, we investigated the mechanism by which PAR-2 contributes to hypertrophic growth of cardiomyocytes in vitro. Furthermore, we determined the effect of cardiomyocyte-specific overexpression of PAR-2 on heart remodeling and function. Finally, we used an in vivo mouse model of myocardial infarction, induced by permanent occlusion of coronary artery, to further determine the effect of PAR-2 deficiency on the long term heart remodeling.

**Materials and Methods**

**Mice**

PAR-2<sup>−/−</sup> mice were backcrossed at least 11 generations onto a C57Bl/6j background and bred to generate PAR-2<sup>−/−</sup> and PAR-2<sup>+/−</sup> litters [23]. Mice overexpressing PAR-2 on cardiomyocytes were generated by construction of a transgene that contained the cardiomyocyte-specific α-myosin heavy chain (αMHC) promoter and the mouse PAR-2 cDNA. Briefly, a 1.2-kbp DNA fragment containing the coding sequence of mouse PAR-2 was cloned into a vector containing the αMHC promoter, and the mouse PAR-2 cDNA. Next, an 8.5-kbp NcoI fragment, containing the αMHC-promoter, the mouse PAR-2 coding sequence, and the human growth hormone polyA sequence, was purified and injected into the pronucleus of fertilized mouse embryos (C57Bl/6j genetic background) by The Scripps Transgenic Core Facility (La Jolla, CA). Transgenic mice were identified by PCR using primers specific for the human genomic DNA amplification and have been previously published (IACUC ID 10-069) and complied with National Institute of Health guidelines.

**Isolation and culturing of rat neonatal cardiomyocytes**

Neonatal rat cardiomyocytes were isolated using a commercial isolation kit (Worthington, Lakewood, NJ) based on the method by Torasen et al. [24]. Cardiomyocytes were separated from non-myocytes by discontinuous Percoll density gradient centrifugation and cultured as described [25]. To analyze the effects of PAR-2 activation on intracellular signaling and gene expression, cells were starved for 48 hours and stimulated with PAR-2 AP (150 μM SLIGRL, Tocris Bioscience, Ellisville, MO) or control peptide (LSIGRL, Tocris Bioscience) under serum-free conditions. Cells were also pre-incubated for 30 minutes with PD90859 (10 μM) or SB203580 (10 μM) to inhibit the activation of MEK1 and p38 MAPKs, respectively [26,27].

**Isolation and culturing of mouse embryonic cardiomyocytes**

Cardiomyocytes from hearts of embryonic (E14) WT (C57Bl/6j) or αMHC-PAR-2 mice were isolated as described [27,28]. For αMHC-PAR-2 mice, each embryo was genotyped. Cardiomyocytes of the same genotype were combined and seeded in 24 well cell culture dishes [28]. An enriched cardiomyocyte population was prepared by the pre-plating method [28]. Changes in murine cardiomyocyte size were analyzed 72 hours after stimulation with 200 μM PAR-2 AP or 200 μM control peptide as well as MEK1 and p38 inhibitor as described above.

To analyze cytokine release, cells were treated with PAR-2 AP (200 μM) or control peptide (200 μM) for 24 hours and MCP-1 and IL-6 release into the supernatant were analyzed by specific Duo-Spot IC Kits (R&D Systems, Minneapolis, MN) [27] and adjusted for the total cell protein concentration.

**Analysis of cardiomyocyte hypertrophic growth**

To determine changes in cell surface area, rat and mouse cardiomyocytes were visualized with a Leica inverted microscope and surface area was quantified by imaging the complete boundary using digitized image analysis software (Image J, version 1.21). After stimulation for 72 hours, 5 frames per dish were captured at ×20 magnification and the cell surface of the cardiomyocytes was averaged for each frame; in total 45 to 65 cells were analyzed per treatment [15,29]. In addition, mRNA expression of ANF and BNP was analyzed using real time PCR as described below.

**Analysis of ERK1/2 and p38 phosphorylation**

Phosphorylation of ERK1/2 and p38 MAPKs was analyzed by ELISA using Duo-Spot IC Kits (R&D Systems) [20]. Cells from 12 well plates were lysed in 200 μL ice cold lysis buffer containing 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 1 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 100 μM PMSF, 5 μg/mL aprotinin in PBS, pH 7.2–7.4 (Sigma Aldrich). All further steps were performed according to the manufacturer’s instructions. Data were normalized with the total ERK1/2 protein expression (Duo-Spot IC Kit, R&D Systems).

**Real-time PCR**

Total mRNA from mouse hearts was reverse transcribed into cDNA and analyzed by real-time PCR using RealMasterMix and realplex<sup>®</sup> Mastercycler (Eppendorf, Hamburg, Germany). Primers were designed for the SYBR-green method to prevent genomic DNA amplification and have been previously published [30] (alltral natriuretic factor [ANF] 5'-CAT CAC CCT GGG CTT CCT CCT and 5'-TGG CCT GCA ATC CTG TGA ATC-3'; B-type natriuretic peptide [BNP] 5'-GCG GCA TGG ATC TCC TGA AGG-3' and 5'-CCC AGG CAG AAA GCC CTG T-3'; connective tissue growth factor (CTGF) 5'-GCA TCT CCA CCC GAG TTA-3' and TGG ACA GGC TTG GAG ATT-3'; transforming growth factor [TGFβ1] 5'-GAC GTG ACT GGA GTT TGA CCGG-3' and 5'-GCT GAA TCG AAA GGC CTG T-3'; TGFβ3 5'-TTG AGC TCT TCC AGA ATC TTT G-3' and 5'-TTC TTG CCA CCT ATG TAG CG-3'; αMHC 5'-TGA TTC CCA ACG AGC GAA A-3' and 5'-GCC GGA AGT CCC CAT AGA GA-3'; βMHC 5'-GAT GGA CAA TCC TGC CTT GGT-3' and 5'-CGG AAA GTC CCC CAT AAG AT-3'). The expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) 5'-GTT GTG AAA AGG ACC TCT CG-3' and 5'-TGA AGT ACT CAT TAT AGT CAA GGG GA-3') was used as internal control. To analyze the expression of MMP-2, MMP-3, MMP-8, TIMP-1, MCP-1, IL-β and IL-6 we used predesigned probe sets (Integrated DNA Technologies, Coralville, IA). Variations in loading were adjusted using GAPDH mRNA expression.

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Histology
Fibrosis was assessed on formalin-fixed, paraffin-embedded heart sections stained with Masson’s Trichrome [25].

Echocardiography
Echocardiography on conscious mice was performed using a VisualSonics Vevo2100 system (VisualSonics, Toronto, ON) as previously described [20,27].

Northern blot analysis
Samples from mouse hearts were collected, frozen in liquid nitrogen and stored at −80°C. Total mRNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) [31,32]. Levels of PAR-2 mRNA and GAPDH mRNA were determined by Northern blotting as previously described [19].

Myocardial infarction model - permanent ligation of the left anterior descending (LAD) coronary artery
Male mice were anesthetized with pentobarbital (45 mg/kg), intubated, and ventilated with a small rodent ventilator (Harvard Apparatus, Holliston, MA) at a rate of 110 cycles/minute with a tidal volume of 2 ml/minute and a positive end-expiratory pressure of 2 cmH2O. A left side thoracotomy was performed, and the pericardium was incised. Myocardial infarction was then induced through permanent ligation of the LAD coronary artery with an 8-0 silk suture proximal to its bifurcation from the main stem. The incision was subsequently closed with a 5-0 silk suture. Mice were then allowed to recover in a temperature-controlled environment. After surgery, mice were administrated with post-operative dose of buprenorphine every 12 hours for 2 days. Mice were closely monitored and all efforts were made to minimize suffering. Four weeks later heart function was analyzed using echocardiography as described above. In addition, cardiac troponin I plasma levels were analyzed from the separate sets of sham and LAD artery occluded animals 24 hours after surgery by using a highly sensitive mouse cardiac troponin I ELISA kit (Life Diagnostics, West Chester, PA) as recently described [27] to analyze initial cardiac injury.

Statistical analysis
All statistical analyses were performed using GraphPad Prism (version 5.0; GraphPad Software Inc., La Jolla, CA). Data are represented as mean ±SEM, unless otherwise indicated in figure legends. For 2-group comparison of continuous data, 2-tailed Student’s t test was used. For multiple-group comparison, normally distributed data were analyzed by 1- or 2-way ANOVA and were Bonferroni corrected for repeated measures over time. A p-value ≤0.05 was regarded as significant.

Results
Inhibition of ERK1/2 and p38 MAPKs reduces PAR-2 induced hypertrophic growth of rat neonatal cardiomyocytes
Consistent with the previous study by Sabri and colleagues [15], we found that stimulation of rat neonatal cardiomyocytes with PAR-2 agonist peptide (SLIGRL, 150 μM) led to ERK1/2 and p38 MAPK phosphorylation (Figure S1A) and hypertrophic growth measured by increased ANF and BNP mRNA expression and total cell area (Figure 1A–C). Importantly, the PAR-2-mediated increase in cardiomyocyte size was attenuated by inhibition of either the ERK1/2 or the p38 pathways significantly reduced hypertrophic growth of mouse embryonic cardiomyocytes (Figure 1A–C). These data indicate that activation of these two pathways is required for PAR-2 induced hypertrophic growth of both rat and mouse cardiomyocytes.
Generation of transgenic mice with cardiomyocyte-specific PAR-2 overexpression

To directly investigate the role of PAR-2 in the heart, we generated mice overexpressing PAR-2 on cardiomyocytes using the alpha myosin heavy chain (αMHC) promoter (αMHC-PAR-2 mice). We used this promoter previously to express PAR-1 on cardiomyocytes [19]. Germline transmission of the transgene was observed in six different lines of αMHC-PAR-2 mice and four of them (lines 11, 12, 18 and 32) demonstrated a significant increase in the heart weight to body weight (HW:BW) ratio by 2–3 months of age compared to control mice. We observed a dramatic increase in HW:BW ratio in αMHC-PAR-2 mice line 11 (10.0±0.39 vs. 4.68±0.22; p<0.005; n = 3 per group) and line 32 (7.30±0.41 vs. 4.92±0.40; p<0.01; n = 3 per group) compared to wild type (WT) littermates, which was associated with premature death of these mice around 2–3 months of age. A moderate increase in the HW:BW ratio was observed in line 12 at the age of 2 months (5.54±0.54, n = 7 vs. 4.85±0.20, n = 5; p<0.01) and line 18 at the age of 3 months (5.17±0.38 vs. 4.69±0.24; n = 6; p<0.05). Northern blot analysis demonstrated that the PAR-2 transgene was specifically expressed in the heart of αMHC-PAR-2 mice line 18 whereas the expression of the PAR-2 transgene in αMHC-PAR-2 mice line 12 was detected not only in the heart but also in the lung (Figure 2A). Therefore, we used mice from line 18 to study the long-term effect of cardiomyocyte-specific overexpression of PAR-2 on heart remodeling and function.

Cardiomyocyte-specific overexpression of PAR-2 leads heart hypertrophy

Since we recently demonstrated that PAR-2 contributes to heart remodeling after cardiac ischemia/reperfusion injury [20] and that activation of PAR-2 leads to hypertrophic growth of cardiomyocytes in vitro [15], we investigated if cardiomyocyte-specific overexpression of PAR-2 induced heart hypertrophy and HF in mice. First, we analyzed the effect of the PAR-2 overexpression on 1 year old mice from line 18. Gross morphological analysis demonstrated that αMHC-PAR-2 mice had larger hearts compared to WT littermates (Figure 3A). Real-time PCR analysis showed that mRNA expression of ANF, BNP and β-myosin heavy chain (βMHC) were significantly increased, whereas mRNA expression of αMHC was significantly decreased in the hearts of αMHC-PAR-2 mice compared to the WT littermates (Figure 3B). Consistent with visibly larger hearts and altered hypertrophic gene mRNA expression, we observed an increase in HW:BW ratio in αMHC-PAR-2 mice compared to the WT littermates (Figure 3C). In addition to heart hypertrophy, αMHC-PAR-2 mice had an increased lung weight to BW ratio (LW:BW, Figure 3C), suggesting lung edema secondary to HF, a common co-morbidity of congestive HF. The ratio of kidney weight to BW was not changed in αMHC-PAR-2 mice (Figure 3C). The ratio of heart weight as well as lung weight to the tibia length was also significantly increased (data not shown). These data indicate that overexpression of PAR-2 in cardiomyocytes leads to heart hypertrophy in mice.

PAR-2 dependent cardiac inflammation

One year old αMHC-PAR-2 mice also showed increased expression of IL-6 and MCP-1 in the heart compared to age matched littermate controls (Figure 4A). In addition, we found that PAR-2 stimulation leads to increased MCP-1 and IL-6 protein expression in embryonic murine cardiomyocytes isolated from WT mice (Figure 4B–C). Importantly, PAR-2 stimulation of cardiomyocytes isolated from αMHC-PAR-2 mice resulted in significantly higher expression of MCP-1 and also slightly higher levels of IL-6 compared to that observed in cell isolated from the hearts of WT littermate mice (Figure 4B–C). The data suggest that acute and chronic PAR-2 activation leads to cardiac inflammation.

αMHC-PAR-2 mice develop cardiac fibrosis

We showed that PAR-2 deficiency reduced cardiac fibrosis after cardiac ischemia/reperfusion injury [20]. Therefore, we investigated whether the overexpression of PAR-2 in cardiomyocytes led to cardiac fibrosis. Masson’s Trichrome staining of heart sections from one year old αMHC-PAR-2 mice showed increased interstitial fibrosis (Figure 5A). Furthermore, a significant up-regulation of the mRNA expression of known pro-fibrotic genes, including TGFβ1, TGFβ3, collagen III, and CTGF, was observed in the hearts of one year old αMHC-PAR-2 hearts compared to their WT littermates (Figure 3B). Cardiac fibrosis is often associated with dysregulation of the MMP/TIMP system [3,6]. Consistent with this notion, hearts from αMHC-PAR-2 mice exhibited increased mRNA expression of MMP-2 and TIMP-1, decreased levels of MMP-9, MMP-13 and TIMP-4 and no change in TIMP-2 compared to littermate controls (Figure 5C). These data indicate that cardiomyocyte-specific overexpression of PAR-2 resulted in pathologic heart fibrosis and remodeling due to increased matrix deposition and dysregulated MMP/TIMP system.

Cardiac hypertrophy, inflammation and fibrosis are associated with impairment of heart function in αMHC-PAR-2 mice

Pathologic heart hypertrophy, inflammation, and fibrosis lead to heart dysfunction and HF. Therefore, transthoracic
echocardiography was used to measure LV function. Consistent with the gross histological analysis, we found that the diameter and volume of the LV was increased significantly in αMHC-PAR-2 mice compared with littermate controls at 12 months of age (Table 1). Moreover, the thickness of the anterior and posterior LV wall at systole but not diastole was reduced in the αMHC-PAR-2 mice (Table 1). As expected, LV function measured by percentages of fractional shortening and ejection fraction was significantly reduced in αMHC-PAR-2 mice compared with WT littermates (Table 1).

PAR-2 contributes to the heart remodeling after permanent occlusion of the LAD coronary artery

To further explore the role of PAR-2 in heart remodeling independent of reperfusion injury, we used a mouse model of heart failure induced by a permanent occlusion of LAD coronary artery.
First we analyzed the myocardial infarction 24 hours after permanent occlusion in PAR-2+/+ and PAR-2−/− mice. Plasma levels of cardiac troponin I were significantly increased in both groups of mice compared to the levels observed in sham operated mice (Figure 6A). There was no difference between PAR-2+/+ and PAR-2−/− mice indicating that PAR-2 does not contribute to the initial ischemic injury in this model. Four weeks after permanent occlusion, we used echocardiography to analyze heart remodeling and function in PAR-2+/+ and PAR-2−/− mice. As shown in Figure 5, occlusion of the LAD resulted in significant dilation of LV and dramatic reduction of heart function. Importantly, both these parameters were significantly attenuated in PAR-2+/+ mice compared to PAR-2−/− mice (Figure 6B–D). Heart weights of PAR-2+/+ mice were increased compared to PAR-2−/− mice 4 weeks after LAD occlusion (208.6±16.1 mg vs. 172.4±8.2 mg, p<0.05). Since PAR-2−/− mice body weights were slightly lower than PAR-2+/+ mice (27.98±0.56 g vs. 29.63±0.95 g, p=0.12) whereas the tibia length were equal between the groups (22.66±0.18 mm vs. 22.55±0.10 mm, PAR-2+/+ vs. PAR-2−/−, p=0.57), we used tibia length to calculate heart weight:tibia length ratio. PAR-2 deficient mice exhibited reduced heart hypertrophy compared to PAR-2+/+ mice as demonstrated by lower heart weight:tibia length ratios (Figure 6E). Representative cross-sections of the hearts from the PAR-2+/+ and PAR-2−/− mice 4 weeks after LAD occlusion are shown in Figure 6F.

**Discussion**

In this study, we demonstrated that cardiomyocyte-specific overexpression of PAR-2 led to pathologic heart hypertrophy associated with cardiac fibrosis. Pathologic remodeling of the heart in zMHC-PAR-2 mice was accompanied by increased ANF, BNP and βMHC expression and decreased zMHC expression. Importantly, BNP is a strong predictor of cardiac hypertrophy and dysfunction in both mouse models and in humans [24]. During heart hypertrophy, an initial increase in LV wall thickness is usually followed by wall thinning and dilatation of the LV chamber [5]. Echocardiography analysis revealed that the diameter and volume of the LV were significantly increased, whereas the thickness of LV walls was significantly reduced at systole but not diastole in zMHC-PAR-2 mice. Moreover, we observed a significant decrease in the heart function in zMHC-PAR-2 mice compared to littermate controls. These data indicate that cardiomyocyte-specific overexpression of PAR-2 results in pathologic heart remodeling which leads to systolic HF in mice.

Hypertrophic growth of cardiomyocytes is one of the processes that contribute to heart remodeling. Activation of PAR-2 in vitro leads to the hypertrophic growth of rat cardiomyocytes and increased phosphorylation of ERK1/2 and p38 [15]. However, the role of these MAPKs in PAR-2 induced cardiomyocyte hypertrophy has not been investigated. We demonstrated that inhibition of these two MAPK pathways significantly attenuated PAR-2-mediated growth of both rat neonatal and mouse embryonic cardiomyocytes in vitro. These data suggest that activation of PAR-2 might contribute to heart remodeling, in part, via MAPK-dependent hypertrophic growth of cardiomyocytes.

Dilated cardiomyopathy caused by pathologic hypertrophy is often associated with inflammation and fibrosis [3,7,8]. Cardiomyocyte-specific overexpression of PAR-2 resulted in cardiac fibrosis. The fibrotic area and levels of TGFβ, collagen III, and CTGF mRNA expression were increased in the hearts of zMHC-PAR-2 mice. In addition, mice with PAR-2 overexpression on cardiomyocytes exhibited increased cardiac inflammation seen as elevated IL-6 and MCP-1 expression. Furthermore, we demonstrated that stimulation of PAR-2 on cardiomyocytes leads to...
Figure 5. Cardiomyocyte-specific overexpression of PAR-2 results in heart fibrosis. A: Representative cross-sections of one year old WT and αMHC-PAR-2 hearts stained with Masson’s Trichrome. B–C: mRNA expression of pro-fibrotic genes and MMPs and TIMPs in the heart of one year old WT (open boxes) and αMHC-PAR-2 (grey filled boxes) mice (7–8 mice per group). * p<0.05.

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increased expression of MCP-1 in vitro. MCP-1 signaling leads to aberrant cardiac fibrosis and induction of HF [7,10]. Importantly, it has been shown that inflammation influences cardiac fibrosis [7,8]. Increased expression of MCP-1 caused changes in the balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system [6,7,9]. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system. End stage failing human hearts show increased expression of MMP2 and TIMP-1, and a balance between matrix synthesis and degeneration by interacting with the MMP/TIMP system.

Table 1. Heart function analysis by echocardiography on one year old wild-type and αMHC-PAR-2 mice.

|            | Wild-type | αMHC-PAR-2 | p-value |
|------------|-----------|------------|---------|
| LVID;d (mm) | 2.90±0.35 | 3.44±0.45  | 0.01    |
| LVID;s (mm) | 1.80±0.33 | 2.62±0.55  | 0.001   |
| LVAW;d (mm) | 1.22±0.09 | 1.21±0.14  | N.S.    |
| LVAWWs (mm) | 1.69±0.15 | 1.48±0.15  | 0.01    |
| LPW;d (mm)  | 1.23±0.16 | 1.16±0.13  | N.S.    |
| LPWWs (mm)  | 1.36±0.17 | 1.21±0.23  | 0.08    |
| LV Vol;d (µL) | 33.07±9.71 | 49.99±15.30 | 0.01 |
| LV Vol;s (µL) | 10.35±4.77 | 26.87±13.04 | 0.001 |
| EF (%)      | 69.80±6.39 | 48.91±11.51 | 0.001 |
| FS (%)      | 38.34±5.11 | 24.46±6.73  | 0.001 |

LVID left ventricle internal diameter, LVAW left ventricle anterior wall, LPW left ventricle posterior wall, Vol volume, d diastole, s systole, EF ejection fraction, FS fractional shortening.
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One limitation of our study is that the phenotype of αMHC-PAR-2 mice is the result of overexpressing PAR-2. It has been previously demonstrated that overexpression may lead to the generation of non-specific effects. For example, cardiomyocyte-specific overexpression of green fluorescent protein results in HF [45]. On the other hand, cardiomyocyte-specific overexpression of various genes has significantly contributed to our understanding of heart diseases, and the results obtained from overexpression studies have been confirmed by complementary studies using knockout mice. For example, cardiomyocyte-specific overexpression of either wild type or constitutively active forms of Gq, induced dilated cardiomyopathy [46,47], whereas mice with cardiomyocyte-specific deletion of Gq/G11 are resistant to ventricular hypertrophy induced by pressure overload [48].

In contrast to our studies with PAR-2 deficient and αMHC-PAR-2 mice, it has been reported that activation of PAR-2 with a PAR-2 agonist peptide has a beneficial effect in both ex vivo and in vivo models of heart ischemia/reperfusion injury [49–51]. The protective mechanism involved vasodilation of coronary vessels, mediated by activation of PAR-2 on endothelial cells [51]. Similar discrepancy between treatment with PAR-2 agonist peptide and PAR-2 deficiency has been observed in the mouse model of colitis induced by intrarectal injection of trinitrobenzene sulfonic acid [52]. Interestingly, PAR-2 plays different roles in different organs subjected to ischemia/reperfusion injury. For example, PAR-2 deficiency increases the infarct volume in the brain [53], has no effect on kidney function [54] and reduces infarct size in the heart [20]. Furthermore, in a mouse model of Alzheimer disease PAR-2 signaling had opposite effects in different cell types within the brain [55]. These apparently contrasting results strongly suggest that PAR-2 mediated effects may be not only organ but even cell type-specific. Therefore, it is possible that after myocardial infarction the activation of PAR-2 on endothelial cells may be protective, whereas PAR-2 signaling on other cell types, such as cardiomyocytes or infiltrating leukocytes may be detrimental. Another possible explanation could be the fact that PAR-2 is differentially activated by tethered versus soluble ligands, such as an agonist peptide [56]. These two types of ligands differentially bind and stabilize different conformations of the receptor, leading to the activation of distinct subsets of signaling pathways [56].
better understanding of the cell type- and ligand-specific responses of PAR-2 after myocardial infarction is needed.

In conclusion, our study suggests that PAR-2 contributes to the pathogenesis of heart hypertrophy and failure. Further studies investigating the effectiveness of specific PAR-2 inhibitors in various mouse models of heart hypertrophy and failure are warranted and will validate if PAR-2 is a good target to attenuate heart failure.

Supporting Information

Figure S1  Activation of PAR-2 leads to ERK1/2 and p38-dependent rat neonatal cardiomyocyte hypertrophy in vitro. A: Activation of ERK1/2 and p38 signaling pathway in cardiomyocytes in response to PAR-2 agonist peptide (PAR-2 AP, 150 μM). (N = 5 each time point). B: Expression of ANF and BNP in cardiomyocytes after 72 h of PAR-2 AP stimulation. (N = 8–11)

Figure S2  Heart specific PAR-2 overexpression. Northern blot analysis of PAR-2 mRNA expression in different organs from αMHC-PAR-2 (line 12 and 18) and littermate controls (WT) mice. Arrow head indicates endogenous expressed PAR-2 mRNA. (TIF)
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Conceived and designed the experiments: SA NM RP. Performed the experiments: SA EMS MT RP. Analyzed the data: SA NM RP. Contributed reagents/materials/analysis tools: MT. Wrote the paper: SA EMS NM RP.
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