Overexpression of cGMP-dependent protein kinase I (PKG-I) attenuates ischemia-reperfusion-induced kidney injury

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Overexpression of cGMP-dependent protein kinase I (PKG-I) attenuates ischemia-reperfusion-induced kidney injury. Am J Physiol Renal Physiol 302: F561–F570, 2012. First published December 7, 2011; doi:10.1152/ajprenal.00355.2011.— cGMP-dependent protein kinase (PKG) is a multifunctional protein. Whether PKG plays a role in ischemia-reperfusion-induced kidney injury (IRI) is unknown. In this study, using an in vivo mouse model of renal IRI, we determined the effect of renal IRI on kidney PKG-I levels and also evaluated whether overexpression of PKG-I attenuates renal IRI. Our studies demonstrated that PKG-I levels (mRNA and protein) were significantly decreased in the kidney from mice undergoing renal IRI. Moreover, PKG-I transgenic mice had less renal IRI, showing improved renal function and less tubular damage compared with their wild-type littermates. Transgenic mice in the renal IRI group had decreased tubular cell apoptosis accompanied by decreased caspase 3 levels/activity and increased Bcl-2 and Bag-1 levels. In addition, transgenic mice undergoing renal IRI demonstrated reduced macrophage infiltration into the kidney and reduced production of inflammatory cytokines. In vitro studies showed that peritoneal macrophages isolated from transgenic mice had decreased migration compared with control macrophages. Taken together, these results suggest that PKG-I protects against renal IRI, at least in part through inhibiting inflammatory cell infiltration into the kidney, reducing kidney inflammation, and inhibiting tubular cell apoptosis.

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ISCHEMIA-REPERFUSION (IR) injury is a major cause of acute renal failure with high morbidity and mortality in patients in shock, renal transplantation, and other clinical settings. Currently, there are still no pharmacological agents available to prevent/treat this severe syndrome. Renal IR injury is a very complex process that involves a variety of pathophysiological mechanisms. Renal IR injury is initiated by the cellular depletion of energy substrates such as ATP during the ischemic phase. The proximal tubule is the major target of IR-induced injury (29). Following reperfusion, a complex series of events occurs including generation of reactive oxygen and nitrogen species, generation of proinflammatory cytokines, alteration of microvascular activity, inflammation, and eventually tubular cell death (3). Morphological changes of kidney IR injury include effacement and loss of proximal tubule brush border, proximal tubular dilation and distal tubular casts, necrosis, and apoptosis of proximal tubular cells (6, 17, 39). In addition, rapid accumulation of massive neutrophils and monocyte/macrophages, and fewer leucocytes in the interstitium of injured kidney is another feature of renal IR injury (3, 22, 25).

The role of nitric oxide (NO) in renal IR injury has been extensively investigated (8, 26, 28, 34, 41, 46, 50). However, the involvement of the NO downstream signaling pathway, cGMP and cGMP-dependent protein kinase (PKG), in renal IR injury has not been investigated. PKG is a serine/threonine kinase consisting of a regulatory and a catalytic domain within one polypeptide chain (48). In mammalian cells, two genes encoding PKG have been identified, type I and type II (18). Type I is alternatively spliced at the first exon to encode two isoforms, Io and Ib. These enzymes contain identical catalytic domains (2, 38). PKG-I is expressed in vascular smooth muscle cells, cardiomyocytes, endothelial cells, mesangial cells, renal tubular cells, macrophages, and other cell types (9). Recent studies demonstrated the protective effect of the cGMP/PKG signaling pathway on IR injury in the heart as well as in cardiomyocytes through several mechanisms such as regulation of mitochondria KATP channels or enhanced phosphorylation of Akt, ERK, and glycogen synthase kinase (GSK)-3β (5, 11, 13). However, whether PKG displays a protective effect on kidney IR injury is unknown.

In this study, we determined how renal IR injury affects PKG-I levels in the kidney and further determined whether overexpression of PKG-I in the kidney and other tissues attenuates renal IR injury by using PKG-I transgenic mice generated by our laboratory (33). We assessed the renal function and structural changes in transgenic mice and wild-type littermates undergoing renal IR injury. In addition, inflammatory cell infiltration into kidney in vivo and macrophage migration in vitro were investigated.

MATERIALS AND METHODS

Experimental animals and protocol. PKG-I transgenic mice were generated by our laboratory previously (33). Eight-week-old male PKG-I transgenic mice and sex- and age-matched wild-type littermates were used in the studies. All these mice were on a B6C3H background and were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. There were four groups of animals: 1) transgenic mice, sham; 2) transgenic mice, IR; 3) wild-type littermates, sham; and 4) wild-type littermates, IR. Each group contained 10 mice. For studies involving inhibitors (Sigma), MEK1 inhibitor PD98059 (10 mg/kg), GSK-3β inhibitor SB216763 (10 mg/kg), and phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (0.75 mg/kg) were administered to PKG-I transgenic mice (ip) 30 min before renal IR injury. To perform renal IR injury, mice were anesthetized and the left renal pedicle was clamped for 45 min as described in this protocol. To perform renal IR injury, mice were anesthetized and the left renal pedicle was clamped for 45 min as described.
Table 1: Sequences of primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5'→3')</th>
<th>Antisense (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S RNA</td>
<td>AG AGT CGG CAT C GT TTA TGG TG</td>
<td>CGA AAG CAT TTG CCA AGA AT</td>
</tr>
<tr>
<td>IL-6</td>
<td>CAG CAA GAG ACT TGC ATC CAG TT</td>
<td>GAA GGA GGA AAG GCC GTG G</td>
</tr>
<tr>
<td>TNF-α</td>
<td>AGG CCA TGG GCT GTA CCT</td>
<td>TGA GTT GGT CCG CCT TCT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CAG CCA GAT GCA GCT AAC GC</td>
<td>GCC TAC TCA TTG GCA TCA TCT TG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGG AGA GTG TGG ATG CCA AGC AAT</td>
<td>GT CTC GAC CAC TGT TTG TGT CCA</td>
</tr>
<tr>
<td>CCR2</td>
<td>AGA GAG CAG CTC CAA AAA GG</td>
<td>GGA AAG AGG CAG TGG CAA AG</td>
</tr>
<tr>
<td>PKG-Ia</td>
<td>AAA CTC CAC AAA TGG CAC CAG TGG GTG</td>
<td>TTT AGT GAA CTT CCG GAA GGC CTC</td>
</tr>
<tr>
<td>PKG-Iβ</td>
<td>TAC AGT ATG CCG TCG AGG AGA AGA</td>
<td>TCA CCG AGC GAT ACT TGT CCA GTT</td>
</tr>
<tr>
<td>PAI-1</td>
<td>GCC TCT CAG CTC CTC CAC CAG TCC</td>
<td>GTA CTG CCG ATG CCA TCT TT</td>
</tr>
</tbody>
</table>

MCP, monocyte chemoattractant protein; PKG, cGMP-dependent protein kinase; PAI, plasminogen activator inhibitor.
The cells were taken from the Percoll interface, washed for two times with sorting buffer containing 1% FBS in D-PBS buffer, and incubated with FITC-conjugated anti-CD11b antibody (1:50, BD Pharmingen) for 30 min at room temperature. The labeled cells were analyzed by flow cytometry using the Flow Cytometry Service Facility at the University of Kentucky.

Macrophage migration assays. Macrophage migration assays were performed using a 24-well Transwell plate (8-um pore size; Costar, Corning, NY). Peritoneal macrophages were isolated from male PKG transgenic mice and wild-type littermate controls using the methods as described previously (31). Peritoneal macrophages at a density of $1 \times 10^6$ cells were loaded into the upper chambers, and the lower chamber...
was filled with either DMEM with 0.2% BSA or DMEM with 0.2% BSA and monocyte chemoattractant protein-1 (MCP-1; 50 ng/ml) and incubated at 37°C for 5 h. Media was removed from the upper chamber. Cells in the bottom chamber were then fixed in methanol and stained with Giemsa solution (Dade Behring, Marburg, Germany). Cell counts were performed by two different observers who were blinded to the study design. Migration was expressed as the number of cells per field.

**Statistical analysis.** All data are expressed as means ± SE. ANOVA was used to analyze variations within the group. Student’s t-tests were used to compare variations between groups. Statistical significance was accepted at $P < 0.05$.

**RESULTS**

**Renal IR injury downregulates kidney PKG-I levels.** To determine the effect of IR injury on kidney PKG-I levels, control mice underwent renal ischemia (45 min)-reperfusion (24 h) injury as described in **MATERIALS AND METHODS**. This has been considered to be a moderate acute kidney failure animal model (15, 35). We demonstrated that mice from the IR group exhibited a significant increase in plasma creatinine levels compared with the sham group (Fig. 1A). In accordance with the renal functional analysis, renal histology revealed severe tubular damage including loss of brush border and vacuolation or necrosis in mice from the IR group (Fig. 1B). Together, these results demonstrated that mice from the IR group exhibited severe kidney damage. In this renal IR injury model, we found that expression of PKG-I (mRNA and protein levels) in the kidney was significantly decreased in the IR group compared with the sham group (Fig. 1, C and D). To further determine whether the IR-induced decrease in the levels of PKG-I is specific to proximal or distal tubules or both, we performed microdissection of proximal tubules from wild-type mice under sham surgery or IR injury. The glomeruli, proximal tubules, and distal tubules plus collecting ducts were isolated and collected to determine the alteration of PKG mRNA and protein levels by real-time PCR or immunoblotting, respectively. As shown in Fig. 1, E–G, IR injury-induced PKG-I reduction (mRNA and protein levels) occurred in glomeruli, proximal tubules, and distal tubules.

**PKG-I transgenic mice have reduced IR injury.** The above results demonstrated that endogenous PKG-I levels in the kidney were downregulated in renal IR injury. In the following studies, we used PKG-I transgenic mice generated by our laboratory previously (33) to determine whether overexpression of PKG-I prevents/attenuates renal IR injury. In PKG transgenic mice, constitutively active PKG-I was overexpressed in the kidney as well as in other tissues (33). Transgenic mice and wild-type control littermates underwent renal...
Fig. 3. PKG-I transgenic mice had reduced tubular cell apoptosis and decreased caspase 3 levels and activity in the kidney in a renal IR injury model. A: a terminal transferase dUTP nick-end labeling (TUNEL) assay was used to analyze apoptosis in kidney sections from 4 groups of mice using an In Situ Cell Death Detection Kit Fluorescein from Roche as described in MATERIALS AND METHODS. Representative fluorescence micrographs are shown. Apoptotic cells are labeled as green. Cell nuclei were stained with 4,6-diamidino-phenylindole (DAPI) and shown as blue. Scale bars = 100 μm. Original magnification ×100. B: TUNEL-positive nuclei were calculated. Values are means ± SE (n = 3). C: representative light micrographs of active caspase 3 immunostaining in kidney sections from 4 groups of mice. The positive staining is shown as brown (indicated by arrows). Original magnification ×40. D: caspase 3 activity in the kidneys from 4 groups of mice were analyzed. Values are means ± SE (n = 5). *P < 0.05 vs. WT IR group.
IR injury (45-min ischemia/24-h reperfusion). Renal function and histology were examined. As shown in Fig. 2A, plasma creatinine levels were significantly reduced in transgenic mice from the IR group compared with wild-type controls. Consistently, renal histology revealed significantly less tubular damage in transgenic mice after ischemic injury (Fig. 2, B and C). In this study, a PKG-I knockout mouse was not used due to its very limited lifespan (~4 wk) (20).

Several PKG-associated downstream targets have been demonstrated in the kidney or heart, such as ERK, GSK-3β, and PI3K/Akt (10–13). To determine which PKG-associated downstream targets might play a role in the protection of our PKG-I transgenic mice under IR injury, MEK1 inhibitor PD98059 (10 mg/kg), GSK-3β inhibitor SB216763 (10 mg/kg), and PI3K inhibitor wortmannin (0.75 mg/kg) were administered to PKG-I transgenic mice (ip) 30 min before IR injury. The results demonstrated that administration of the PD compound abolished the protective effect of overexpression of PKG-I on kidney IR injury. However, the GSK-3β inhibitor or PI3K inhibitor had no effect (Fig. 2D). These data suggest that protection of PKG-I transgenic mice under IR injury is mediated by the ERK pathway. Consistent with this result, phospho-ERK levels were increased in the IR-injured kidneys from PKG-I transgenic mice compared with wild-type mice (see Fig. 4C).

In addition to acute necrotic damage, tubular apoptosis contributes to the development of ischemic acute kidney injury (17). To determine whether there is an alteration in tubular apoptosis in transgenic mice in the above IR injury model, we examined renal tissues by TUNEL assay. In kidneys from both sham groups, apoptotic cells were rare. However, in kidneys from the IR group, apoptotic cells were significantly increased in wild-type mice compared with transgenic mice (Fig. 3, A and B). Consistently, active caspase 3 staining (Fig. 3C) or caspase 3 activity was significantly reduced in transgenic mice from the IR group compared with wild-type mice (Fig. 3D).

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Moreover, we determined the expression of antiapoptotic, proapoptotic, or survival proteins in the kidney. We found that expression (mRNA and protein levels) of antiapoptotic genes Bcl-2 and Bag-1 was significantly decreased in wild-type (IR) kidneys, which was inhibited in transgenic mice (Fig. 4, A and B). The expression of Bax in the kidney (in cytosol or mitochondria) was similar between wild-type and transgenic mice (data not shown). In addition, IR injury increased p-ERK levels in the kidneys from both wild-type and transgenic mice. However, p-ERK levels were increased to a greater extent in transgenic mice (IR) than in wild-type mice (IR) (Fig. 4C). Taken together, these results demonstrated that PKG-I transgenic mice have reduced tubular apoptosis in renal IR injury partially through upregulation of antiapoptotic or survival signaling molecules in the kidney.

PKG-I transgenic mice have reduced expression of renal cytokines in a renal IR injury model. Accumulating evidence suggests that an inflammatory response plays a role in ischemic acute kidney injury (17). To determine the effect of overexpression of PKG-I on local inflammation in renal IR injury, the mRNA (Fig. 5A) and protein levels (Fig. 5B) of proinflammatory cytokines including IL-1β, IL-6, TNF-α, and PAI-1 were...
examined. The results demonstrated that IR significantly increased expression of all of these four cytokines in the kidney from both wild-type and transgenic mice compared with their sham groups. However, IL-1β, IL-6, and TNF-α protein levels were significantly reduced in the transgenic mice IR group compared with the wild-type IR group, suggesting that PKG-I represses IR injury-induced local kidney inflammation.

PKG-I transgenic mice have reduced macrophage infiltration into the kidney in a renal IR injury model. To determine the mechanism of a reduced inflammatory response in kidneys from transgenic mice after IR, we investigated the accumulation of neutrophils and macrophages in the injured kidneys. First, macrophage infiltration into the kidney was determined by immunohistochemical staining renal tissues with the macrophage marker anti-F4/80 antibody or by flow cytometry analysis using an anti-CD11b antibody, another murine macrophage marker. Second, neutrophil infiltration was determined by flow cytometry analysis. As shown in Fig. 6A, there was no significant difference of F4/80-positive cells in the sham group between wild-type mice and transgenic mice. However, in the IR group, F4/80-positive cells were significantly increased in wild-type mice compared with transgenic mice. Consistently, CD11b-positive macrophages were significantly decreased in transgenic IR kidneys compared with wild-type IR kidneys as demonstrated by flow cytometry (Fig. 6B). Neutrophil infiltration in the kidney was similarly increased in IR mice from both wild-type and transgenic groups (Fig. 6C). Furthermore, we examined the expression of MCP-1 and its cognate receptor CCR2 in the kidneys from sham or IR groups. IR similarly increased renal MCP-1 expression in both wild-type and transgenic mice (data not shown). Kidney CCR2 levels were not altered by IR and were similar in the sham and IR groups for both wild-type and transgenic mice (data not shown). Together, these data suggest that PKG transgenic mice have reduced IR injury-induced macrophage infiltration into the kidney possibly through a MCP-1-independent mechanism.

Peritoneal macrophages isolated from PKG transgenic mice show decreased migration. To further determine the effect PKG-I on macrophage function, peritoneal macrophages were isolated from wild-type and PKG-I transgenic mice and underwent migration to the known macrophage chemoattractant (MCP-1; 50 ng/ml) in vitro. The results demonstrated that peritoneal macrophages isolated from transgenic mice had decreased ability to migrate toward MCP-1 (Fig. 7).

DISCUSSION

In this study, the role of PKG-I in renal IR injury was investigated. Using an acute kidney injury mouse model, we first demonstrated that IR injury downregulated PKG-I levels in the kidney. Moreover, overexpression of PKG-I attenuated renal IR injury, which was accompanied by reduced tubular cell apoptosis partially due to increased expression of antiapoptotic genes (Bcl-2 and Bag-1) or increased levels of phosphorylated ERK. Inhibitor studies further support the involvement of an ERK pathway in PKG-I-mediated renal IR protection. Additionally, decreased accumulation of macrophages and reduced expression of proinflammatory cytokines in the injured kidneys were demonstrated in PKG-I transgenic mice, which is consistent with the observed decreased mobility of macrophages from transgenic mice. Together, these results suggest that PKG-I has a protective effect on renal IR injury partially through inhibiting tubular cell apoptosis and suppressing kidney inflammation.

PKG is a downstream signaling mediator of NO and cGMP. It is a serine/threonine kinase, consisting of a regulatory and a
catalytic domain. Binding of cGMP by the regulatory domain leads to activation of the catalytic domain and increases PKG activity (48). PKG levels/activity have been shown to be modulated in many disease conditions. For example, PKG expression is downregulated in diabetes or cancer (7, 16, 21). Our previous studies demonstrated that the NO and cGMP levels were decreased in kidney mesangial cells under high-glucose conditions, resulting in decreased PKG kinase activity (45, 48). In vascular smooth muscle cells, glucose decreases PKG mRNA and protein levels through PKC-dependent activation of NAD(P)H oxidase-derived superoxide production (30). In the current studies, we found that mRNA and protein levels of PKG-I in the kidney were also downregulated in IR-induced kidney injury. The increased production of reactive oxygen species or inflammatory cytokines under IR conditions may contribute to the decreased PKG levels in IR kidneys (4, 32). The cellular and molecular mechanisms of renal IR injury induced downregulation of PKG-I expression in the kidney need to be further investigated.

In this study, we demonstrated that overexpression of PKG-I attenuates IR-induced kidney injury. One mechanism is possibly due to decreased apoptosis and necrosis in tubular cells. We observed a significant increase in antiapoptotic genes including Bcl-2 and Bag-1 levels (mRNA and protein) and a decrease in caspase 3 levels/activity in the kidneys from transgenic mice. Bcl-2 is a membrane protein that blocks a step in a pathway leading to cell apoptosis (43). BAG1 (or Bcl-2-associated athanogene 1) was identified by Takayama et al. in 1995 and has been described as a multifunctional protein able to delay cell death (42). Therefore, PKG-I-mediated upregulation of both Bcl-2 and BAG1 may greatly contribute to the decreased renal IR injury-induced cell death.

![Fig. 6](image_url) Macrophage infiltration into kidney was decreased in Tg mice undergoing IR-induced kidney injury. A: kidney sections from 4 groups of mice were stained with anti-F4/80 antibody. The positive staining is shown as brown. Representative light micrographs are shown. Scale bars = 50 μm. Original magnification ×40. F4/80-positive cells were also calculated. Values are means ± SE (n = 5). *P < 0.05. B: isolated kidney mononuclear cells (KMNC) from 4 groups of mice were stained with anti-CD11b antibody (B), or kidney cells isolated from 4 groups of mice were stained with anti-neutrophil antibody (C) and analyzed by flow cytometry. The percentage of CD11b+ cells within KMNCs or neutrophil+ cells within the whole kidney cell populations was calculated. Values are means ± SE (n = 3). *P < 0.05.

![Fig. 7](image_url) Macrophages from Tg mice had decreased migration. Basal or monocyte chemotactic protein-1 (MCP-1; 50 ng/ml)-stimulated peritoneal macrophage migration was determined as described in MATERIALS AND METHODS. Values are means ± SE (n = 5). *P < 0.05 vs. WT (basal), #P < 0.05 vs. WT (MCP-1).
In summary, PKG transgenic mice and wild-type controls were subjected to IR injury in a single-kidney model which mimics the kidney transplant setting. We found that PKG levels in the kidney were downregulated in IR-induced kidney injury in wild-type mice. PKG-I transgenic mice developed less IR-induced kidney injury, which was associated with significantly decreased macrophage infiltration into the kidney, decreased kidney inflammation, and tubular cell apoptosis. The results of this study are relevant to the field of kidney transplantation and may lead to the development of novel therapies to prevent the development of renal IR injury.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES
PKG-I ATTENUATES KIDNEY IRI


