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Review

Role of Prokineticin Receptor-1 in Epicardial Progenitor Cells

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Abstract: G protein-coupled receptors (GPCRs) form a large class of seven transmembrane (TM) domain receptors. The use of endogenous GPCR ligands to activate the stem cell maintenance or to direct cell differentiation would overcome many of the problems currently encountered in the use of stem cells, such as rapid *in vitro* differentiation and expansion or rejection in clinical applications. This review focuses on the definition of a new GPCR signaling pathway activated by peptide hormones, called "prokineticins", in epicardium-derived cells (EPDCs). Signaling via prokineticin-2 and its receptor, PKR1, is required for cardiomyocyte survival during hypoxic stress. The binding of prokineticin-2 to PKR1 induces proliferation, migration and angiogenesis in endothelial cells. The expression of prokineticin and PKR1 increases during cardiac remodeling after myocardial infarction. Gain of function of PKR1 in the adult mouse heart revealed that cardiomyocyte-PKR1 signaling activates EPDCs in a paracrine fashion, thereby promoting *de novo* vasculogenesis. Transient PKR1 gene therapy after myocardial infarction in mice decreases mortality and improves heart function by promoting neovascularization, protecting cardiomyocytes and mobilizing $WT1^+$ cells. Furthermore, PKR1 signaling promotes adult EPDC proliferation and differentiation to adopt endothelial and smooth muscle cell fate, for the induction of *de novo* vasculogenesis. PKR1 is expressed in the

proepicardium and epicardial cells derived from mice kidneys. Loss of PKR1 causes deficits in EPDCs in the neonatal mice hearts and kidneys and impairs vascularization and heart and kidney function. Taken together, these data indicate a novel role for PKR1 in heart-kidney complex via EPDCs.

Keywords: GPCR; prokineticin; EPDCs; angiogenesis; cardiomyopathy; renal defects; cardiac progenitor cells; receptor; signaling; kidney progenitor cells

1. Introduction

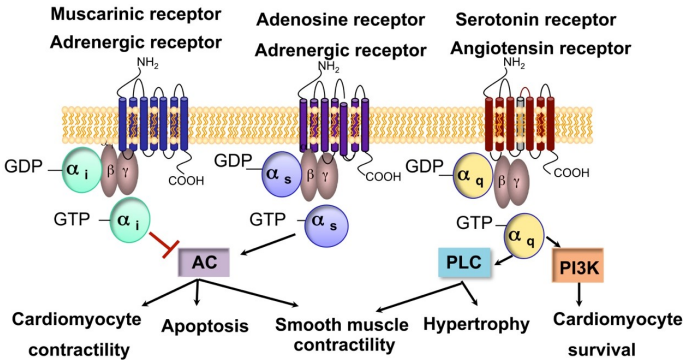
Over the last decade, stem/progenitor-cell therapy has emerged as an innovative approach to instigating cardiac repair and regeneration [1]. Treatments based on cardiac progenitor cells have recently emerged as promising potential therapies for heart conditions [2]. For cardiovascular therapy, pluripotent cardiac progenitor cells resident in the epicardium have several distinct advantages over other adult stem-cell types. They are autologous, tissue-specific and already committed to a cardiac fate [3,4]. Epicardium-derived cells (EPDCs) are present in the heart in several species, including zebrafish, mice and humans [5,6]. EPDCs offer an optimal target, since they give rise to cardiomyocytes and coronary vascular cells and provide a cocktail of growth factors that may contribute to regeneration of heart. However, the race is still on to find the "best" factors or drugs to control cell fate decisions within adult EPDCs for reconstitution of the myocardium and improving vascularization and function after myocardial damage.

G protein-coupled receptors (GPCRs) form a large class of seven-transmembrane domain receptors that transmit extracellular signals to cells by coupling to guanine nucleotide-binding proteins (G proteins) [7]. GPCRs are the targets of about 20 to 50% of the drugs currently on the market and their genes account for 3 to 5% of the human genome. Many hormones and neurotransmitters use GPCRs to exert their cardiovascular effects [8,9]. Following the binding of a ligand on the extracellular side of the GPCR, conformational changes are induced, causing the intracellular loops to bind and activate the heterotrimeric G protein. The activated G protein dissociates from the receptor and its subunits (α and βγ) to induce second messenger signaling mediated by effector molecules, such as kinases, phospholipases, enzymes or channels. GPCRs are also involved in non G protein-mediated signaling via scaffold proteins, such as beta-arrestin [7]. The role of GPCRs in the heart is illustrated in Figure 1. GPCRs may promote cardiac hypertrophy [10] or protect cardiomyocytes against hypoxic challenge [11,12] via Gαq signaling. Gα12 signaling interacts with the cytoplasmic domain of cadherins, resulting in the release of the transcriptional activator, β-catenin, which is a regulator of epicardial development [13]. Gα13 signaling is involved in vessel formation [14]. Gαs signaling regulates heart rate and contractility in response to catecholamine stimulation, but excessive Gαs signaling in the heart eventually induces myocardial hypertrophy, fibrosis and necrosis [15].

Relatively little is known about the role of GPCRs in the functional activities of EPDCs in both normal and disease conditions. Given the important roles played by GPCRs in cardiac regulation, it is vital to decipher the functions of GPCRs in EPDCs if we are to develop novel regenerative therapies to limit cardiovascular disease.

Only two factors have been identified as capable of stimulating adult EPDCs. Stimulation of the adult epicardium by thymosin beta 4 resulting in the differentiation of EPDC into vasculogenic cell types and cardiomyocytes has been demonstrated [16]. More recently, prokineticin-2, a potent angiogenic hormone, has been shown to stimulate adult EPDCs [17]. This review aims to provide an overview of what is currently known about the regulation of EPDC proliferation and differentiation by prokineticin receptor-1 (PKR1), a GPCR.

Figure 1. Many hormones and neurotransmitters use G protein-coupled receptors (GPCRs) to maintain cardiovascular homeostasis. Gαs signaling via adenylyl cyclase (AC) regulates heart rate and contractility in response to catecholamine stimulation, but excessive Gαs signaling in the heart eventually induces myocardial hypertrophy, fibrosis and necrosis. Gαq signaling promotes cardiac hypertrophy or protects cardiomyocytes against hypoxic challenge.



2. Prokineticins and Cognate Receptors

Prokineticins are small secreted proteins [18,19] . They were first identified in the gastrointestinal tract as potent agents mediating muscle contraction [20] and have been isolated from bovine milk [21]. They comprise two classes: prokineticin-1 (PK1), originally called endocrine gland-derived vascular endothelial growth factor (EG-VEGF), based on the functional similarity to VEGF [22], and prokineticin-2 (PK2, also called Bv8). An alternatively spliced product of the PK2 gene encoding 21 additional amino acids compared with PK2 is designated PK2L (for PK2 long form) [23]. Both PK2 and PK2L are expressed in heart, and only PK2 is expressed in kidney. PK1 and PK2 are approximately 50% homologous and contain carboxyl-terminal cysteine-rich domains that form five disulfide bridges. N terminal hexapeptide (AVITGA) and cysteine residues in the carboxy-terminal domain are crucial for their biological activities. Prokineticins are widely distributed in mammalian tissues [24]. Prokineticins induce cell excitability (gut spasmogen, pain sensitization [25], circadian rhythm [26] and sleep [27]), cell motility (angiogenesis [28], neurogenesis [29], hematopoiesis [30], neovasculogenesis [17]) and complex behaviors (feeding [31], drinking [31], anxiety [32]). Prokineticin signaling has been implicated as a survival/mitogenic factor for various cells, including endothelial cells [33], neuronal cells, [29] enteric neural crest cells [34], granulocytic [35] and monocytic lineage [36], lymphocytes and hematopoietic stem cells [30].

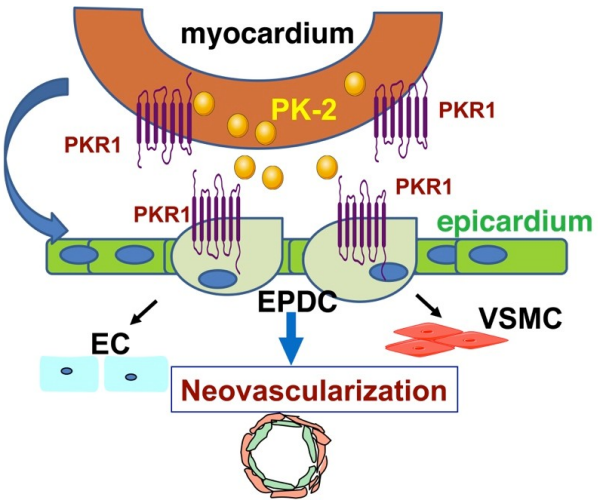
Prokineticins bind to two cognate 7-transmembrane GPCRs. PKR1 and PKR2 share about 85% amino acid identity and encoded within distinct chromosomes in both mouse and human [21].

Prokineticin-2 is the most potent agonist for both receptors [21]. PKR2 is the dominant receptor in the adult brain, particularly in the hypothalamus, the olfactory ventricular regions and the limbic system. However, PKR1 is widely distributed in the periphery. These receptors couple to Gαq, Gαi and Gαs to mediate intracellular calcium mobilization, activation of MAPK, Akt kinases and cAMP accumulation, respectively [37]. In cultured capillary endothelial cells derived from heart, prokineticin-2 via PKR1 induces proliferation, migration and vessel-like formation, activating Gα11/MAPK and Akt kinases [33]. In cardiomyocytes, activation of overexpression of PKR1 protects cardiomyocytes against hypoxic insult, activating the PI3/Akt pathway [38].

3. Prokineticin Signaling in Myocardium and Epicardium Interaction

Transgenic mice overexpressing *PKR1* in cardiomyocytes under the control of an α-MHC promoter display no spontaneous cardiomyocyte abnormalities [17]. However, they have abnormally large numbers of epicardin-positive EPDCs, high capillary density and large numbers of coronary vessels. This study demonstrated that cardiomyocyte PKR1 signaling upregulates the ligand of PKR1, prokineticin-2, which in turn acts as a paracrine factor, inducing the proliferation and differentiation of EPDCs into endothelial and smooth muscle cells, thereby promoting neovascularization. This study described a new cardiomyocyte-epicardium interaction involving prokineticin/PKR1 signaling (Figure 2). Neonatal PKR1-null mutant mice have low levels of angiogenesis in the heart. These findings suggest that PKR1 is involved in postnatal *de novo* vascularization, rather than vasculogenesis during embryogenesis [17].

Figure 2. A novel cardiomyocyte-epicardium interaction via prokineticin/PKR1 signaling. Cardiomyocyte PKR1 signaling upregulates its own ligand, prokineticin-2 (PK-2), as a paracrine factor, to induce the proliferation of EPDCs and their differentiation into endothelial (EC) and vascular smooth muscle cells (VSMC), thereby promoting neovascularization.



Prokineticin/PKR1 signaling also plays a cell-autonomous role in EPDC activation. Prokineticin/PKR1 signaling promotes the proliferation and differentiation of EPDCs. During normal development, EPDCs give rise to vascular precursors and adventitial fibroblasts. Prokineticin/PKR1 signaling reprograms neonatal and adult EPDCs, causing them to differentiate into a subset of

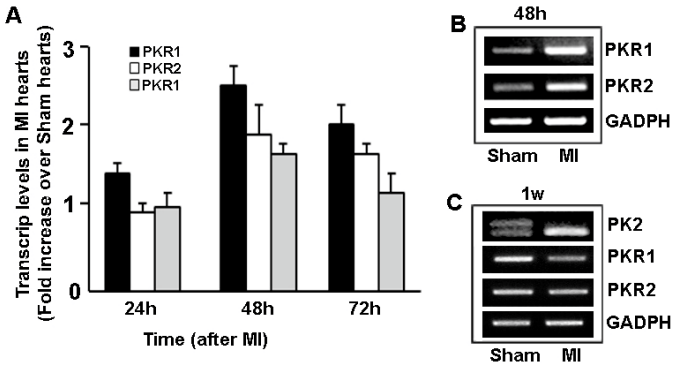
embryonic cells, such as endothelial and smooth muscle cells, rather than fibroblasts. These data suggest that the PKR1 signaling inherent to EPDCs may determine the lineage of these cells [17].

4. Prokineticin Signaling in Myocardial Infarction (MI)

The expression of prokineticins and their receptors is upregulated within 48 h after MI in mice (Figure 3A,B). Accordingly, Akt phosphorylation levels that are 30% higher in ischemic than in non-ischemic hearts indicate an activation of the compensatory cardioprotective signaling pathway, triggering the endogenous wound-healing process [38]. However, the PKR1 expression was reduced, while PKR2 expressions remain unaltered one week after MI (Figure 3C). Indeed, PK2 long form (PK2L) has not been detected in MI hearts one week after MI (Figure 3C). Whether the alternative splicing of PK2L occurred during the heart remodeling after the MI needs to be studied.

In the mouse coronary ligation of MI model, the intracardiac PKR1 gene transfer utilizing adenovirus (Adv) carrying PKR1 cDNA (Adv-PKR1) induced a three fold increase in the PKR1 level 24 h after MI, rising to a four fold increase 48 h after MI, remaining elevated after 1 week after MI [38].

Figure 3. (**A**) Quantitative analyses of expression levels of prokineticin 2 (PK2) and its receptors, PKR1 and PKR2 in MI and sham operated mice hearts, 24, 48 and 72 hours after the operations. (**B**) Representative illustration of PCR analyses of the PKR1 and PKR2 transcripts 48h after MI. (**C**) Expression of transcripts of PK2 long form (PK2L, double bands) and short form (single bands), and PKRs in MI and sham operated hearts one week after MI.

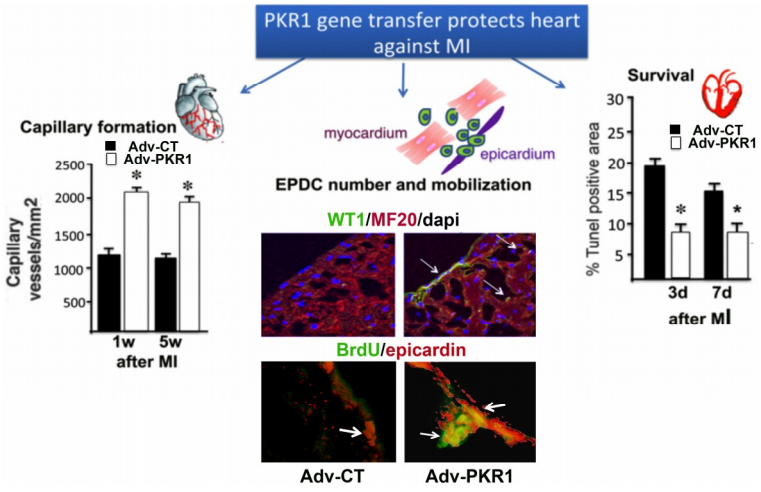


PKR1 gene transfer decreased MI size, improved left ventricular performance and resulted in lower mortality than in untreated control mice [38]. Neither VEGF protein nor VEGF-A transcripts, 164 and 188, displayed differences in abundance between PKR1-treated hearts and vehicle-treated hearts after MI, consistent with observations in cultured endothelium cells after prokineticin-2 treatment. Thus, transient PKR1 transfection induces capillary network growth without increasing VEGF levels. The cardioprotector signaling pathway, involving Akt activation, has been shown to be significantly more active *in vivo* in PKR1-treated hearts than in Adv-control vector-treated hearts one week after coronary ligation [38]. These data thus suggest that transient PKR1 gene transfer has beneficial effects on recovery from myocardial infarction.

Transient gene therapy with PKR1 enhances angiogenesis and decreases apoptosis after MI [38]. The PKR1-mediated cardioprotective effects may involve the prevention of cardiomyocyte apoptosis.

The promotion of collateral vessel formation, to ensure sufficient tissue oxygenation, would also preserve myocardial function. PKR1-mediated angiogenesis may, therefore, play an important role in keeping sufficient numbers of cardiomyocytes alive for successful cardioprotection, after its initial anti-apoptotic effect. A third mechanism might involve a contribution of the inflammation itself to PKR1-mediated angiogenesis in infarcted hearts. Indeed, PKR1 signaling is involved in inflammation, monocyte activation and macrophage differentiation [36], and PKR1-knockout mice display a lack of inflammation in response to PK2 stimulation [25,39]. However, the number of cells infiltrating the scar area after myocardial infarction did not differ between mice transfected with the PKR1 gene and control mice after MI, arguing against an indirect effect of PKR1 through the inflammatory response. Finally, PKR1 could also preserve myocardial function by inducing WT1 and epicardin positive progenitor cell expansion and migration, as shown in Figure 4.

Figure 4. Mechanisms by, which PKR1 gene therapy may protect the heart against damage caused by myocardial infarction. One of the possible mechanisms involves the direct activation of endothelial cells to promote angiogenesis. Capillary numbers were increased in Adenovirus (Adv)-PKR1 treated hearts as compared to Adv-control treated hearts one week and five weeks after the coronary ligation as a mouse model of myocardial infarction (MI). Alternatively, EPDCs may be mobilized from the epicardium to the scar area, to regenerate the heart. WT1 staining of cryosectioned hearts revealed that WT1 positive (green) cell number in the niche and their mobilizations towards MF-20 positive (red) cardiomyocytes were increased in the Adv-PKR1 treated hearts as compared to Adv-control treated hearts one week after MI. A third possible mechanism involves the inhibition of apoptosis in cardiomyocytes. Tunel assay revealed that Adv-PKR1 treated hearts had lower levels of apoptotic cardiomyocytes as compared to Adv-control treated hearts three and seven days after MI.

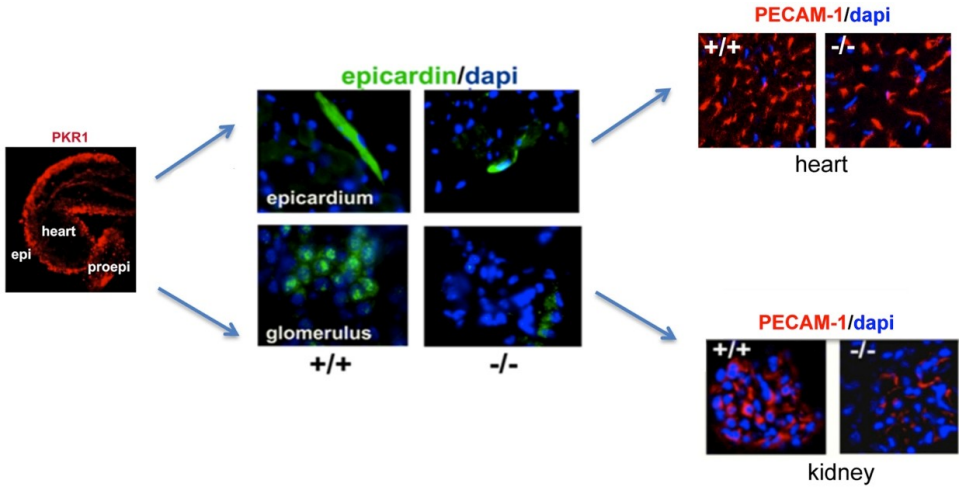


5. Prokineticin Signaling in the Epicardium-Kidney Axis

Several studies have shown that many genes are expressed in both epicardium and kidney progenitors. The deletion of these proepicardial genes, such as WT1 [40], tcf21 (epicardin) [41,42], the nephrin [43] gene and T-box transcription factor 18 (Tbx18) [44,45] in mice has been shown to lead to both heart and

kidney defects. WT1 induces epithelial-to-mesenchymal transition (EMT) during the formation of cardiac progenitors from the epicardial field [46]. WT1 regulates the reverse process (MET) in kidney mesenchyme. Similarly inactivating mutation of WT1 in humans leads to disease(s) associated with both [47] and heart functions [48]. These findings suggest that the development of the epicardium and kidney may be connected via the proepicardium [49]. The proepicardium has been shown to expresses PKR1 (Figure 5), which is required for epicardial and glomerular differentiation [40,50]. Moreover, the expression of PKR1 genes has been demonstrated in both epicardial and kidney progenitors. In particular, PKR1 expression has been demonstrated in epicardin-positive progenitor cells resident in the heart and kidney. Neonatal PKR1-null mutant mice have lower capillary numbers and densities in the heart and kidneys than their wild-type littermates (Figure 5). Epicardin-positive progenitor cells are significantly less abundant in the PKR1-null mutant epicardium, subepicardium and glomerulus than in the corresponding wild-type tissues [51]. In epicardin positive progenitor cells from wild-type kidneys (prepared on postnatal day 1), prokineticin-2 induces the differentiation of these cells into both endothelial and smooth muscle cells. By contrast, these effects of prokineticin are completely abolished in these cells derived from mutant kidneys (Figure 5). These data demonstrate that PKR1 signaling controls the differentiation of the epicardin positive progenitor cells potentially involved in glomerular and epicardial angiogenesis. Adult PKR1-null mutant mice develop abnormalities of cardiac and renal function [52]. PKR1 loss leads to cardiomegaly, severe interstitial fibrosis and cardiac dysfunction under stress conditions, accompanied by renal tubular dilation, small numbers of glomerular capillaries, urinary phosphate excretion and proteinuria in older individuals. Thus, PKR1 inactivation in mice leads to defects of progenitor cell proliferation and differentiation, potentially linking cardiac and renal damage.

Figure 5. Expression of PKR1 in epicardium and proepicardium of mice embryo. PKR1-specific antibody has shown that PKR1 (red) expresses in mouse epicardium and proepicardium at 8.5 dpc. Neonatal PKR1-null mutant mice had lower levels of epicardin positive cells (green) in their hearts and glomerular structures in the kidneys that were correlated with reduced PECAM-1 positive endothelial cells (red). Note: the epicardin/TCF21 antibody that was raised against synthetic peptide from N-terminus labels cytoplasmic and the nuclear epicardin protein that has a transduction domain (PTD) sequence the same as the family member, *BETA2*/NeuroD [53].



6. Concluding Remarks

Many studies have focused on angiogenesis in cancer and circadian clock regulation, whereas explorations of the role of the prokineticin signal transduction pathway in cardiovascular disease have only recently begun. Such studies in cardiovascular diseases have suggested that PKR1 plays an important role in EPDCs, endothelial cells and cardiomyocytes. The EPDC pool is a mixture of cells with different gene expression profiles**.** Several recent lineage-tracing experiments have demonstrated the presence of multipotent progenitor cells within the activated adult epicardium, but there have been few functional studies directly manipulating gene expression specifically in the adult epicardium, to evaluate its contribution to cardiac regeneration and repair. Current studies on cardiac regeneration via EPDCs have revealed discrepancies, with several possible origins: (1) different EPDC pools may generate different types of cardiac cells; (2) all the EPDCs are multipotent and the genetic engineering of these cells or the provision of particular growth stimuli, such as PKR1, can elicit the myogenic or vasculogenic potential of these cells; (3) different classes of vertebrates may have different systems of EPDC regulation. $WT1^+$ epicardial progenitor cells give rise to smooth muscle cells of the coronary vasculature and to fibroblasts and contribute 4% of the total number of cardiomyocytes [54]. Similar results have been obtained for EPDCs expressing the Tbx18 gene [55]. $Tcf21^+$ epicardial cells in mouse [56] and zebrafish [57] give rise to intracardiac fibroblasts, but not to coronary smooth muscle or endothelial cells. However, scleraxis- and semaphorin3D-expressing EPDCs in the developing mouse heart give rise to endothelial cells of the coronary vasculature and contribute 6.6% and 0.36% of the total number of cardiomyocytes, respectively [58]. We hypothesize that although EPDCs are not necessarily a natural source of both vasculogenic cells and cardiomyocytes; the activation of PKR1 or genetic engineering of these cells with the PKR1 gene can be used to elicit the myogenic and vasculogenic potential of EPDCs and can induce the secretion of prosurvival factors in these cells, contributing to the maintenance/regeneration of damaged hearts. We are currently carrying out experimental studies in our laboratory to assess the feasibility of this approach. We will then consider the role of PKR1 in renal epicardin positive progenitor cell differentiation into renal cells. Overall, the available data show that PKR1 is involved in postnatal cardiac and renal neovascularization, but the role of prokineticins in the pathophysiology of human heart and kidney diseases remains to be explored.

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Conflict of Interest

The authors declare no conflict of interest.

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