Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2010 November ; 30(11): 2136–2142. doi:10.1161/ATVBAHA.110.213280.

Protease-Activated Receptor 2 Deficiency Reduces Cardiac Ischemia/Reperfusion Injury

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Abstract

Objective—Protease-activated receptor-2 (PAR-2) signaling enhances inflammation in different diseases. The effect of PAR-2 deficiency in cardiac ischemia/reperfusion (I/R) injury is unknown. We investigated the effect of PAR-2 deficiency on I/R injury-induced infarct size, inflammation, heart remodeling and cardiac function.

Methods and Results—PAR-$^{-/-}$- mice and wild-type (WT) littermates were subjected to 30 minutes of ischemia and up to 4 weeks of reperfusion. Infarct size, oxidative/nitrative stress, phosphorylation of mitogen-activated protein kinases (MAPK) and inflammatory gene expression were assessed 2 hours after reperfusion. Changes in heart size and function were measured by echocardiography up to 4 weeks after reperfusion. Infarct size was significantly reduced in hearts of PAR-2$^{-/-}$ mice compared to WT littermates. In addition, oxidative/nitrative stress, phosphorylation of MAPK and expression of pro-inflammatory genes were significantly attenuated in injured hearts of PAR-2$^{-/-}$ mice. Finally, PAR-2$^{-/-}$ mice were protected from post-infarction remodeling and showed less impairment in heart function compared with WT littermates up to 4 weeks after I/R injury.

Conclusion—PAR-2 deficiency reduces myocardial infarction and heart remodeling following I/R injury.

Keywords

Protease-activated receptor 2; myocardial infarction; inflammation; cardiac remodeling; oxidative/nitrative stress

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Disclosures:
None.

Introduction

Protease-activated receptors (PARs) are a family of seven transmembrane domain G protein–coupled receptors activated by proteolytic cleavage[1]. The PAR family consists of four members: PAR-1 to -4. PAR-2 is activated by several proteases, including trypsin, mast cell tryptase, matriptase and the coagulation proteases factor VIIa (FVIIa) and FXa1−4. PAR-2 can be also activated by synthetic agonist peptides corresponding to the tethered ligand sequence1. Activation of PAR-2 leads to an elevation of intracellular Ca^{2+} and stimulation of multiple intracellular signaling pathways, including MAPK pathways[1, 5, 6].

PAR-2-dependent signaling has been shown to play an important role in many animal models of inflammatory diseases. Recent studies demonstrated a role of PAR-2 in leukocytes rolling, arthritis, inflammatory pain and allergic inflammation, as well as in skin disorders[7–12]. In contrast, other studies support a protective role of PAR-2 in airway inflammation, ischemic brain injury, and influenza virus infection[13–15]. Furthermore, in a mouse model of Alzheimer's disease, PAR-2 protects neurons from beta-amyloid induced toxicity, but its activation in microglia cells led to neurotoxicity[16]. These studies indicate that PAR-2 activation on different cell types may have detrimental or protective effects in different disease models.

Myocardial infarction (MI) is one of the leading causes of mortality and morbidity in the Western world17. Coronary vessel occlusion and subsequent ischemia results in myocardial cell death18. Early restoration of blood flow within the coronary vessels is necessary to provide oxygen and nutrients to the ischemic area. However, reperfusion itself contributes to injury of the heart by initiating a local inflammatory response, which leads to further myocardial damage17, 18. This is known as ischemia/reperfusion (I/R) injury. With time, areas of the initial infarct are replaced by collagen-rich scar tissue. The acute loss of myocardial cells results in abnormal loading conditions that involve not only the border zone of infarction, but also the remote myocardium. These abnormal loading conditions lead to hypertrophy, aberrant cardiac remodeling, and ultimately heart failure[17, 18].

PAR-2 is express by numerous cell types within the cardiovascular system. Functional PAR-2 expression has been demonstrated on vascular endothelium, smooth muscle cells and cardiomyocytes but not on cardiac fibroblasts[5, 6]. In addition, PAR-2 is expressed on activated neutrophils[19]. *In vitro* studies demonstrated that PAR-2 activation on cultured cardiomyocytes results in a series of molecular and morphological changes that lead to hypertrophic growth[6]. Furthermore, several recent papers 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 cardiac I/R injury[20–22]. The protective mechanism involved vasodilation of coronary vessels, which was mediated by activation of PAR-2 on endothelial cells[21]. However, the effect of global deficiency of PAR-2 on myocardial infarction has not been investigated.

In this study, we investigated the role of PAR-2 in cardiac I/R injury. We found that PAR-2 deficiency resulted in a significant reduction in inflammation and infarct size, as well as attenuation of pathologic heart remodeling.

Methods

Human study

PAR-1 and PAR-2 mRNA levels were determined in heart samples from the LV (LV) free wall (toward the apex) of 5 male patients in end-stage HF of ischemic origin (mean age 57+/−9) at the time of left ventricular assist device placement. Non-failing tissue was obtained from the LV free wall (toward the apex) of five male non-failing organ donor hearts rejected for transplant for physical incompatibilities (mean age 48+/−7). LV tissue obtained from surgery

was immediately frozen in liquid nitrogen and stored at −80°C. All surgical procedures and tissue harvesting were performed with informed patient consent and are concordant with NIH and University of Rochester Institutional Review Board guidelines.

Mice

PAR-2$^{+/-}$ mice were backcrossed onto a C57Bl/6J background and intercrossed to generate PAR-2$^{-/-}$ and wild-type (WT) littermate mice[23]. Male mice (8–12 weeks of age) were used for experiments. All experiments were approved by the Animal Care and Use Committees of the different institutions and complied with National Institute of Health guidelines.

Cardiac I/R injury model

For the short-term I/R model (30 minutes of ischemia and 2 hours of reperfusion), the surgical protocol and infarct-size determination were performed as previously described[24] with some modifications. Briefly, intraperitoneal injection of pentobarbital (100 mg/kg; Abbott Laboratories, Abbott Park, IL) was used for anesthesia. Mice were intubated orally to provide artificial ventilation (0.3 mL tidal volume, 120 breaths/min). The left anterior descending coronary artery was occluded with a 7-0 silk suture (US Surgical Corp, Norwalk, Conn) passed through PE tubing (US Surgical Corp) to make a Rumel snare. After 30 minutes of ischemia, the snare was released, and the heart was reperfused for 2 hours. Finally, the artery was reoccluded, and 4% Evans Blue dye was injected into the aortic root to delineate the area at risk from not-at-risk myocardium (blue). Hearts were then explanted, rinsed in 0.9% normal saline, and placed in 1% agarose gel (UltraPure agarose, Life Technologies, Gaithersburg, MD) in PBS (pH 7.4). Hearts were sectioned parallel to the AV groove in ≈1-mm sections. Viable and necrotic sections of the area at risk were identified by incubating the hearts in 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St Louis, Mo) for 10 minutes at 37°C followed by 10% neutral buffered formaldehyde for 24 hours. Each section was weighed and photographed. The LV, area at risk, and infarct areas were traced and calculated by computer planimetry (Image J, version 1.21). Infarct volumes were calculated as $[(A_{1x}W_1)+(A_{2x}W_2)+(A_{3x}W_3)+(A_{4x}W_4)+(A_{5x}W_5)]$, where A is the area of infarct for the slice denoted by the subscript and W is the weight of the respective section.

The long-term model (30 minutes of ischemia and 4 weeks of reperfusion) was performed in a similar manner. Ischemia was validated via ECG recordings. After 30 minutes of ischemia, occlusion of the left anterior descending coronary artery was released, the wound was closed and animals were returned to their cages. Surgery was performed in a blinded fashion.

TaqMan® Real-time Polymerase Chain Reaction (PCR)

Total RNA was isolated using Trizol reagent and reverse transcribed into cDNA using RETROscript® Kit (Applied Biosystems, Foster City, California). Levels of different mRNAs were analyzed by real-time PCR using RealMasterMix and realplex^2 Mastercycler (Eppendorf AG, Hamburg, Germany). TaqMan primer and probe sets (Applied Biosystems) were used to analyze the mRNA expression of: mouse IL-1β (Mm01336189_m1), mouse IL-6 (Mm99999064_m1), mouse TNF-α (Mm00443259_g1), mouse KC (Mm00433859_m1), mouse MCP-1 (Mm00441242_m1), mouse MIP-2 (Mm00436450_m1), mouse PAR-1 (Mm00438851_m1), mouse PAR-2 (Mm00433160_m1), human PAR-1 (Hs00169258-m1) and human PAR-2 (Hs00608346-m1). The mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (4352339E) was used as internal control.

ELISA

Protein extracts were generated from mouse hearts as previously described[25]. Cytokine and chemokine protein expression in the hearts was analyzed by Quantikine ELISAs (R&D

Systems, Minneapolis, MN, USA). Levels of 3-nitrotyrosine, a marker of oxidative/nitrative stress, was analyzed using commercial elisa assay (Hbt Hycult Biotechnology, Uden, The Netherlands). Phosphorylation of the MAP kinases ERK1/2, JNK and p38 as well as AKT was analyzed in heart lysates with the Duo-Set IC Kit (R&D Systems). All ELISAs were performed according to the manufactures' instructions. Data were normalized to the protein concentration in respective lysates and compared to non-injured WT hearts. Protein concentration was measured using Bio Rad D_c Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Echocardiography

Echocardiography was performed using a VisualSonics Vevo 660 ultrasound system as described[24]. Diameter of LV and LV wall thickness at the end of systole and diastole were measured digitally on M-mode tracings and averaged from 4 cardiac cycles. Fractional shortening (FS) was then calculated from measured ventricle dimensions.

Heart weight to body weight ratio

Hearts were stopped at diastole by intra-ventricular injection of 1mL 20mM KCl solution. Heart weight to body weight ratio (HW:BW) was calculated by dividing the HW (mg) by BW (g).

Histology

Hearts were fixed with 10% formalin solution and embedded in paraffin. Deparaffined sections were stained with the Masson's trichrome.

Statistic analysis

All statistical analyses were performed using GraphPad Prism version 5.01. Data are represented as means ± SEM. For two group comparison of parametric data, a Student's t-test was performed. For multiple group comparison, one-way ANOVA or two-way ANOVA tests were performed followed by Bonferroni post test analysis.

Results

MI upregulates PAR-1 and PAR-2 expression

We used short-term (30 minutes ischemia and 2 hours of reperfusion) and long-term (30 minutes ischemia and 4 weeks of reperfusion) mouse models of I/R injury to determine if the expression levels of PAR-1 and PAR-2 mRNA are altered during MI and heart remodeling, respectively. We found that levels of PAR-2 but not PAR-1 mRNA expression were upregulated in injured hearts 2 hours after reperfusion (Figure 1A and B). In the long-term model, we observed a significant increase in both PAR-1 and PAR-2 mRNA expression in the hearts (Figure 1A and B).

Next, we analyzed the levels of PAR-1 and PAR-2 mRNA expression in heart biopsies obtained from patients with ischemic heart failure. PAR-1 and PAR-2 mRNA expression were significantly upregulated in failing hearts compared to the levels observed in the biopsies from healthy donors (Figure 1C and D).

PAR-2 deficiency reduces infarct size

To investigate the role of PAR-2 deficiency in MI, PAR-$2^{-/-}$ and WT littermate mice were subjected to 30 minutes of ischemia and 2 hours of reperfusion. We found that infarct size was significantly reduced in hearts of PAR-$2^{-/-}$ mice compared to WT littermates (24.3±1.7% *vs*. 35.8±3.4%) with no significant changes in the area at risk (Figure 2A).

Oxidative/nitrative stress is attenuated in the injured hearts of PAR-2$^{-/-}$ mice

It has been demonstrated that formation of peroxynitrite ($ONOO^-$), a product of nitric oxide and superoxide interaction, contributes to the MI by inducing oxidative /nitrative stress[26–28]. Nitrotyrosine is a stable end product of peroxynitrite oxidation and has been used as a marker of oxidative/nitrative stress in a mouse model of cardiac I/R injury[26–28]. After 30 minutes of ischemia and 2 hours of reperfusion, the level of nitrotyrosine in myocardial tissue was significantly increased in the hearts of WT mice (Figure 2B). Importantly, the level of nitrotyrosine in injured hearts of PAR-2$^{-/-}$ mice was significantly reduced compared to the level observed in injured hearts of WT mice (Figure 2B).

PAR-2 deficiency reduces activation of MAPK pathways

It has been demonstrated that activation of various MAPK pathways contributes to inflammation in the setting of I/R injury29, [30]. PAR-2 deficiency had no effect on basal phosphorylation levels of ERK-1/2, p38 and JNK in mouse hearts. I/R injury (30 minutes of ischemia and 2 hours of reperfusion) resulted in increased phosphorylation of all three MAPKs in the hearts of WT mice (Figure 3A–C). Importantly, activation of ERK-1/2, p38 and JNK was significantly attenuated in the hearts of PAR-2$^{-/-}$ mice (Figure 3A–C). In contrast, PAR-2 deficiency had no effect on phosphorylation of AKT (Figure 3D).

PAR-2 deficiency attenuates inflammatory gene expression in the heart

Cardiac I/R injury induced the expression of a variety of inflammatory mediators, including IL-1β, IL-6, TNF-α and KC 2 hours after reperfusion at both mRNA and protein levels (Figure 4A and B). Importantly, injured hearts of PAR-2$^{-/-}$ mice exhibited significantly less IL-1β, TNF-α and KC mRNA expression compared with injured hearts of WT mice (Figure 4A). The IL-6 mRNA expression was also attenuated in PAR-2$^{-/-}$ mice but did not reach statistical significance (Figure 4A). Consistent with changes of mRNA expression, protein levels of IL-1β, IL-6, TNF-α and KC were significantly reduced in the injured hearts of PAR-2$^{-/-}$ mice compared with injured hearts of WT mice (Figure 4B). PAR-2 deficiency did not effect expression of MCP-1 and MIP-2 in injured hearts (data not shown).

PAR-2 deficiency reduces cardiac remodeling and heart dysfunction

To study the role of PAR-2 in heart remodeling, PAR-2$^{-/-}$ and WT littermate mice were subjected to 30 minutes of ischemia followed by 4 weeks of reperfusion period. Gross morphological analysis of heart cross-sections demonstrated that injured hearts of WT mice were larger with visible dilatation of the LV compared to injured hearts of PAR-2$^{-/-}$ mice (Figure 5A). I/R injury resulted in a significant increase in the HW:BW ratio in WT mice indicating hypertrophic remodeling of the hearts (Figure 5B). Importantly, the increase in HW:BW ratio was significantly smaller in PAR-2$^{-/-}$ mice compared with WT littermates (Figure 5B).

To analyze the heart function, echocardiography was performed before and up to 4 weeks after cardiac I/R injury in PAR-2$^{-/-}$ and WT littermate mice. Before surgery, the morphological and functional parameters of hearts were similar between WT and PAR-2$^{-/-}$ mice (day 0) (Figure 6). After cardiac I/R injury, PAR-2$^{-/-}$ mice showed significantly less ventricular dilatation compared to WT littermates, as demonstrated by changes in LV diameter and volume at the end of systole (Figure 6A and B). Moreover, 3 weeks after the I/R injury, thinning of the LV posterior wall was significantly attenuated in the heart of PAR-2$^{-/-}$ mice at the end of systole compared to WT mice (Figure 6C). I/R injury or PAR-2 deficiency had no effect on these three parameters at the end of diastole (data not shown). Importantly, PAR-2$^{-/-}$ mice showed significantly less impairment in the heart function after cardiac I/R injury, measured by

percentage of fractional shortening (Figure 6D). These data indicate that PAR-2 deficiency protects against systolic heart dysfunction induced by I/R injury.

Discussion

In this study, we found that PAR-2 deficiency reduces MI in a mouse model of cardiac I/R injury. Interaction between nitric oxide and superoxide anion, one of the reactive oxygen species (ROS) generated during cardiac I/R injury, results in the formation of highly cytotoxic molecule peroxynitrite[31]. Peroxynitrite mediated toxicity involves direct oxidative damage to lipids, proteins and DNA, the activation of metalloproteinases, the impairment of mitochondrial respiration, and the nitration of tyrosine residues within proteins32. The reduction of nitrotyrosine levels in the myocardium of PAR-2 deficient mice suggests that oxidative/nitrative stress is one of the mechanisms by which PAR-2 may enhances MI. This concept is supported by recent publications indicating that activation of PAR-2 leads to generation of both nitric oxide and ROS, including superoxide anion19, 33, 34.

During I/R injury, release of ROS results in the activation of MAPKs[35]. Furthermore, activation of PAR-2 also leads to phosphorylation of MAPKs in various cell types, including cardiomyocytes and endothelial cells[6, 36]. Recent studies demonstrated that activation of MAPKs enhance inflammation and contribute to MI. For example, overexpression of a dominant-negative p38 MAPK or inhibition of JNK MAPK results in significant protection from I/R injury[30, 37]. Moreover, reduced activation of MAPK was correlated with smaller infarcts in mice deficient for the lectin-like receptor for oxidize-LDL[38]. In our study, we demonstrated that the smaller infarcts observed in the hearts of PAR-2$^{-/-}$ mice after I/R injury was associated with reduced phosphorylation of ERK-1/2, p38 and JNK and attenuated expression of various inflammatory mediators. Therefore, we propose that PAR-2 contributes to MI, in part, by enhancing inflammation in a MAPK-dependent manner.

Many cell types can contribute to a PAR-2-mediated inflammatory response within injured myocardium. One of them is endothelial cells. It has been demonstrated that activation of PAR-2 on endothelial cells induces the expression of IL-1β, IL-6 and IL-8 (human homolog of mouse KC)[39, 40]. The production of chemokines leads to the recruitment of leukocytes, including neutrophils and monocytes, into the injured myocardium41. Both cell types have been shown to express PAR-219, 42. It has been shown that PAR-2 signaling plays a role in leukocyte adhesion, rolling and migration[7, 43]. Furthermore, monocytes/macrophages and neutrophils expresses multiple inflammatory cytokines, and may be a source of TNF-α and IL-1β in the injured myocardium[42, 44–46]. Therefore, PAR-2-dependent signaling in endothelial cells and leukocytes will increase the expression of inflammatory mediators.

There is a growing body of evidence that, in addition to endothelial cells and leukocytes, cardiomyocytes can also contribute to the expression of inflammatory mediators. Activation of Toll-like receptors (TLRs) in cardiomyocytes, including TLR-4, leads to the expression of IL-6, KC and MIP-247. Importantly, TLR4 signaling has been shown to play a role in MI and inflammation after I/R injury48. Our preliminary data indicate that activation of PAR-2 in cultured neonatal mouse cardiomyocytes also results in the expression of IL-6 and KC but not TNFα or MIP-2 protein (S. Antoniak, N. Mackman and R. Pawlinski - unpublished data). Therefore, during I/R injury, activation of PAR-2 on cardiomyocytes may contribute to the expression of IL-6 and KC. Interestingly, it has been demonstrated that PAR-2-mediated expression of IL-1β was significantly attenuated in TLR4 deficient macrophages49. Furthermore, the TLR/IL-1 receptor-like domain of TLR4 has been found to interact with the cytoplasmic C-terminus of PAR-250. A mutation of this domain abolished PAR-2 dependent activation of NF-κB in macrophages50. Further studies are required to determine if TLR4 and

PAR-2 cooperate in a similar manner in cell types within the injured heart to enhance inflammation.

We also demonstrated that PAR-2 deficiency reduced heart remodeling and heart dysfunction up to 4 weeks after injury. This is may be due to a smaller initial infarct size in the hearts of PAR-2$^{-/-}$ mice. It is known that the size of the initial infarct affects the extent of heart remodeling after MI[17, 18]. In addition, activation of PAR-2 has been shown to induce hypertrophic growth of cardiomyocytes *in vitro*[6]. Since hypertrophic growth is a major component of post-MI remodeling, it is possible that PAR-2 may also contribute to heart remodeling by increasing the hypertrophic growth of cardiomyocytes.

In contrast to the reduced infarct size observed in PAR-2$^{-/-}$ mice, we have previously demonstrated that deficiency of PAR-1 has no effect on infarct size in the same short-term model of cardiac I/R injury24. This indicates that there are differences between PAR-1 and PAR-2 dependent signaling in MI. In the present study, we showed that short-term cardiac I/R injury led to the significant increase of PAR-2 but not PAR-1 mRNA expression. This may explain, in part, the different effects of PAR-1 and PAR-2 deficiency on MI. Inducible expression of PAR-2 is likely to play pathologic role in the inflammatory response during I/R injury. We also showed that PAR-1 and PAR-2 mRNA expression in the heart is significantly increased in long-term model of I/R injury, as well as in heart samples from patients who developed ischemic heart failure. It is uncertain if the increased expression of these two genes contributes to the development of heart failure or is just an effect of heart remodeling. Our previous data indicate that increase PAR-1 signaling in cardiomyocytes leads to the development of eccentric heart hypertrophy and reduction of heart function[24]. We also found that cardiomyocyte-specific overexpression of PAR-2 led to heart hypertrophy (S. Antoniak, N. Mackman and R. Pawlinski – unpublished data). These data, together with the observation that not only PAR-2 deficiency, but also PAR-1 deficiency significantly reduced long-term heart remodeling[24], suggest that both PAR-1 and PAR-2 may contribute to ischemic heart failure in humans.

Our study raises the question of what protease(s) activate PAR-2 during cardiac I/R injury. We and others have previously demonstrated that tissue factor (TF), the primary initiator of coagulation cascade, contributes to MI in rabbit and mouse models of cardiac I/R injury[51–53]. Subsequently, it has been shown that TF-dependent thrombin generation and fibrin deposition enhances MI24, 54. Importantly, the TF:FVIIa complex can activate PAR-2, which is one possible mechanism of PAR-2 activation during I/R injury[2, 3]. Further studies are required to determine if TF:FVIIa and/or FXa activate PAR-2 in injured hearts. In addition, PAR-2 can be activated by other proteases, such as mast cell tryptase[5]. Mast cells have been found between muscle fibers in the heart and mast cell deficient mice exhibit reduced infarct size and inflammation after cardiac I/R injury[55, 56]. This may be due, in part, to a reduction in PAR-2 activation.

In contrast to our results, several recent publications have reported that activation of PAR-2 with a synthetic PAR-2 agonist peptide has a beneficial effect in both *ex vivo* and *in vivo* rat models of cardiac I/R injury[20–22]. How can administration of PAR-2 agonist peptide be protective whereas a global deficiency of PAR-2 results in a smaller infarct? It has been demonstrated that depending on the disease model activation of PAR-2 may have either protective or detrimental effects. Furthermore, recent studies in mouse model of Alzheimer's disease suggest that PAR-2-dependent signaling may have opposite effects in different cell types within the brain[16]. It is possible that a similar scenario may occur in the heart after I/R injury. In the short-term I/R injury model activation of PAR-2 on endothelial cells may be protective, whereas PAR-2 signaling on other cell types, like cardiomyocytes or infiltrating leukocytes may lead to the detrimental effects. Another possible explanation for these different

results came from a recent paper demonstrating that PAR-2 is differentially activated by tethered ligands versus soluble ligands, such as an agonist peptide[57]. These two types of ligands differentially bind and stabilized different conformations of the receptor resulting in activation of distinct subsets of signaling cascades[57]. A better understanding of the cell type-specific responses of PAR-2 during cardiac I/R injury is needed. Interestingly, PAR-2 deficiency and treatment with PAR-2 agonist peptide had beneficial effects not only in heart I/R injury but also in the mouse model of colitis induced by intrarectal injection of trinitrobenzene sulfonic acid[58, 59].

In summary, we demonstrated that PAR-2 deficiency reduces inflammation, myocardial infarction and cardiac remodeling after I/R injury. Further studies are required to determine the effect of inhibition of PAR-2 in WT mice before considering PAR-2 as a potential target for therapy to prevent myocardial damage.

Acknowledgments

We thank Christine Rothnie for excellent technical assistance and Patricia Andrade-Gordon for providing PAR-2 deficient mice.

Sources of funding:

This study was supported by grants from the American Heart Association (S.A. and R.P.) and the NIH (N.M., B.C.B.).

References

1. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000;407:258–264. [PubMed: 11001069]
2. Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. Proc Natl Acad Sci U S A 2001;98:7742–7747. [PubMed: 11438726]
3. Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. Proc Natl Acad Sci U S A 2000;97:5255–5260. [PubMed: 10805786]
4. Camerer E, Barker A, Duong DN, Ganesan R, Kataoka H, Cornelissen I, Darragh MR, Hussain A, Zheng YW, Srinivasan Y, Brown C, Xu SM, Regard JB, Lin CY, Craik CS, Kirchhofer D, Coughlin SR. Local protease signaling contributes to neural tube closure in the mouse embryo. Dev Cell 2010;18:25–38. [PubMed: 20152175]
5. Steinberg SF. The cardiovascular actions of protease-activated receptors. Mol Pharmacol 2005;67:2–11. [PubMed: 15371558]
6. Sabri A, Muske G, Zhang H, Pak E, Darrow A, Andrade-Gordon P, Steinberg SF. Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. Circ Res 2000;86:1054–1061. [PubMed: 10827135]
7. Lindner JR, Kahn ML, Coughlin SR, Sambrano GR, Schauble E, Bernstein D, Foy D, Hafezi-Moghadam A, Ley K. Delayed onset of inflammation in protease-activated receptor-2-deficient mice. J Immunol 2000;165:6504–6510. [PubMed: 11086091]
8. Ferrell WR, Lockhart JC, Kelso EB, Dunning L, Plevin R, Meek SE, Smith AJ, Hunter GD, McLean JS, McGarry F, Ramage R, Jiang L, Kanke T, Kawagoe J. Essential role for proteinase-activated receptor-2 in arthritis. J Clin Invest 2003;111:35–41. [PubMed: 12511586]
9. Vergnolle N, Bunnett NW, Sharkey KA, Brussee V, Compton SJ, Grady EF, Cirino G, Gerard N, Basbaum AI, Andrade-Gordon P, Hollenberg MD, Wallace JL. Proteinase-activated receptor-2 and hyperalgesia: A novel pain pathway. Nat Med 2001;7:821–826. [PubMed: 11433347]
10. Cenac N, Andrews CN, Holzhausen M, Chapman K, Cottrell G, Andrade-Gordon P, Steinhoff M, Barbara G, Beck P, Bunnett NW, Sharkey KA, Ferraz JG, Shaffer E, Vergnolle N. Role for protease activity in visceral pain in irritable bowel syndrome. J Clin Invest 2007;117:636–647. [PubMed: 17304351]

11. Schmidlin F, Amadesi S, Dabbagh K, Lewis DE, Knott P, Bunnett NW, Gater PR, Geppetti P, Bertrand C, Stevens ME. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J Immunol 2002;169:5315–5321. [PubMed: 12391252]
12. Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, Luger TA, Schmelz M. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 2003;23:6176–6180. [PubMed: 12867500]
13. Cocks TM, Fong B, Chow JM, Anderson GP, Frauman AG, Goldie RG, Henry PJ, Carr MJ, Hamilton JR, Moffatt JD. A protective role for protease-activated receptors in the airways. Nature 1999;398:156–160. [PubMed: 10086357]
14. Jin G, Hayashi T, Kawagoe J, Takizawa T, Nagata T, Nagano I, Syoji M, Abe K. Deficiency of PAR-2 gene increases acute focal ischemic brain injury. J Cereb Blood Flow Metab 2005;25:302–313. [PubMed: 15647743]
15. Khoufache K, LeBouder F, Morello E, Laurent F, Riffault S, Andrade-Gordon P, Boullier S, Rousset P, Vergnolle N, Riteau B. Protective role for protease-activated receptor-2 against influenza virus pathogenesis via an IFN-gamma-dependent pathway. J Immunol 2009;182:7795–7802. [PubMed: 19494303]
16. Afkhami-Goli A, Noorbakhsh F, Keller AJ, Vergnolle N, Westaway D, Jhamandas JH, Andrade-Gordon P, Hollenberg MD, Arab H, Dyck RH, Power C. Proteinase-activated receptor-2 exerts protective and pathogenic cell type-specific effects in Alzheimer's disease. J Immunol 2007;179:5493–5503. [PubMed: 17911636]
17. White HD, Chew DP. Acute myocardial infarction. Lancet 2008;372:570–584. [PubMed: 18707987]
18. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? J Clin Invest 1985;76:1713–1719. [PubMed: 4056048]
19. Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/Factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. J Clin Invest. 2008
20. Napoli C, Cicala C, Wallace JL, de Nigris F, Santagada V, Caliendo G, Franconi F, Ignarro LJ, Cirino G. Protease-activated receptor-2 modulates myocardial ischemiareperfusion injury in the rat heart. Proc Natl Acad Sci U S A 2000;97:3678–3683. [PubMed: 10737808]
21. McLean PG, Aston D, Sarkar D, Ahluwalia A. Protease-activated receptor-2 activation causes EDHF-like coronary vasodilation: selective preservation in ischemia/reperfusion injury: involvement of lipoxygenase products, VR1 receptors, and C-fibers. Circ Res 2002;90:465–472. [PubMed: 11884377]
22. Jiang R, Zatta A, Kin H, Wang N, Reeves JG, Mykytenko J, Deneve J, Zhao ZQ, Guyton RA, Vinten-Johansen J. PAR-2 activation at the time of reperfusion salvages myocardium via an ERK1/2 pathway in in vivo rat hearts. Am J Physiol Heart Circ Physiol 2007;293:H2845–H2852. [PubMed: 17720772]
23. Damiano BP, Cheung WM, Santulli RJ, Fung-Leung WP, Ngo K, Ye RD, Darrow AL, Derian CK, de Garavilla L, Andrade-Gordon P. Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. J Pharmacol Exp Ther 1999;288:671–678. [PubMed: 9918574]
24. Pawlinski R, Tencati M, Hampton CR, Shishido T, Bullard TA, Casey LM, Andrade-Gordon P, Kotzsch M, Spring D, Luther T, Abe J, Pohlman TH, Verrier ED, Blaxall BC, Mackman N. Protease-activated receptor-1 contributes to cardiac remodeling and hypertrophy. Circulation 2007;116:2298–2306. [PubMed: 17967980]
25. Liu L, Tsai JC, Aird WC. Egr-1 gene is induced by the systemic administration of the vascular endothelial growth factor and the epidermal growth factor. Blood 2000;96:1772–1781. [PubMed: 10961876]
26. Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ, Ma XL. Adiponectin cardioprotection after myocardial ischemia/reperfusion involves the reduction of oxidative/nitrative stress. Circulation 2007;115:1408–1416. [PubMed: 17339545]
27. Ji L, Fu F, Zhang L, Liu W, Cai X, Zheng Q, Zhang H, Gao F. Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress. Am J Physiol Endocrinol Metab 2010;298:E871–E880. [PubMed: 20124508]

28. Iliodromitis EK, Andreadou I, Prokovas E, Zoga A, Farmakis D, Fotopoulou T, Ioannidis K, Paraskevaidis IA, Kremastinos DT. Simvastatin in contrast to postconditioning reduces infarct size in hyperlipidemic rabbits: possible role of oxidative/nitrosative stress attenuation. Basic Res Cardiol 2010;105:193–203. [PubMed: 20066537]
29. Clerk A, Sugden PH. Inflame my heart (by p38-MAPK). Circ Res 2006;99:455–458. [PubMed: 16946139]
30. Ferrandi C, Ballerio R, Gaillard P, Giachetti C, Carboni S, Vitte PA, Gotteland JP, Cirillo R. Inhibition of c-Jun N-terminal kinase decreases cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats. Br J Pharmacol 2004;142:953–960. [PubMed: 15210584]
31. Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. Br J Pharmacol 2003;138:532–543. [PubMed: 12598407]
32. Liu B, Tewari AK, Zhang L, Green-Church KB, Zweier JL, Chen YR, He G. Proteomic analysis of protein tyrosine nitration after ischemia reperfusion injury: mitochondria as the major target. Biochim Biophys Acta 2009;1794:476–485. [PubMed: 19150419]
33. Park GH, Jeon SJ, Ryu JR, Choi MS, Han SH, Yang SI, Ryu JH, Cheong JH, Shin CY, Ko KH. Essential role of mitogen-activated protein kinase pathways in protease activated receptor 2-mediated nitric-oxide production from rat primary astrocytes. Nitric Oxide 2009;21:110–119. [PubMed: 19527794]
34. Lim SY, Tennant GM, Kennedy S, Wainwright CL, Kane KA. Activation of mouse protease-activated receptor-2 induces lymphocyte adhesion and generation of reactive oxygen species. Br J Pharmacol 2006;149:591–599. [PubMed: 16981001]
35. McCubrey JA, Lahair MM, Franklin RA. Reactive oxygen species-induced activation of the MAP kinase signaling pathways. Antioxid Redox Signal 2006;8:1775–1789. [PubMed: 16987031]
36. Syeda F, Grosjean J, Houliston RA, Keogh RJ, Carter TD, Paleolog E, Wheeler-Jones CP. Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways. J Biol Chem 2006;281:11792–11804. [PubMed: 16467309]
37. Kaiser RA, Bueno OF, Lips DJ, Doevendans PA, Jones F, Kimball TF, Molkentin JD. Targeted inhibition of p38 mitogen-activated protein kinase antagonizes cardiac injury and cell death following ischemia-reperfusion in vivo. J Biol Chem 2004;279:15524–15530. [PubMed: 14749328]
38. Hu C, Chen J, Dandapat A, Fujita Y, Inoue N, Kawase Y, Jishage K, Suzuki H, Li D, Hermonat PL, Sawamura T, Mehta JL. LOX-1 abrogation reduces myocardial ischemia-reperfusion injury in mice. J Mol Cell Cardiol 2008;44:76–83. [PubMed: 18022184]
39. Niu QX, Chen HQ, Chen ZY, Fu YL, Lin JL, He SH. Induction of inflammatory cytokine release from human umbilical vein endothelial cells by agonists of proteinase-activated receptor-2. Clin Exp Pharmacol Physiol 2008;35:89–96. [PubMed: 18047634]
40. Shpacovitch VM, Brzoska T, Buddenkotte J, Stroh C, Sommerhoff CP, Ansel JC, Schulze-Osthoff K, Bunnett NW, Luger TA, Steinhoff M. Agonists of proteinase-activated receptor 2 induce cytokine release and activation of nuclear transcription factor kappaB in human dermal microvascular endothelial cells. J Invest Dermatol 2002;118:380–385. [PubMed: 11841560]
41. Birdsall HH, Green DM, Trial J, Youker KA, Burns AR, MacKay CR, LaRosa GJ, Hawkins HK, Smith CW, Michael LH, Entman ML, Rossen RD. Complement C5a, TGF-beta 1, and MCP-1, in sequence, induce migration of monocytes into ischemic canine myocardium within the first one to five hours after reperfusion. Circulation 1997;95:684–692. [PubMed: 9024158]
42. Johansson U, Lawson C, Dabare M, Syndercombe-Court D, Newland AC, Howells GL, Macey MG. Human peripheral blood monocytes express protease receptor-2 and respond to receptor activation by production of IL-6, IL-8, and IL-1{beta}. J Leukoc Biol 2005;78:967–975. [PubMed: 16000389]
43. Zoudilova M, Min J, Richards HL, Carter D, Huang T, DeFea KA. beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. J Biol Chem 2010;285:14318–14329. [PubMed: 20207744]
44. Xing Z, Kirpalani H, Torry D, Jordana M, Gauldie J. Polymorphonuclear leukocytes as a significant source of tumor necrosis factor-alpha in endotoxin-challenged lung tissue. Am J Pathol 1993;143:1009–1015. [PubMed: 8213996]

45. Feiken E, Romer J, Eriksen J, Lund LR. Neutrophils express tumor necrosis factoralpha during mouse skin wound healing. J Invest Dermatol 1995;105:120–123. [PubMed: 7615965]
46. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T, Ziegler-Heitbrock L. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J Immunol 2002;168:3536–3542. [PubMed: 11907116]
47. Boyd JH, Mathur S, Wang Y, Bateman RM, Walley KR. Toll-like receptor stimulation in cardiomyoctes decreases contractility and initiates an NF-kappaB dependent inflammatory response. Cardiovasc Res 2006;72:384–393. [PubMed: 17054926]
48. Chong AJ, Shimamoto A, Hampton CR, Takayama H, Spring DJ, Rothnie CL, Yada M, Pohlman TH, Verrier ED. Toll-like receptor 4 mediates ischemia/reperfusion injury of the heart. J Thorac Cardiovasc Surg 2004;128:170–179. [PubMed: 15282452]
49. Rallabhandi P, Nhu QM, Toshchakov VY, Piao W, Medvedev AE, Hollenberg MD, Fasano A, Vogel SN. Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. J Biol Chem 2008;283:24314–24325. [PubMed: 18622013]
50. Ramachandran R, Hollenberg MD. Proteinases and signalling: pathophysiological and therapeutic implications via PARs and more. Br J Pharmacol 2008;153 Suppl 1:S263–S282. [PubMed: 18059329]
51. Erlich JH, Boyle EM, Labriola J, Kovacich JC, Santucci RA, Fearns C, Morgan EN, Yun W, Luther T, Kojikawa O, Martin TR, Pohlman TH, Verrier ED, Mackman N. Inhibition of the tissue factor-thrombin pathway limits infarct size after myocardial ischemia-reperfusion injury by reducing inflammation. Am J Pathol 2000;157:1849–1862. [PubMed: 11106558]
52. Golino P, Ragni M, Cirillo P, Scognamiglio A, Ravera A, Buono C, Guarino A, Piro O, Lambiase C, Botticella F, Ezban M, Condorelli M, Chiariello M. Recombinant human, active site-blocked factor VIIa reduces infarct size and no-reflow phenomenon in rabbits. Am J Physiol Heart Circ Physiol 2000;278:H1507–H1516. [PubMed: 10775128]
53. Loubele ST, Spek CA, Leenders P, van Oerle R, Aberson HL, van der Voort D, Hamulyak K, Petersen LC, Spronk HM, ten Cate H. Active site inhibited factor VIIa attenuates myocardial ischemia/reperfusion injury in mice. J Thromb Haemost 2009;7:290–298. [PubMed: 19036066]
54. Petzelbauer P, Zacharowski PA, Miyazaki Y, Friedl P, Wickenhauser G, Castellino FJ, Groger M, Wolff K, Zacharowski K. The fibrin-derived peptide Bbeta15–42 protects the myocardium against ischemia-reperfusion injury. Nat Med 2005;11:298–304. [PubMed: 15723073]
55. Patella V, Marino I, Arbustini E, Lamparter-Schummert B, Verga L, Adt M, Marone G. Stem cell factor in mast cells and increased mast cell density in idiopathic and ischemic cardiomyopathy. Circulation 1998;97:971–978. [PubMed: 9529265]
56. Bhattacharya K, Farwell K, Huang M, Kempuraj D, Donelan J, Papaliodis D, Vasiadi M, Theoharides TC. Mast cell deficient W/Wv mice have lower serum IL-6 and less cardiac tissue necrosis than their normal littermates following myocardial ischemia-reperfusion. Int J Immunopathol Pharmacol 2007;20:69–74. [PubMed: 17346429]
57. Ramachandran R, Mihara K, Mathur M, Rochdi MD, Bouvier M, Defea K, Hollenberg MD. Agonist-biased signaling via proteinase activated receptor-2: differential activation of calcium and mitogen-activated protein kinase pathways. Mol Pharmacol 2009;76:791–801. [PubMed: 19605524]
58. Hyun E, Andrade-Gordon P, Steinhoff M, Vergnolle N. Protease-activated receptor-2 activation: a major actor in intestinal inflammation. Gut 2008;57:1222–1229. [PubMed: 18460552]
59. Fiorucci S, Mencarelli A, Palazzetti B, Distrutti E, Vergnolle N, Hollenberg MD, Wallace JL, Morelli A, Cirino G. Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. Proc Natl Acad Sci U S A 2001;98:13936–13941. [PubMed: 11717450]

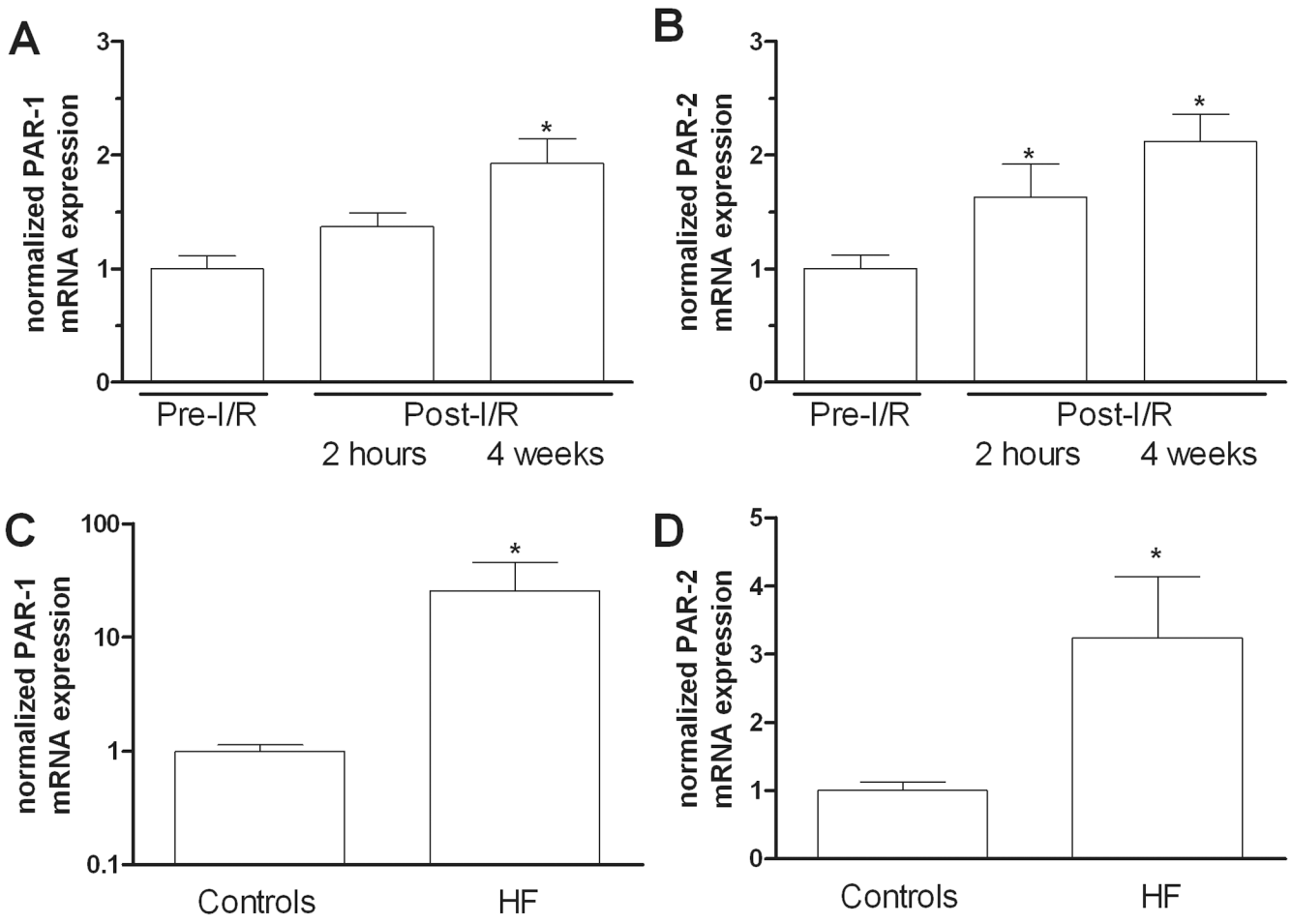

Figure 1. PAR-1 and PAR-2 mRNA expression in injured mice and humans hearts

Murine PAR-1 **(A)** and PAR-2 **(B)** mRNA expression was analyzed using real-time PCR in C57BL/6 WT mice hearts before (n=8) and after 30 minutes of ischemia and 2 hours (n=5) or 4 weeks (n=7) of reperfusion. Human PAR-1 **(C)** and PAR-2 **(D)** mRNA expression was analyzed by real-time PCR in human heart samples from patients with ischemic HF (n=5) and compared to mRNA expression in healthy controls n=5). * $P<0.05$ *vs.* un-injured or healthy controls

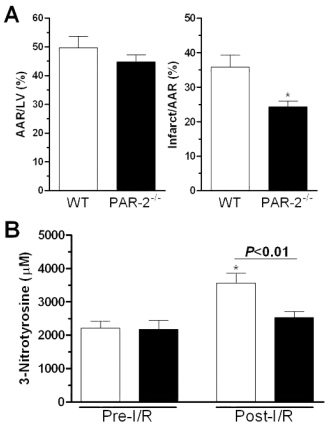

Figure 2. PAR-2 deficiency reduces myocardial infarction and oxidative/nitrative stress after short term I/R injury

A: Area at the risk (AAR), determined as a percentage of LV area and infarct size, determined as a percentage of AAR in WT (white bars, n=7) and PAR-$2^{-/-}$ (black bars, n=13) mice subjected to 30 minutes of ischemia and 2 hours of reperfusion. * $P<0.05$ *vs.* injured WT mice. **B:** Levels of 3-nitrotyrosine in heart lysates of control mice (n=4 for WT and PAR-$2^{-/-}$) and mice subjected to 30 minutes of ischemia and 2 hours of reperfusion (n=8 for WT (white bars) and n=8 for PAR-$2^{-/-}$ (black bars)). * $P<0.05$ *vs.* pre-I/R injury group within the same genotype.

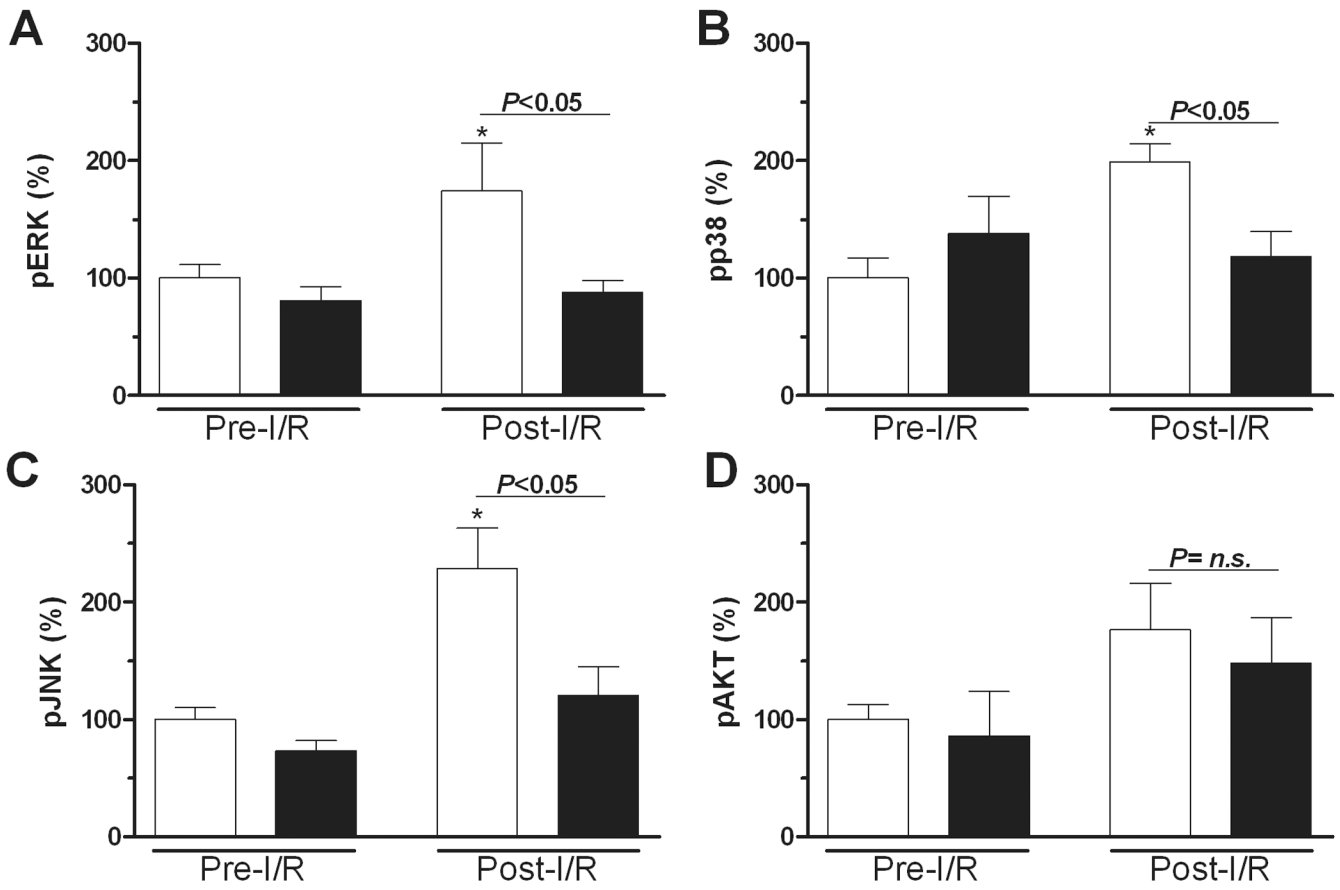

Figure 3. PAR-2 deficiency results in reduced intracellular signaling in heart after I/R injury

Activation of MAPK (**A:** ERK1/2; **B:** p38; **C:** JNK) and AKT **(D)** were analyzed in heart lysates of control mice (n=4 for WT and PAR-2$^{-/-}$) and mice subjected to 30 minutes of ischemia and 2 hours of reperfusion (n=8 for WT (white bars) and n=8 for PAR-2$^{-/-}$ (black bars)). The phosphorylation state of the non-injured WT littermates was set to 100%. * *P*<0.05 *vs.* pre-I/R injury group within the same genotype.

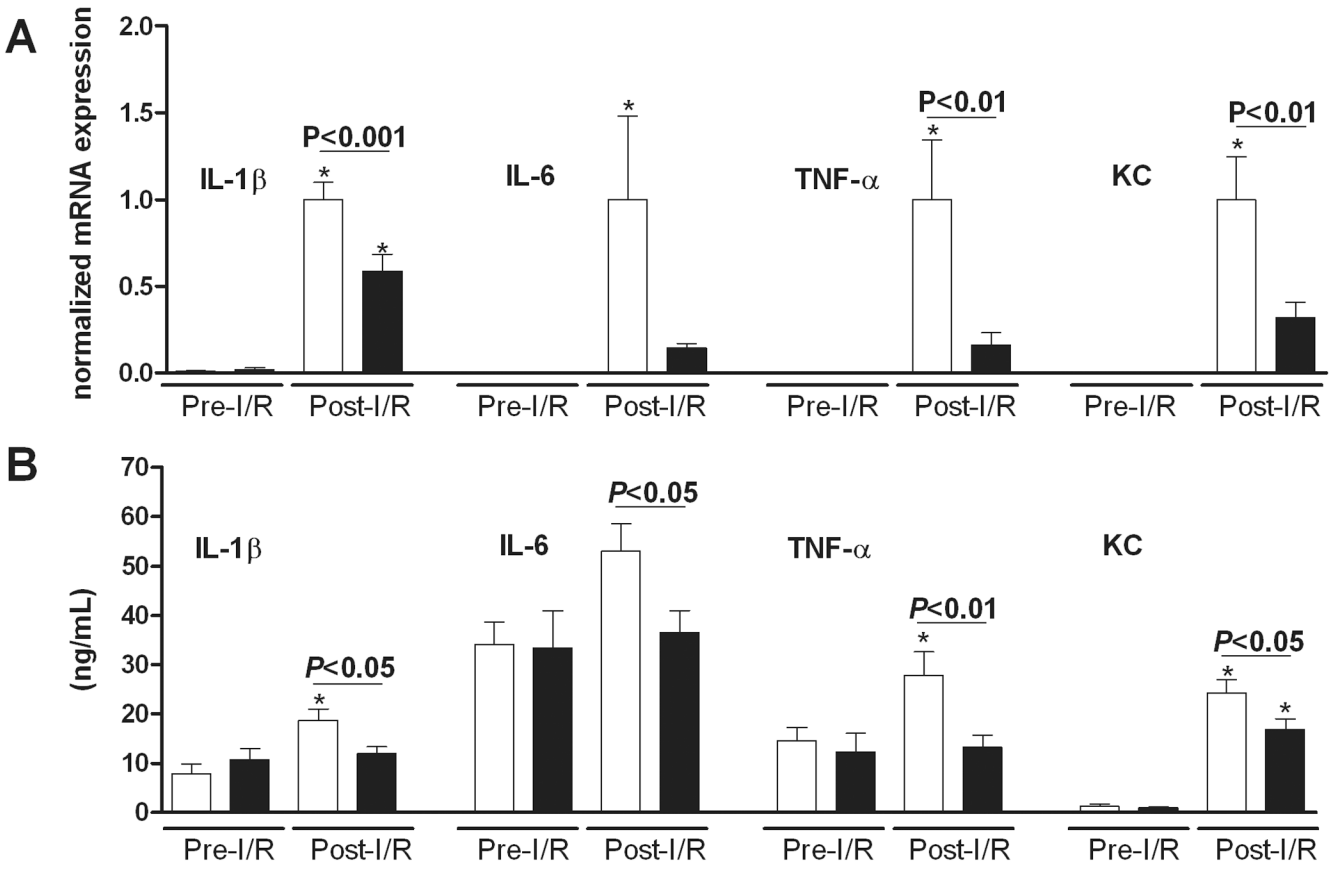

Figure 4. PAR-2 deficiency reduces myocardial inflammation after I/R injury

A: Levels of pro-inflammatory cytokine and chemokine mRNAs in the hearts of control mice (n=4 for WT and PAR-$2^{-/-}$) and mice subjected to 30 minutes of ischemia and 2 hours of reperfusion (n=10 for WT (white bars) and n=8 for PAR-$2^{-/-}$ (black bars)). mRNA expression in the injured WT hearts were set to 1. **B:** Protein levels of inflammatory mediators IL-1β, IL-6, TNF-α and KC in hearts before and after short-term I/R injury (n=8 for WT and n=8 for PAR-$2^{-/-}$). * $P<0.05$ *vs.* pre-I/R injury group within the same genotype.

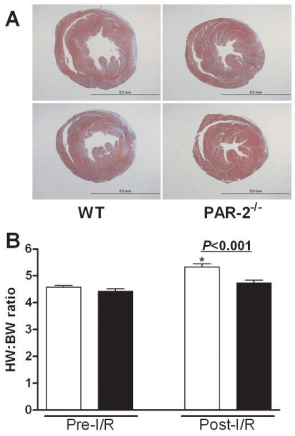

Figure 5. Effects of a PAR-2 deficiency on heart remodeling 4 weeks after cardiac I/R injury

A: Representative cross-sections from WT and PAR-2$^{-/-}$ hearts 4 weeks after cardiac I/R injury cut at the level of papillary muscles and stained with Masson's Trichrome. The scar tissue is stained blue. **B:** HW:BW ratio of non-injured controls (n=4 each group) and injured WT (white bars, n=7) and PAR-2$^{-/-}$ (black bars, n=6) mice. * $P<0.05$ *vs.* pre-I/R injury group within the same genotype.

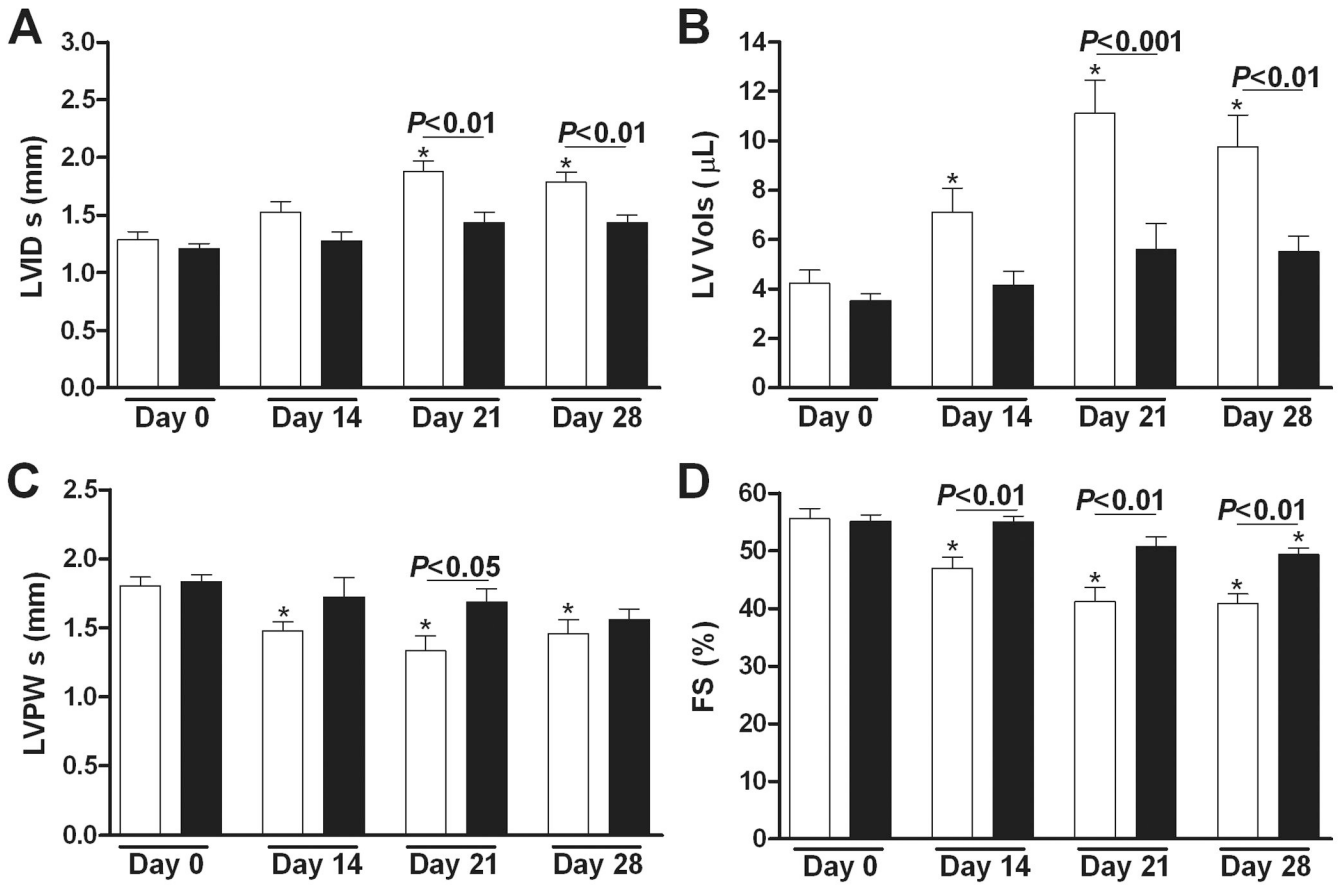

Figure 6. Echocardiographic analysis of heart size and function

A: Systolic left ventricular internal diameter (LVID s), **B:** systolic left ventricular volume (LV Vols), **C:** thickness of LV posterior wall at the end of systole (LVPW) and **D:** Percentage of fractional shortening (FS) was analyzed by echocardiography before and up to 4 weeks after cardiac I/R injury in WT (n=7) and PAR-$2^{-/-}$ (n=6) mice. * $P<0.05$ *vs.* pre-I/R injury group within the same genotype.