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Protease-activated Receptors and Myocardial Infarction

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Abstract

Protease activated receptors (PARs) are widely expressed within the heart. They are activated by a myriad of proteases, including coagulation proteases. *In vitro* studies showed that activation of PAR-1 and PAR-2 on cardiomyocytes induced hypertrophy. In addition, PAR-1 stimulation on cardiac fibroblasts induced proliferation. Genetic and pharmacologic approaches have been used to investigate the role of the different PARs in cardiac ischemia/reperfusion (I/R) injury. In mice and rats PAR-1 is reported to play a role in inflammation, infarct size and remodeling after cardiac I/R injury. However, there are notable differences between the effect of a deficiency in PAR-1 and inhibition of PAR-1. For instance, inhibition of PAR-1 reduced infarct size whereas there was no effect of a deficiency of PAR-1. These differences maybe due to off-target effects of the inhibitor or PAR-4 compensation of PAR-1 deficiency. Similarly, a deficiency of PAR-2 was associated with reduced cardiac inflammation and improved heart function after I/R injury, whereas pharmacologic activation of PAR-2 was found to be protective due to increased vasodilatation. These differences maybe due to different signaling responses induced by an endogenous proteases versus an exogenous agonist peptide. Surprisingly, PAR-4 deficiency resulted in increased cardiac injury and increased mortality after I/R injury. In contrast, a pharmacological study indicated that inhibition of PAR-4 was cardioprotective. It is possible that the major cellular target of the PAR-4 inhibitor is platelets, which have been shown to contribute to inflammation in the injured heart, whereas PAR-4 signaling in cardiomyocytes may be protective. These discrepant results between genetic and pharmacological approaches indicate that further studies are needed to determine the role of different PARs in the injured heart.

Keywords

Myocardial Infarction; I/R injury; Protease-activated Receptors; Coagulation; Heart Failure; Review

2. Introduction

Myocardial infarction is one of the leading causes of mortality and morbidity in the western world (1). Acute myocardial infarction is caused by thrombotic occlusion of a coronary artery after disruption of an atherosclerotic plaque. Thrombogenic factors from the plaque promote platelet activation, adhesion and aggregation, as well as activation of the coagulation cascade (2). Although the early restoration of circulation within the coronary vessels is necessary to provide oxygen and nutrients to the ischemic area, reperfusion itself also exacerbates myocardial damage by inciting a local inflammatory response (1). This process is known as ischemia/reperfusion (I/R) injury and was first described by Jennings et al. (3). The molecular and cellular events underlying I/R injury are complex. They include

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ion accumulation, mitochondrial dysfunction, reactive oxygen species (ROS) formation, activation of coagulation, apoptosis, endothelial dysfunction, complement activation and leukocyte accumulation (1). Expression of adhesion receptors on endothelial cells (EC) promotes invasion of inflammatory cells, particularly neutrophils. Neutrophils are toxic to the myocardium because they release proteases and generate ROS (1). Besides the neutrophil mediated effects, there is evidence that T-cells and macrophages have a role in the pathogenesis of myocardial damage during reperfusion (1).

Myocardial infarction leads to the structural remodeling of the heart. Cardiac fibroblasts proliferate and form collagen-rich scar tissue that replace viable myocardium (1). The heart compensates for the loss of myocardial tissue by the hypertrophic growth of the remaining cardiomyocytes. However, this leads to pathologic remodeling resulting in weakening of the ventricular wall and heart failure.

3. Protease activated receptors

PARs belong to the family of seven membrane spanning G-protein coupled receptors. There are 4 PARs known (PAR-1 to -4) (4; 5). Proteolytic cleavage of the N-terminus of the receptor exposes a tethered ligand which then activates the receptor (5). Numerous proteases, including coagulation proteases have been shown to activate PARs *in vitro* and *in vivo* (Table) (6). Binding of coagulation factor VIIa (FVIIa) to tissue factor (TF) on cell membranes leads to the generation of coagulation proteases FXa and thrombin. Thrombin cleaves fibrinogen to fibrin and activates PAR-1, -3 and -4 on a variety of cells (Figure). Additionally, FXa can induce PAR-1-dependent signaling. Trypsin, tryptase and matriptase are the major activators of PAR-2, although this receptor is also activated by FVIIa and FXa (6; 7). PAR-2 is not activated by thrombin.

Small synthetic activating peptides (AP), corresponding to the tethered ligand, can also be used to selective activate the different PARs (6). Importantly, several groups observed a difference in the cellular responses of PARs activated with APs versus proteases, and even between the different activating proteases (6).

Pharmacologic PAR-specific inhibition is mediated by antagonists or pepducins (6, 8). Pepducins are cell-permeable peptides derived from the third intracellular loop of either PAR-1 or PAR-4 and they disrupt signaling between the receptors and the specific G-proteins (8). The PAR-4 pepducin, P4pal10, inhibits intracellular PAR-4 signaling in platelets (8).

Interestingly, receptor dimerization has been described for the different PARs. PAR-1 can form homodimers as well as heterodimers with either PAR-2, PAR-3 or PAR-4 (8). Furthermore, activation of PAR-1 can lead to trans-activation of PAR-2 or PAR-4 (8; 9). Recently, it was shown that thrombin activation of PAR-3 in human lung epithelial cells led to Rho- and calcium dependent ATP release which was PAR-1 and PAR-4 independent (10). Mouse PAR-3 does not signal (6; 8) but acts as cofactor and induces signaling in combination with PAR-1, PAR-2 or PAR-4 (8).

4. Tissue factor, coagulation proteases and myocardial infarction

Golino and colleagues (11) observed that rabbit hearts had increased TF activity after I/R injury and that administration of anti-TF antibodies restored coronary blood flow in the injured heart. The authors proposed that the observed effects were due to ROS-mediated induction of TF expression in the endothelium of the heart without a disruption of its integrity (11). Later, the same group used active site-inhibited human FVII (FVIIai) to inhibit the TF:FVIIa complex during cardiac I/R injury (12). Administration of human

FVIIai reduced infarct size, fibrin deposition and platelet accumulation within the damaged rabbit hearts (12). In addition, administration of human FVIIa during cardiac I/R injury resulted in an increase in infarct size (12). In this study, the authors did not exclude the possibility that pre-existing subendothelial TF played a role in activation of coagulation.

Our group observed a reduction in infarct size when anti-TF antibodies were administrated to rabbits subjected to cardiac I/R injury (13). In our model, rabbit hearts exhibited an increase in cardiomyocyte TF expression but not EC TF. Importantly, we observed extra-vascular fibrin depositions after I/R injury that co-localized with TF-positive cardiomyocytes (13). Further, inhibition of TF reduced myocardial cytokine expression and cellular infiltration (13). Due to the presence of fibrin within the myocardium, we proposed that I/R injury disrupts the integrity of the endothelium and allows leakage of clotting factors from the plasma into the myocardium (13). Subsequent activation of the clotting cascade results in fibrin deposition within the myocardium (13; 14).

Recently, Loubele et al. (15) reported a reduction in cardiac injury and neutrophil infiltration in the myocardium of injured mice treated with murine FVIIai. In addition, FVIIai reduced NFκB activation and inflammatory gene expression (15). However, it is not clear whether the observed results were due to reduced thrombin generation and fibrin deposition, or reduced TF:FVIIa signaling via PAR-2 (16).

Our group showed that inhibition of thrombin with hirudin decreases infarct size in rabbits and mice (13; 17). This was most likely due to reduced fibrin generation because we found that PAR-1 deficiency had no effect on infarct size (17). In addition, a reduced infarct size was observed in fibrinogen deficient mice (18). The authors showed that the deleterious effects of fibrin are mediated by a degradation product of fibrin called E1 (Figure). This fibrin fragment facilitates neutrophil/EC interaction which leads to neutrophil infiltration into the myocardium after I/R injury (18).

Despite older studies suggesting that platelets play no or a minimal role in cardiac I/R (19), more recent studies showed that platelet activation and accumulation within the damaged heart contribute to local inflammation, ventricular remodeling and rupture (20).

5. Role of PARs in myocardial infarction and remodeling

In the following sections we will present a summary of the roles of PAR-1, -2 and -4 in hypertrophy of cardiomyocytes, proliferation of cardiac fibroblasts and in cardiac I/R injury.

5.1 PAR-1

In the heart, PAR-1 is expressed by cardiomyocytes, fibroblasts, smooth muscle cells (SMC) and EC. Furthermore, PAR-1 is also expressed on circulating cells, such as leukocytes, and in human platelets. PAR-1 expression is increased in the hearts of patients with ischemic and idiopathic dilated heart failure (21; 22). In addition, PAR-1 expression is increased in the left ventricle in a mouse model of chronic heart failure and after cardiac I/R injury (21; 22). *In vitro* studies with cardiomyocytes demonstrated that activation of PAR-1 led to calcium influx, increased protein synthesis, cell size, rearrangement of sarcomere organization, and expression of the pro-hypertrophic atrial natriuretic factor (ANF) (7; 23). These are all characteristic of hypertrophic growth. Cardiac fibroblasts respond to PAR-1 activation with a transient increase of intracellular calcium levels and enhanced cell proliferation (7; 23). In addition, PAR-1 activation leads to trans-activation of the epidermal growth factor receptor (EGFR) in cardiac fibroblast via increased phospholipase C and Src kinase activity (23).

We showed that an absence of PAR-1 did not affect infarct size in mouse model of heart I/R injury. We also did not observe any differences in the expression of inflammatory mediators, such as interleukin (IL)-1, IL-6, macrophage inflammatory protein 2 and monocyte chemotactic protein-1 (17). However, PAR-1 deficient mice had reduced cardiac remodeling and dilatation of the left ventricle 2 weeks after ischemia (Figure) (17).

In contrast to our observations with PAR-1 deficient mice, administration of the PAR-1 antagonist SCH79797 was shown to reduce the infarct size in rat hearts after cardiac I/R injury *in vivo* (24). This was associated with activation of cardioprotective pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt, nitric oxide synthase (NOS), and potassium channels (24). In this model, PAR-1 AP had no effects on the functional recovery or infarct size (24).

The differences between this observation and our results with PAR-1 deficient mice may be explained by off-target effects of SCH79797. One study reported that SCH79797 interfered with the growth of human and mouse cell lines and that the anti-proliferative activity of SCH79797 was PAR-1 independent (25). An alternate explanation for the different findings might be that PAR-4 may compensate for PAR-1 deficiency (26).

Recently, it has been shown that 41-amino acid peptide released after thrombin cleavage of PAR-1 N-terminus (parstatin) can inhibit PAR-1 signaling (27). Strande et al. (28) found that parstatin mediates cardioprotective effects on cardiomyocytes and coronary flow after I/R injury in rats. These protective effects were linked to activation of the G_i-protein pathway, which includes p38, extracellular signal-regulated kinase (ERK) 1/2, NOS, and potassium channels (28). A shorter version of parstatin (1–26) exhibited beneficial G_i-protein-dependent effects in I/R injury, including improved mitochondria function and activation of Akt and NOS (29). This leads to increased nitric oxide and cyclic guanosine monophosphate levels in the heart. However, it is unclear if all the effects of parstatin are due to inhibition of PAR-1 or some may be due to off-target effects of this inhibitor.

To analyze the role of PAR-1 in heart hypertrophy, we generated mice with cardiomyocyte specific overexpression of PAR-1. These transgenic mice develop eccentric hypertrophy with dilated cardiomyopathy manifestation at 12 months of age (17). Eccentric hypertrophy results from an increase in serial but not parallel assembly of sarcomeres in cardiomyocytes (17). Interestingly, intercrossing of PAR-1 overexpressing mice with mice lacking TF in cardiomyocytes reduced the eccentric heart hypertrophy in the PAR-1 transgenic mice (17). This suggests that TF-dependent coagulation proteases activate the overexpressed PAR-1 and induces hypertrophy.

5.2. PAR-2

Expression of PAR-2 within the heart was first described by Sabri and colleagues and localized to cardiomyocytes, SMC and EC (30). At present, it is not clear whether cardiac fibroblasts express PAR-2. Sabri and coworkers (30) were not able to detect PAR-2 mRNA in rat cardiac fibroblasts. However, a recent publication claimed that trypsin and PAR2 AP induce the proliferation of avian cardiac fibroblasts (23). PAR-2 activation on cardiomyocytes promotes inositol trisphosphate accumulation, stimulates mitogen-activated protein kinases (MAPKs), such as p38 and ERK1/2, and elevates calcium influx (30). Furthermore, PAR-2 stimulation induces cardiomyocyte hypertrophy *in vitro* (30). We found that PAR-2 AP stimulated hypertrophy of neonatal rat cardiomyocytes was blocked by inhibition of the MAPKs p38 and ERK1/2 (Antoniak, Mackman and Pawlinski unpublished data).

We observed increased expression of PAR-2 in human hearts with ischemic heart failure and increased PAR-2 expression after cardiac I/R injury in mice (Figure) (21). In addition to myocardial cells, PAR-2 is expressed on neutrophils, mast cells, and other circulating leukocytes. PAR-2 activation contributes to the inflammatory response by activating neutrophils, inducing mast cell degranulation and stimulating inflammatory gene expression (31).

Recently, we showed that PAR-2 deficiency results in reduced cardiac I/R injury in mice. PAR-2 deficient mice had a reduced oxidative stress and infarct size compared to wild-type littermates (21). We also observed reduced expression of inflammatory cytokines/chemokines and MAPK activation. PAR-2 deficient mice had less remodeling and exhibited less impairment in heart function after cardiac I/R injury (21). However, this may be due to a smaller infarct in PAR-2 deficient mice (21).

At present, the proteases that activate PAR-2 during cardiac I/R injury are not known. One candidate is FVIIa (7; 8). The inhibition of the TF:FVIIa complex may be protective, in part, by reducing PAR-2 signaling (16). Treatment with FVIIai during I/R injury reduced infarct size and inflammation (12; 15). However, PAR-2 can be activated by other proteases, such as mast cell tryptase (7; 8). 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 (32). This may be due, in part, to a reduction in PAR-2 activation (33).

In contrast to studies showing a pathologic contribution of PAR-2 in cardiac I/R injury, studies with PAR-2 AP in wild-type mice showed that PAR-2 mediates protective effects in cardiac I/R injury. Napoli et al. (34; 35) were the first to describe that PAR-2 activation protects the heart from experimental I/R injury *in vivo*, and in Langendorff-perfused hearts. PAR-2 AP improved the recovery of myocardial function and decreased oxidative I/R injury. The ischemic risk zone, creatine kinase release, and cardiac inflammation were also reduced after PAR-2 AP treatment. The effects of PAR-2 AP on the coronary flow were nitric oxide and tyrosine kinase independent. However, in this model the authors observed increased expression of tumor necrosis factor-α and induction of PAR-2 expression (34; 35). An independent study showed that PAR-2 activation causes endothelium-dependent coronary vasodilatation. PAR-2 activation led to the release of endothelium derived hyperpolarizing factor and activation of vanilloid receptor on C-fibers (36). The vanillioid receptor activation was due to PAR-2-dependent activation of the protein kinase (PK) C-epsilon and PKA pathways (37). The protective effects of PAR-2 AP were further associated with enhanced ERK1/2, B-cell lymphoma 2 (Bcl-2)-associated death promoter (BAD) expression, and PKC phosphorylation (38). PAR-2 activation with AP in rats before reperfusion reduced the myocardial apoptosis by upregulating of Bcl-2 and PAR-2, and downregulating Bcl-2–associated X protein (Bax) (39).

How can we explain the contradictory results with PAR-2 deficient mice and PAR-2 activation in wild-type mice? PAR-2 is not only coupled to G-proteins, but also to other proteins, such as β-arrestins (6). β-arrestins were first described as adaptor proteins which maintain receptor internalization and desensitization (40). Recent studies suggest that β-arrestins are responsible for the observation that different ligands acting at the same G-protein coupled receptor can produce distinct signaling responses (40). This so-called ‘biased agonism’ is dependent on the conformational changes within the receptor after activation (40). PAR-2 activation with AP may lead to a different change in the receptor’s three dimensional structure compared to activation by proteases. These changes could lead to a different phosphorylation pattern of intracellular components of the receptor and altered G-protein coupling or β-arrestin activation (6; 40). β-arrestin dependent signaling is often restricted to the cytoplasm and involves different MAPKs, such as ERK1/2 (6; 41). It is

slower and more sustained than G-protein signaling (6; 41). Furthermore, β-arrestin prevents phosphorylation and degradation of IκBα and thus attenuates activation of NFκB and transcription of NFκB-target genes (41). β-arrestins have been shown to mediate anti-inflammatory and cytoprotective effects (41). Further, the activity of β-arrestins was linked to cardio-protection via trans-activation of EGFR (42). It is possible that the described biased signaling might be responsible for the protective effects of PAR-2 AP stimulation in wild-type mice during cardiac I/R injury.

5.3. PAR-4

Steinberg's group first described the present of functional PAR-4 on cardiomyocytes (43). Later, PAR-4 expression was also confirmed in SMC but not fibroblasts and EC in human and rodent hearts (44; 45). Hearts of patients with ischemic cardiomyopathy exhibit elevated levels of PAR-4 (44). Activation of PAR-4 on cardiomyocytes produces a unique signaling signature that is different to that produced by the activation of PAR-1. Activation of PAR-4 leads to enhanced p38 phosphorylation (43). The increased p38 activity was due to Src and EGFR kinase activity and human epidermal growth factor receptor 2 (ErbB2/HER2) via a PP1 sensitive pathway (43). Importantly, platelets are the main cellular source of PAR-4 in the circulation in the mouse. In addition, leukocyte sub-populations also respond to PAR-4 APs with increased chemotaxis and release of inflammatory mediators (46; 47).

Considering the fact that PAR-4 activation leads to platelet aggregation in mice and that, further, PAR-4 contributes to inflammation, it could be hypothesizes that inhibition of PAR-4 would improve outcome during cardiac I/R injury due to reduce platelet activation and inflammation. However, PAR-4 deficiency in mice resulted in a significantly lower survival rate due to increased left ventricular dilatation and decreased contractility after I/R injury (Figure) (44). PAR-4 deficient mice had larger infarcts and more myocardial apoptosis compared to wild-type littermates. The authors concluded that a lack of PAR-4 exacerbates myocyte loss, fibrosis, ventricular remodeling, and decline of function after I/R injury (44). In addition, our group observed increased levels of cardiac troponin I in plasma from PAR-4 deficient mice compared to wild-type littermates after short term I/R injury (Antoniak and Mackman unpublished data). These observations suggest that PAR-4 activation during cardiac I/R injury mediates a protective signal in the heart, possibly on cardiomyocytes, and that this signal may overcome any beneficial effects of reduced platelet activation.

In contrast to results with PAR-4 deficient mice, rat cardiac I/R injury experiments with different PAR-4 antagonists had the opposite effect (45). The authors used a pepducin against PAR-4 in their study (45). P4pal10 treatment of rats reduced infarct size at any time point of administration *in vivo*. In addition to P4pal10, PAR-4 inhibition by trans-cinnamoyl-YPGKF-amide (tc-Y-NH[2]) before ischemia decreased infarct sizes of rat hearts *ex vivo* under platelet free conditions. The authors proposed that the protective effects of P4pal10 and tc-Y-NH(2) were due to unmasking of adenosine receptor signaling (45). Further, they hypothesized that PAR-4 inhibition may uncouple adenosine receptors and allow adenosine signaling, that conferred a cardioprotective effect (45). However, these authors did not show that the PAR-4 antagonists were ineffective in PAR-4 deficient mice or that PAR-4 activation increases cardiac injury.

Again, there are significant differences between a genetic absence of PAR-4 and a pharmacologic inhibition of this receptor. The differences may be due to accessibility of the PAR-4 inhibitor to the target cells. The PAR-4 pepducin may not reach all myocardial cells and the authors may see only effects of a partial PAR-4 inhibition which does not correlate with a global knock-out of PAR-4. Further, the PAR-4 pepducin may interfere with G-protein binding and shifts the coupling from one G-protein subtype to another. More

importantly, Hollenberg et al. (48) showed that tc-Y-NH(2) and P4-pal10 were potent antagonists of both thrombin and the PAR-4 AP in a PAR-4 dependent rat platelet aggregation assay *in vitro*. However, both investigated antagonists were quite active as agonists in an endothelium-dependent nitric oxide mediated rat aorta relaxation assay and in a gastric longitudinal muscle contraction assay (48). Furthermore, the authors claimed that one needed to be caution in interpretation of *in vivo* experiments with the two PAR-4 antagonists due to their complex pharmacological properties (48).

6. Conclusions and perspectives

These studies demonstrate multiple roles for PARs in the injured heart. Further studies are needed to determine if PARs are viable targets for drugs to treat myocardial infarction, cardiac remodeling and heart failure.

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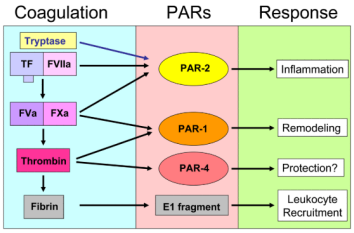

Figure 1. Model showing a summary of the effects of activation of different PARs by coagulation proteases after cardiac I/R injury

The model is based on the phenotype of mice deficient in the different PARs and does not represent the different results observed with pharmacologic inhibitors. It should be noted that model uses pathways established from *in vitro* experiments with culture cells. At present, the proteases that activate the different PARs *in vivo* are unknown. Tryptase is shown as an example of a protease not involved in coagulation that can activate a PAR-2.

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Table 1

PARs in experimental cardiac I/R injury

Receptor	Activating Proteases	Genotype	Observation	Reference
PAR-1	**Thrombin** Trypsin, Plasmin, FXa Granzyme A, APC, Cathepsin G KLK-1, -4, -5, -6, -14 MMP-1, -3, -8, -9, -13, RgpB Proatheriocytin, Pen c 13	PAR-1 deficient mice	No changes in infarct size, reduced remodeling	(17)
		PAR-1 agonist in rats	No effect on infarct size	(24)
		PAR-1 antagonist (SCH79797) in rats	Reduced infarct size, PI3K/Akt and NOS activation	(24)
		PAR-1 antagonist (Parstatin) in rats	Cardioprotection due to increased coronary flow, NOS and Akt activation	(27; 28)
PAR-2	**Trypsin, Tryptase Matriptase** FXa, TF:FVIIa, Acrosin Granzyme A, HAT, Trypsin IV TMPRSS2, Chitinase KLK-2, -4, -5, -6, -14, RgpB Pen c 13, Der P1, Der p3, Der p9	PAR-2 deficient mice	Reduced infarct sizes, reduced remodeling with reduces left ventricular dilatation	(21)
		PAR-2 AP in wild-type mice and rats	Cardioprotection due to increased blood flow, increased NOS activity and reduced apoptosis	(34–39)
PAR-4	**Thrombin** Trypsin, Plasmin, Cathepsin G Trypsin IV, FXa MASP-1, KLK-1, -14, RgpB	PAR-4 deficient mice	Increased infarct size, increased apoptosis, increased ventricular dilatation	(44)
		PAR-4 antagonists (Pepducin, antagonist peptide) in rats	Cardioprotection with reduced infarct size and increased adenosine receptor signaling	(45)

APC: activated protein C; F: factor; MMP: matrix metalloproteinase; TF: tissue factor; KLK: kallikrein-related peptidase; RgpB: cysteine protease from *Porphyromonas gingivalis*; HAT: human airway trypsin-like protease; MASP-1: complement pathway protease;

PI3K: phosphatidylinositol 3-kinase; NOS: nitric oxide synthase;