Updates on Endothelial Functions of Proangiogenic Prokineticin

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Coronary and renal blood flows are important for maintaining cardiac and renal function. Endothelial cells (ECs) play critical roles in forming new vessels and regulating vessel functions. ECs also protect the surrounding tissues by secreting a variety of biologically active substances. Endothelial dysfunction occurs in various forms of cardiovascular and renal diseases. It plays a fundamental role in the pathogenesis of acute coronary syndromes, hypertension, type 1 and type 2 diabetes mellitus, coronary artery disease, congestive heart failure, and chronic renal failure. Microvascular endothelial dysfunction correlates with reduced coronary flow reserve in patients with coronary artery disease. These findings support the existence of a link between endothelial dysfunction and the development of structural changes in coronary circulation. Endothelial dysfunction, together with inflammation, is involved in the loss of kidney function among subjects with chronic kidney failure. Endothelial dysfunction may serve as a marker for the initiation and progression of insulin resistance, chronic kidney failure, and cardiovascular diseases.

Microvascular endothelial dysfunction correlates with reduced coronary flow reserve in patients with coronary artery disease. New insights into the regulation of endothelial function will be obtained through greater understanding of the signaling pathways within ECs. New pharmacological strategies that specifically target ECs hold promise for the prevention and treatment of cardiovascular and renal diseases. Here, we summarize recent findings on the proangiogenic factors, prokineticins, and their receptors in endothelial function/dysfunction and discuss the potential implications toward novel therapeutic targets in cardiovascular and renal diseases.

Prokineticins and Their Receptors

Prokineticins are angiogenic hormones (from 80 to 120 amino acids) released principally by macrophages and reproductive organs. They are called prokineticins because of their contractile properties in the gastrointestinal tract for which they were first described. They have a highly conserved N-terminal AVITGA motif essential for their biological activity. Two prokineticin isoforms have been identified: prokineticin-1 (prokineticin-1), originally called endocrine gland–derived vascular endothelial growth factor (EG-VEGF) based on its potent angiogenic roles similar to those of VEGF, and prokineticin-2 (also called Bv8). Although prokineticin1 and prokineticin2 are ≈50% identical, both contain carboxyl terminal cysteine-rich domains to form 5 disulfide bridges. Prokineticin-2 (81 amino acid peptide) has a long form with 21 additional amino acids called PK2L. Prokineticins and their receptors exist in all mammalian tissues. In reproductive tract, estrogen, progesterone, and human chorionic gonadotrophin, as well as hypoxia-inducible factor-1α, upregulates expression of prokineticin-1. In olfactory bulbs, neurogenin1 and Mash1 upregulates prokineticin-2, whereas homeobox transcriptional factors (distal-less homeobox 1 and 2) downregulates prokineticin-2 expression. Transcription factor activating protein-1 and granulocyte colony-stimulating factor also regulate the expression of the prokineticin-2. It is possible that stimuli such as circadian in the central nervous system and remodeling/injury in the cardiovascular system are the regulators of the control prokineticin-2 expression.

Prokineticins exert their biological functions through binding to 2 G-protein–coupled receptors: prokineticin receptor-1 and 2 (PKR1 and PKR2), which couple to different G proteins. Prokineticin-induced Ca2+ mobilization, and phosphatidylinositol turnover are consistent with coupling to Goq/i/o pathways. Prokineticin receptor signaling is also associated with activation of the Goq,i/o pathways.

Prokineticin receptor signaling regulates vascular structure and functions in a paracrine and autocrine fashion that may have beneficial effects on tissue repair by inducing angiogenesis/neovasculogenesis to improve tissue circulation.

Autocrine Regulation of Endothelial Function by Prokineticin Receptors

Several studies have shown that ECs of the vascular and microvascular beds of various organs exhibit structural and functional heterogeneity that may be altered by pathological conditions. The divergent roles of PKR1 and PKR2 are because of their coupling to different G proteins, expression profile, and their localization, determining the functional heterogeneity of ECs. Activation of PKR1 on fetal ECs within the stroma increases their proliferation, migration, invasion, and branching. PKR2 activation, however, induces transcellular permeability because of activation of different signaling pathway.

Similarly, the high levels of PKR1 on coronary ECs or overexpression of PKR1 on these cells promotes the formation of vessel-like structures. PKR1-mediated angiogenesis results principally from the stimulation of EC

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proliferation and migration. Activation of mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase are involved in the prokineticin-mediated angiogenesis signaling pathway. Gα11 seems to regulate the activity of MAPK and Akt in angiogenic signaling of PKR1.26 Gα11 proteins have been shown to be involved in EC proliferation via tyrosine phosphorylation of VEGFR-2 on HUVEC (human umbilical vein endothelial cell) cells.20

The overexpression of PKR2 compared with PKR1 in coronary ECs results in a fenestrated EC phenotype. The overexpression of PKR2, unlike that of PKR1, does not induce angiogenesis.28 PKR2 overexpression increases the formation of vesiculo-vacuolar permeability organelles and caveolae, small invaginations of the plasma membrane that facilitate vesicular transport of small proteins through the cytoplasm of an individual EC, thereby increasing the transcellular permeability of these cells. PKR2 signaling leads to the abnormal organization of ECs: the cells disconnect because of loss of the cell–cell adhesion molecule ZO-1 (Zonula occludens protein-1) at tight junctions.28 PKR2 regulates the paracellular permeability between cells via Gα12-ZO-1 protein interactions as described for VEGF signaling.31 Gα12 signaling has also been recently shown to increase cell permeability and tight junctional disassembly via its interaction with ZO-1 in Madin-Darby canine kidney cells.32 Indeed, PKR2 signaling is involved in the activation of Gα12, which then interacts with ZO-1, thereby downregulating ZO-1-mediated cell–cell adhesion. PKR2 is strongly expressed in fenestrated ECs, such as those found in the endocrine glands,17 corpus luteum, kidney, and liver,33 suggesting the involvement of PKR2 in EC fenestration. The PKR1 is mainly localized in the capillary ECs to promote angiogenesis.

The expression levels of prokineticin receptors that also determine the functional heterogeneity of capillary ECs are affected by cellular aging.28 PKR1 levels in capillary ECs decrease after several passages (>10–15), whereas PKR2 predominates in capillary ECs, promoting cellular detachment and altering the morphology of the EC (Figure 1A). The role of PKR1 in vascular aging was clearly demonstrated in the aortas of 24-week-old endothelial-specific PKR1-deficient (PKR1ec−/−) mice.35 The mutant aortas already showed substantial lipid deposition in their intima at the age of 12 weeks. By the age of 24 weeks, abundant microaneurysms and considerable macrophage infiltration were observed. The aortas of PKR1ec−/− mice displayed the progressive impairment of endothelium-dependent relaxation. Relaxation of aortic rings from the mutant mice in response to sodium nitroprusside (endothelium-independent relaxation) was unaltered. However, acetylcholine-mediated (endothelium-dependent relaxation) was significantly impaired in the aortas of 24-week-old PKR1ec−/− mice because of low NO synthesis (eNOS) activity.35 The endothelium-specific loss of PKR1 also did not alter aortic vasomotor contraction responses to phenylephrine or a synthetic thromboxan A2 receptor agonist (U46619). Mice lacking the eNOS gene develop modest hypertension at a young age, but blood pressure in male NOS−/− mice decreases with age, because of cardiac dysfunction.36 Blood pressure changes are probably masked by the cardiac dysfunction in PKR1ec−/− mice, despite the presence of low levels of NOS in the endothelium.

Whether PKR1 expression is altered after abdominal aortic rupture in humans need to be explored.

**PKR1-Dependent Paracrine Regulation of Vascular Function in Heart**

The role of PKR1 in ECs has been studied in vivo by examining how the cardiovascular and renal system was affected in PKR1ec−/− mice at the ages of 12 and 24 weeks relative to wild-type mice. In agreement with the in vitro findings, the specific loss of PKR1 from mouse ECs resulted in defective
angiogenesis, leading to necrosis/apoptosis in the surrounding tissues in several organs, including the heart and kidneys.\textsuperscript{35}

There was significantly less capillary formation in PKR1\textsuperscript{ec−/−} hearts. The posterior walls of PKR1\textsuperscript{ec−/−} hearts were thinner at the age of 12 weeks. No detectable changes in heart function were found in the PKR1\textsuperscript{ec−/−} mice at this age, despite an increase in left ventricular end-systolic diameter. However, shortening fractions (indicators of left ventricular contractility) were markedly lower in mutant mice at the age of 24 weeks. Hence, the loss of ventricular mass in PKR1\textsuperscript{ec−/−} mice is because of the loss of capillary formation and a high level of apoptosis. The remaining viable heart muscle is subject to greater biomechanical stress, triggering hypertrophy.\textsuperscript{35} In summary, PKR1\textsuperscript{ec−/−} hearts displayed EC deregulation, capillary refraction, apoptosis, fibrosis, and ectopic lipid deposition, resulting in impaired diastolic function (Figure 1B).

Paracrine Regulation of Vascularization by Cardiomyocyte Prokineticin Receptors

Transgenic mice overexpressing PKR1 in the cardiomyocytes (transgenic-PKR1) displayed no spontaneous abnormalities in cardiomyocytes but an increased number of epithelial derived progenitor cells (EPDCs), with an increase of capillary density and coronary arterioles.\textsuperscript{37} In transgenic-PKR1 hearts, the increased angiogenesis and vasculogenesis is not the result of acute cardiac dysfunction because no cardiomyopathy has been found in these mice. The mechanism involved in these events in the transgenic-PKR1 hearts is because of a activation of a paracrine mechanism. The cardiac PKR1 signaling upregulates its own ligand prokineticin-2, which in turn acts as a paracrine factor to stimulate the EPDC differentiation into endothelial and smooth muscle cells to promote neovascularization. This paracrine signaling can also activate EC-PKR1 signaling to promote angiogenesis (Figure 1C). However, cardiomyocyte PKR1 is essential for cardiomyocyte function. PKR1-null mice had cardiomyocyte contractile defects and apoptosis partially because of lack of PKR1 signaling in cardiomyocytes (autonomous effect of PKR1).\textsuperscript{38}

Transgenic mice overexpressing PKR2 in the heart (transgenic-PKR2) exhibited eccentric hypertrophy and vascular leakage.\textsuperscript{39} Eccentric hypertrophy in transgenic-PKR2 mice was accompanied by a striking increase in the size of blood vessels without increasing the number of vessels. Indeed, enhanced vascular leakiness was resulted from swollen ECs with increased caveolae-like vacuoles and fenestrae. Cardiac PKR2 signaling releases an unknown substance, which promotes tight junction defects between cardiac ECs, resulting from reduced ZO-1 expression.\textsuperscript{39} In summary, overexpression of PKR2 in cardiomyocytes leads to eccentric hypertrophy via an autocrine signaling and provokes fenestration in the ECs, leading to vascular leakage by paracrine signaling. Inhibition of PKR2 signaling could be beneficial for the treatment of cardiomyopathy.

Paracrine Regulation of Vascularization by Epicardial Prokineticin Receptors

PKR1 regulates epicardial–mesenchymal transition to form EPDC during heart development.\textsuperscript{40} Genetic ablation of PKR1 in epicardium leads to a diminished ventricular expansion and septal defects during embryogenesis and severe hypoplasia. PKR1 via activating Akt signaling changes cell morphology, actin cytoskeleton remodeling, and epicardial–mesenchymal transition gene expression profile. Similarly, activation of the phosphatidylinositol 3-kinase/Akt promotes epicardial–mesenchymal transition in developing heart.\textsuperscript{41} In contrast, inhibition of phosphatidylinositol 3-kinase/Akt signaling blocks epicardial–mesenchymal transition in mice.\textsuperscript{32} Epicardial PKR1 contributes to (1) proliferation of EPDC and cardiomyocyte, involving in development of ventricular wall, (2) formation of coronary circulation, and (C) regulation of cardiac rhythmicity. Prokineticin-2/PKR1 signaling induces EPDC proliferation and differentiation into vasculogenic cell type.\textsuperscript{33} Thus, impaired vasculogenesis in the epicardial specific PKR1 mutant mice hearts is because of impaired EPDC proliferation and a defective EPDC differentiation into endothelial and smooth muscle cell type (Figure 1D). These mice models provide genetic models for congenital dysfunction of the heart and should facilitate studies of both pathogenesis and therapy of cardiac disorders in humans.

PKR1-Dependent Regulation of Insulin Uptake in Heart and Kidney

The ECs of these organs exhibited severely decreased fluorescein isothiocyanate (FITC)-insulin uptake. The transcapillary transport of insulin in the vascular wall of older mice was thus defective. Isolated ECs from the mutant cardiac and renal tissues exhibited little insulin uptake, confirming that the loss of PKR1 from EC decreased insulin transport. Overexpressing PKR1 in these ECs promoted FITC-insulin passage. Indeed, the primary defect linking insulin resistance and endothelial dysfunction is believed to be NO deficiency of endothelial origin.\textsuperscript{43} In agreement, insulin uptake and insulin-mediated eNOS activation were impaired in all mutant ECs. Similarly, altered eNOS activation and low insulin action have recently been demonstrated in the endothelium of patients with diabetes mellitus.\textsuperscript{44} Thus, impaired insulin delivery to ECs may lead to defective NOS and eNOS activation in PKR1\textsuperscript{ec−/−}aortas, consequently impairing endothelium-dependent relaxation. These data highlight the role of PKR1 as a positive regulator of insulin uptake (Figure 1E).\textsuperscript{45}

Kidney structure and function in PKR1\textsuperscript{ec−/−} mice have also determined at the age of 12 and 24 weeks.\textsuperscript{38} Twelve-week-old PKR1\textsuperscript{ec−/−} mice displayed dilatation of the Bowman spaces in most glomeruli, a compact glomerulus, fibrosis, and enlarged tubular structures with a swollen necrotic nucleus, abnormal mitochondria, and aberrant organization of podocytes. Abnormal tubular function was evident with higher levels of absolute renal phosphate (P) excretion in the PKR1\textsuperscript{ec−/−} mice because of lower levels of sodium–calcium and sodium–phosphate exchanger transcripts. By 24 weeks of age, the morphological changes in the PKR1\textsuperscript{ec−/−} kidneys were associated with higher levels of apoptosis and impaired insulin-mediated, Akt activity–dependent lipid accumulation. Mutant mice displayed high levels of creatinine clearance and urine volume at 24 weeks of age and proteinuria by 36 weeks of age.\textsuperscript{38} The PKR1\textsuperscript{ec−/−} mouse model provides insight into endothelial dysfunction as the common mechanism that partially underlies the pathological features of heart and kidney (Figure 2). Role
of endothelial PKR2 in vivo on heart and kidney functions has not been studied yet.

Similarly, global PKR1 knockout mice have peripheral obesity accompanied by a diabetes mellitus–like syndrome at the late ages (36 weeks), mainly because of endothelial dysfunction and impaired adipose tissue functions. However, these mice exhibited cardiomegaly, severe interstitial fibrosis, and cardiac dysfunction under stress conditions. They had also renal tubular dilation, reduced glomerular capillaries, urinary phosphate excretion, and proteinuria. PKR1-null mice also exhibited impaired hyperalgesic responses to prokineticin-2 but normal hyperalgesic responses to bradykinin and prostaglandin E2. PKR2 is predominantly expressed in the olfactory bulb. PKR2 knockout mice had abnormal development of the olfactory bulb and reproductive system, similar to the clinical features of Kallmann syndrome, a human disease characterized by association of hypogonadotropic hypogonadism and anosmia. Indeed, loss-of-function mutation in the prokineticin-2 gene causes Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism. Whether PKR2 deficiency leads to cardiovascular diseases under the stress condition remains to be determined.

Conclusions and Perspectives

The structural and functional integrity of the endothelium is required to maintain vascular homeostasis and prevent cellular death in the surrounding tissues. This is highlighted by studies showing that endothelial dysfunction increases the risk of developing target organ damage and cardiovascular and kidney events. Several cardiovascular drugs improve compromised endothelial function presumably because of their pleiotropic and ancillary properties, such as lowering blood pressure, lowering lipid levels, or improving insulin sensitivity. Nonetheless, the endothelium is increasingly becoming a surrogate end point for therapeutic approaches to reduce cardiovascular risk, as demonstrated by its inclusion among markers of organ damage in the latest European hypertension guidelines.

Proangiogenic prokineticins can be an important marker of hypoxic damage in the heart and kidneys. Moreover, prokineticin receptors can be novel target for the treatment of cardiovascular and renal diseases.

Prokineticin Levels as a Marker of Hypoxic Disease and Organ Damage

Prokineticin levels have been associated with many endothelial dysfunction–related diseases. The reduced prokineticin-2 mRNA or protein levels are associated with reduced levels of its receptor PKR1 as compared with control nonfailing human heart tissues. It is likely that prokineticin pathology associated with reduced capillary density in patients with heart failure lead to impaired contractility. The difference of prokineticin-1 levels between failing and nonfailing human hearts has been subtle; however, the changes in prokineticin-2 and its receptor PKR1 levels may be relevant. High prokineticin-2 levels are associated with various cardiometabolic risk factors including high blood pressure and high blood lipid, blood glucose, and uric acid levels. High prokineticin-2 levels are also independently associated with metabolic syndrome. High levels of prokineticin have been found in the white adipose tissue of obese humans.

Choke et al also showed that prokineticin-2 expression increases after abdominal aortic rupture in humans. Prokineticin-2 via PKR2 can be activated by pathological stimuli such as hypoxia–ischemia and excitotoxic glutamate. High levels of prokineticin-2 can also contribute to cerebral ischemia. In agreement, we have shown that the level of prokineticin-2 and its receptor are elevated in ischemic hearts. The upregulated prokineticins and their receptor levels in mice heart 24 hours after myocardial ischemia indicates an activation of the cardioprotective signaling pathway. Whether prokineticin levels in serum of acute myocardial ischemia patients can be marker of wound-healing process needs to be studied.

Prokineticins are important factors for placental development during pregnancy. Increased expression of serum prokineticin-1 during pregnancy may contribute to the development
of preeclampsia and/or intrauterine growth restriction. Sustained high levels of prokineticin-1 beyond the first trimester of pregnancy contribute to the development of pregnancy-induced hypertension.

Prokineticin-1 and PKR1 expression peak during the first trimester of pregnancy, controlling the formation of pseudovascular networks and feto–maternal circulation. Prokineticin-1 is also a key factor in the success of embryo implantation. The deregulation of prokineticin-1 expression is associated with early pregnancy pathologies, such as ectopic pregnancy and repeated miscarriage. Indeed, polymorphisms in the prokineticin-1, PKR1, and PKR2 genes are associated with repeated miscarriage. Circulating prokineticin-1 level in pregnant women is higher during the first trimester than during the second and third trimesters. Prokineticin-1 may be a new non-invasive predictive marker of successful embryo implantation for conventional in vitro fertilization-embryo transfer.

### Prokineticins in Treatment Strategies of Cardiovascular Disease

Several studies have shown that PKR1 signaling in cardiomyocyte is beneficial; however, PKR2 is detrimental, indicating that the beneficial effects of PKR1 signaling counteract those of PKR2. Thus, PKR1 agonist or PKR2 antagonist can be used for treatment of heart and kidney diseases (Table).

<table>
<thead>
<tr>
<th>Ligands</th>
<th>PKR1</th>
<th>PKR2</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PC 1: [2-(5-(4-ethylbenzyl)-1-(4-methoxy-benzyl)-1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazin-2-ylamino)ethyl]guanidine</td>
<td>IC50: 100 nmol/L</td>
<td>IC50: 100 nmol/L</td>
<td>Balboni et al.</td>
</tr>
<tr>
<td>PC-7: N-[2-[5-(4-fluoro-benzyl)-1-(4-methoxy-benzyl)-4,6-dioxo-1,4,5,6-tetrahydro-[1,3,5]triazin-2-ylamino]-ethyl]-guanidine</td>
<td>IC50: 33 nmol/L</td>
<td>IC50: 5230 nmol/L</td>
<td>Jacobson et al.</td>
</tr>
<tr>
<td>A-24 peptide</td>
<td>antagonist</td>
<td>agonist</td>
<td>Miele et al.</td>
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AGN013782 indicates the half maximal effective concentration; IC50, the half maximal inhibitory concentration; PKR1, prokineticin receptor 1; and PKR2, prokineticin receptor 2.

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### Disclosures

None.

### References

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