Renal PKC epsilon deficiency attenuates acute kidney injury and ischemic allograft injury via TNF alpha dependent inhibition of apoptosis and inflammation

Song Rong¹,³, Katja Hueper⁶, Torsten Kirsch¹, Robert Greite¹, Christian Klemann⁸, Michael Mengel⁴, Matthias Meier¹, Jan Menne¹,², Michael Leitges¹,⁵, Nathan Susnik¹, Martin Meier⁷, Hermann Haller¹, Nelli Shushakova¹,², Faikah Gueler¹

Correspondence to: Prof. Faikah Gueler,
Department of Nephrology
Medical School Hannover,
Carl-Neuberg-Str.1, 30625 Hannover,
Tel: 0049-511-532 3722, Fax: 0049-511-552366
email: gueler.faikah@mh-hannover.de

¹Department of Nephrology, Hannover Medical School, Hannover, Germany
²Phenos GmbH, Hannover, Germany
³The Transplantation Center of the affiliated hospital, Zunyi Medical College, China
⁴Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada
⁵The Biotechnology Centre of Oslo, University of Oslo
⁶Institute for Diagnostic and Interventional Radiology, Medical School Hannover, Hannover, Germany

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Abstract

Acute kidney injury (AKI) increases the risk of morbidity and mortality after major surgery and transplantation. We investigated the effect of protein kinase C (PKC) epsilon deficiency on AKI and ischemic allograft damage after kidney transplantation (ktx). PKC epsilon deficient and wild type (WT) control mice were subjected to 35 minutes of renal pedicle clamping to induced AKI. PKC epsilon deficiency was associated with a marked improvement in survival and an attenuated loss of kidney function. Furthermore, functional MRI studies revealed better renal perfusion in PKC epsilon deficient than WT mice one day after IRI. Acute tubular necrosis and neutrophil infiltration were markedly reduced in PKC epsilon deficient mice. To determine whether this resistance to IRI resulted from changes in local renal cells or infiltrating leukocytes, we studied a life supporting renal transplant model of ischemic graft injury. We transplanted kidneys from H₂b PKC epsilon deficient mice (129/SV) and their corresponding WT littermates into MHC-incompatible H₂d recipients (BALB/c) and induced ischemic graft injury by prolonged ischemia time. Recipients of WT allografts developed severe renal failure and died within 10 days of transplantation. Recipients of PKC epsilon deficient allografts had better renal function and survival. They had less
generation of reactive oxygen species and up-regulation of pro-inflammatory proteins (i.e. ICAM-1, i-NOS, TNF-alpha) and showed less tubular epithelial cell apoptosis and inflammation in their allografts. These data suggest that local renal PKC epsilon expression mediates pro-apoptotic and pro-inflammatory signaling and that an inhibitor of PKC epsilon signaling could be used to prevent hypoxia-induced AKI.
Introduction

Long cold ischemia time (CIT) increases the risk of delayed graft function after kidney transplantation, acute rejection and progressive interstitial fibrosis (31). Members of the protein kinase C (PKC) family of serine/threonine kinases regulate many processes that contribute to renal damage and recovery during acute kidney injury including adhesion, inflammation, apoptosis, migration, and proliferation (5, 8, 16). Following LPS administration, PKC epsilon inhibition markedly decreases renal macrophage activation and pro-inflammatory cytokine production (1, 4). The effects of PKC epsilon depends on the type of cell and injury studied (36, 41). PKC epsilon activation has been linked to protection from ischemic injury in heart and other organs (3, 6, 45, 50, 51). In contrast, Nowak and coworkers showed that PKC epsilon activation had also negative effects by inducing mitochondrial dysfunction and fragmentation in renal proximal tubular cells (33, 34). Beneficial effects of PKC epsilon inhibition with better cardiac outcome have been shown in animal models of cardiac hypertrophy and cardiac dysfunction (14, 22, 38). PKC epsilon deficient mice had less inflammation in sepsis models due to alteration of macrophage signaling (46). The aim of our study was to assess the role of PKC epsilon in renal IR injury. First, we investigated PKC epsilon deficient mice in a model of renal ischemia and reperfusion (IR) injury. Next, we looked at the impact of local renal PKC epsilon deficiency by investigating a live supporting kidney transplant model of ischemia-induced renal allograft damage. PKC epsilon deficiency resulted in reduced IR injury with improved renal perfusion, renal function and better survival. In recipients of PKC epsilon deficient allografts attenuated TNF-alpha signaling was associated with a decrease in apoptosis and inflammation. Reduced expression of the adhesion molecule ICAM-1 and attenuated infiltration with monocytes/macrophages contributed to the superior allograft survival that we observed in PKC epsilon deficient allografts.
Materials and Methods

Animals

PKC epsilon deficient mice were generated as previously described (25). Adult PKC epsilon deficient mice are healthy and do not show any overt abnormalities. Homozygous male PKC epsilon deficient mice (background: 129SV; H2b) and WT littermates of the heterozygous cross were used in the renal IR injury model. In addition, PKC epsilon deficient and WT mice served as kidney donors in the life supporting kidney transplant model. BALB/c (H2d) mice were used as recipients and were supplied by Charles River (Sulzfeld, Germany). PCR genotyping verified PKC epsilon deficiency. Mice weighing between 25-30g (approximately 12 weeks of age) were used for all experiments. Animals were cared for in accordance with our institutions guidelines for experimental animals. They had a 12 hour day and night cycle and free access to food and drinking water. The local animal protection committee approved these experiments.

Renal ischemia reperfusion injury

Renal IR-injury was induced in homozygous male PKC epsilon-/- mice and corresponding WT littermate controls as described previously (18). Briefly, mice were anesthetized with isoflurane. Then, after median laparatomy, the renal pedicles were bluntly dissected and a non-traumatic vascular clamp was applied to both pedicles for 35 min. Survival and renal function were studied in n=8 mice for four weeks and histology for acute kidney injury was performed after 24 hours after surgery in additional n=9 mice each group.

Functional magnetic resonance imaging (MRI)

MRI examinations were done 24h after unilateral clamping of the right renal pedicle for 35 min using a 7 Tesla small animal scanner (Bruker, Pharmascan) and a circular polarized volume coil (Bruker T10327V3). Animals were anesthetized by isoflurane inhalation and the
respiration was monitored and kept between 30-50 breaths/min during the entire examination. For visualization of renal morphology respiratory triggered, fat-saturated T2-weighted sequences were acquired in axial and coronal planes that covered both kidneys. The coronal plane was adjusted to the long axis of the kidney. Renal blood flow (RBF) was measured without administration of contrast agent using a fat-saturated flow alternating inversion recovery (FAIR) arterial spin labelling (ASL) sequence. Sequence parameters were as follows: TR/TE = 10,000/16.4 ms, 13 inversion times (30, 100, 200, 300, 500, 700, 1000, 1200, 1500, 2000, 3000, 5000, 8000 ms), matrix = 128x128, FOV = 35x35 mm², slice thickness = 2 mm, number of slices = 1. Parameter maps of RBF were then calculated on a pixel-by-pixel basis. Regions of interest (ROI) were placed manually into the renal cortex of both kidneys on RBF maps by one reader who was blinded to the animal group identity and mean RBF values were determined separately for the right kidney with IRI and the other kidney without IRI. Furthermore, relative RBF of the kidney with IRI was calculated as the percentage RBF compared to the contra lateral control kidney without IRI (20).

Kidney Transplantation

Kidney transplantation from PKC epsilon deficient mice and their WT littermates to BALB/c mice was performed as described previously (17, 18). In brief, the animals were anesthetized with isoflurane, and the left donor kidney attached to a cuff of the aorta and the renal vein with a small caval cuff and the ureter were removed en bloc. After left nephrectomy of the recipient, the vascular cuffs were anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. The ureter was directly anastomosed to the bladder (19). Prolonged ischemia times was used to induce ischemic allograft damage (60 min cold and 60 min warm ischemia time, respectively). The right native kidney was removed through a flank incision four days later. The general physical condition of the animals was monitored and kidney function was estimated at designated time points by
measuring serum creatinine level using an automated method (Olympus Analyzer, Germany). For survival studies, 12 mice per group were followed for four weeks.

Renal Morphology

Kidneys were harvested 24h after IR-injury and six days after transplantation. Half of each allograft was immediately fixed in buffered formalin and embedded in paraffin, and the other half was shock frozen in liquid nitrogen. Three µm paraffin sections were cut and stained with PAS. Evaluation of acute tubular necrosis was performed using a semi-quantitative grading system: 0= no ATN, 1= focal ATN with <10% of tubuli of the cortex affected, 2= moderate ATN with 10-25% of tubuli affected, 3 = severe ATN with 25-50% of tubuli affected, 4 = very severe with >50% of the tubuli affected. Analysis was done by a nephropathologist without knowledge of the animal group identity. Cryosections were immunostained using the following primary antibodies: rat anti-mouse GR-1 (for granulocyte detection, Serotec, Oxford, UK), monoclonal rat anti-mouse ICAM-1 (Serotec, Oxford, UK), rat anti-mouse monocyte/macrophage ´(F4/80, Serotec, Oxford, UK), polyclonal goat anti-mouse TNF-alpha (Biotechnology Santa Cruz, CA, USA), polyclonal rabbit anti-mouse active Caspase 3 (BD Pharmingen, Heidelberg, Germany). For indirect immunofluorescence, nonspecific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch Lab, West Grove, USA) for 30 min. Thereafter, cryosections were incubated with the primary antibody for 1 hour in a humid chamber at room temperature. For fluorescent visualization of bound primary antibodies, sections were further incubated with Cy3 conjugated secondary antibodies (Jackson Immuno Research Lab, West Grove, USA) for 1 hour. Sections were analyzed using a Zeiss Axioplan-2 imaging microscope with the computer program AxioVision 4.3 (Zeiss, Jena, Germany). The analysis was performed without knowledge of the animal assignment. To assess leukocyte cell infiltration 10 different view fields per renal section were analysed in each kidney. For granulocyte infiltration cell
numbers were counted. For inflammation and for monocyte/macrophage infiltration a semiquantitative grading system was used: score 0: <5% leukocytes per view field, 1: 5% to 25%, 2: 26% to 50%, 3: 51% to 75% and 4: marked infiltration with more than 75% of the tubule interstitial area affected. For ICAM-1 and iNOS evaluation semiquantitative scoring was 0: no expression, 1: mild expression, 2: moderate, 3: marked, 4: intense staining >75% of the glomeruli affected.

*Generation of reactive oxygen species (ROS)*

The redox-sensitive fluorophore hydroethidine (DHE) was used to evaluate $\text{O}_2^-$ production in the kidney in situ (19). Six µm thick cryosections of frozen tissue were incubated with 0.1 mM DHE dissolved in Hapes-Tyrode buffer solution (132 mM NaCl, 4 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 9.5 mM HEPES, 5 mM Glucose) for 12 minutes at room temperature. After incubation, images were obtained with the use of Leica imaging system IM 500 (Ex: 520 nm, Em: 605 nm).

*RNA extraction and real time quantitative PCR*

Frozen kidneys were ground to a coarse powder in liquid nitrogen and total RNA was extracted using Trizol reagent (Invitrogen, Germany). For quantitative PCR (qPCR), 1 µg of DNase-treated total RNA was reverse transcribed using Superscript II Reverse transcriptase (Invitrogen) and qPCR was performed on an SDS 7700 system (Applied Biosystems, Germany) using Rox dye (Invitrogen, Germany), FastStart taq Polymerase (Roche diagnostics, Germany) and gene specific primers, and Fam-Tamra-labeled TaqMan probes (BioTez, Germany). PCR amplification was initiated at 96°C (10 min); then 40 cycles followed: 10 sec 95°C and 1 min at 60°C. For normalization we used the beta-actin signal. The sequences of the TaqMan sets read as follows (5’-3’):

$\beta$-actin: Fam-AGGGCTATGCTCTCCCTACGCCAT-Tamra,
fwd 5’- TCACCCACACTGTGCCCAT-3’, rev 5’-AGCCAGGTCCAGACGCAG-3’
fwd 5’-GTGACCAGGCTGTCGCTACA-3’, rev 5’-AGGGCAATTACAGTCACGGC-3’.
Quantification was carried out using qgene software (20).

Statistical analysis

After verifying normal distribution by the Klosogorov-Smirnov-test, we compared treatment groups by analysis of variance (ANOVA) and post hoc Scheffe test. Differences were considered as significant at p < 0.05. Relative RBF measured by MRI was compared between PKC epsilon deficient mice and WT mice using unpaired t-tests. Data are presented as mean± standard error (SEM). For statistical evaluation SPSS 12.01 software was used.

Results

PKC epsilon deficiency attenuates IR injury

PKC isoforms are participants in a variety of signaling cascades, which mediate IR injury. To elucidate the role of PKC epsilon we performed bilateral renal pedicle clamping in PKC epsilon -/- mice and corresponding WT controls. All WT controls died within 3 days of IR injury. In contrast, 60% of PKC epsilon deficient mice lived longer than 28 days post-ischemia (Fig.1A). Furthermore, the loss of renal function in PKC epsilon deficient mice was significantly less than that in WT mice at d1 post-ischemia (Fig.1B). Functional MRI revealed significant renal perfusion impairment one day after IRI in WT mice of 40% compared to the contra lateral unclipped kidney. PKC epsilon deficient mice showed also a mild but not significant decrease of renal perfusion (Fig. 1C, **p<0.01). The renal perfusion of the unclipped kidney of PKC epsilon deficient mice was lower than of the WT mice. IR-induced acute tubular necrosis (ATN, Fig. 2A, C) and inflammatory cell infiltration with granulocytes
(GR-1 positive cells, Fig. 2B, D) was significantly reduced in PKC epsilon deficient mice compared to WT controls.

PKC epsilon deficiency of the allograft improved survival and renal function after kidney transplantation

To investigate the impact of local renal PKC epsilon expression on ischemic graft injury we transplanted PKC epsilon deficient donor kidneys after prolonged ischemia time into WT recipients. We used a life-supporting allogenic transplant model in which the remaining endogenous kidney was removed on day four to study the extent of ischemia-induced graft injury on apoptosis and inflammation in vivo. All recipients of WT allografts died within 10 days after transplantation, but 60% of PKC epsilon deficient allograft recipients survived more than 10 days (Fig. 3E). Due to severe ischemic injury WT allograft recipients showed severe loss of renal function with a significant s-creatinine elevation six days after transplantation (160 ± 30 µmol/l). This increase in serum (s)-creatinine was markedly attenuated in PKC epsilon deficient allograft recipients (77 ± 8 µmol/l; p<0.05 vs. WT; Fig. 3D).

ROS generation and apoptosis were markedly reduced in PKC epsilon deficient allografts

Formation of reactive oxygen species (ROS) is an early event in IR injury and it drives apoptosis of renal cells and acute tissue damage. Therefore, we looked for reactive oxygen species (ROS) by DHE staining in renal allografts (Fig. 4 A, D). Within 4 hours after transplantation WT allografts exhibited substantial ROS generation in the tubulo-interstitium (A) and the glomeruli (data not shown). Markedly less ROS generation was detected in PKC epsilon deficient allografts (D). Since ROS generation due to severe hypoxia induces apoptosis and, consequently, acute tubular necrosis (ATN) we examined PAS stained renal tissue one day after transplantation. PKC epsilon deficient allografts had significantly less
ATN and inflammation than allografts from WT controls (Fig. 3A and 4G). Expression of active caspase-3, an apoptosis marker, was elevated in WT allografts in the cytoplasm of damaged proximal tubuli mainly in the outer stripe of the outer medulla (OSOM), an area which is very sensitive to hypoxic damage due to the corticomedullary oxygen gradient (2) (Fig 4B, E). PKC epsilon deficient allografts had reduced active caspase-3 staining (Fig 4E).

TNF alpha expression was significantly reduced in PKC epsilon deficient allografts

TNF alpha, an important mediator of apoptotic cell death due to IR injury (10), has been shown to be regulated by some PKC isoforms(24). To investigate whether the anti-apoptotic phenotype of PKC epsilon deficient allografts is associated with altered TNF alpha signaling, we performed immunohistochemistry and qPCR for TNF alpha. We localized TNF alpha protein expression to the media of the vessel wall (Fig. 4 C, F) and to the tubules of the OSOM (data not shown). PKC epsilon deficient allografts showed less TNF alpha protein expression than WT allografts (Fig. 4F). Furthermore, we found significantly lower levels of TNF alpha mRNA in PKC epsilon deficient allografts than in WT allografts (*p<0.05, Fig. 4H).

PKC epsilon deficiency of the allograft reduced inflammation

Ischemic allograft injury leads to acute inflammation with monocyte/macrophage infiltration that results from activation of adhesion molecules and up-regulation of inducible NO-synthase (i-NOS). Therefore, we asked whether PKC epsilon deficiency altered inflammation after ischemic allograft injury. To answer this question, we stained monocytes/macrophages with F4/80 six days post transplantation (Fig. 5). Many F4/80 positive monocytes /macrophages were present in the perivascular and the tubulo-interstitial compartment in WT allografts (Fig. 5A). Monocyte/macrophage infiltration into PKC epsilon deficient allografts was much lower (Fig. 5D). Since adhesion molecules are important
mediators of cell infiltration, we investigated ICAM-1 expression. We found intense staining of ICAM-1 in the glomeruli and the intima of the vessel wall, as well as in the tubulo-interstitium in WT allografts (Fig. 5B), in PKC epsilon deficient allografts (Fig. 5E), ICAM-1 up-regulation was less pronounced than in WT allografts.

Another marker of inflammation and rejection is i-NOS. It has been shown that PKC epsilon affects i-NOS expression in macrophages (9). In addition, data from cardiac transplantation using i-NOS deficient allografts showed that NO derived from i-NOS contributed to the inflammatory response during acute cardiac allograft rejection (30). We found intense staining for i-NOS six days after transplantation in the glomeruli and in the vessel walls of rejecting WT allografts (Fig. 5C); the i-NOS signal was reduced in PKC epsilon deficient mice (Fig. 5F).

Discussion

Several PKC isoforms are expressed in renal tissue and some of them are up-regulated following IR injury. PKC epsilon is among the up-regulated isoforms (37). The exact role of PKC epsilon in IR injury is still debated however. In cardiac pre-conditioning experiments, up-regulation of PKC epsilon has been shown to be protective (7, 35). On the other hand, PKC inhibition in a model of acute heart allograft rejection improved survival (26) and in other animal models of cardiac hypertrophy PKC epsilon inhibition has been shown to attenuate cardiac dysfunction (14, 22). In our study, we showed that PKC epsilon deficiency resulted in improved kidney function and survival after renal IR injury. In PKC epsilon deficient kidneys, there was less renal perfusion impairment after IR injury than in WT kidneys measured by arterial spin labeling (ASL) with non-invasive functional MRI (20). The renal perfusion of the unclipped kidney of PKC epsilon deficient mice was lower than of the WT mice, the reason for that is not known and needs further investigation. In previous studies, we showed that renal perfusion impairment correlates with severity of IR injury in
mice and rats (53) and with inflammation and progression to chronic kidney disease (21). In healthy volunteers ASL has been shown to be a reproducible and robust way to assess renal perfusion (15). Due to the quality of renal blood flow assessment by ASL, we think that our results with better renal perfusion stress the protective effect of PKC epsilon deficiency after IR injury. Renal microcirculatory dysfunction causes alterations in renal tissue oxygenation, nitric oxide bioavailability and oxygen radical homeostasis(13). It has been shown that preservation of renal blood flow by protecting peritubular capillaries with COMP-angiopoetin 1 treatment improved outcome after renal IR injury and reduced neutrophile infiltration (23). Similarly, we found that tissue damage and inflammatory cell infiltration with granulocytes was reduced in PKC epsilon deficient mice. This is in line with previous studies, showing that PKC epsilon deficiency leads to compromised host defense against bacterial infection due to a significant reduction in the generation of TNF alpha, NO and interleukin (IL)-1beta (4). Nowak and coworkers showed that PKC epsilon activation mediated mitochondrial dysfunction and fragmentation in proximal tubular epithelial cells (RPTC) (34). Furthermore, their data showed that PKC-epsilon activation after oxidant injury in RPTC decreases mitochondrial function and active Na+ transport and that inhibition of PKC-epsilon activation improves these functions(33). To determine if the beneficial effects of PKC epsilon deficiency is due to PKC epsilon deficiency of the local renal cells or circulating leukocytes, we investigated PKC epsilon deficiency in a model of ischemia-induced allograft damage after kidney transplantation. This allowed us to compare PKC deficient and WT allografts. Recipients in all studies were BalbC mice with functional PKC epsilon genes and therefore normal leukocyte function.

We found that PKC epsilon deficient allografts had improved survival and less loss of renal function. One of the mechanisms by which PKC epsilon may improve survival is by attenuating generation of ROS which contribute to apoptosis of kidney cells. We observed that PKC epsilon deficiency resulted in reduced ROS generation in allografts after
transplantation. These results are in line with previous data by Li and coworkers (28) showing that ROS generation in vascular smooth muscle cells (VSMCs) was PKC epsilon- and PKC alpha-dependent. They also demonstrated that down-regulation of PKC reduces the cleavage of caspase-3 in VSMCs. In line with this observation, we found that PKC epsilon deficiency of the renal allograft resulted in diminished active caspase-3 expression in the tubular epithelial cells of the allograft after ischemic injury.

Depending on the stress model and cell type, PKC epsilon regulates different pro- and anti-apoptotic signalling pathways. Pro-apoptotic enhancement of peripherin aggregation by PKC epsilon activation has been observed in neuroblastoma cells (48). In contrast, PKC epsilon activation had anti-apoptotic effects resulting from inhibition of TNF-alpha-related apoptosis-inducing ligand (TRAIL) (42, 43) and prevention of activation and translocation of Bax to the mitochondria (29) in MCF-7 breast cancer cells. In our study, PKC epsilon deficiency had anti-apoptotic effects in renal tubular epithelial cells which exhibited less acute tubular necrosis and attenuated caspase-3 activation. Since TNF alpha has been shown to be an important mediator of tubular epithelial cell apoptosis in the model of unilateral ureter obstruction (UUO) (32), we suspected that PKC epsilon deficiency might affect TNF alpha activation in renal allografts as well. Indeed, we found significantly reduced TNF alpha mRNA and protein expression in PKC deficient allografts, but the exact mechanism of TNF alpha and PKC epsilon interaction remains to be explored.

Hypoxic renal allograft damage is characterized by inflammatory cell infiltration with neutrophils and later macrophages and lymphocytes. Macrophages constitute 40 to 60% of infiltrating cells during acute allograft rejection. In patients with acute rejection, interstitial macrophage infiltration was significantly higher than in non-rejecting patients (47). Furthermore, macrophage infiltration three months after transplantation correlated inversely with graft survival (44). In our study, we identified markedly reduced interstitial inflammation by monocytes/macrophages in PKC epsilon deficient allografts. Monocyte/macrophage
Infiltration is mediated by up-regulation of adhesion molecules such as ICAM-1. Previously, we showed that blockade of ICAM-1 up-regulation by antisense RNA improved renal isograft survival, attenuated IR injury, and enhanced immediate graft function (12). In previous studies, we also observed improved allograft survival in uPA receptor deficient mice (18) and mice in which complement activation had been blocked (17). In both models, ICAM-1 levels were lower than in WT or untreated animals. In line with these results, we observed decreased ICAM-1 expression in PKC epsilon deficient allografts. This should contribute to a decrease in monocyte/macrophage infiltration and improved allograft survival. IR injury, as well as acute renal allograft rejection has been shown to be associated with increased expression of the pro-inflammatory i-NOS (49, 52). iNOS expression was also linked to transplant rejection in a rat model of allogenic kidney transplantation, where macrophage depletion resulted in reduced acute rejection and i-NOS expression. However, the effect of iNOS inhibition is contradictory and might be organ and cell type specific. In hypoxic lung injury selective iNOS inhibition has been shown to attenuate hypoxia induced lung injury (39) whereas in cardiac IR injury the same iNOS inhibitor exacerbated peroxidative and apoptotic damage (39, 40). In our study, diminished renal i-NOS expression in PKC epsilon deficient allografts was accompanied by protective effects with less inflammation and apoptosis.

IR injury initiates and induces the alloimmune responses leading to acute and chronic allograft rejection (11, 27). Here, we identified PKC epsilon as important mediator of transplant induced IR injury that results from up-regulation of ICAM-1 and inflammatory cell infiltration. Our findings are at odds with data published by Tanaka and colleagues (50) who showed that treatment with a PKC epsilon activator plus a PKC delta inhibitor reduced cardiac injury and reduced graft coronary artery disease in a murine model of allogenic heart transplantation. In their study, however, the different effects of the PKC epsilon activator and the PKC delta inhibitor could not be distinguished. In contrast, in another study treatment with a PKC epsilon inhibitor caused improved survival in experimental heart transplantation.
by reducing inflammatory cell infiltration (26). The latter supports the findings of our study which showed that PKC epsilon deficiency resulted in better allograft survival and reduced macrophage infiltration.

Taken together, we delineated the distinct role of local renal PKC epsilon expression in ischemia-induced allograft damage. The fact that PKC epsilon allograft deficiency attenuates IR-induced apoptosis and blunts the generation of pro-inflammatory agents including TNF alpha, ICAM-1, and i-NOS suggests that PKC epsilon antagonists could prove useful in preventing ischemia-induced graft injury.

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References


**Legends:**

**Figure 1. PKC epsilon deficiency attenuates IR injury**

All WT mice (white triangles) died within 3 days after IR injury (A). PKC epsilon deficiency (black square) markedly improved survival to 60% survival over 28 days. IR-injury caused severe renal dysfunction with elevation of s-creatinine levels (B) in WT mice which was markedly attenuated by PKC epsilon deficiency (*p<0.05 vs. WT at day one). Renal perfusion was measured by arterial spin labelling with functional MRI at day 1 after IRI. The contra lateral unclipped kidney (control, left column) was compared to the clipped kidney (IRI, right
column). WT mice (black bar) showed significant renal perfusion impairment due to IRI (grey bar, C, **p<0.01). The PKC epsilon deficient mice (white bar) had only a mild but not significant decrease of renal perfusion after IRI (striped bar) compared to the contra lateral kidney. Renal perfusion of the unclipped kidney of PKC epsilon deficient mice was lower than of WT mice.

**Figure 2. PKC epsilon deficiency attenuated acute tubular necrosis an inflammatory cell infiltration**

WT mice (A) exhibited diffuse severe acute tubular necrosis (ATN) after IR injury. In contrast, the PKC epsilon deficient mice (C) had only mild focal ATN (PAS stain, scoring E **p<0.01, magnification 200 fold). Infiltration of GR-1 positive neutrophils stained in red (B, D) after IR injury was significantly reduced in PKC epsilon deficient compared to WT mice (p<0.001, F, magnification 400 fold). Renal cells show green autofluorecence.

**Figure 3. PKC epsilon deficiency improved survival and renal function after kidney transplantation**

WT allograft recipients (dotted line) died within 10 days after transplantation, whereas 60% of PKC epsilon deficient allografts (black line) were still alive at that time and had prolonged allograft survival (E). PKC epsilon deficient allograft recipients (white bars) showed significantly less s-creatinine elevation compared to WT allograft recipients (black bars) at six days after transplantation (D, *p<0.05 vs. WT). Inflammation at one day after transplantation was significantly reduced in PKC epsilon allografts (A-C, *p<0.05, magnification 200 fold)

**Figure 4. PKC epsilon deficiency reduced ROS generation and apoptosis**

Within 4h after ischemic transplant injury increased ROS generation in the tubulo-interstitial compartment (A) of WT allografts was detected. In contrast, PKC epsilon deficient allografts...
showed markedly reduced generation of ROS (D, magnification 200 fold). Up-regulation of cleaved caspase-3 activation as a marker of apoptosis was reduced in tubular epithelial cells (E) in PKC epsilon deficient allografts compared to WT allografts (B). TNF-alpha protein expression was up-regulated in WT allografts (C) but not in PKC epsilon deficient allografts (F, bar represents 50um). Furthermore, TNF-alpha mRNA expression was significantly reduced PKC epsilon deficient allografts compared to WT allografts (*p<0.05, H). Acute tubular necrosis was significantly reduced in PKC epsilon deficient allografts compared to WT allografts (**p<0.01, G).

**Figure 5. PKC epsilon deficiency reduced inflammation**

WT allografts (A) showed severe F4/80 positive monocytes/macroage infiltration (F4/80 positive cells are stained in red, auto fluorescence of the tubular epithelial cells is green) in the tubulo-interstitium six days after transplantation. PKC epsilon deficiency (D) reduced macrophage infiltration significantly (G, **p<0.01). WT allografts showed intense staining for ICAM-1 (B) and i-NOS (C) which were markedly reduced in PKC epsilon deficient allografts (E/H for ICAM-1 and F/I for i-NOS). Magnification for all images is 400 fold.