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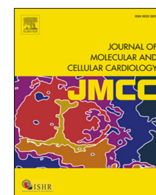

Protease-activated receptor 4 deficiency offers cardioprotection after acute ischemia reperfusion injury

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ABSTRACT

Protease-activated receptor (PAR)4 is a low affinity thrombin receptor with less understood function relative to PAR1. PAR4 is involved in platelet activation and hemostasis, but its specific actions on myocyte growth and cardiac function remain unknown. This study examined the role of PAR4 deficiency on cardioprotection after myocardial ischemia–reperfusion (IR) injury in mice. When challenged by in vivo or ex vivo IR, PAR4 knockout (KO) mice exhibited increased tolerance to injury, which was manifest as reduced infarct size and a more robust functional recovery compared to wild-type mice. PAR4 KO mice also showed reduced cardiomyocyte apoptosis and putative signaling shifts in survival pathways in response to IR. Inhibition of PAR4 expression in isolated cardiomyocytes by shRNA offered protection against thrombin and PAR4-agonist peptide-induced apoptosis, while overexpression of wild-type PAR4 significantly enhanced the susceptibility of cardiomyocytes to apoptosis, even under low thrombin concentrations. Further studies implicate Src- and epidermal growth factor receptor-dependent activation of JNK on the proapoptotic effect of PAR4 in cardiomyocytes. These findings reveal a pivotal role for PAR4 as a regulator of cardiomyocyte survival and point to PAR4 inhibition as a therapeutic target offering cardioprotection after acute IR injury.

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1. Introduction

Restoration of blood flow after acute myocardial infarction limits infarct size and reduces mortality. However, reestablishing blood flow is often followed by a second set of stresses, a phenomenon referred to as ischemia–reperfusion (IR) injury, which can result in additional myocardial damage and account for up to half of total infarct size [1]. The factors contributing to IR injury are complex and include microvascular obstruction, inflammation, release of oxygen radicals, myocardial stunning, and activation of mitochondrial apoptosis and necrosis [1,2]. Extensive research has explored the mechanisms responsible for the activation of inflammatory-derived cytokines/chemokines and reactive oxygen species and their roles in the infarcted heart. However, there is a paucity of information regarding the role of inflammatory serine proteases on cardiac injury post-IR [3]. These proteases not only cleave extracellular substrates, but also mediate cell signal transduction, in part, through protease-activated receptors (PARs) [4,5].

PARs are a family of seven transmembrane spanning domain G protein-coupled receptors that are activated by cleavage of the receptor N-terminal domain thereby exposing a new, previously cryptic sequence [6,7]. The exposed sequence remains tethered to the receptor and further acts as a receptor-activating tethered ligand. Four members of PARs have been characterized, PAR1, PAR3 and PAR4 are activated by thrombin, whereas PAR2 is activated by other proteases such as trypsin and tryptase, but not by thrombin [6,7]. PAR1 is the prototypical receptor to which most of the cellular and platelet actions of thrombin are attributed, and its role in fibroblast proliferation and cardiac remodeling has been well established [7–10]. In contrast, knowledge of PAR4 expression and function in the heart is still quite limited. PAR4 is generally recognized as a low affinity thrombin receptor [6,7], although it can also be activated by other proteases including trypsin [11], tissue kallikrein [12], and the neutrophilic granule protease cathepsin G (Cat.G) [13]. Synthetic peptides based on the receptor-activating sequence of the

Abbreviations: AAR, area at risk; AP, agonist peptide; Cat.G, cathepsin G; cTnI, cardiac troponin I; EGFR, epidermal growth factor receptor; IA, infarct area; IR, ischemia–reperfusion; KO, knockout; LAD, left anterior descending; LV, left ventricular; MPO, myeloperoxidase; NRCMs, neonatal rat cardiac myocytes; PAR, protease-activated receptor; TTC, triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; WT, wild-type.

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tethered ligand (e.g., GYPGKF for murine PAR4) are also capable of activating PAR4 by direct binding to the receptor [6,7]. PAR4-deficient mice show a normal phenotype, but hemostasis is impaired due to defective platelet thrombin signaling [14,15]. On the other hand, when administered locally at high doses, PAR4 agonists produce inflammation [16, 17]. Although these results suggest that the predominant role of PAR4 is in platelet activation [7,9], these receptors are also expressed in cardiomyocytes [18] with their contribution to myocyte growth and function remaining largely unknown.

We previously showed that PAR4 stimulation is slower in onset but more prolonged in cardiac myocytes relative to PAR1 [18]. Moreover, pharmacological inhibition of PAR4 using the pepducin antagonist of PAR4, P4pal 10, or the trans-cinnamyol-YPGKF-amide peptide have suggested that antagonism of this receptor is protective in response to 2 h of myocardial ischemia reperfusion injury [19]. However, the interpretation of data using antagonist peptides is potentially confounded by limitations of the bioavailability of these agents in vivo as well as the observation that both behave as agonists in some in vitro model systems [16]. The specific functions of PAR4 on cardiac function are still not well understood and no loss-of-function cardiac phenotypes of PAR4 gene have yet been described. In the present study, we show that PAR4-deficient mice display normal cardiac function but present reduced inflammation, less myocyte death and improved cardiac function in response to acute IR injury. Reciprocally, activation of PAR4 leads to myocyte apoptosis through activation of JNK signaling pathway. Our results suggest that inhibition of PAR4 could be a potential therapeutic target to protect the heart from acute IR injury.

2. Methods

2.1. Myocardial IR injury procedure

All mice were maintained in accordance with protocols approved by the Animal Care and Use Committee of Temple University. PAR4 knockout (KO) mice were generated and genotyped as described previously [15]. Twelve week-old male wild-type (WT) and PAR4 KO mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) to perform a left thoracotomy under mechanical ventilation. Body temperature was maintained by a heated surgical platform and was monitored throughout surgery using a rectal sensor. A 6-0 suture with a slipknot was tied around the left anterior descending (LAD) coronary artery to produce ischemia. Consistent elevation of the ST segment was observed in lead II tracings following occlusion of the LAD coronary vessel. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Nikon) by discoloration of the occluded distal myocardium. The ligation was released after 30 min of ischemia and the heart was allowed to reperfuse as confirmed by visual inspection. The chest wall was closed with 8–0 silk and then the animal was removed from the ventilator and kept warm in the cage maintained at 37 °C overnight. A sham procedure constituted the surgical incision without LAD ligation. Hearts were harvested after 24 h of reperfusion.

2.2. Data analysis

Data are expressed as the mean ± SEM. We used a 2-tailed, unpaired Student's t test for all pair-wise comparisons (GraphPad Prism version 5). P values of less than 0.05 were considered significant. All in vitro experiments were performed at least three times from three different cultures and the data values were scaled to controls. A value of $P < 0.05$ was considered statistically significant.

An Expanded Methods section is provided in the Supplemental material.

3. Results

3.1. PAR4 expression is upregulated after acute myocardial IR

PAR4 expression was investigated in WT hearts after IR injury. In the absence of IR, PAR4 immunostaining was weak and was homogenously distributed throughout the myocardium (Fig. 1A). At 24 h after IR injury, PAR4 staining was strongly evidenced in inflammatory cells which occupied most of the damaged area, and in surviving cardiac muscle fibers

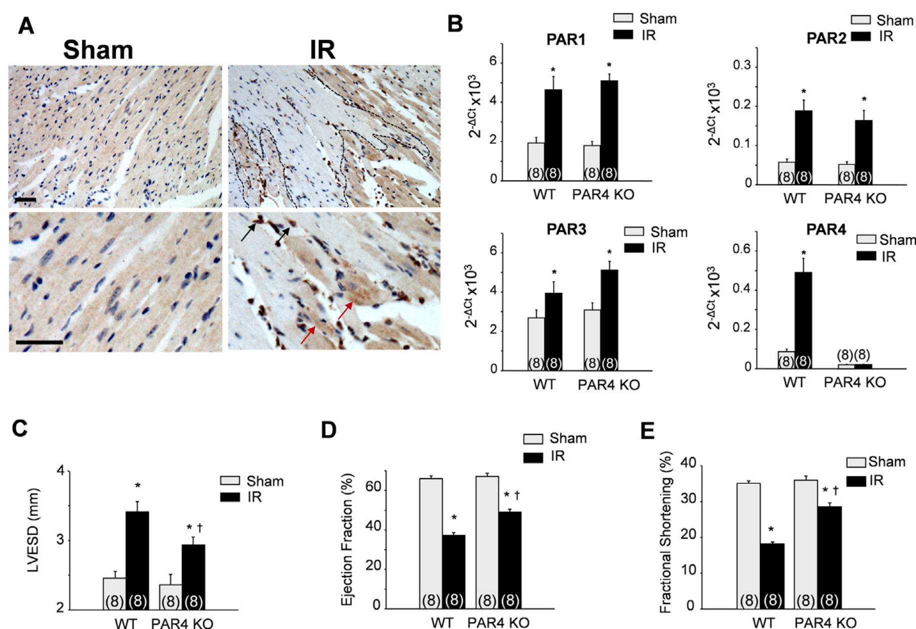

Fig. 1. PAR4 ablation protects against acute IR. (A–E) The left anterior descending artery was ligated for 30 min to induce ischemia and the heart was subsequently reperfused for 24 h (IR). (A) Representative immunostaining of paraffin-embedded heart sections of wild-type (WT) sham or after IR injury stained for PAR4 and counterstained with hematoxylin. PAR4 staining was detected in infiltrating inflammatory cells (black arrows) and in surviving cardiomyocytes (red arrows). Scale bar: 40 μm. (B) PARs mRNA levels in the hearts of WT and PAR4 KO as assessed by real-time qPCR. Data were normalized to GAPDH and were expressed relative to levels in WT sham hearts. (C–E) Echocardiography measurement of left ventricular (LV)-end systolic dimension (C), LV ejection fraction (D), and LV fractional shortening (E) in WT and PAR4 KO animals. Values are presented as mean ± SEM, $^*P < 0.05$ vs. WT shams, $^†P < 0.05$ vs. WT IR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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