Genetic Inactivation of Prokineticin Receptor-1 Leads to Heart and Kidney Disorders

Mounia Boulberdaa, Gulen Turkeri, Kyoji Urayama, Mojdeh Dormishian, Cécilia Szatkowiski, Luc Zimmer, Nadia Messaddeq, Virginie Laugel, Pascal Dollé, Canan G. Nebigil

Objective—Prokineticins are potent angiogenic hormones that use 2 receptors, prokineticin receptor-1 (PKR1) and PKR2, with important therapeutic use in anticancer therapy. Observations of cardiac and renal toxicity in cancer patients treated with antiangiogenic compounds led us to explore how PKR1 signaling functioned in heart and kidney in vivo.

Methods and Results—We generated mice with a conditional disruption of the PKR1 gene. We observed that PKR1 loss led to cardiomegaly, severe interstitial fibrosis, and cardiac dysfunction under stress conditions, accompanied by renal tubular dilation, reduced glomerular capillaries, urinary phosphate excretion, and proteinuria at later ages. Abnormal mitochondria and increased apoptosis were evident in both organs. Perturbation of capillary angiogenesis in both organs was restored at the adult stage potentially via upregulation of hypoxia-inducible factor-1 and proangiogenic factors. Compensatory mechanism could not rerove the epicardial and glomerular capillary networks, because of increased apoptosis and reduced progenitor cell numbers, consistent with an endogenous role of PKR1 signaling in stimulating epicardin + progenitor cell proliferation and differentiation.

Conclusion—Here, we showed for the first time that the loss of PKR1 causes renal and cardiac structural and functional changes because of deficits in survival signaling, mitochondrial, and progenitor cell functions in found both organs. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: angiogenesis ■ cardiomyopathies ■ kidney ■ pharmacology ■ receptors

Pathological angiogenesis is characterized by either excessive (eg, cancer) or inadequate (eg, coronary artery disease) neovascularization. Identification of molecular and cellular components involved in promoting or inhibiting angiogenesis is emerging to develop angiogenesis based treatments.

Prokineticins 1 (also called EG-vascular endothelial growth factor [EG-VEGF]) and 2 (also called Bv8) have been identified as potent angiogenic factors. These 2 secreted proteins from the AVIT secreted protein family are widely distributed in mamma-

lian tissues and human blood cells. In addition to being strong angiogenic factors, prokineticins act as survival/mitogenic fac-
tors in various cells, including endothelial cells, neuronal cells, lymphocytes and hematopoietic stem cells, and cardiomyocytes. They exert their biological effects by stimulating 2 closely related receptors, prokineticin receptor-1 (PKR1) and PKR2. Prokineticin-2 is the most potent agonist of both these receptors. Both receptors use the various G protein signaling pathways and are ubiquitously expressed in mammalian tissues.

Aberrant prokineticin signaling may also cause hypervascu-

larity in various tissues and is strongly associated with the development of polycystic ovary syndrome; neuroblastoma and colorectal, testicular, and prostate cancer. It has recently been demonstrated that prokineticin-2 is secreted by tumor-infiltrating myeloid cells and strongly promotes tumor angiogenesis. Antiprokineticin-2 treatment significantly decreases myeloid cell mobilization, thereby inhibiting tumor growth. Thus, the use of immunoneutralizing antibodies or antagonists of prokineticin-2 and its receptors may be a useful strategy for cancer treatment. However, antiangiogenesis treatments targeting platelet-derived growth factor (PDGF), VEGF and its recep-
tors, or RTK signaling have been associated with clinical renal and cardiac toxicity. Thus, antiangiogenesis treatment-related cardiovascular and renal side effects and impaired tissue repair increase the risk of heart and kidney failure in cancer patients. However, little is known about the role of PKR1 signaling in the kidney and cardiovascular system.

The low levels of PKR1 and prokineticin-2 transcripts and proteins in cardiac samples from humans with end-stage heart failure suggest that prokineticin-2/PKR1 signaling is particularly important in the heart. Intracardiac transient PKR1 gene transfer decreases mortality rate and preserves left ventricular function by promoting angiogenesis and cardiomyocyte survival in a mouse model of myocardial infarction. Interestingly, PKR1

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From the Centre National de la Recherche Scientifique, Université de Strasbourg, UMR, Ecole Supérieure de Biotechnologie de Strasbourg, Illkirch, France (M.B., G.T., K.U., M.D., C.S., C.G.N.); CERMES, Imagerie du vivant and Université Lyon 1 (L.Z.); Institut de Génétique et de Biologie Moleculaire et Cellulaire, UMR 7104 Centre National de la Recherche Scientifique, U964 Institut National de la Santé et de la Recherche Médicale, Illkirch, France (N.M., V.L., P.D.).
Correspondence to Canan G. Nebigil, ESBS, Bd Sébastien Brandt BP 10413, F-67412 Illkirch, France. E-mail canan.nebigil-desaubry@unistra.fr © 2011 American Heart Association, Inc.
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overexpression in mouse heart does not lead to spontaneous abnormalities in cardiomyocytes but to an increase in capillary density and the number of coronary arterioles. Cardiac PKR1 signaling leads to the upregulation of the ligand of this receptor, prokineticin-2, as a paracrine factor inducing the proliferation and differentiation of epicardin+, epicardium-derived progenitor cells, thereby regulating postnatal coronary angiogenesis and vasculogenesis. PKR1 signaling via Akt protects cardiomyocytes, as well as endothelial cells, against apoptosis. A divergent role of prokineticin-2 in endothelial cells has recently been described, with the activation of PKR1 signaling promoting angiogenesis and the activation of PKR2 inducing fenestration in endothelial cells.

On the basis of the occurrence of cardiac and renal conditions in patients treated with angiogenesis inhibitors, we hypothesized that PKR1 signaling might play a key role in heart and kidney function. We show here that the loss of PKR1 causes structural and functional changes in the kidney and heart reminiscent of adverse cardiovascular and renal events induced by treatments inhibiting angiogenesis.

Methods

Generation of Knockout Mice

For conditional disruption of the GPR73 gene encoding PKR1, mice transgenic for the germline null allele of PKR1 were generated in the transgenic core facility of the Mouse Clinical Institute, Illkirch, France, as previously described (Supplemental Material, available online at http://atvb.ahajournals.org). Loss of PKR1 in heart and kidney was verified at the transcriptional and protein levels. The PKR2 mRNA expression remained unchanged in heart and kidney (Supplemental Figure I). All animal experimentation was performed in accordance with institutional guidelines of the French Animal Care Committee, with European regulation–approved protocols. Unless otherwise mentioned, analyses were performed on mice aged 24 weeks.

Histological and Electron Microscopy Analyses

Hearts were removed from neonatal or 24-week-old mice, dissected, and frozen for the cutting of frozen sections (10 μm), which were stained with Mallory tetrachrome. Echocardiography and Hemodynamic Assessment

Systolic function in 24-week-old male mice (n = 12) was assessed by echocardiography in M-mode (Sonos 5500, Hewlett-Packard, with a 15-MHz linear transducer) and 2-dimensional measurements (Supplemental Material).

Isolation of Cardiomyocytes

Ventricular cardiomyocytes were isolated from adult mice as previously described.

Terminal dUTP Nick-End Labeling Assay, Immunohistochemistry, and Western Blot Analysis

Terminal dUTP Nick-End Labeling (TUNEL) assays, immunofluorescence, Oil Red O staining, and Western blot analyses were performed according to the manufacturer’s instructions (Supplemental Material). The intensity of staining of each section was quantified with ImageJ software.

RNA Extraction, Quantification, and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was isolated from neonatal and adult mouse hearts and kidneys with TRI Reagent (Molecular Research Center) and treated with DNase, using the RNase-Free DNase Set, as previously described. Primer sequences are shown in Supplemental Table I.

In Situ Hybridization

Serial cryosections (10 μm) were collected on gelatin-coated (Superfrost Plus, Kindler) slides. In situ hybridization was performed as described in the gene expression section of the EMPReSS web site (http://empress.har.mrc.ac.uk/browser) (see Supplemental Material).

Biochemical Analyses on Blood and Urine Samples

Blood samples were collected and analyzed as outlined on the EMPReSS web site (http://www.empress.har.mrc.ac.uk) (see Supplemental Material).

Kidney Explants and Isolation of Epicardin+ Progenitor Cells

Renal explants and neonatal renal–epicardium-derived progenitor cell cultures were performed as previously described for cardiac explants and cardiac–epicardium-derived progenitor cell cultures (Supplemental Material).

Statistical Analysis

Data are expressed as means±SEM. Multigroup comparisons were carried out by 1-way ANOVA with post hoc correction. The Student t test was used for pairwise comparisons between groups. For all analyses, values of P<0.05 were considered significant.

Results

PKR1-Null Mutant Mice Display Histological and Morphological Alterations to the Heart

Mutant mice developed dilated cardiomyopathy, as demonstrated by the presence of cardiomegaly and chamber enlargement (Figure 1A and 1B). Mallory tetrachrome staining revealed a severe interstitial fibrosis (blue), cellular disorganization, and an abnormal alignment of myofibers in the epicardial and subepicardial area of mutant hearts (Figure 1C). A corresponding decrease in LV mass was also detected in mutant hearts (Figure 1D). The ratio of heart weight with blood in the ventricular cavities to body weight was slightly but significantly (P<0.05) higher in mutant mice (6.1±0.01 mg/g, n=6) than in wild-type mice (5.3±0.01 mg/g, n=6). Reverse transcription–polymerase chain reaction analysis of mRNA from the hearts of 24-week-old mutant mice showed the levels of expression of the collagen 1a1 and 1a2 genes to be particularly high in mutant mice, consistent with the development of severe cardiac fibrosis (Supplemental Figure IIB). A reduced level of the contractile sarcomeric protein troponin C was evident in the mutant hearts (Supplemental Figure IIC). No changes in expression levels of intracellular Ca2+ cycling genes were observed in mutant hearts (Supplemental Figure III).

Morphometric analysis confirmed that isolated cardiomyocytes from 24-week-old mutant hearts were significantly (33%) larger than those from wild-type hearts (Figure 1E). Mean cardiomyocyte length was 405.3±16.7 μm for the wild-type and 512.09±22.7 μm for the mutant (n=420, P<0.001). Dystrophin staining of cryosectioned hearts confirmed an increase in cardiomyocyte length (Supplemental Figure II). Thus, PKR1-null mice develop physiologically significant, but compensated, moderate dilated cardiomyopathy.
PKR1 Deficiency–Induced Cardiac Dysfunction

Echocardiographic analysis revealed a significant decrease in interventricular septal thickness and LV posterior wall thickness was observed in mutants (Supplemental Table II). LV end-diastolic and end-systolic dimensions under basal conditions did not differ significantly between mutant and wild-type mice. However, in conditions of stress, such as the response to β1-adrenergic stimuli (dobutamine injection), mutant mice displayed significantly a reduction in fractional shortening and in ejection fraction (Figure 1F). An increase in both LV end-diastolic and end-systolic dimensions was observed after dobutamine treatment. No change in expression of the β1-adrenergic receptor was detected in mutant hearts, ruling out the possibility of changes in the β-adrenergic sensitivity of the heart (Supplemental Figure III). The clinical course of the disease was analyzed by examining mutant hearts at neonatal stages (P1). Histological analyses provided evidence of dilated cardiomyopathy in neonatal mutant hearts (n=6, Figure 1G).

PKR1 Deficiency-Induced Cardiac Dysfunction Is Associated With Abnormal Mitochondrial Structure and Function and an Increase in Apoptosis

Electron microscopy showed that the hearts of 24-week-old mutants had a thin epicardium with an abnormal subepicardial accumulation of cardiomyocytes and high levels of collagen deposition (Figure 2A). The mutant cardiomyocytes had undulating cellular membranes and displayed focal myofibril disorganization, a decrease in sarcomere length and aberrant mitochondria, myocyte fragmentation, a modified distribution of mitochondria between myofibrils, and myofibril disarray (Figure 2B). Staining for a cell adhesion molecule, N-cadherin, confirmed the disassembly of intercalated disc structures and abnormal structure of the mutant heart. Furthermore, mutant hearts had more mitochondria than wild-type hearts, and these mitochondria were densely packed and appeared to be fused (Figure 2B). Mitochondrial function was assessed by carrying out a histochemical analysis of the enzymes involved in respiratory chain function, such as succinic dehydrogenase, and Oil Red O staining. Succinate dehydrogenase (SDH) activity in the subepicardial area of mutant hearts was significantly lower than that in wild-type hearts, consistent with the defects observed in mitochondrial morphology (Figure 2C). Oil Red O staining showed that mutant myocytes contained larger numbers of lipid droplets, with levels of lipid accumulation within these cells 3 times higher than in the wild-type (Figure 2D).

To investigate the role of programmed cell death in the dilated cardiomyopathy of the mutants, we carried out TUNEL analyses on heart sections from mutant and wild-type mice (Figure 2E). The abundance of TUNEL-positive epicardial and myocardial cells (green) was detected in the mutant hearts.

PKR1 Deficiency-Induced Renal Abnormalities Accompanied Apoptosis and Reduced Capillary Lumen in Glomeruli

Macroscopic inspection showed the kidneys of mutant mice to be massively enlarged, with an irregular surface presenting multiple cortical swellings (Figure 3A). In some cases, 1 kidney was atrophied or displayed massive urine retention (Figure 3A and 3B). Overall, the ratio of kidney weight with urine in the cortical swellings to body weight significantly increased in PKR1-null mice as compared with wild-type mice at the age of 24 weeks (Figure 3C). We also examined neonatal kidneys to determine the clinical course of the disease. Kidney hypoplasia was also evident in neonatal mutants (Figure 3D). TUNEL assays in kidney sections from mutant and wild-type mice revealed that the glomeruli of
mutant kidneys contained significantly more TUNEL-positive cells (green) than those of wild-type kidneys, at the age of 24 weeks (Figure 3D). Accordingly, mutant glomeruli contained fewer PECAM-1-positive visible capillary endothelial cells (Figure 3F).

Renal Ultrastructural Analysis and Abnormal Mitochondria

Systematic histological examinations of mutant mice showed dilatation of the Bowman’s spaces in glomeruli (Figure 4A, upper panels). Electron micrographs showed considerable thickening of the lamina densa of the glomerular base membrane, with the presence of numerous protrusions on the podocytic side (Figure 4A, lower panels). Mallory tetrazolium staining revealed dilated renal tubules and severe interstitial fibrosis between the tubules (Figure 4B, upper panels). In the mutant kidney, peritubular capillaries were occupied by structures with multiple lumens and prominent interstitial cells, often in loose associations, corresponding to dysmorphogenic capillaries (Figure 4B, middle panels). The number and density of mitochondria in proximal tubule cells were significantly lower in mutant animals than in wild-type animals. These abnormalities in mitochondrial density were accompanied by changes in mitochondrial structure, such as mitochondrial swelling, and decreases in the numbers of cristae and granulocytes (Figure 4C, lower panels). Consistent with the ob-
served defects in mitochondrial morphology, mutant kidneys displayed only 75% of the SDH activity of wild-type kidneys (n=6, Figure 4D).

PKR1 Deficiency-Induced Renal Dysfunction
Renal functions of 24-week-old mice were assessed by blood and urine chemistry. The 24-hour urine flow of mutant mice was significantly lower than that of wild-type mice. However, no differences in water and food intake and blood glucose levels were observed (Table). No significant changes in urinary potassium, sodium, and chloride excretion rates were observed between mutant and wild-type mice, consistent with the values obtained for serum (Table). Mutant mice had a significantly lower glomerular filtration rate, as estimated by creatinine clearance. Lower creatinine concentrations (mmol/24-hour per g) in the urine of mutant mice were associated with higher concentrations in the serum of these mice. However, the urine phosphate/creatinine ratio but not calcium/creatinine ratio was significantly higher in mutant mice than in wild-type controls. Mutant mice displayed higher levels of absolute renal phosphate excretion, associated with hypophosphatemia (Table), indicating that mutant mice displayed poor absolute renal phosphate retention in the kidney and poor stabilization of serum absolute renal phosphate concentration. Note that mutant mice exhibit protein urea at the age of 36 weeks (total proteins in urine were 60.625±0.70 and 10.72±2.4 g/L for wild-type and mutant, respectively, n=5, P<0.05), consistent with fenestrated endothelial cell structures at this age (Supplemental Figure VIC).

Characterization of Cardiac and Renal Function Impairment
We determined the potential defect in vascularization by determining the numbers of PECAM-1+ capillary density in hearts and kidneys. Neonatal PKR1-null mutant mice exhibit reduced numbers of capillary number and density as detected by PECAM-1 staining in the heart and kidneys compared with their wild-type littermates (Figure 5A). However, at the age of 24 weeks, no difference in the level of angiogenesis was observed in the mutant hearts and kidneys at the expense of epicardial and glomerular zones compared with wild-type hearts and kidneys (Supplemental Figure IV). A reduction of angiogenesis was observed in mutant epicardium by 12% (n=4, P<0.05) and in mutant glomerulus by 39% (n=4, P<0.05). Hypoxia-inducible factor-1α (HIF-1α) transcript levels were significantly increased in the mutant hearts but not in the kidneys at the postnatal day 1. However, at the age of 3 weeks, inductions of HIF-1α transcript and protein were observed in both heart and kidneys of mutants (Figure 5B and 5C). Parallel to HIF-1α induction at this age, the proangiogenic factors PDGF-B and fibroblast growth factor-2 transcripts were increased in both mutant heart and kidneys. VEGF transcripts were enhanced slightly in the heart but significantly in the kidneys of the mutants (Figure 5D). These data indicate the initiation of compensatory signaling at these time points. Next, we investigated whether the PKR1 survival pathway is impaired in the neonatal heart and kidney. Indeed, activation of Akt kinase appeared reduced in mutant both kidneys and hearts compared with wild-type, indicated by 60% or greater reductions in phospho-Akt (Figure 5F), confirming the increased cell death in these organs at the early stage (Supplemental Figure IV). Costaining of the apoptotic marker active caspase-3 and cell-specific markers illustrated that cardiomyocytes (troponin+), epicardial and glomerular progenitor cells (epicardin+), and glomerular endothelial cells (PECAM+) were also positive for active caspase-3 (Figure 5F) only in mutant hearts and kidneys at the age of 24 weeks. These data clearly indicate that the angiogenic defects in the mutant are ameliorated over time but cannot compensate for the survival deficit in these cells. Supporting these findings, we found that the VEGF level was increased in the extraglomerular area but not in the glomerular zone of the mutant kidneys (Figure 5G).

Interestingly, epicardin+ cells in the mutant epicardium-subepicardium and glomerulus were significantly lower than those in the wild-type tissues (Figure 6A). To assess the possible direct effect of PKR1 signaling on renal-epicardin+ cells as shown for cardiac-epicardin+ cells,19 we used explant cultures...
from wild-type and mutant kidneys from postnatal day 1 (P1). In untreated kidney explants, 50±6% of the cells proliferate, as shown by Ki67 staining (Figure 6B). The treatment of kidney explants with prokineticin-2 (5 to 10 mmol/L) stimulated the extensive outgrowth of cells (Figure 6B, histogram). Of the emerging epithelial cells, 90% were positive for epicardin (red). Prokineticin-2 induced differentiation of renal-epicardin+ cells into PECAM-1+ endothelial (red) and α-smooth muscle actin+ vascular smooth muscle cells (green) that were blocked in the mutant renal-epicardin+ cells (Figure 6B, histogram, and Supplemental Figure VA). Similar results were obtained in epicardin+ isolated cell cultures derived from kidney explants (Supplemental Figure VB).

**Discussion**

Several angiogenic factors have been shown to regulate heart and kidney functions. Mice lacking Pdgf-B display renal and cardiovascular abnormalities.25,26 VEGF has been shown to be involved in heart development,27 glomerulogenesis, and tubulogenesis in the kidney.28 Fibroblast growth factor is also involved in heart development and function29 and in glomerulus formation.30 Here, we provide the first evidence that angiogenic PKR1 signaling regulates heart and kidney functions that are correlated with the expression pattern of PKR1 in heart and kidney (Figure 6 and Supplemental Figure IE). Acute or chronic dysfunction in the heart or kidney may lead to dysfunction in the other organ, resulting in the cardiorenal syndrome.31 However, in PKR1-null mutant mice, both organs displayed morphological abnormalities at the neonatal stage, eliminating the possibility of a cardiorenal syndrome. The cardiomyopathy induced by the absence of PKR1 was associated with decreases in LV mass and LV posterior and interseptal diameters, cardiomyocyte elongation, and an increase in apoptosis, together with abnormal mitochondrial structure and function, with no change in indices of cardiac function in basal conditions. However, systolic and diastolic dysfunctions, as determined by measuring the percentage of fractional shortening and ejection fraction, were evident under stress conditions, such as those induced by adrenergic stimuli. Impaired contractility in mutant hearts could result from reduced levels of contractile protein TnTC, because changes in TnTC lower contractile force by reducing strong myosin-actin binding in human dilated cardiomyopathy.32 The lack of increase in arterial blood pressure in PKR1-null mutants compared with wild-type mice (110±7 and 105±8 mm Hg, respectively; Supplemental Material) strongly indicates that cardiomyopathy was not a consequence of the hemodynamic alterations responsible for cardiac overloading, instead resulting directly from the loss of PKR1 in the heart.

We show here that PKR1-null mutant mice also developed renal functional abnormalities. Creatinine clearance was weaker in mutant mice than in the wild-type, and mutant mice had a low glomerular filtration rate. Increased thickness of glomerular base membrane and reduced filtration rates may explain the lower protein levels in the 24-week-old mutants. However, mutant mice exhibit high levels of proteinuria at the age of 36 weeks, probably because of progressive glomerular endothelial dysfunction33 (Supplemental Figure VIC). Ablation of PKR1 resulted in the enlargement of tubules and an increase in the excretion of phosphate into urine. The effects of PKR1 loss-of-function on urinary phosphate excretion were accompanied by hypophosphatemia. It is clear that the hypercreatinemia in mutant mice resulted from the hypoexcretion of creatinine. PKR1 ablation did not cause a renal calcium leak, eliminating the possibility of coordinated ef-
PKR1 also serve as survival factors for corpus luteum–derived induction of apoptosis by hypoxic stimuli. Prokineticins via bodies in cardiomyocytes (cm), epicardium (epi), and glomerulus. G, VEGF immunostaining of glomerulus (glm) and tubules (tbl).

HIF-1α transcription levels in RNA derived from neonatal (left) and 3-week-old (right) heart and kidneys (n=4, P<0.05). C, Western blot analyses with HIF-1α and GAPDH antibodies on hearts and kidneys confirm an increase in HIF-1α levels in 3-week-old mutant heart and kidneys (n=3, duplicated, P<0.05). D, Histogram represents reverse transcription–polymerase chain reaction analysis showing that expression of proangiogenic factors PDGF, fibroblast growth factor (FGF), and VEGF was differentially altered in mutant heart and kidneys at the age of 3 weeks (n=4). E, Western blot analysis and histogram show that diminished levels of phosphorylated (p-)Akt form in neonatal mutant both hearts and kidneys (n=4). F, Immunostaining of heart and kidney sections with active caspase-3 antibody and cardiomyocyte-specific tropo- nin, endothelium-specific PECAM-1, and progenitor cell–specific epicardin anti-

Our findings raise the possibility that abnormal mitochondrial structure and low oxidative capacity of the mitochondria in mutant mice accelerate the onset of the observed cardiac and renal abnormalities and may trigger a subset of apoptotic signals and adaptive mechanisms for the inhibition of angiogenesis. Recent reports have demonstrated that mitochondrial dysfunction is involved in either the etiology or the underlying pathological feature of various renal diseases and is a major cause of cardiac dysfunction. Profound reductions in Akt activation might increase mitochondrial apoptosis, possibly accounting for the loss of epicardial cells and cardiomyocytes in the mutant hearts, leading to the thinning of the ventricular wall. The kidney cortex in PKR1 mutant mice appeared thinner than that of wild-type mice as a consequence of urinary retention, which is consistent with the lower Akt activity and increased apoptosis. The data presented here are the first in vivo report that PKR1 signaling prevents apoptosis in cardiomyocytes, epicardium, and glomerular endothelial and progenitor cell consistent with the previous in vitro findings that PKR1 via activates Akt signaling, protecting cardiomyocytes and endothelial cells against the induction of apoptosis by hypoxic stimuli. Prokineticins via PKR1 also serve as survival factors for corpus luteum–derived and bovine aortic and human placental endothelial cell and umbilical vein–derived macrovascular endothelial cells.

Neonatal and 3-week-old PKR1 mutant mice have fewer capillaries than normal in heart and kidney, potentially establishing hypoxic conditions with impaired mitochondrial function. Mitochondrial activity has also been shown to be involved in HIF-1α activation in mildly hypoxic conditions. In the mutant heart and kidneys, these defects in angiogenesis were compensated by an increase in HIF-1α production accompanying an increase in proangiogenic factors. Interestingly, compensated mechanism reverts the extraepicardial and extravascular capillary networks in mutant hearts and kidneys. Interstitial cells and tubular epithelium also provide signals such as VEGF for maintenance of the peritubular capillary network, independent of the glomerular capillary network. Our findings clearly demonstrated that VEGF level indeed was increased in mutant tubular epithelium, possibly restoring extravascular angiogenesis. Currently, we are investigating critical components of compensatory angiogenesis in mutant hearts.

Restoration of the angiogenic pathways could not reverse damages in PKR1 mutant hearts and kidneys because of defective survival pathways, such as reduced Akt activity, increased caspase-3 activity, and impaired common progenitor cell functions. Moreover, an abundance apoptotic epicardin+ cells in mutant epicardium and glomeruli can be considered a failure of endothelial repair mechanisms in epicardial and glomerular angiogenesis. Recently, we have shown that prokineticin-2 via PKR1 induces the proliferation and differentiation of epicardin+, cardiac epicardin+ cells into endothelial and smooth muscle cells, thereby regulating postnatal coronary angiogenesis and vasculogenesis. Renal epicardin+ cells may play an important role in vascular remodeling during glomerular development.
(Pod1)–null mice have been shown to have heart defects and a complex kidney phenotype. Recently, a relationship between the epicardium and the glomerulus was identified, originating in a similar progenitor tissue, and expressing similar genes. We show here that PKR1 signaling controls progenitor cell differentiation that may be involved in glomerular and epicardial angiogenesis. We currently initiate the cell-specific inactivation of PKR1 in the mouse model to further and fully elaborate the mechanism underlying pathology in PKR1 mutant heart and kidneys.

Several anticancer drugs inhibiting PDGF receptor, VEGF, or multiple receptor tyrosine kinase signaling have recently been associated with cardiac and renal impairments, including proteinuria, hemorrhage, thrombosis, hypertension, and impairment in wound healing and tissue repair. Moreover, the activation or upregulation of other proangiogenic signaling pathways as a form of evasive resistance to angiogenesis inhibitors has been shown to circumvent antiangiogenic treatment, triggering neovascularization in due course. Here we showed that angiogenic PKR1 inhibition provokes cardiac and renal impairments, proteinuria, and rebound development of angiogenesis. PKR1-null mutant mice exhibit progressive fibrosis in their remodeling hearts and kidneys, possibly because of hypoxia, which may also drive fibrosis. On the contrary, anti-VEGF therapy reduces fibrosis and collagen deposits in the lungs and the liver with unknown mechanism; it is not evident that it is due to inhibition of VEGF signaling, because administration of VEGF had a clear therapeutic benefit, slowing the development of renal fibrosis. Note that some of the adverse effects of antiangiogenic therapeutics could be due to off-target effects of the drugs.

Collectively, schematic illustration (Supplemental Figure VII) shows that PKR1 inactivation in mice leads to deficits in Akt survival signaling, mitochondrial functions, and progenitor cell functions that could be the link between cardiac and renal damage. This study may provide new insight into the pathogenesis of cardiovascular and renal diseases and lead to improvements in the management of adverse effects of antiangiogenic therapy.

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Disclosures
None.

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Figure 6. A, Epicardin + progenitor cells (green) in heart and glomeruli. Magnification, ×40. Quantification of epicardin positive cells was calculated as numbers of epicardin positive cells/4’,6-diamidino-2-phenylindole (dapi+) cells corrected with 100 per high-power field (n=5, P<0.05). B, Representative illustration of epicardin+, Ki67+, PECAM-1-, and α-smooth muscle actin (SMA)-positive cell number in the renal epicardium-derived progenitor cells derived from kidney explants treated with vehicle (veh) or prokineticin-2 (PK-2) (5 or 10 nmol/L) for 24 hours. Magnification, ×63. Histograms show the quantitative changes between wild-type (+/−) and null mutants (−/−). Quantification of the specific cell number/dapi positive cell number corrected with 100 per high-power field (n=4, P<0.05).
Genetic inactivation of prokineticin receptor-1 leads to heart and kidney disorders

Short title: Loss of PKR1 induces heart and kidney abnormalities

Mounia Boulberdaa, Gulen Turkeri, Kyoji Urayama, Mojdeh Dormishian, Cécilia Szatkowiski, Luc Zimmer, Nadia Messaddeq, Virginie Laugel, Pascal Dollé and Canan G. Nebigil

1Centre National de la Recherche Scientifique (CNRS), Université de Strasbourg, UMR, Ecole Supérieure de Biotechnologie de Strasbourg, Illkirch France, 2CERMEP, Imagerie du vivant and Université Lyon 1, 3Institut de Génétique et de Biologie Moléculaire et Cellulaire, UMR 7104 CNRS, U 964 INSERM, Illkirch France

Supplemental Material

Generation of knockout mice:
To conditionally disrupt the GPR73 gene encoding PKR1, embryonic stem (ES) cells were electroporated with the targeting vector pGPR73 L3 that encompasses GPR73 exon 2 and contains a loxP site in the intron located upstream of exon 2, whereas a loxP-flanked (floxed) neomycin (neo) selection cassette is present in the intron downstream of exon 2. To excise floxed DNA segments of the GPR73 L3 allele, GPR73 L3/+ mice were bred with CMV-Cre transgenic mice that express Cre recombinase in germ cells. PCR analysis of tail DNA revealed a Cre-dependent GPR73 L- allele excision, yielding GPR73 +/L– heterozygous animals. After heterozygous interbreeding, GPR73 L-/L- mice were recovered at Mendelian frequency, survived to adulthood, and were fertile. These mice have a reduced life span by 6% as compare to wild type.

Echocardiographic and hemodynamic analyses:
Echocardiographic analyses were performed on slightly anesthetized animals as that allows the maintenance of heart rate close to the physiological values (450-500 beats/min), a condition necessary for accurate measurements of LV diameters. End diastole was defined as the maximal left ventricle diastolic dimension and end systole was defined as the peak of posterior wall motion. Single outliers in each group were omitted for statistical analysis. Fractional shortening (FS), a surrogate of systolic function, was calculated from left ventricle dimensions as follows: FS = ((EDD - ESD)/EDD) times 100%. Ejection fraction (EF) was calculated as follows: (LV diastolic volume-LV systolic volume)/LV diastolic volume. We calculated mean measurements for six selected cardiac cycles from at least two independent scans performed in a randomized blind fashion, with papillary muscles used as a point of reference to ensure that all scans were carried out at the same level.

Blood pressure measurements.
Systolic blood pressure was measured in the morning in trained conscious mice by indirect tail-cuff sphygmanometer (Visitech BP-2000 Blood Pressure Analysis System).

TUNEL Analysis:
Briefly, frozen sections were fixed in 4% freshly prepared paraformaldehyde in PBS for 20 minutes and incubated in permeabilization solution (0.1% Triton, 0.1% sodium citrate), followed by incubation with TUNEL reaction mixture at 37°C for 60 minutes in a dark, humidified chamber. Sections were then washed, counterstained with DAPI (0.5 µg/mL in PBS), and mounted with VECTASHIELD medium (Vector Laboratories, Inc, Burlingame, Calif).

For staining the tissue section for succinate dehydrogenase activity, the hearts and kidneys were frozen, and 5-µm sections were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M sodium succinate
and 0.1% nitro blue tetrazolium at 37 °C for 5 min. The staining intensity (blue color) of each section was quantified by using Image software. We acquired 6 to 10 images per heart or kidney (3 to 4 animals per genotype group) with a fluorescence microscope (Leica). Results are expressed as the percentage of apoptotic cells. The TUNEL labeling index was calculated as the mean number of DAPI-stained TUNEL-positive nuclei/glomerulus, for 50 glomeruli per kidney (n=3) or of DAPI-stained TUNEL-positive nuclei in 10 high-power microscopic fields (x 40) per heart section (n=3), for the various groups of mice. Glomerular pathology analyses were based on the assessment of 50 glomeruli/kidney cross-section. Tubule damage (consisting of at least one of the following: dilatation, atrophy, necrosis) was assessed by scoring 100 renal proximal tubules/kidney cross-section on randomly selected high-power microscopy fields (x40).

Frozen tissue sections for the immunofluorescence staining of structural proteins were fixed, blocked and stained with primary antibodies against PECAM-1, podocin, NaPi, Wt1, Dystrophin, N-cadherine and PKR2, (Santa Cruz); LC3, and epicardin (abcam), α-SMA (sigma), active capase-3 (Millipore), and PKR1 (IGBMC, Illkirch). Antibody binding was detected by incubation with Fluorescein, Alexa 555- or Alexa 488-conjugated secondary antibodies. Finally, the nuclei were stained with DAPI. Fluorescence was analyzed on a Leica Microsystems TCS SP5 laser scanning confocal microscope. We determined the number of PECAM-1-positive, DAPI-stained cells per glomerulus, by counting 50 glomerular fields per kidney section for each group of mice.

For staining the tissue section for oil red O, cryosectiones (5 µm) were fixed in 10% formalin for 30 min, and then washed in distilled water and stained with 0.5% oil red O in 36% Triethylphosphat (Sigma-Aldrich) for 30 min to identify neutral lipids, cholesterol, and fatty acids (red colour). After rinsing with water, nuclei were counterstained (blue) with Mayer’s hematoxylin for 5 min and washed in distilled water for 3 times. Finally, slides were covered in aqueous mount under a coverslip for viewing with a light microscope at equal light intensity. Images from the stained slides of mice were initially acquired using a 24-bit file format. ImageJ 1.37v software (National Institutes of Health) was used to convert bright-field (24-bit) images of Oil red O stainings to 8-bit images. Threshold values were chosen that maximize selection of the Oil red O positive tissue while minimizing background interference. Thus, the total number of lipid drops from each image (percent) was quantified.

**RNA Extraction, Quantification, and Reverse-Transcription Polymerase Chain Reaction Analyses.**
A minimum of 3 mouse heart or kidney samples for each of the genotype groups were analyzed in each experiment. Extracted RNA samples were used as the template for cDNA prepared via a reverse-transcription polymerase chain reaction that was performed in duplicate for all RNA samples. Semi-quantitative or real-time reverse-transcription polymerase chain reaction (MiQ, Biorad) was then used to determine the mRNA expression of indicated genes. The GAPDH housekeeping gene was used as a control. Results were analyzed with Bio-Rad qPCR software version 2.0.

**In situ hybridization:**
DNA templates for producing mouse PKR1 riboprobes were generated from PCR expression vector encoding full length PKR1 after the linearization with BamHI or EcoRV and transcription with T7 or Sp6 RNA polymerases to generate the antisense and sense probes, respectively. Digoxigenin-labeled (DIG-RNA labeling Mix, Roche) riboprobes were transcribed in vitro in antisense or sense orientations using T7, Sp6, or T3 RNA polymerases as indicated above. Some of the experiments were performed using a Tecan GenePaint Robot and a Tyramide signal amplification method (for details, see www.eurexpress.org and www.genepaint.org). In each hybridization experiment, control sense probes were included, allowing the determination of possible false-positive signal.

**Western blot analysis**
Tissues were lysed in lysis buffer. Total protein was separated by SDS-PAGE in 7 or 12% acrylamide gels and blotted onto PVDF membranes. The membrane was blocked and incubated with primary antibodies, troponin C, HIF1α (Santa Cruz); phosho-Akt and total Akt (Cell Signaling). It was then incubated with
horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary immunoglobulins. The signal was then detected by enhanced chemiluminescence (Pierce, Rockford, IL), according to the manufacturer's protocol. Akt activity was evaluated and quantified by Western blot analysis and densitometry, using antibodies specific for Akt kinase at the residues (Ser-473) that are phosphorylated upon activation.

**Biochemical analyses on blood and urine samples:**
After an overnight fast, blood was collected at 8 a.m. by retro-orbital puncture after a short anesthesia with isoflurane (Forene®, Abbott France). 300 µl of blood was collected into a heparinized tube for biochemical analysis. Urine was obtained from individual mice housed in metabolic cages for 24 hours. Urine and plasma creatinine concentrations were determined by a modification of Jaffé’s reaction method. The parameters were determined using an Olympus analyzer with kits and controls supplied by Olympus or other suppliers (see EMPReSS Website).

**Kidney explants, Isolation of Epicardin + progenitor cells**
Kidneys were removed from P1 neonate C57BL/B6 mice, cut into pieces (approximately 1 mm³), rinsed in PBS to remove excess blood and plated onto 0.1 % gelatin-coated culture chamber in DMEM containing GlutaMax™, 4.5 g /L glucose, penicillin, streptomycin and 15 % fetal calf serum (FCS) in the presence of prokineticin-2 (5 nM-10 nM) or its vehicle (PBS only). Cultures were maintained with minimum disturbance to allow explants to adhere. After 2 or 4 days of culture, explants were removed and cells were maintained in DMEM containing Glutamax, 4.5g/L glucose, penicillin streptomycin without serum during 12hours at 37°C in a humidified air 5% CO₂ atmosphere. Next, cells were cultured in the presence or absence of prokineticin-2 (5 or 10nM) during 24 hours. Cells were fixed in 3.7% formaldehyde at 4°C and cell types were assessed by immunofluorescence.

Primary renal-EPDC cells migrated away to form a monolayer surrounding the remaining kidney explants were utilized and further cultured (16 days) in DMEM containing GlutaMax™, 4.5 g /L glucose, penicillin, streptomycin and 10% FCS, and trypsinized and resuspend in the isolation Buffer (0.1%BSA, 2mM EDTA in PBS) and incubated with biotynilated anti-TCF21 (N-terminus, Santa Cruz) for 20 minutes at room temperature then incubated with 85µl of FlowComp Dynabeads (Dynabeads R FlowCompTM Flexi, invitrogen ) for 30 minutes at room temperature and then placed in the magnet for 2 minutes. The cells attached to Dynabeads were collected with 1mL FlowComp Release Buffer and then cells were resuspend in medium containing 10% Fetal calf serum with DMEM 4.5g/L glucose, glutamax, pencilline, streptomycin. After 12 hours cells were washed with PBS and fresh medium without serum were added during 12 hours. Next, cells were cultured in the presence or absence of prokineticin-2 (5 or 10nM) during 48 hours. Cells were fixed in 3.7% formaldehyde at 4°C and cell types were assessed by immunofluorescence staining. In some setting of experiment, detergent was eliminated in the buffers to see plasma membrane localization of the protein.

A) TCF21 labeling of the renal EPDCs without permeabilization, showing cell membrane localization of TCF21 as observed for BETA2/NeuroD protein*.

B) RT-PCR analysis of expression of PKR1 in TCF21 positive renal-EPDCs.

### Supplemental Tables

**Table I. Oligos for reverse transcriptase-polymerase chain reaction**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>Col1a1 Forward</td>
<td>TGTCCCAACCCCCAAAGAC</td>
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<tr>
<td>Col1a1 Reverse</td>
<td>TCGGTGTCCCTTCTATTCC</td>
</tr>
<tr>
<td>Col1a2 Forward</td>
<td>GAACGGTCCACGATTGCATG</td>
</tr>
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<td>Col1a2 Reverse</td>
<td>CCTGCAGGACCCTGTGGGACC</td>
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<tr>
<td>Phospholamban Forward</td>
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<td>SERCA2a Forward</td>
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**Table II. Echocardiographic analyses (24-week-old mice, n=6 for each group)**

<table>
<thead>
<tr>
<th>Echocardiographic data (before dobutamine injection)</th>
<th>Wild type</th>
<th>Null mutant</th>
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<tr>
<td></td>
<td>PKR1 (+/+)</td>
<td>PKR1 (-/-)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>444.78±28.21</td>
<td>452.33±20.62</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>112.97±8.01</td>
<td>92.94±4.16 *</td>
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<tr>
<td><strong>Diastolic parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVS (mm)</td>
<td>0.809±0.054</td>
<td>0.612±0.021 *</td>
</tr>
<tr>
<td>LVID (mm)</td>
<td>3.794±0.135</td>
<td>4.047±0.279</td>
</tr>
<tr>
<td>LVPW (mm)</td>
<td>1.064±0.044</td>
<td>0.953±0.034 *</td>
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<tr>
<td>LV vol (µl)</td>
<td>62.33±5.59</td>
<td>72.71±6.66</td>
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<tr>
<td><strong>Systolic parameters</strong></td>
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<tr>
<td>IVS (mm)</td>
<td>1.166±0.075</td>
<td>0.990±0.053 *</td>
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<td>LVID (mm)</td>
<td>2.598±0.155</td>
<td>2.823±0.148</td>
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<tr>
<td>LVPW (mm)</td>
<td>1.417±0.074</td>
<td>1.310±0.055</td>
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<tr>
<td>LV vol (µl)</td>
<td>25.51±3.90</td>
<td>32.02±4.31</td>
</tr>
<tr>
<td></td>
<td>Wild type</td>
<td>Null mutant</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td>PKR1 (+/+) PKR1 (-/-)</td>
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</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>474.86±32.80</td>
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<td><strong>Diastolic parameters</strong></td>
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<td>LVPW (mm)</td>
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<td>LV vol (µl)</td>
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<tr>
<td><strong>Systolic parameters</strong></td>
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<tr>
<td>IVS (mm)</td>
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<td>LVPW (mm)</td>
<td>1.754±0.069</td>
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<tr>
<td>LV vol (µl)</td>
<td>8.34±1.48</td>
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<tr>
<td>Ejection fraction (%)</td>
<td>86.65±2.02</td>
<td>78.53±1.47*</td>
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<tr>
<td>Fractional shortening (%)</td>
<td>54.31±2.67</td>
<td>46.27±1.47*</td>
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<tr>
<td>E wave (mm/s)</td>
<td>884.40±18.77</td>
<td>861.66±54.60</td>
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<tr>
<td>A wave (mm/s)</td>
<td>590.11±69.19</td>
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<tr>
<td>E/A ratio</td>
<td>1.513±0.061</td>
<td>1.630±0.104</td>
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<tr>
<td>IVRT (ms)</td>
<td>20.36±1.49</td>
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<tr>
<td>IVCT (ms)</td>
<td>13.57±1.65</td>
<td>18.75±1.91*</td>
</tr>
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</table>

IVS: interventricular septum, LVID: left ventricular internal diameters
LVPW: left ventricular posterior wall, LV vol: left ventricular volume
IVRT: isovolumetric relaxation time, IVCT: isovolumetric contraction time
* P<0.05
**Supplemental Figures**

**Figure 1.** A) Reverse transcriptase–polymerase chain reaction analyses for prokineticin receptors and house-keeping gene GAPDH on RNA extracts from heart and kidney. B) Histogram demonstrated that PKR1 (R1) expression was found only in the wild type heart and kidney. PKR2 (R2) transcripts were not altered by ablation of PKR1 in heart and kidney (n=4). C) Immunostaining analysis on cryosectioned hearts and kidney revealed that PKR1 expression (green) is found only in wild heart and kidney. D) Immunostaining analysis on cryosectioned hearts and kidney revealed that PKR2 protein levels remained unaltered (n=4, p>0.05). E) Histogram and western blot analyses demonstrated that PKR2 protein level was not similar between wild and mutant hearts (n=3, p>0.05). F) PKR1 expression was found in PECAM-1 positive endothelial cells of the coronary artery, dystrophin positive myocardium, and WT1 positive epicardium. Despite these defects, PKR1–null mice remained fertile and survived without evident cardiac failure (pulmonary edema, liver enlargement) for up to 12 months (data not shown).
Figure 2. A) Semi-thin sections colored with toluidin blue demonstrated cellular disarray in the subepicardium and myocardium of the mutant mice (left). RT-PCR analysis and histogram represents a RT-PCR analyses, showing increased levels of collagen transcripts in the mutant heart (n=4, p<0.05). B) Dystrophin staining on the cryosectioned hearts revealed an increase in length of the mutant cardiomyocytes (Original magnification=X63). C) Troponin C (TnT) staining and western blot analyses revealed a decrease in TnT levels in mutant heart. GAPDH (gph) was used as an internal control protein to quantify the TnT protein levels (n=4, p<0.05) (Original magnification=X63).
Figure 3. Histogram represents a real-time PCR analyses, showing that no significant changes were obtained in the expression of phospholambadan (PLB), serca 2a, beta-1 adrenergic receptors (β-1AR) or ryonidine receptor-2 (RYR2) in mutant hearts as compared to wild type mice.

Figure 4. A) Representative illustrations of PECAM-1 positive cells in epicardium and myocardium of hearts. A slight reduction of angiogenesis (12 %, n=4, p<0.05) was observed in mutant epicardium as compare to wild epicardium. Note that angiogenesis in mutant endocardium and myocardium were similar to wild endocardium and myocardium. Histogram shows no significant changes on total PECAM-1 positive capillary density in between wild type (+/+) and mutant hearts (-/-) at the age of 24 weeks, n=4. B) Representative illustrations of PECAM-1 positive cells in kidneys. No significant changes in the extraglomerular capillary formation were observed between wild and mutant kidneys at the age of 24 weeks, n=4. C) Tunel positive apoptotic cells in neonatal hearts, n=3, p<0.01 (right).
Figure 5. A) Representative illustration of epicardin, Ki67, Original magnification=X63), PECAM-1 and α-SMA positive cell number (Original magnification=X40) in the renal EPDCs derived from mutant kidney explants treated with vehicle or prokineticin-2 (5 or 10 nM) for 24 h. Histograms show the quantitative changes in null mutants (-/-). Quantification of the specific cell number/dapi positive cell number corrected with 100 per high power field. B) Representative illustration of Flk-1, (VEGF receptor), an endothelial specific marker and α-SMA positive cell number in the isolated epicardin positive renal EPDCs derived from kidney explants treated with vehicle or prokineticin-2 (5 or 10 nM) for 48 h. Original magnification=X63. Histograms show quantification of the specific cell number/dapi positive cell number corrected with 100 per high power field (n=4, p<0.05).
Figure 6. Expression of PKR1 in mouse kidney. A) In the kidney, glomeruli and blood vessels were strongly labeled with the antibody directed against PKR1. Confocal analyses showed that PKR1 colocalized with PECAM-1 [an endothelial cell marker in the glomeruli] and NaPi [sodium-phosphate transporter, a marker of tubules], but not with podocin [a podocyte marker] or vimentin [a mesangial cell marker]. Thus, within the glomeruli, PKR1 is expressed in endothelial cells, but not in podocytes and mesangial cells. PKR1 expression was also detected in the tubular structures positive for NaPi. B) In situ hybridization on cryosectioned kidneys showing PKR1-specific staining (blue) in glomerular and tubular structures, detected with antisense-PKR1 probes but not with sense-PKR1 probes. C) Impaired glomerular endothelial cells (ec) with fenestration and podocyte structures (pod) in 36 weeks old mutant mice kidneys (+/-) demonstrated by electron microscopic analysis.
Figure 7. Schematic representation of pathological consequences of PKR1 inactivation in mice. Inactivation of PKR1 in mice leads to impaired survival signaling, angiogenesis and mitochondrial defects that potentially induce apoptosis in cardiomyocytes and epicardin⁺ cells in heart and glomerular endothelial and epicardin⁺ cells in kidney. However, hypoxia in heart and kidney restores impaired angiogenesis at the expense of apoptotic cells. Overall these mutant mice exhibit severe cardiomyopathy and renal disorders at the 24 week old age.