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RGS4 Prevents the Development of Renal Dysfunction in Response to Ischemia/Reperfusion Injury

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Abstract

Acute kidney dysfunction after ischemia-reperfusion injury may be a consequence of persistent intrarenal vasoconstriction. Regulators of G protein Signaling (RGS) proteins are GTPase activating proteins for heterotrimeric G proteins that can regulate vascular tone. RGS4 is expressed in vascular smooth muscle cells in the kidney. However, RGS4 protein levels are low in many tissues as a consequence of N-end rule-mediated polyubiquination and proteasomal degradation. In this work, the role of RGS4 in the renal response to ischemia/reperfusion injury (IRI) was investigated. A murine model of IRI was employed and RGS4-null mice (R4KO) were highly sensitized to the development of renal dysfunction after IRI. Furthermore, R4KO mice exhibited reduced renal blood flow after IRI. Kidneys were studied for intrinsic RGS4 function by ex vivo isolation. R4KO kidneys exhibited increased renal vasoconstriction in response to endothelin-1 infusion. The intrinsic renal activity of RGS4 was examined in an in vivo model of syngeneic renal transplantation. Transplanted R4KO kidneys exhibited significantly reduced reperfusion blood flow and increased renal cell death. To increase RGS4 activity, wild type mice were administered the proteasomal inhibitor MG-132 and this resulted in increased renal RGS4 protein. Furthermore, MG-132 treatment inhibited the development of renal dysfunction after IRI in wild type - but not R4KO - mice. These results demonstrate that RGS4 antagonizes the development of renal dysfunction in response to IRI.

Keywords

acute kidney injury; ischemia/reperfusion; G protein; RGS; proteasome

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DISCLOSURE

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INTRODUCTION

Acute kidney injury (AKI) affects almost 15% of patients admitted to the hospital in the United States and results in significant mortality and morbidity [1–3]. AKI in humans is often due to episodic hypotension, such as occurs with septic, hypovolemic or cardiogenic shock. In shock, renal hypoperfusion is followed by reperfusion in surviving patients. Deceased donor organ transplants are also subject to ischemic kidney injury[4]. These forms of kidney ischemia-reperfusion injury (IRI) often result in acute renal dysfunction due to acute tubular necrosis. Although the intra-renal release of reactive oxygen species is thought to be one cause of reperfusion injury, persistent reductions in renal blood flow and increased renal vascular resistance after reperfusion may also contribute to the renal dysfunction [5]. The concentration of ligands that bind to transmembrane G protein coupled receptors (GPCR), including thromboxane A_2, angiotensin II and endothelin-1, is increased in ischemic kidney injury[6, 7].

Vascular smooth muscle cells regulate renal blood flow in a manner that depends on their contractile state. Endothelin-1 and angiotensin II regulate vascular tone by binding to transmembrane receptors on smooth muscle cells[8, 9]. Endothelin-1 acts on the vascular bed of the kidney and this contributes to vaso-occlusive crisis, cyclosporine nephrotoxicity and experimental IR I[10–13]. Endothelin-1 binds to the endothelin-1A (ET-1A) GPCR in vascular smooth muscle cells and activates the Gαq subunit which, in turn, directly stimulates phospholipase Cβ, to generate inositol triphosphate (IP3) which opens IP3-dependent calcium channels resulting in vasoconstriction[14].

G protein α subunits have intrinsic GTPase activity that is relatively weak[15]. This intrinsic GTPase activity results in the hydrolysis of GTP to GDP, resulting in deactivation of the α subunit and reformation of the heterotrimer with βγ subunits. Regulator of G protein signaling (RGS) proteins are GTPase activating proteins (GAPs) that promote the deactivation of GTP-bound α subunits by stabilizing the transition state [15, 16]. RGS4 is a relatively small RGS family member that has a 120 amino acid RGS box with GAP activity[17–22]. RGS4 abundance is limited by N-end pathway-mediated polyubiquination and proteasomal degradation[23, 24]. RGS4 is a vascular-associated protein however its expression outside of the brain remains controversial[25]. RGS4 has the ability to promote the deactivation of both Gq/11 and Gi/o family members dependent on the cell type where it acts [20, 26, 27].

We previously generated mice with targeted disruption of the gene encoding RGS4 (R4KO) [28]. R4KO mice had normal blood pressure and renal function at baseline along with several other hemodynamic parameters. In previous work, we showed that R4KO mice were sensitized to cyclosporine A (CyA)-induced nephrotoxicity[28]. CyA-treated R4KO mice rapidly developed severe renal dysfunction and exhibited increased mortality when compared to CyA-treated wild type mice. Administration of the endothelin-1 antagonist bosentan to CyA-treated R4KO mice reversed the development of renal failure and significantly reduced mortality[28].

To determine whether R4KO mice are also sensitized to a more generalized form of kidney injury, we used a murine model of ischemia-reperfusion injury. By use of this model system, we performed experiments that suggest that R4KO mice are highly sensitized to IRI of the kidney. Because of the potential therapeutic benefit of enhancing RGS activity during renal injury, it is important to understand the role of RGS4 in acute renal ischemia.

RESULTS

RGS4-null mice are sensitized to renal ischemia-reperfusion injury

The localization and intensity of RGS4 expression outside of the central nervous system is not well-established[25]. To precisely determine the expression pattern of RGS4 in the kidney, mice with targeted insertion of the *lacZ* gene encoding β-galactosidase downstream of the promoter region of *rgs4* was analyzed. The RGS4 reporter mouse (*rgs4*$^{tm1Dgen/+}$) showed intense X-gal staining in the arterial vasculature of the kidney with minimal staining in tubular structures (Figure 1a,b).

To model renal ischemia-reperfusion injury in genetically-modified mice, we initially performed warm ischemic renal injury (IRI) by a technique previously described in rats and mice[29, 30]. Both the plasma urea and serum creatinine levels in *rgs4*$^{-/-}$ mice were significantly elevated when compared to WT mice 24 hours after ischemic injury (Figure 2a, b).

To determine whether the increased plasma urea and creatinine levels in *rgs4*$^{-/-}$ mice after IRI was associated with intra-renal injury, kidney sections were evaluated by histological methods 24 hours after surgery. *Rgs4*$^{-/-}$ kidneys exhibited massive tubular injury most prominently in the cortico-medullary junction 24 hours after IRI, whereas WT kidneys exhibited moderate injury measured by standardized tubular injury scoring (Figure 3).

To further evaluate renal injury after IRI surgery, animal survival was monitored for 15 days in R4KO and wild type mice. R4KO mice exhibited significantly reduced survival after IRI surgery compared to wild type mice. Fifteen days after IRI, 5 of 8 wild type animals were alive, but all 8 *rgs4*$^{-/-}$ mice died within 7 days of surgery (Mantel-Cox log rank analysis $P<0.0001$) (Figure 4).

Increased renal vaso-constriction after IRI in *RGS4*$^{-/-}$ mice

The increased sensitivity of *rgs4*$^{-/-}$ mice to renal IRI could be due to vasomotor or renal tubular abnormalities since RGS proteins are known to be expressed in both vascular smooth muscle cells and tubular epithelial cells [31]. To address this mechanistic issue, we examined blood flow with laser doppler in the minutes following kidney reperfusion[32–34]. The recovery of renal blood flow immediately after the end of the ischemic period was impaired in *rgs4*$^{-/-}$ kidneys (Figure 5). Persistently reduced R4KO intra-renal blood flow after IRI may therefore be responsible for the abnormal phenotype.

RGS4$^{-/-}$ mice are sensitized to ligand-stimulated renal vaso-constriction and Gq activation

RGS4 promotes the deactivation of Gαq/11 and Gαi/o and is expressed in vascular smooth muscle cells[19, 20, 25]. Our previous work implicated a cyclosporine-dependent release of endothelin-1 in the renal vasculature. We therefore hypothesized that *rgs4*$^{-/-}$ mice would be sensitized to the vascular effects of ligands that activate Gαq, such as endothelin-1. *Rgs4*$^{-/-}$ and wild type kidneys were rapidly isolated and placed on an ex vivo perfusion apparatus capable of measuring intra-renal perfusion pressures. Endothelin-1 (50pmol) was administered by bolus injection into the renal artery and perfusion pressures were measured. In wild type mice, the renal artery perfusion pressure increased and peaked 6 minutes after endothelin-1 injection, but rapidly decreased to baseline by 18 minutes post-injection. In contrast, *rgs4*$^{-/-}$ kidneys exhibited a sustained increase in renal artery perfusion pressures after endothelin-1 administration that did not decline for 25 minutes (Figure 6a).

To confirm that *rgs4*$^{-/-}$ kidneys were sensitized to Gq-mediated signaling in response to endothelin-1 infusion, we measured renal IP3 levels. Ex vivo perfused WT and *rgs4*$^{-/-}$ kidneys were treated with endothelin-1 or buffer for 10 minutes. Endothelin-1-treated *rgs4*$^{-/-}$ kidneys had significantly higher IP3 levels than endothelin-1-treated WT kidneys (Figure 6b).

RGS4 has intrinsic renal activity in an in vivo model of cold ischemia-reperfusion injury

To identify the renal-specific effect of RGS4 in vivo, we performed syngeneic kidney transplantation surgery, a model of cold renal ischemia-reperfusion injury, between *rgs4*$^{-/-}$ and wild type mice. This model allowed us to exclude the possibility that systemic factors in *rgs4*$^{-/-}$ mice were responsible for the abnormal renal vasomotor tone, such as circulating systemic factors or input from the autonomic nervous system[35, 36]. Kidneys were surgically isolated from wild type and *rgs4*$^{-/-}$ mice and were kept in buffer for 3 hours prior to implantation into wild type recipient mice. In a subpopulation of kidneys kept in cold storage, organs were cannulated and arterial perfusion pressure was measured in isolation (Suppl. Figure 1). Perfusion pressure stabilized in all kidneys after fifteen minutes of perfusion. Steady state perfusion pressure was increased in procured *rgs4*$^{-/-}$ kidneys. Renal blood flow in transplanted *rgs4*$^{-/-}$ kidneys was dramatically reduced in comparison to transplanted wild type kidneys in the early organ reperfusion phase (Figure 7).

To determine whether the reduced blood flow observed in transplanted *rgs4*$^{-/-}$ kidneys was associated with intra-renal injury, kidney sections were evaluated by histological methods 12 hours after transplantation into wild type mice. *Rgs4*$^{-/-}$ kidneys exhibited increased tubular cell death most prominently in the cortico-medullary junction whereas wild type kidneys exhibited minimal tubular cell death (Figure 8).

Role of RGS4 proteasomal degradation in renal IRI response

RGS4 protein levels are minimized in many cell types as a result of N-end rule pathway-mediated proteolysis[23, 24]. We hypothesized that acute treatment of WT mice with inhibitors of the N-end rule pathway-mediated proteasomal degradation would result in RGS4 protein up-regulation and that increased RGS4 protein levels would lead to resistance to IRI.

To increase RGS4 protein levels in kidney, we treated mice with the synthetic peptide aldehyde proteasomal inhibitor, MG-132, and then subjected animals to IRI. MG-132 treatment of WT resulted in increased RGS4 protein levels and reduced plasma urea 24 hours after IRI (Figure 9).

Although cytosolic RGS4 levels are distinctly sensitive to proteasomal inhibition the half-life of other beneficial proteins may also persist. To determine whether the effect of MG-132 on the response to renal IRI was specifically due to stabilization of RGS4 protein, we used *rgs4*$^{-/-}$ mice in similar experiments. Administration of MG-132 to *rgs4*$^{-/-}$ mice did not have a beneficial effect on IRI-mediated renal dysfunction (Figure 7b).

DISCUSSION

Acute renal failure (ARF) frequently occurs in the hospital setting and is associated with a high incidence of morbidity, unremitting kidney injury and death[37]. The extent of kidney injury is dependent on the duration of ischemia and has been incorporated into risk assessment scores because of the prognostic value of tissue ischemia[38–40].

RGS proteins are hypothesized to play a major role in the regulation of vascular tone throughout the body by modulating the activation of heterotrimeric G proteins[19, 20]. The

role of RGS4 in the regulation of vascular tone has not been previously studied. This is in part due to the low RGS4 mRNA levels in many tissues outside of the brain, except under periods of stress or inflammation [17, 25, 41, 42]. Furthermore, the basal protein levels for RGS4 in most tissues are very low. This is likely a consequence of constitutive N-end rule-mediated proteasomal degradation [23, 24] that can be manipulated to increase RGS4 levels in greater proportion than other vascular-associated proteins such as RGS2 and RGS5[24]. In the N-end rule pathway, the amino-terminal methionine of RGS4 is removed by a methionine aminopeptidase, and the uncovered second residue, a cysteine, is first oxidized and then arginylated, before RGS4 is poly-ubuiqinated and degraded by the proteasome [23, 24, 43, 44]. The dependence on oxygen or nitric oxide for the modification of the cysteine prior to arginylation suggests that kidney ischemia may result in RGS4 protein stabilization.

Heterotrimeric G protein signaling is a key feature in the physiologic regulation of vascular tone. In response to ischemia, vascular endothelial cells in the kidney are injured, leading to the release of endothelin-1, angiotensin-II, thromboxane A_2, adenosine and other ligands that stimulate seven transmembrane Gq protein-coupled receptors in smooth muscle cells. Serum concentrations of these ligands persist during the reperfusion phase and prolong the ischemic event beyond the period of extrinsic reduction in renal blood flow. RGS proteins may play a critical role in extinguishing the persistent intracellular signal initiated by extracellular GPCR ligands.

In previous work, we generated mice lacking RGS4 and found that *rgs4*$^{-/-}$ mice exhibited a markedly abnormal response to cyclosporine, with reduced renal blood flow, increased renal dysfunction, and mortality [28]. In the current work, we found that R4KO mice were highly sensitized to renal IRI. Isolated kidney experiments and syngeneic renal transplantation suggest this sensitivity is due to the intrinsic renal function of RGS4. The absence of RGS4 may prolong G protein-mediated vasoconstriction in the renal vasculature. This is evident in both our ex vivo and in vivo model systems and is consistent with the known function of RGS proteins as a deactivator of the G protein signaling cascade. RGS proteins enhance the GTPase activity of the Gα subunit to which they bind. Our studies are the first to identify RGS protein activity in the renal vasculature linked to physiologic function. RGS2 was the first RGS protein recognized to influence vascular hemodynamics in a mammalian model system. R*gs2*$^{-/-}$ animals exhibit baseline hypertension and increased collagen matrix deposition in elastic arteries of the kidney [45] in contrast to *rgs4*$^{-/-}$ which are normotensive. Similar to *rgs4*$^{-/-}$ animals, *rgs2*$^{-/-}$ exhibit end organ failure only after significant hemodynamic perturbation is initiated. Cardiomyocytes in *rgs2*$^{-/-}$ mouse hearts are highly responsive to supraphysiologic cardiac afterload and generate cardiac hypertrophy out of proportion to that identified in wild type controls [46]. RGS2 expression has not been identified in renal vasculature suggesting RGS proteins have discriminant activity in diverse vascular beds of the mammalian system. Agents that promote RGS4 expression, RGS4 protein stabilization, or that antagonize Gq/11 signaling in renal vascular smooth muscle cells are likely to reduce the development and progression of acute kidney injury after IRI. Agents that block the N-end rule pathway are likely to stabilize RGS4 protein and may have salutary effects on kidney function in the setting of ischemia/reperfusion injury.

METHODS

Mice with targeted disruption of the *RGS4* genes

Murine 129/SvJ embryonic stem cells with targeted disruption of one allele of the *rgs4* gene were generated as described [29]. Homozygous *rgs4*$^{-/-}$ mice in the C57Bl/6J genetic background (R4KO) were bred and were found to be viable and fertile. All research

involving the use of mice were performed in strict accordance with protocols approved by the Animal Studies Committee of Washington University School of Medicine.

Construction of Targeting Vector and Generation of RGS4 Null Mice

The RGS4 null mice expressing beta-galactosidase under the control of the RGS4 promoter were generated by Deltagen, Inc. (San Mateo, CA) with techniques described previously[47]. Briefly, a genomic fragment including protein coding regions of the RGS4 gene was isolated from a mouse genomic library and subcloned into the SK(−) vector. A segment of the protein coding region was replaced with an IRES-lacZ reporter and neomycin (G418) resistance cassette. This mutation was designed to produce a loss-of function mutation by deletion of an amino acid sequence.

Murine warm renal ischemia/reperfusion surgery

Adult male wild type and R4KO mice were anesthetized with a mixture of xylazine (16 mg/kg) and ketamine (80 mg/kg). We determined that R4KO mice were overly sensitive to simultaneous bilateral renal pedicle clamping. Therefore, a sequential pedicle clamp technique was employed. First, a left flank incision was made and the kidney was exposed. A pedicle clamp was placed on the left main renal artery and the clamp was adjusted so that the renal blood flow was reduced to 0.–1.5 mL/min/100g by Laser Doppler Flowmeter. After 25 minutes, the clamp was released and removed. The pedicle clamp was then placed and tightened on the main right renal artery for 25 minutes with confirmation by Laser Doppler Flowmeter that the right renal blood flow was reduced. After 25 minutes, the clamp was released and removed. The incisions were closed and the animal was allowed to recover.

Ex vivo isolated kidney perfusion

Our ex vivo kidney perfusion technique is an adaptation of the original protocol described by Stegbauer and colleagues[48]. We modified the technique for unilateral isolation of the left kidney to reduce warm ischemia time. Once extracted, the kidney is maintained at a constant pressure using a programmable peristaltic pump and in-line pressure monitor. Average flow of perfusate is 7.4 ml/min per gram of kidney tissue. Pressure was recorded digitally (2 kHz) by a pressure recording system composed of an ETH-256C amplifier and PB-100 probe (iWorx, Dover, NH), and the resulting pressure recordings were interfaced with a PC using an A/D converter (National Instruments, Austin, TX) and a home made program developed using LabView 7.1 (National Instruments, Austin TX) to control signal acquisition, data saving and off-line analysis. The kidney was maintained in a thermo-controlled chamber and infused with Krebs-Henseleit buffer at 37°C.

In a subset of animals designated for kidney procurement and transplantation organs were procured and instead cannulated for ex vivo measurement of perfusion pressure. Immediately after extraction, organs were flushed with organ preservation buffer and stored for three hours at 2C° and then analyzed with ex vivo kidney perfusion apparatus. Flow rate was gradually increased over the first 2 minutes of perfusion then kept constant at 2.5 mL/min per g while perfusion pressure was measured.

Syngeneic renal transplantation

At twelve weeks of age, donor mice were randomized to undergo left nephrectomy. The surgical operator was blinded to the animal's genotype. Mice were anti-coagulated with systemic heparin 1000 U/kg. Mice were anesthetized with isoflurane. Aortic branches including the spermatic arteries and lumbar arteries were ligated with suture. Ligatures were placed around the aorta proximal to the left renal artery and proximal to the femoral artery

bifurcation. Aortic ligation was then performed with dissection of the left kidney from perinephric fascia. The ureter was kept intact along with a 10 mm diameter bladder patch. The procured kidney was immediately flushed with chilled Collins organ preservative buffer. Recipient mice at 20 weeks of age, weighing between 25–30 g, were selected as syngeneic renal allograft recipients. Microvessel clamps with 60 g/mm^2 of force were placed at the transverse level inferior to the spermatic arteries and proximal to the femoral artery bifurcations. Clamping these locations helps to avoid the need to ligate arterial branches and reduces the number of lumbar vein ligations. An aortotomy was performed with a curved 6-0 surgical needle. Orifice diameter was enlarged with iridectomy scissors to match the diameter of the donor allograft's artery. Two running stitches were used for the arterial anastomosis. Venotomy was used to perform similar technique for the venous anastomosis. Donor venotomy was made proximal to the outflow of the renal vein. Two running stitches were used for the venous anastomosis. The microvessel clamps were removed. The contents of the recipient's bladder was aspirated with a 31 gauge needle. The bladder wall was incised to a diameter matching the donor's bladder patch. Two running stitches were used to anastomose the bladder walls. The abdomen was rinsed with sterile normalized saline. The fascial and then skin layers were closed with 6-0 silk and the animal was allowed to recover in a clean, isolated cage.

Renal histology and morphometry

Mice were weighed, and kidneys isolated. Tissue was fixed in 10% formalin and paraffin-embedded. 5-μm sections were microtome sectioned and exposed to Schiff's reagent to perform Periodic Acid Schiff (PAS) staining. Tubular injury scoring was completed on each kidney at the maximum longitudinal section in the sagittal plane. For each specimen, 10 consecutive fields at a magnification of 200× were analyzed Individual sections were evaluated and considered positive for injury by the presence of tubule flattening, necrosis, apoptosis, or lumenal casts. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was performed by the Washington University histology core. For each specimen, 10 consecutive fields at a magnification of 200× were analyzed for green fluorescent nuclear staining which designated TUNEL positivity. X-gal staining and co-staining with alpha-smooth muscle actin monoclonal mouse antibody (Abcam, Cambridge, MA) was performed by the Washington University histology core. Frozen tissue sections were microtomed (6μm) and formalin-fixed for 10 minutes in preparation for X-gal staining. Tissue was then washed with PBS and incubated with X-gal working solution (X-gal [Boehringer Mannheim, Germany] + N,N- Dimethylformamide) for 24 hours and washed again. Co-staining with alpha-smooth muscle actin monoclonal mouse antibody was then performed. Finally 4',6-diamidino-2-phenylindole (DAPI) solution (Molecular Probes Inc., Eugene, OR) staining verified cell viability DAPI staining

Protein analysis

Cytosolic protein lysates from kidney tissue were obtained as previously described[28]. In brief, tissue was harvested from mice, and lysates were processed in the presence of protein lysis buffer. Once processed, protein bound to nitrocellulose filters were washed and incubated with murine anti-RGS4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-β actin (Sigma Aldrich, St. Louis, MO). Bands were visualized by use of the ECL system (GE-Healthcare Bio-Sciences Corp., Piscataway, NJ). Densitometry was performed using Image J software v1.24.

Inositol-1,4,5-triphosphate tissue measurement

IP3 can now be assayed directly with a radioreceptor assay (Perkin Elmer, Waltham, MA) avoiding indirect calculation of IP3 levels by measurement of the activity of inositol polyphosphate precursors[49]. Tissue was rapidly isolated, bivalved, snap frozen in liquid

nitrogen, rapidly pulverized on dry ice. The pulverized tissue was immediately resuspended in ice cold 1M trichloroacetic acid (TCA). TCA solution includes 1mM EDTA to chelate magnesium prevent phosphatase interference and minimize the degradation of IP3. After centrifugation, the supernatant was removed. 1,1,2-Trichloro-1,2,2-trifluoroethane (TCTFE)-trioctylamine was added in 2:1 proportion for each volume of supernatant. The aqueous layer was aspirated and stored on ice. A working receptor/tracer solution was prepared by diluting the concentrated receptor preparation tracer in 1:15 proportion. The working receptor/tracer solution was thoroughly mixed in 4:1 proportion with each sample and centrifuged at 2000×g for 17 minutes. Samples were then decanted and resuspended with 0.15M NaOH and mixed with 5mL of Ultima Gold scintillation fluid (Packard Instruments, Meridian, CT). Scintillation counts were measured in a Beckman scintillation counter.

In vivo hemodynamic monitoring

Renal artery blood velocity was measured immediately following the release of the left renal artery pedicle clamp in our model of warm renal ischemia/reperfusion injury. We used an Advanced Laser [Doppler] Flowmeter 21 (ALF21, ADVANCE Co., Tokyo, Japan) which measures instantaneous fluid flow and was directly applied to the medial aspect of the left kidney's cortical surface at the level of the midpole. The timing of measurement after the first pedicle clamp release avoided the possibility of preconditioning. Mice were anesthetized with a mixture of xylazine (16 mg/kg) and ketamine (0.080 mg/g). A sham procedure was performed exposing the right kidney but maintaining its blood supply. After renal transplant the same technique was used to measure renal blood flow. Control blood flow in transplant recipients was performed on the right native kidney after the transplant anastomoses were complete.

A volume pressure recording (VPR) device (Kent Scientific Corp., Torrington, CT) was used for tail cuff blood pressure measurements of mice during and after surgical procedures. Five cycles were completed per animal and then averaged.

MG-132 administration

The synthetic peptide aldehyde proteasomal inhibitor, MG-132, was administered 48 hours and 24 hours prior to surgery. Mice were treated with MG-132 (2 mg/kg) or vehicle (DMSO) (100 μL) 48 hours prior to IRI. Mice were then re-dosed with MG-132 (1 mg/kg) or vehicle (50 μL) 24 hours prior to surgery.

Quantitation of body fluid constituents

Laboratory values were obtained 24 hours after warm ischemia/reperfusion injury was performed. Levels of blood plasma urea nitrogen and serum creatinine were measured in the Department of Comparative Medicine Diagnostic Laboratory at Washington University School of Medicine.

Survival studies

Mice were monitored after warm ischemia/reperfusion injury on a perpetual 8 hour interval. Mice that were determined moribund after one time interval were monitored more frequently on a four hour basis. Mice that remained moribund after one eight hour interval and then one four hour interval were euthanized. Mice were defined as moribund if they were immobile, or lethargic with raised hair, or lethargic and tremulous.

Statistical analysis

All data are presented as mean values ± standard error of the mean. Static group analysis was performed with SigmaStat® 3.1 (Systat Software, Inc., San Jose, CA). Statistical relationships were determined by the student's T-test. One-way ANOVA analysis was used in comparisons of greater than 2 groups, and Holm-Sidak pairwise comparisons were calculated between groups. Kaplan Meier survival curves and Mantel Cox log-rank analysis was performed with SPSS v13.0 (SPSS Inc., Chicago, IL).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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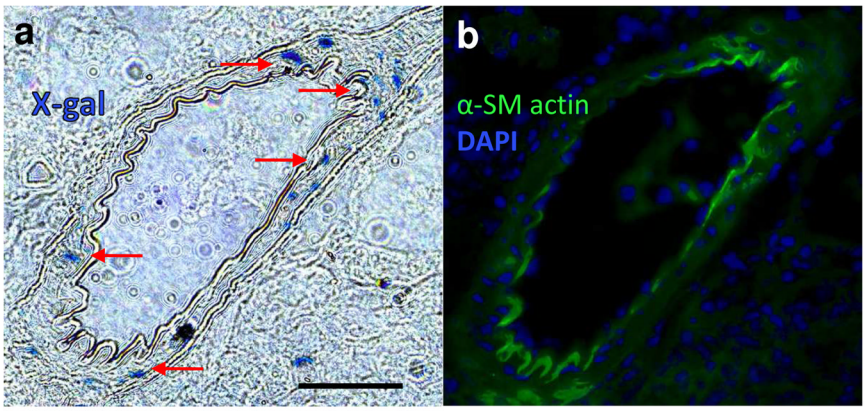

Figure 1. X-gal staining in the RGS4 reporter mouse (rgs4$^{tm1Dgen/+}$)

(a) X-gal Staining is focused to the nuclear compartment in arterial vascular smooth muscle cells of the kidney **(b)** Tissue was co-stained with anti-alpha-smooth muscle actin (α-SM actin) mouse antibody to confirm that β-galactosidase is expressed in vascular smooth muscle cells. 4',6-diamidino-2-phenylindole (DAPI) staining verified cell viability. (scale bar = 20μm).

Figure 2a

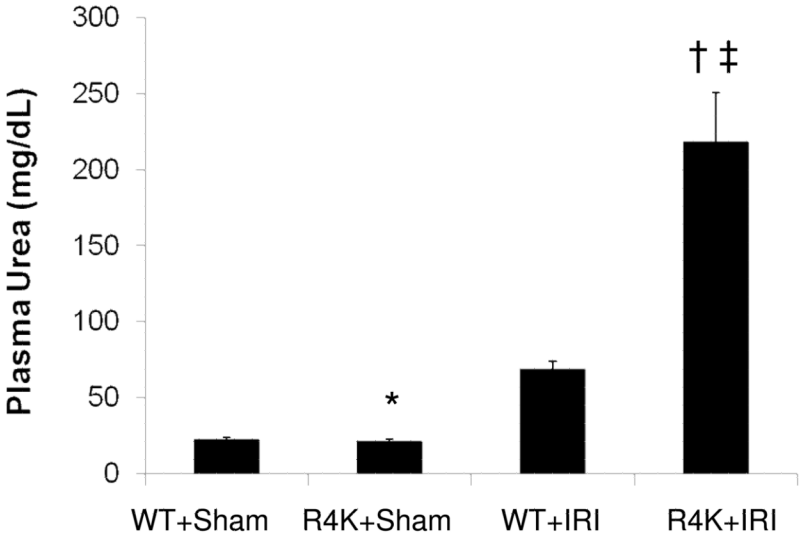

Figure 2b

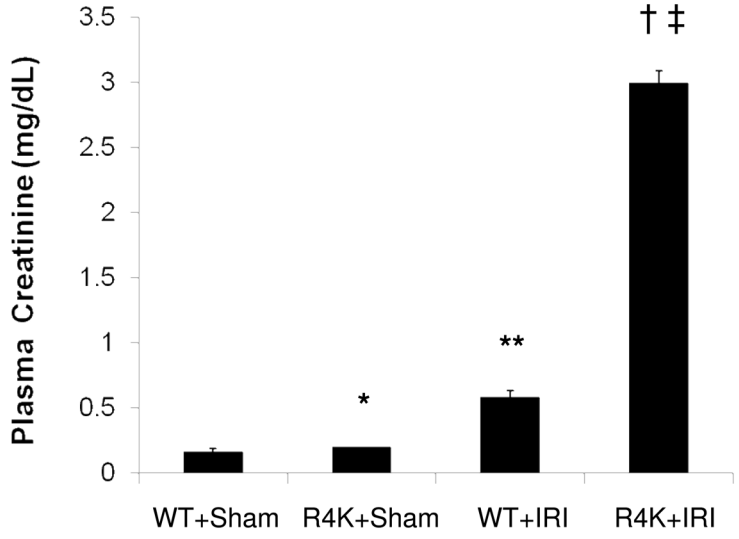

Figure 2. *RGS4* $^{-/-}$ mice are susceptible to renal ischemia/reperfusion injury

(a) Plasma urea was measured in *rgs4*$^{-/-}$ (R4K) (n=10) and wild type congenic mice (WT) (n=10). Plasma urea was increased in R4K after ischemia-reperfusion injury (IRI) compared to other groups by one-way independent ANOVA pair-wise comparison: composite p $=4\times10^{-5}$; WT+Sham (n=5) vs R4K+Sham (n=5) = * (p = 0.68); R4K+Sham (n=5) vs R4K +IRI (n=5) = † ($p=2\times10^{-4}$); WT+IRI vs R4K+IRI = ‡ ($p=3\times10^{-4}$). **(b)** Plasma creatinine was measured in *rgs4*$^{-/-}$ (R4K) (n=10) and wild type congenic mice (WT) (n=10). Plasma

creatinine was increased in R4K after ischemia-reperfusion injury compared to other groups by one-way independent ANOVA pair-wise comparison: composite $p = 1\times10^{-10}$; WT+Sham (n=5) vs R4K+Sham (n=5) = * (p = 0.7); WT+Sham (n=5) vs WT+IRI (n=5) = ** ($p=1\times10^{-4}$) ; R4K+Sham (n=5) vs R4K+IRI (n=5) = † ($p=1\times10^{-11}$); WT+IRI vs R4K+IRI = ‡ ($p=1\times10^{-7}$).

Figure 3a-d

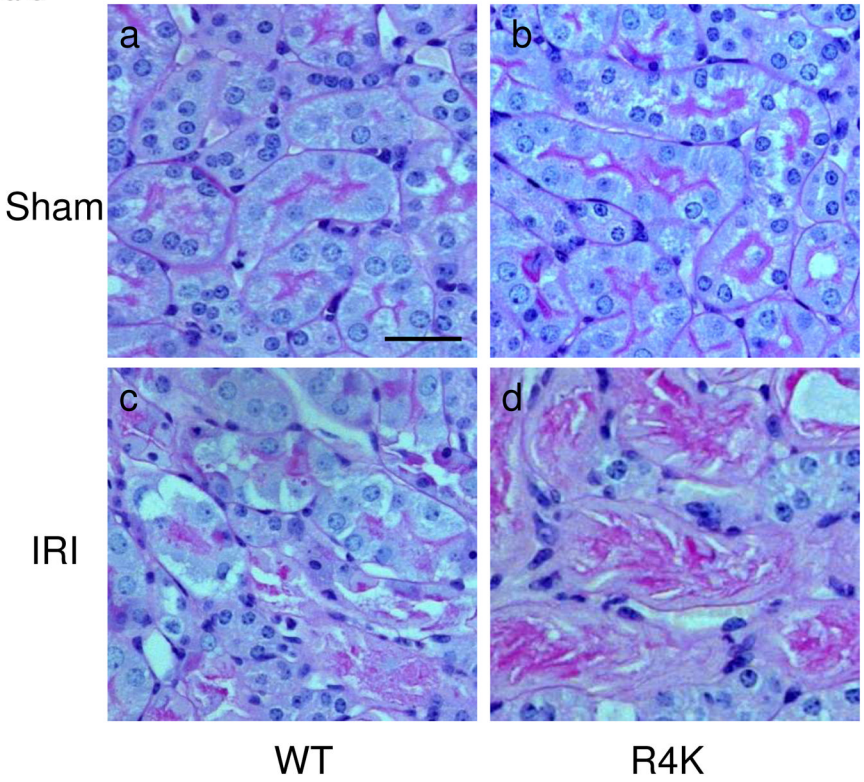

Figure 3e

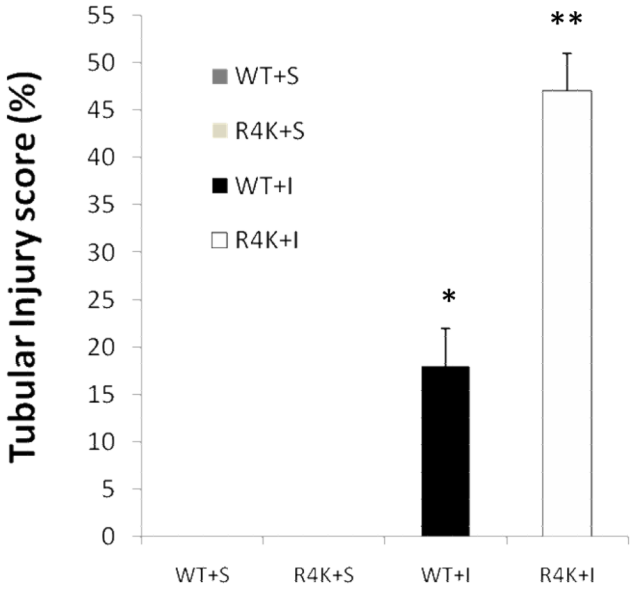

Figure 3. Absence of RGS4 leaves the kidney susceptible to acute tubular necrosis due to schemia/reperfusion injury (IRI)

Periodic Acid Schiff stained kidney sections from (**a,c**) wild type and (**b,d**) RGS4 knockout underwent sham surgery or IRI. (**d**) R4K+I kidneys had an increased tubular injury score of 47±6%, 2.6-fold more tubular damage than WT+I (**p=2×10^{-8}, Student's t-test). WT+I vs WT+S,*p<0.001. Kidney sections were processed after 24hrs of reperfusion and were evaluated for tubule flattening, necrosis, apoptosis, or presence of casts. (scale bar = 100μm).

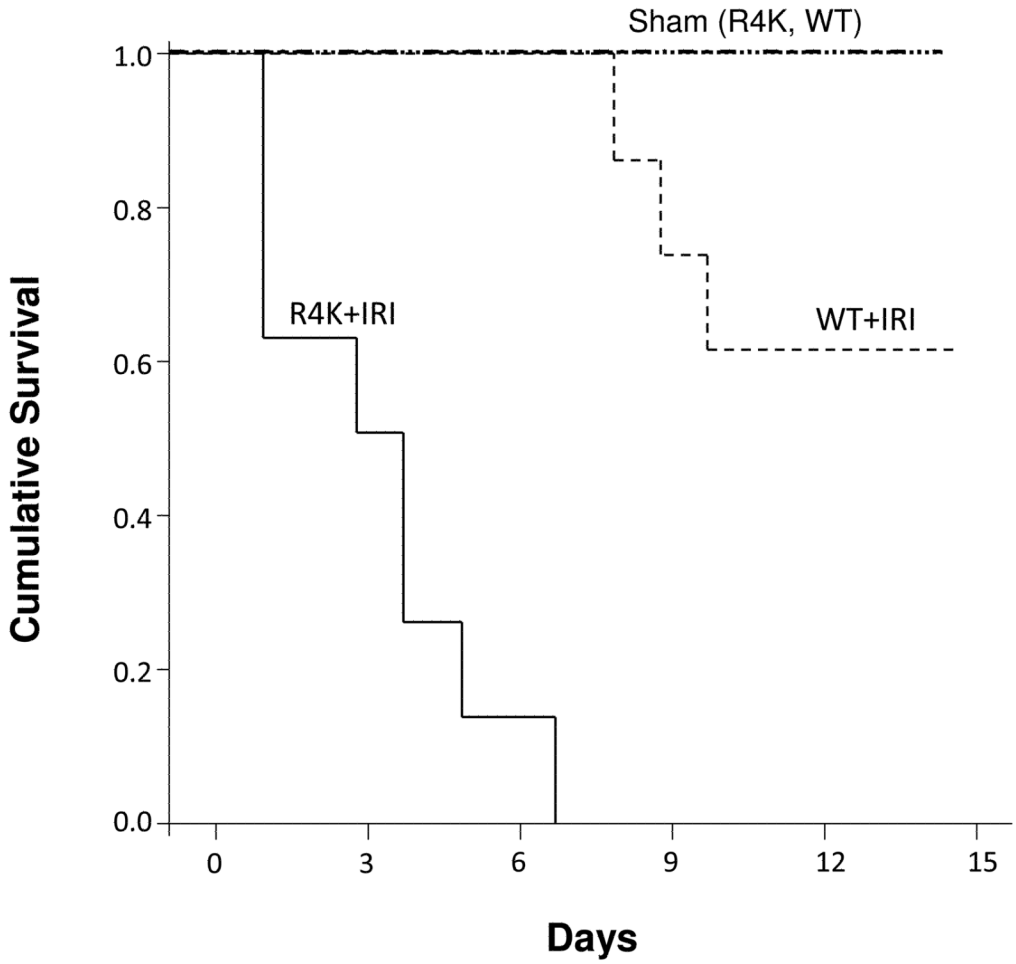

Figure 4. Increased mortality after standardized ischemia/reperfusion injury

Rgs4$^{-/-}$ mice (n = 8) versus congenic wild type controls (WT) (n = 8) underwent the same surgical procedure. Survival after IRI was monitored for 15 days. (Mantel–Cox log rank test , $p=1\times10^{-6}$).

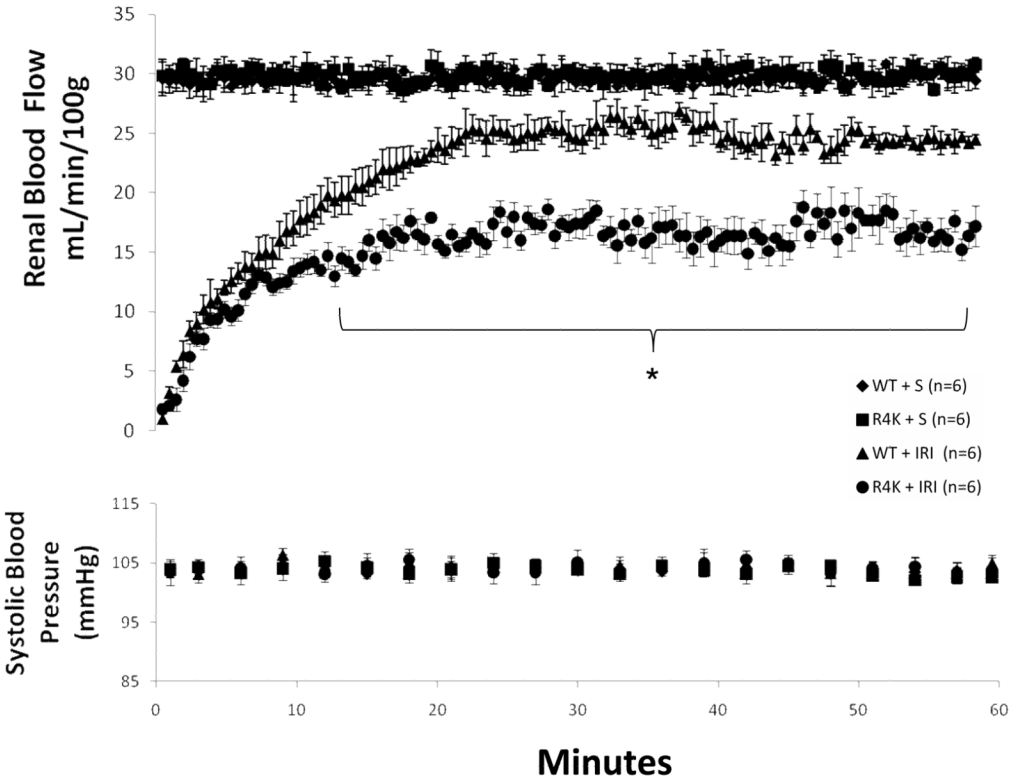

Figure 5. Reduced recovery of renal blood flow was observed in *RGS4*$^{-/-}$ mice after renal ischemic injury

Renal blood flow in wild type (WT) and *rgs4*$^{-/-}$ (R4K) kidneys was analyzed by use of a laser Doppler flowmeter placed on the midpole of the left kidney in the minutes following the ischemic phase of IRI (IRI) surgery or after a sham operation (S). Blood flow measurement was obtained in wild congenic control mice undergoing sham surgery (WT +S), *rgs4*$^{-/-}$ undergoing sham (R4K+S), congenic control mice undergoing ischemia/ reperfusion injury (WT+IRI), or *rgs4*$^{-/-}$ undergoing ischemia/reperfusion injury (R4K+IRI). ANOVA pair-wise comparison: *,composite $p=1\times10^{-11}$. Systemic blood pressure was measured simultaneously with tail-cuff volume pressure measurement device.

Figure 6a

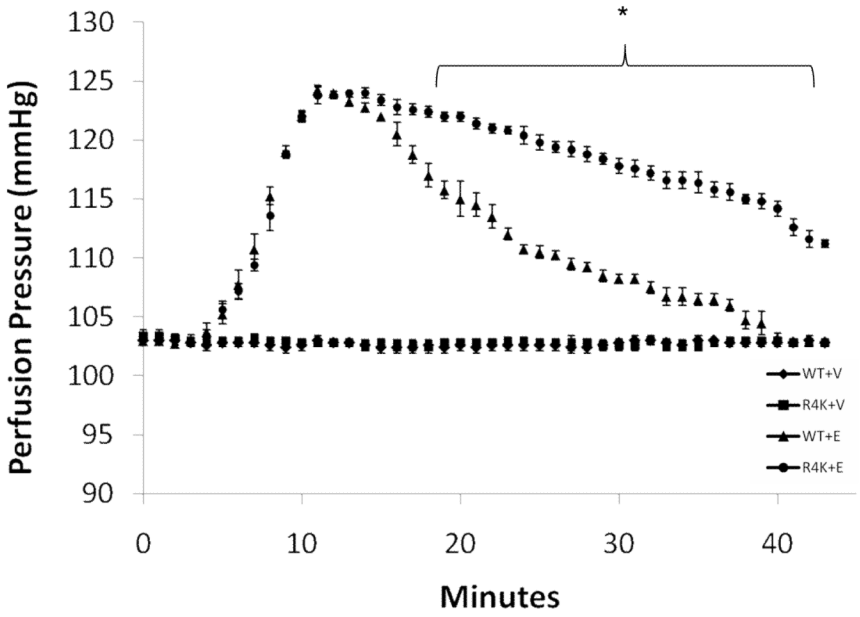

Figure 6b

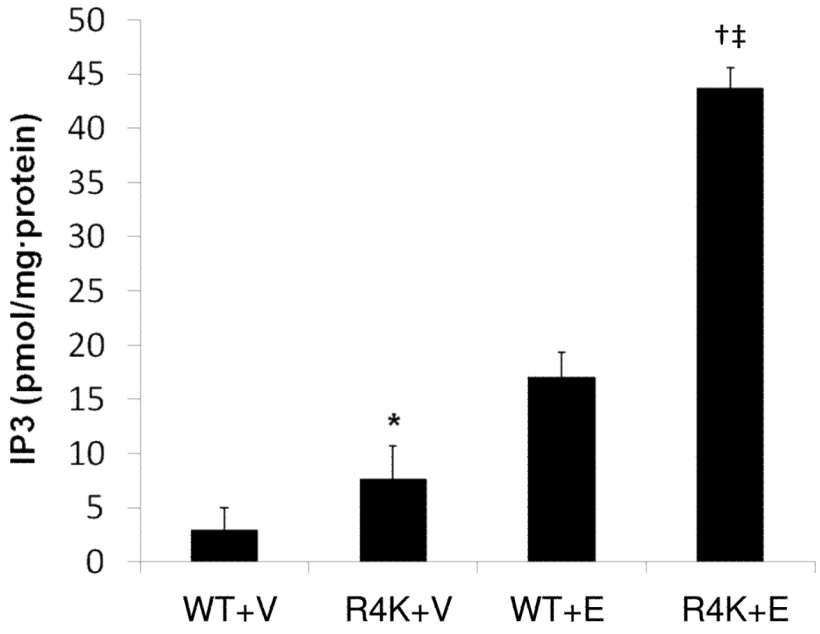

Figure 6. An enhanced response to endothelin-1 was observed in RGS4-null kidneys

(a) Persistent vasoconstriction observed in ex vivo perfused *rgs4*$^{-/-}$ kidneys after infusion of endothelin-1. Kidneys were isolated from wild type or *rgs4*$^{-/-}$ mice and were placed on a perfusion apparatus with constant monitoring of the perfusion pressures. Kidneys were administered a bolus injection of saline vehicle (V) or endothelin-1 (E), and the renal perfusion pressures were monitored for 45 minutes. Endothelin bolus (50pmol) was injected to the ex vivo kidney perfusion system. After 18±2 minutes the rate change of of pressure (mmHg) per unit of time (seconds) was distinct between congenic controls (WT+E)(n=5) and *rgs4*$^{-/-}$ exposed to endothelin (R4K+E) (n=5). ANOVA pair-wise comparison: composite $p=2\times10^{-10}$. Average decrease in pressure / change in time (−dp/dt) was −0.37±0.02 mmHg/min in *rgs4*$^{-/-}$ vs. −0.73±0.01 mmHg/min in WT ($p=1\times10^{-5}$, student's t test). **(b)** Increased inositol triphosphate accumulation in *rgs4*$^{-/-}$ kidneys in response to bolus injection of endothelin-1. Kidneys were isolated, perfused and administered vehicle or endothelin-1 as above. IP3 was increased in *rgs4*$^{-/-}$ exposed to endothelin (R4K+E) (n=5) compared to wild type congenic mice (WT+E) (n=5) by one-way ANOVA pair-wise comparison: composite $p = 2\times10^{-12}$; WT+V (n=5) vs R4K+V (n=5) = * (p = 0.16); R4K+E (n=5) vs WT+E (n=5) = † ($p=4\times10^{-9}$) ; R4K+E vs R4K+S (n=5) = ‡ ($p=1\times10^{-9}$).

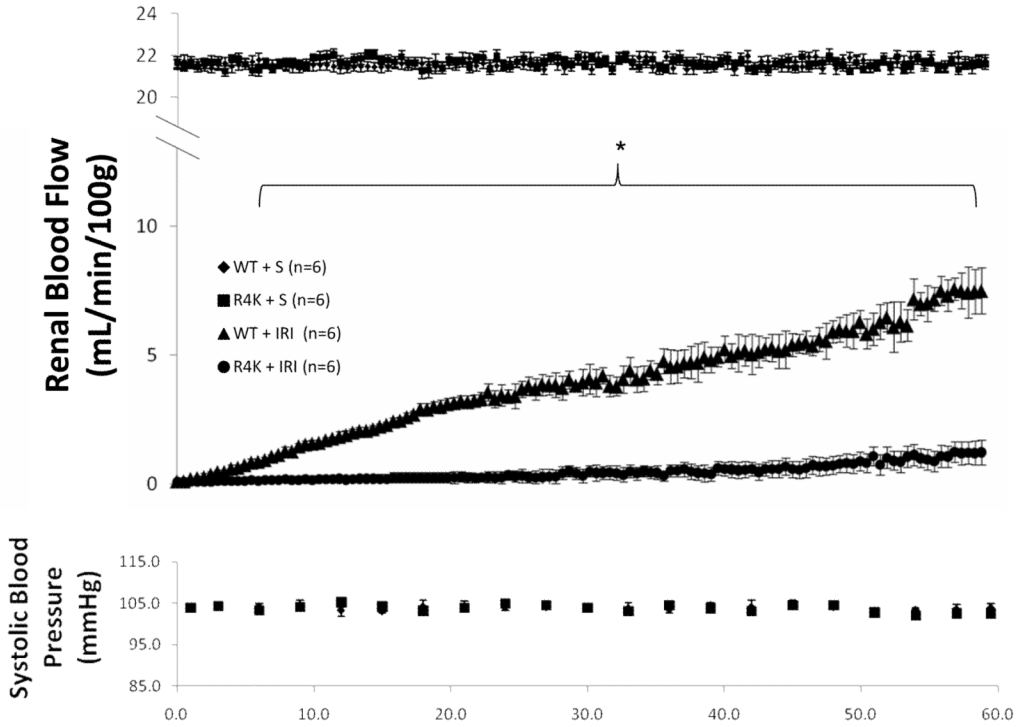

Figure 7. Reduced renal blood flow in kidney transplants with RGS4 deletion

Renal blood flow was measured immediately after transplant anastomosis in wild type (WT +TX) and *rgs4*$^{-/-}$ (R4K+TX) kidney allografts. The rate change of reperfusion blood flow was depressed in R4K allografts compared to WT allografts during reperfusion (*, $p=1\times10^{-11}$). Systemic blood pressure was measured simultaneously with tail-cuff volume pressure measurement device.

Figure 8a-d

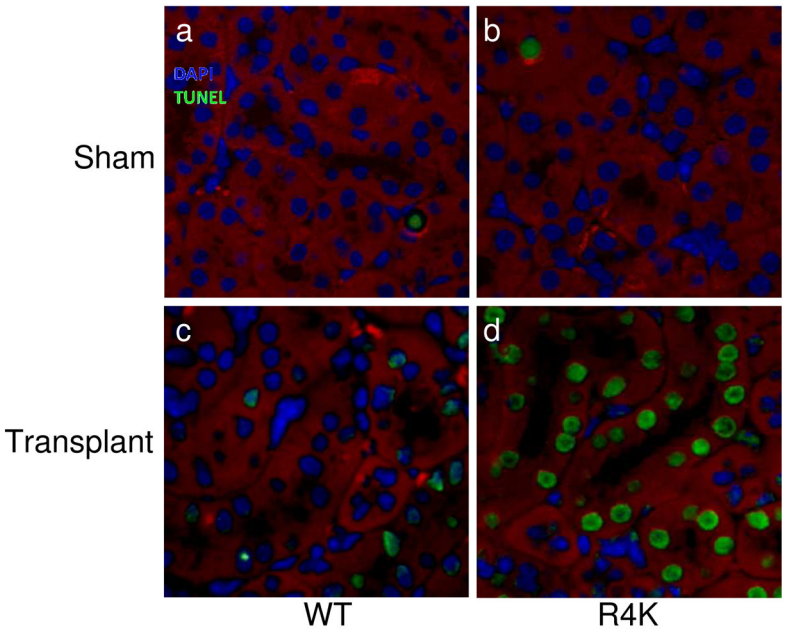

Figure 8e

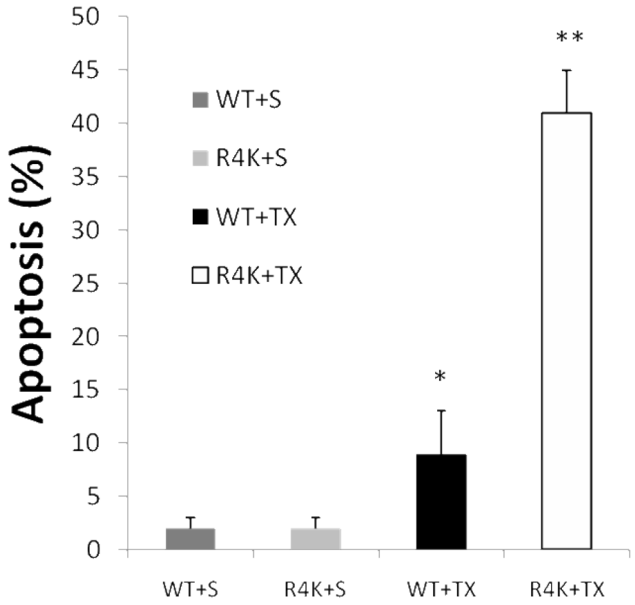

Figure 8. Cell death after syngeneic renal transplantation

TdT-mediated dUTP nick end labeling assay (TUNEL) staining performed on tissue sections from (**a,c**) wild type and (**b,d**) RGS4 knockout kidneys obtained after procurement or 24 hours after allograft implantation, respectively. (**e**) Few TUNEL positive cells (green) were seen in sham WT or R4K kidneys after procurement however there was 5.3-fold more TUNEL positive cells in R4K donor allografts (R4K+TX) compared to WT animals

undergoing the same procedure (WT+TX). (**,$p=1\times10^{-5}$). Increased cell death was present in wild type mice undergoing surgery (WT+TX) compared to shams (WT+S) (*, p=0.012, Student's t-test). Scale bar=25μm.

Figure 9a

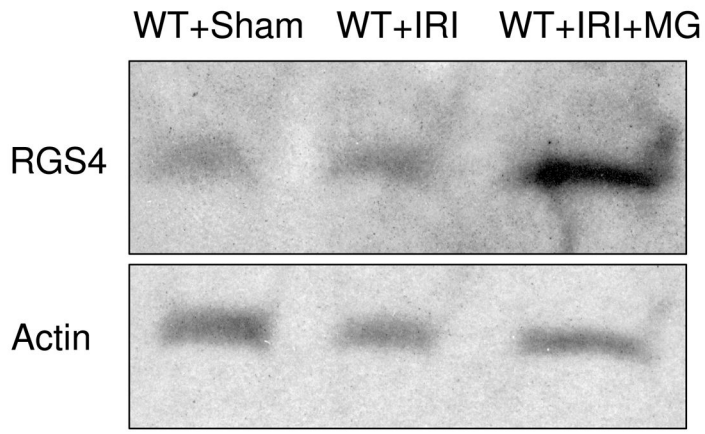

Figure 9b

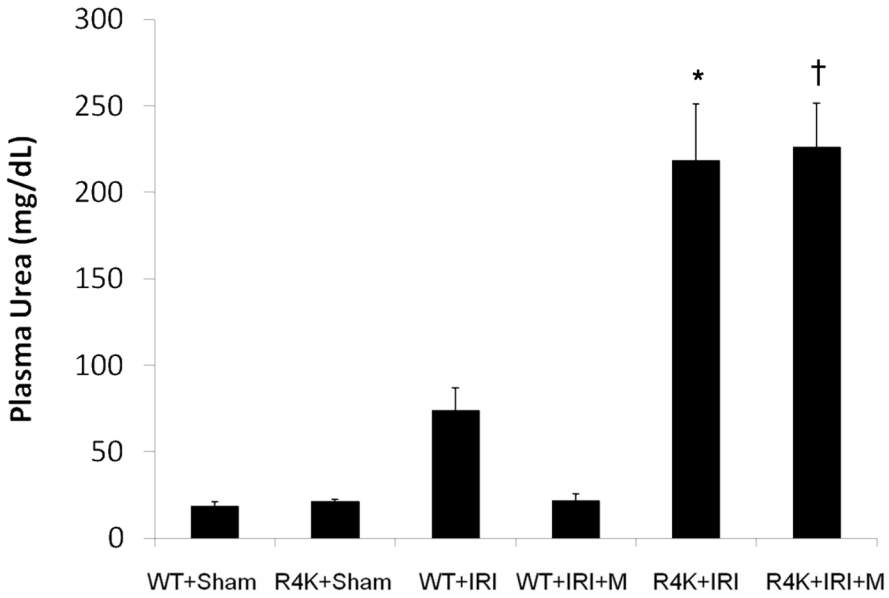

Figure 9. Proteasomal inhibition prevents IRI in kidneys by stabilizing RGS4 protein levels

(a) Administration of MG-132 (M) to wild type mice results in increased renal RGS4 protein levels. Immunoblot is representative of five separate experiments. (**b)** Administration of MG-132 (M) to wild type mice (WT), but not *rgs4*$^{-/-}$ mice (R4K), inhibits renal IRI. 24 hours after IRI surgery, blood was obtained from mice for determination of plasma urea nitrogen levels. For each group, 5 animals were analyzed. *,$p=5\times10^{-8}$ versus R4K+Sham; †,$p=1\times10^{-11}$ versus R4K+Sham.