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CRRL269: A Novel Particulate Guanylyl Cyclase A Receptor Peptide Activator For Acute Kidney Injury

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Abstract

Rationale: Acute kidney injury (AKI) has a high prevalence and mortality in critically ill patients. It is also a powerful risk factor for heart failure (HF) incidence driven by hemodynamic changes and neurohormonal activation. However, no drugs have been approved by the FDA. Endogenous particulate guanylyl cyclase A receptor (pGC-A) activators were reported to preserve renal function and improve mortality in AKI patients, while hypotension accompanied by pGC-A activators have limited their therapeutic potential.

Objective: We investigated the therapeutic potential of a non-hypotensive pGC-A activator, CRRL269 in a short-term, large animal model of ischemia induced AKI and also investigated the potential of urinary C-type natriuretic peptide (uCNP) as a biomarker for AKI.

Methods and Results: We first showed CRRL269 stimulated cGMP generation, suppressed plasma angiotensin II and reduced cardiac filling pressures without lowering blood pressure in the AKI canine model. We also demonstrated CRRL269 preserved glomerular filtration rate (GFR), increased renal blood flow (RBF) and promoted diuresis and natriuresis. Further, CRRL269 reduced kidney injury and apoptosis as evidenced by ex vivo histology and tissue apoptosis analysis. We also showed, compared to native pGC-A activators, CRRL269 is a more potent inhibitor of apoptosis in renal cells and induced less decreases in intracellular Ca²⁺ concentration in vascular smooth muscle cells. The renal anti-apoptotic effects were at least mediated by

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DISCLOSURES

Mayo Clinic, Y.C. and J.C.B. filed a patent for the use of CRRL269 for acute kidney injury treatment. Mayo Clinic, Y.C., S.J.S. and J.C.B. filed a patent for the use of urinary CNP as a potential biomarker for acute kidney injury diagnosis. The authors declare no conflicts of interests.

cGMP/PKG pathway. Further, CRRL269 inhibited pro-apoptotic genes expression using a PCR gene array. Additionally, we demonstrated AKI increased uCNP levels.

Conclusions: Our study supports developing CRRL269 as a novel renocardiac protective agent for AKI treatment.

Keywords

natriuretic peptide; particulate guanylyl cyclase A receptor; acute kidney injury; urinary CNP; apoptosis; calcium; heart failure; drug research; cardio-renal syndrome

Subject Terms:

Cardiorenal Syndrome; Heart Failure; Ischemia; Nephrology; Kidney

INTRODUCTION

Acute kidney injury (AKI) is a syndrome of impaired renal function, characterized by a reduction of glomerular filtration rate (GFR) or urine output (UV) ^{1, 2}. It has an overall frequency of 6% in community acquired and hospital acquired subjects and is especially common in critically ill patients admitted to intensive care units, with a frequency of 57% ^{3, 4}. Notably, AKI is a common complication after cardiovascular (CV) surgery such as coronary artery bypass grafting and aortic repair, ranging from 13% to 43%, depending on the definition of AKI ⁵⁻⁷. Further, hypotension and decreased renal perfusion also plays a key role in initiating AKI in the surgical setting. In a contemporary matched cohort of 146,941 hospitalized adults, 31,245 (21%) experienced AKI. Importantly, the study observed that AKI was independently associated with a higher risk for heart failure (HF) after discharge ². Despite increasing prevalence of AKI with high mortality and high risk for HF, no drugs for AKI have been approved by the FDA.

Both ischemia and hypovolemia have been implicated in mediating AKI through activation of pathways of cellular injury and death. Indeed, one mechanism for AKI as well as the increased risk for HF is activation of intrarenal and circulating inflammatory cytokines and deleterious hormones such as angiotensin II (ANGII). Notably, experimental AKI and transition to chronic kidney disease is reduced with ANGII receptor type 1 (AT1R) blockade prior to the onset of ischemic AKI ⁸. Moreover, recent studies have also reported that ANGII and the AT1R mediate intrarenal inflammation in models of AKI ^{8, 9}. Additionally, renal cell apoptosis has emerged as an underlying final pathway contributing to AKI initiation and associated decline in GFR ¹⁰.

CRRL269 is a novel designer natriuretic peptide (NP) which was engineered at the Mayo Clinic as an innovative peptide targeting the renocardiac protective particulate guanylyl cyclase A receptor (pGC-A) which synthesizes the second messenger guanosine 3',5' cyclic monophosphate (cGMP) ¹¹. Importantly, CRRL269 is a selective pGC-A activator with minimal particulate guanylyl cyclase B receptor (pGC-B) activity ¹¹. Activation of pGC-A is associated with inhibition of cell apoptosis, suppression of inflammatory cytokines, diuresis and natriuresis, induction of endothelial regeneration, anti-remodeling with inhibition of the

renin-angiotensin system (RAS) ^{12–18}. Specifically, CRRL269 represents a chimeric peptide that integrates key amino acids (AAs) from URO (urodilatin, a renal-derived peptide) with key AAs from BNP (B-type NP, a cardiac derived peptide) ¹¹. CRRL269 possesses enhanced renoprotective properties such as diuresis, natriuresis and increase in GFR compared with native pGC-A activators such as BNP or URO while retaining RAS-suppressing properties with less hypotension. Indeed, CRRL269 mediated superior renal pGC-A activation in vitro and renal protective properties in vivo compared to the two respective NPs. Mechanisms for its enhanced renal actions are due to increased resistance to neprilysin degradation and RAS suppression ¹¹. The mechanism of BP preservation was due to reduced arterial relaxation and greater cardiomyocyte cAMP generation ¹¹. Although it is known that NPs induce vasorelaxation via suppression of intracellular Ca²⁺ in vascular smooth muscle cells (VSMC) ^{19, 20}, the action of CRRL269 on Ca²⁺ levels in VSMC remains undefined.

Given the renoprotective properties of CRRL269 and the need for FDA-approved therapies targeting AKI, here we investigated for the first time the renocardiac protective and RAS suppressing actions of CRRL269 in a large animal model of ischemic AKI produced by the clinically relevant maneuver of aortic cross clamping (ACC) simulating surgical aortic repair ²¹. This included assessing CRRL269 mediated increases in plasma, urinary and renal cGMP. We also sought to define in vitro the role of renal apoptosis in ischemic AKI and the ability of CRRL269 to attenuate cell death compared with BNP and atrial NP (ANP). Additionally, we defined the role of PKG as a mediator of the anti-apoptotic actions of CRRL269. We also explored the molecular mechanism of CRRL269's BP preservation through the actions of CRRL269 on intracellular Ca²⁺ levels in vascular smooth muscle cells. Lastly, we assessed urinary C-type natriuretic peptide (uCNP) as a biomarker for AKI, based upon our previous findings in human HF cohorts and aged animals ^{22–24}.

METHODS

Studies were performed in accordance with the Animal Welfare Act and with approval of the Mayo Clinic Institutional Animal Care and Use Committee (IACUC protocol number: A00002722).

The authors declare that all supporting data are available within the article (and its online supplementary files in the Online Data Supplement).

Ischemic acute kidney injury in canines.

An ischemic AKI model in normal mongrel canines (male, 25–30kg, age 1–2 years old, n=5 per group, Twin Valley Kennel) was created with high dose intravenously indomethacin infusion (10mg/kg with NaHCO₃ [5mg/kg] dissolved in 50ml distilled water) followed by suprarenal aortic cross clamping (ACC), as described previously ²¹. The number of animals in each group was defined by a priori power calculation considering a statistical power of 80% and $\alpha=0.05$. We used male canines only to minimize the experimental variation of the outcomes of ischemic acute kidney injury and we plan to recruit both male and female patients in our future clinical trials. Briefly, canines were anesthetized with intravenous pentobarbital (15–30 mg/kg) and fentanyl (4–12 μ g/kg), intubated and mechanically

ventilated on air with supplemental oxygen, 3 L/min, 12 cycles/min (Harvard Apparatus, Millis, MA). Both femoral artery and carotid artery were accessed for mean arterial pressure (MAP) monitoring and blood sampling. The femoral vein was cannulated for continuous inulin infusion and saline/indomethacin/drug infusions. The left renal artery was attached with a probe for renal blood flow (RBF) measurement and a catheter was placed in the left ureter for passive urine collection. A flow-directed balloon-tipped thermodilution catheter was advanced to the pulmonary artery through the external jugular vein for measurement of cardiac filling pressures and cardiac output (CO). After 60 min equilibration, baseline (BL) characteristics were established. A 45 min infusion of indomethacin (INDO) was then started. A Blalock clamp was applied at the level of the suprarenal aorta (ACC) to occlude blood flow to two kidneys. ACC was maintained for 60 min and 16.3 pmol/kg/min CRRL269 or vehicle (0.9% saline) infusion was initiated 5 min before releasing the clamp. Dogs were randomized to receive CRRL269 or vehicle infusion. Investigators were not blinded to treatments but biochemical analysis was performed by technicians who were blinded to treatment. The dose of CRRL269 was chosen based upon ANP dose used in our previous AKI study²¹ and it is a medium dose between low and high doses we investigated in our previous study in normal canines¹¹. Drug infusion lasted for 120 min and data/samples were collected every 30 min, termed as Clearance 1 (CL1), Clearance 2 (CL2), Clearance 3 (CL3) and Clearance 4 (CL4). Immediately after completion of CL4 the animal was sacrificed, left kidney was harvested, sliced and preserved in 10% formalin solution or slices were snap frozen with liquid nitrogen. GFR values were calculated based on inulin clearance. Urinary sodium was measured with pHox Ultra (Nova Biomedical, Waltham, MA). Dogs were included in the study based upon the effectiveness of renal aortic clamping which was demonstrated by the marked elevation of MAP and lack of urine output. One dog was euthanized early as a result of severe blood loss after unclamping of the aorta, which was torn by the surgical clamp, and therefore was excluded from the analysis.

Hormonal measurements.

Plasma and urinary cGMP were measured with commercially available ELISA kits (Enzo Life Sciences, Farmingdale, NY). Ex vivo renal parenchyma cGMP levels were measured with the same ELISA kits. Approximately 0.1g frozen canine renal cortex or medulla (n=5 Veh, n=4 CRRL269) were homogenized with 2mL 0.1N HCl by a homogenizer (Polytron PT1200E, Fisher Scientific, Waltham, MA). Homogenates were subjected to 600g 10 min centrifugation and supernatants were collected for cGMP assay. Final cGMP levels were adjusted by protein concentration measured by BSA method. Plasma angiotensin II (ANGII) was measured by radioimmunoassay (RIA) (Phoenix pharmaceuticals, Burlingame, CA). Plasma and urinary CNP was measured by CNP RIA (Phoenix pharmaceuticals, Burlingame, CA). Both ELISA and RIA were performed as instructed by the manufacturers' protocols.

Histology and TUNEL analysis.

Fixed renal tissues were embedded with paraffin and sliced. For histology analysis, paraffin-embedded slides in vehicle (n=5) and CRRL269 (n=5) were stained with hematoxylin and eosin (H&E). A renal pathologist who is blinded to the study examined the H&E staining slides. Renal morphologic injury such as tubular dilation and vacuolization was evaluated.

Renal cortical apoptosis in vehicle (n=5) and CRRL269 (n=5) dogs were assessed with TUNEL assay (Promega, Madison, WI) followed the protocol provided by the manufacturer. Normal canines (n=4, same background as the canines used in the current study) which did not undergo AKI or any other surgeries were added as an additional group to evaluate the renal apoptosis. DAPI (Vectashield, Vector Laboratories, Burlingame, CA) was used to label the nucleus. TUNEL results were analyzed by confocal fluorescence microscopy (Nikon A50R, Melville, NY).

Oil immersion induced hypoxia/reoxygenation in vitro.

Human primary renal proximal tubular epithelial cells (HRPTC, ScienCell Research Laboratories, Carlsbad, CA) were seeded in 96-well plates and cultured for 1 day until 70–80% confluency before experiments. Passages 3–5 were used. Caspase 3/7 green apoptosis assay agent (Essen BioScience, Ann Arbor, MI) was added to culture medium for the detection of apoptosis. Plates were then transferred to a time-lapsed, live imaging system IncuCyte (Essen BioScience, Ann Arbor, MI) for apoptosis monitoring for a total of 24 hours. Hypoxia/reoxygenation (H/R) in cells was mimicked with mineral oil (Sigma, St Louis, MO) immersion²⁵. Cells were immersed with oil for 2 hours, washed twice and replaced with fresh medium including vehicle (H/R group) or drugs. ANP, BNP, CRRL269 at concentrations of 10^{-8} , 10^{-7} or 10^{-6} M were used. Protein kinase G (PKG, important cGMP binding protein) activating analogue 8-pCPT-cGMP (specific for PKG and low affinity to phosphodiesterases, Sigma, St Louis, MO) at concentrations of 10^{-7} , 10^{-6} or 10^{-5} M were used. Cells were treated with 10^{-6} , 5×10^{-6} or 1.5×10^{-5} M PKG inhibitor KT-5823 (Abcam, Cambridge, MA) for 30 min before 10^{-6} M CRRL269 was added. Cells without H/R stimulation were labeled as negative control (Neg Ctrl) group. Apoptosis signals (e.g. apoptosis object counts) were analyzed with the software provided by IncuCyte.

Apoptosis Pathway PCR Array and Caspase7 Real Time PCR.

Two groups of HRPTC (vehicle and 10^{-6} M CRRL269) which underwent oil induced H/R stress as described above were grown in 6-well plates and total RNA were collected with the Trizol method (Thermo Fisher Scientific, Waltham, MA). RNA was then reverse transcribed for cDNA for PCR RT2 array experiment. Apoptosis pathway PCR array (Qiagen, Germantown, MD) was then conducted with the SYBR green (Bio-Rad Laboratories, Hercules, CA) method in Lightcycler 480 (Roche Diagnostics, Indianapolis, IN). Raw data was analyzed with online Qiagen RT2 PCR analysis program. The Caspase7 gene was up-regulated by H/R and down-regulated by CRRL269 both at least two-fold and was selected for RT-PCR verification. Caspase7 RT-PCR primer (PPH00110C-200, Qiagen, Germantown, MD) was obtained and RT-PCR reactions were performed with SYBR green method. B2M gene (PPH01094E, Qiagen, Germantown, MD) was used for normalization.

Measurements of intracellular calcium in vascular smooth muscle cells.

Human primary aortic vascular smooth cells (HASMC, ScienCell Research Laboratories, Carlsbad, CA) were cultured and maintained as instructed by the manufacturer and cells at passage 4 or 5 were used. Calcium imaging was performed as previously described²⁶. Briefly, cells were seeded on a 25mm round cover glass (Fisher Scientific, Pittsburgh, PA) with a density of 5×10^4 cells per slide. These glass slides were pretreated with 0.5N NaOH

for 8 hours, rinsed with water and sterilized before use. After overnight culturing, the glass with the cells was assembled in a MS-502 Chamber (ALA Scientific Instrument, Farmingdale, NY) and loaded with 3 μ M fura-2 AM (Invitrogen, Carlsbad, CA) for 30 min at a 37°C incubator. The chamber was then placed on the stage of an inverted Olympus IX71 microscope (Olympus America Inc, Waltham, MA). The cells were maintained in 1 mL HASMC medium and pretreated with or without ANP, BNP or CRRL269 at concentration of 10^{-9} or 10^{-7} M for 5 min before perfused with 5 mL medium containing ANGII (10^{-8} or 10^{-7} M). The intracellular Ca^{2+} fluorescence signals were measured as a ratio of fluorescence intensities at 510 nm from excitations of 340/380 nm using a Hamamatsu ORCA-R2 CCD camera with a Sutter LB-LS/17 light source. Baseline fluorescence was recorded after 5 min of pretreatment and fluorescence images were acquired continuously during the ANGII perfusion. Background fluorescence was subtracted and the calcium signal (F) was normalized to baseline fluorescence (F0) and expressed as a ratio (F/F0) using MetaFluor software (Olympus America Inc, Waltham, MA).

Statistics and data analysis.

Data were expressed as means \pm SEM and significance analysis was performed with Prism 7 (Graphpad, La Jolla, LA). Data normality was assessed with Shapiro-Wilk test. For normally distributed data, one-way ANOVA and two-way ANOVA was used to in the in vivo and in vitro studies in which multiple time points were involved, and two-tailed t-tests were used for comparisons between two groups. Non-parametric Mann-Whitney test was applied for data that were not normally distributed.

RESULTS

Ischemic AKI hormonal and renal function in vivo in canines.

In vivo, continuous infusion for 2 hours with CRRL269 increased plasma, urinary and renal parenchyma cGMP levels consistent with pGC-A activation (Figure 1A, B, C and D). Results in vivo were presented as changes from baseline. Compared to vehicle, CRRL269 treatment markedly elevated cGMP values from CL1 to CL4. Notably, AKI induced by ACC greatly stimulated plasma ANGII levels compared to baseline. CRRL269 inhibited plasma ANGII levels (Figure 1D). Specifically, in vehicle group, ANGII levels remained high during post-ischemia phases from CL1 to CL4, while CRRL269 infusion significantly reduced circulating ANGII levels.

With ACC occluding suprarenal aorta, GFR was markedly reduced during ACC and changes from baseline results are showed in Figure 2A. A marked reduction of RBF was also observed as reported in Figure 2B during ACC. Importantly during post-ischemia phases, CRRL269 maintained GFR and RBF while vehicle did not preserve these two renal hemodynamic parameters (Figure 2A and B). Similarly, urine output (UV) and urinary sodium excretion (UNaV) were reduced by ACC while CRRL269 significantly induced diuresis and natriuresis compared to vehicle, which is consistent with the renal protective actions observed with GFR and RBF (Figure 2C and D).

Cardiovascular function in ischemic AKI canines.

ACC resulted in a marked elevation of mean arterial pressure (MAP) while during post-ischemia reperfusion periods, MAP returned to baseline levels. Notably, CRRL269 induced similar BP effects compared with vehicle infusion (Figure 3A), indicating CRRL269 is not a hypotensive agent and a similar trend was observed in cardiac output (CO) (Figure 3B). Right atrial pressure (RAP) and pulmonary capillary wedge pressure (PCWP) were also markedly elevated during renal ischemia, of which CRRL269 reduced RAP and PCWP after ischemia (Figure 3C and D). The results of CV function parameters support CRRL269 reduced cardiac filling pressure without a hypotensive response.

Renal injury ex vivo analysis.

H&E staining indicated that CRRL269 group presented with less vacuolization compared to vehicle consistent with less renal injury (Figure 4A and B). Additionally, ischemia/reperfusion markedly increased renal cortical apoptotic cell death compared to normal canine kidneys, as illustrated by TUNEL assay using confocal microscopy (Figure 4C and D). Apoptotic cells were mostly tubular epithelial cells and glomerular mesangial cells. Importantly, CRRL269 treatment reduced apoptosis in the renal cortex in comparison with vehicle.

H/R mediated apoptosis in HRPTC.

Recognizing the anti-apoptosis observed in renal tubular cells ex vivo by CRRL269, we employed human renal proximal tubular epithelial cells (HRPTC) to investigate hypoxia/reoxygenation (H/R) induced apoptosis in vitro with a time-lapsed and live imaging system. Here, H/R markedly stimulated apoptosis in HRPTC as evidenced by the sharp increase of apoptotic signals 2 hours after reoxygenation initiated, in comparison with the negative control group (Figure 5A, B and C). CRRL269 at concentrations 10^{-7} and 10^{-6} M significantly reduced apoptotic signals compared to the H/R group alone. More importantly, CRRL269 induced more robust anti-apoptotic effects compared to ANP and a trend of superiority was observed in comparison with BNP (Figure 5A). We next found PKG specific analog, 8-pCPT-cGMP, at concentrations of 10^{-6} and 10^{-5} M mimicked the apoptosis inhibition observed with pGC-A activators (Figure 5B). Furthermore, PKG inhibitor KT-5823 abrogated the anti-apoptotic effects induced by CRRL269 (Figure 5C). In the human apoptosis pathway gene array, 33 out of 84 genes in the apoptosis pathway were up-regulated at least 2 fold by H/R stimulation. In contrast, 8 out of 84 genes (CASP7, FADD, CRADD, DAPK1, HGDC, BCL2L11, BIK, TNFRSF1A) were down-regulated at least 2 fold by CRRL269 treatment (Figure 5D). Caspase7 gene (top gene, up-regulated in H/R and down-regulated in CRRL269) was chosen to be verified by RT-PCR due to its critical function mediating apoptosis and our results support that H/R increased Caspase7 gene by 1.89 fold and CRRL269 treatment significantly reduced its expression level to 1.25 (Figure 5E) ²⁷.

Intracellular Ca²⁺ levels inhibited by pGC-A activators under ANGII stimulation.

Intracellular Ca²⁺ levels in human aortic smooth muscle cells (HASMC) were increased under the stimulation by the vasoconstrictor ANGII at 10^{-8} or 10^{-7} M (Figure 6A and B).

Pretreatment with pGC-A activator ANP, BNP or CRRL269 at 10^{-9} or 10^{-7} M suppressed Ca^{2+} enhancement by ANGII. Notably, CRRL269 presented less Ca^{2+} suppression in comparison with ANP and BNP.

CNP as a compensatory biomarker for AKI.

We found plasma and urinary CNP levels increased starting from the AKI reperfusion phase with elevation in urinary CNP being more pronounced (Figure 7A and B). Infusion with CRRL269 increased urinary CNP levels compared to vehicle.

Furthermore, with RT-PCR, cortical NPPC gene expression levels, although lower compared to medullary expression, were elevated by CRRL269 treatment (Supplemental Figure IA). In vitro, human glomerular microvascular endothelial cells (HGMEC) presented considerably lower NPPC mRNA levels than tubular cells HRPTC (Supplemental Figure IB). Additionally, CNP treatment at 10^{-7} M suppressed human renal fibroblast (HRF) proliferation (Supplemental Figure IC).

DISCUSSION

The current study was designed to advance the therapeutic potential of CRRL269, a novel pGC-A activator, for the treatment of AKI with a special focus on studies in a large animal model with high clinical relevance. The major findings of our study are that CRRL269 exerted renal protective actions in canine model of AKI post-I/R for 2 hours with GFR and RBF preservation, enhanced diuresis and natriuresis, reduced renal cortical vacuolization and suppression of ANGII without inducing hypotension. This occurred in the setting of CRRL269 mediated increases in plasma, urinary and renal tissue cGMP. Moreover, CRRL269 reduced cardiac filling pressures such as RAP and PCWP. Further, CRRL269 reduced apoptosis in the kidney cortex in our AKI model. In vitro, CRRL269 exerted more robust anti-apoptotic effects in human renal tubular cells and less intracellular Ca^{2+} suppression in arterial vascular smooth muscle cells, compared to native pGC-A activators and also attenuated H/R-mediated gene up-regulation of apoptotic pathways. Additionally, we established that urinary CNP may be a novel biomarker for AKI. Taken together, our results support CRRL269 as a promising novel AKI drug.

The lack of use of large animal models of AKI has, in part, limited successful drug discovery in AKI as rat and mice models, compared to canine or porcine models, lack the high functional similarity and hemodynamics profiles common to humans. Further, use of small animal models is also complicated by the difficulty in measuring key hemodynamic data during and after induction of AKI^{28, 29}. We incorporated a large animal model in our study, which highly mimics ischemia induced AKI in the clinical setting resulting from suprarenal aortic cross clamping (ACC) that is highly relevant to vascular surgical reconstruction of the aorta and/or renal arteries. In this model, CRRL269 infusion was started after ischemia and before reperfusion, and resulted in renoprotection which corroborated with the in vitro apoptosis experiments thus confirming and extending previous studies in large animal models^{21, 30, 31}. Specifically, ACC in canines in the presence of inhibition of vasodilating prostaglandins induced reductions in GFR in association with renal vasoconstriction with tubular vacuolization and marked increase in apoptotic cell

death. Though the duration of the study was short and physiological parameters were only collected 2 hour post-ischemia, we observed moderate loss of GFR, which is in line with published studies in AKI long-term studies such as post-ischemia for 48 hours^{32, 33}. We believe the promising results obtained from this short-term study will guide us in the design of more long-term studies, such as 2 or 7 days post-ischemia AKI, to evaluate CRRL269's therapeutic potential more extensively.

Importantly, CRRL269 treatment improved renal function highlighted by GFR preservation and enhanced renal excretory actions in vivo. The enhanced renal actions occurred in the absence of hypotension. The maintenance of BP is especially useful for the safety, therapeutic potential and renal functional preservation in human clinical studies in AKI patients³⁴. Indeed, it was demonstrated that a reduction in renal perfusion pressure leads to abolished natriuretic effects stimulated by ANP administration in rodents, implicating the importance of maintaining BP in pGC-A therapeutics³⁵. A decrease of cardiac filling pressures without significant hypotension would lead to an increase in transrenal perfusion pressure and consequently improve renal function. CRRL269 retains its ability to reduce cardiac filling pressures, consistent with our previous report in normal canines¹¹. The reduction of RAP and PCWP could be due to the decrease of intravascular volume in view of the markedly greater increase in diuresis and natriuresis with CRRL269 treatment in the presence of AKI. Importantly and in contrast to endogenous pGC-A activators such as BNP, CRRL269 was not associated with hypotension¹¹. Here we report that a key mechanism of the more preserved BP is, at least partially, mediated by less intracellular Ca²⁺ suppression in vascular smooth muscle cells stimulated by ANGII in the presence of CRRL269. This data complements the potential enhanced positive cardiac inotropic effects secondary to our findings of cAMP increases in cardiomyocytes and reduced arterial relaxing effects observed previously¹¹. However, future in-depth studies are warranted to elucidate this novel CRRL269 modulation of intracellular Ca²⁺ levels.

In AKI, neurohormonal pathways such as RAS activation are suggested to induce cardiac structural changes and dysfunction³⁶. The pathophysiological consequences by RAS activation include cardiac hypertrophy, fibrosis, inflammation and apoptosis, which further result in cardiac dysfunction and HF. Studies reported that plasma ANGII was significantly increased in ischemic AKI and more importantly angiotensin converting enzyme inhibitor (ACEi) administration was shown to attenuate the initiation and progression of myocardial dysfunction and remodeling in animal models and patients³⁷⁻³⁹. Thus these previous results were not surprising given the enormous cardioprotective effects induced by ACEi and anti-RAS drugs in the treatment of HF. Thus a promising therapeutic agent suppressing RAS especially ANGII activation is critical for the protection of cardiac function in AKI. In our current study, CRRL269 suppressed plasma ANGII levels, hence supporting the potential of cardioprotection in the setting of AKI.

Lines of evidence support that the pGC-A/cGMP signaling pathway plays a critical role maintaining renal physiological integrity and protecting the kidney from injury⁴⁰⁻⁴⁴. One of the key pathophysiological pathways targeted by pGC-A is apoptosis. Repeated studies have established that apoptosis is a key feature of AKI although drugs targeting apoptosis have not been effective in preventing AKI⁴⁵. Recognizing the critical role of I/R induced

apoptosis in tubular cells in our in vivo AKI model, we employed an in vitro a model of acute H/R mediated apoptosis in tubular cells to investigate the molecular mechanism of CRRL269's renal protective action. CRRL269 showed superior anti-apoptotic actions compared to ANP and we demonstrated that this property is mediated by the cGMP/PKG pathway illustrated by a PKG-specific cGMP analogue and a PKG inhibitor. Further, CRRL269 suppressed apoptotic genes with PCR array analysis. Moreover RT-PCR demonstrated down-regulation of Caspase7, which is important in cell apoptosis execution, by CRRL269 in HRPTC thus providing useful evidence for inhibition of renal cell apoptosis through activation of pGC-A/cGMP signaling by CRRL269 in vivo. Indeed, in vivo we observed robust increases in plasma, urinary and renal tissue cGMP. The superior anti-apoptotic effects of CRRL269 observed in vitro support its direct cytoprotective effect, which is independent of its beneficial effects on renal hemodynamics. Further studies on the underlying molecular mechanism of CRRL269 renoprotective effects such as PKG substrates (e.g. cell survival related protein–Akt) phosphorylation are warranted.

Biomarkers such as NGAL and KIM-1 have been used for AKI early prediction and prognosis^{46,47}. However, the use of current biomarker profiles in the clinic has been limited by the specificity and predictive power signified by the average or low AUCs⁴⁸. Novel biomarkers providing an early detection of AKI immediately after injury insult are needed. In our laboratory, we previously documented that CNP is present in the kidney and is predominantly distributed in proximal, distal tubules and collecting ducts, and not in glomeruli^{24,49,50}. Urinary CNP was previously reported to predict patient outcomes in acute decompensated HF which was superior compared to KIM-1 or NGAL²². Furthermore, it was suggested in subjects with impaired renal function, uCNP was increased⁵¹. In the current study we explored, for the first time, the biomarker potential of uCNP in AKI and we showed that uCNP was markedly elevated by I/R. Notably, CRRL269 treatment increased uCNP and renal CNP gene expression levels, which is consistent with report of in vitro CNP mRNA and protein increases by native pGC-A activators⁵². We also confirmed CNP's renal protective effects in which CNP suppressed renal fibroblast proliferation in vitro. It is thus interesting to speculate if the renoprotective actions of CRRL269 may, in part, be also secondary to actions of CNP.

Our study is a pilot, short-term study assessing the renocardiac protective actions of CRRL269 in AKI canines. The current study also advances the understanding of pathophysiological changes caused by AKI, such as apoptosis and renal dysfunction. Our study has limitations as we only assessed the acute effects of CRRL269 in AKI. Specifically, its renoprotective effects in intermediate and long-term periods were not investigated in the current study. Thus, further studies in more long-term model models of AKI are warranted with the use of CRRL269 to evaluate if its renal protective actions are sustained. As described above, renal and cardiac function assessment conducted in 2 day and 7 days post-I/R in AKI model to further confirm CRRL269's sustained renocardiac protection and rule out the possibility of a merely transient improvement by CRRL269 observed in the present study is warranted. Additionally, a preventive strategy with CRRL269 infusion prior to ischemic insult is warranted to explore its potential as a drug for AKI prevention. Moreover, it is important to assess the protective actions of CRRL269 in other models of AKI such as

nephrotoxic drug induced AKI to explore its potential in a wider scope given the heterogeneity observed in the AKI patient population.

Given no drugs have been approved for AKI treatment by the FDA and the connection between the heart and kidney, there is an unmet need for the development of innovative drugs to preserve the kidney function directly and in the meantime protect the heart in the setting of AKI. The connection between the heart and kidney requires single agents targeting both organs thus treating cardiorenal syndrome. Recognizing the renocardiac protective effects of CRRL269 in AKI canines, we believe it may have translational potential for the treatment of AKI as well as HF prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

ACC	aortic cross clamping
ANGII	angiotensin II
AKI	acute kidney injury
BP	blood pressure
cGMP	guanosine 3',5' cyclic monophosphate
GFR	glomerular filtration rate
I/R	ischemia/reperfusion
HF	heart failure
H/R	hypoxia/reoxygenation
HRPTC	human renal proximal tubular epithelial cells
NP	natriuretic peptide
pGC-A	particulate guanylyl cyclase A receptor
RAS	renin-angiotensin-system

uCNP urinary C-type natriuretic peptide

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NOVELTY AND SIGNIFICANCE

What Is Known?

- To date, no drugs has been approved by the FDA for acute kidney injury (AKI) treatment.
- Native particulate guanylyl cyclase A receptor (pGC-A) activators have potential beneficial actions in AKI, but may have limited use as they produce hypotension.

What New Information Does This Article Contribute?

- A innovative designer pGC-A activator CRRL269 preserved renal function and exhibited cardiac protection, without causing hypotension in a large animal model of AKI.
- Its beneficial actions were mediated, at least in part, by anti-apoptosis and reduced calcium suppression.
- We also reported that urinary CNP (uCNP) may serve as a biomarker for identifying AKI.

Native pGC-A activators have the potential to treat AKI due to their renal and cardiac protective actions. However, native activators inevitably reduce blood pressure, which may reduce renal perfusion pressure and thereby compromise their renal function enhancement. Here we employed a novel pGC-A activator, CRRL269 which we previously documented possessed superior renal protective effects than native activators in a large animal model of AKI in canines. We reported that CRRL269 significantly improved renal function compared to vehicle treatment, with less kidney injury, renal apoptosis while importantly maintaining the blood pressure. We also reported for the first time that uCNP was elevated markedly during AKI and thus may represent a novel biomarker for AKI. Taken together, our results support the development of CRRL269 for the treatment of AKI.

Fig.1A

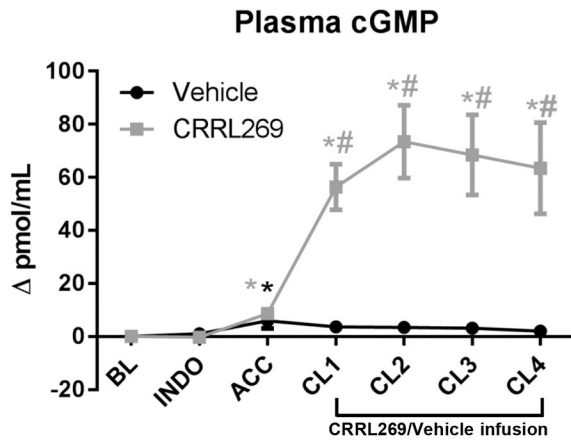


Fig.1B

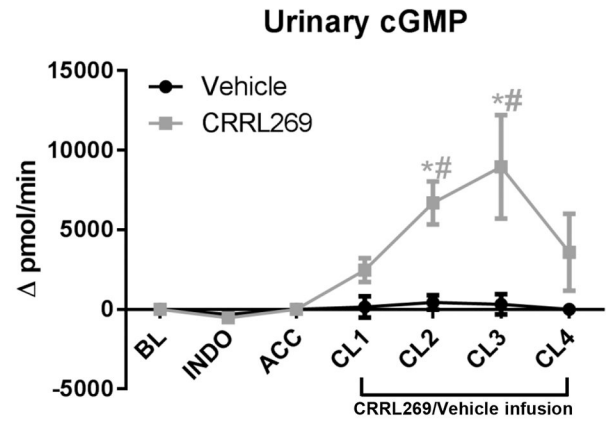


Fig.1C

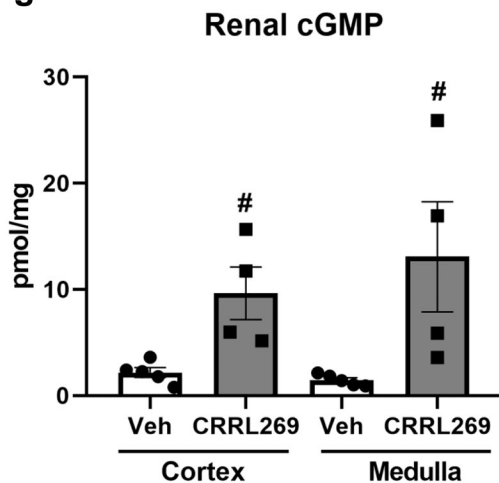


Fig.1D

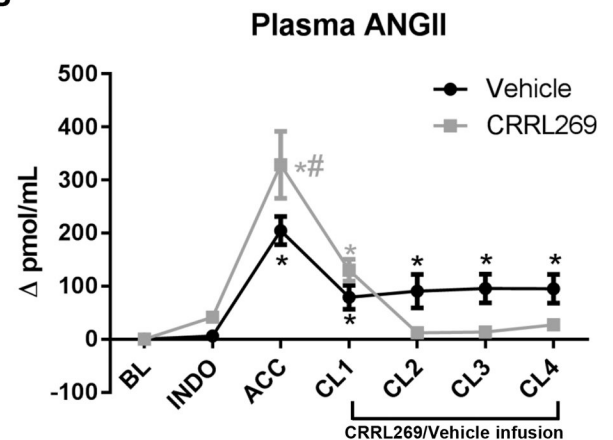


Figure 1. Plasma, urinary, renal cGMP and plasma angiotensin II (ANGII) values modulated by CRRL269.

(A) plasma cGMP, $n=5$ each group, # $p<0.05$ vs vehicle using two-way ANOVA followed by Bonferroni's *post-hoc* test, * $p<0.05$ vs BL using one-way ANOVA followed by Dunnett's *post-hoc* test (B) urinary cGMP, $n=5$ each group, same statistical methods as plasma cGMP (C) renal cortical and medullary cGMP, $n=5$ Veh and $n=4$ CRRL269, # $p<0.05$ vs Veh with unpaired t-test, and (D) plasma ANGII value changes from baseline by CRRL269 or vehicle. $n=5$ each group, same statistical methods as plasma cGMP. AKI protocol in canines was consisted of BL, INDO, ACC, CL1, CL2, CL3 and CL4. After 60 min equilibration, a baseline (BL) was recorded. Then 45 min infusion of indomethacin (INDO) was initiated, and this was followed by 60 min aortic cross clamping (ACC). 16.3 pmol/kg/min CRRL269 or vehicle (0.9% saline) infusion started after ACC and it lasted for 120 min. Data were recorded at BL, INDO, ACC, clearance 1 (CL1), clearance 2 (CL2), clearance 3 (CL3) and clearance 4 (CL4) during CRRL269/vehicle infusion.

Fig.2A

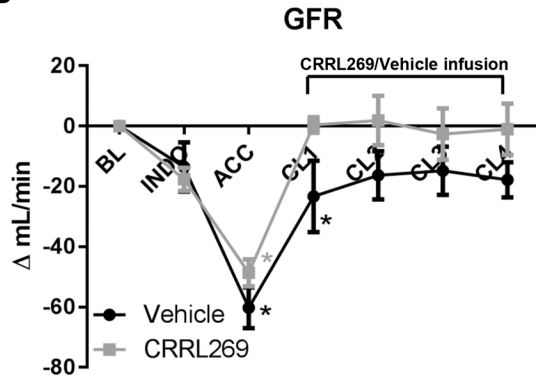


Fig.2B

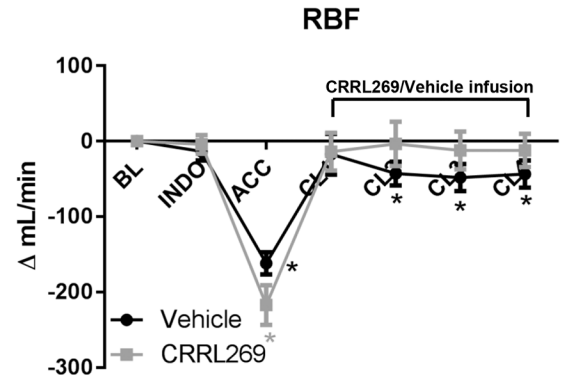


Fig.2C

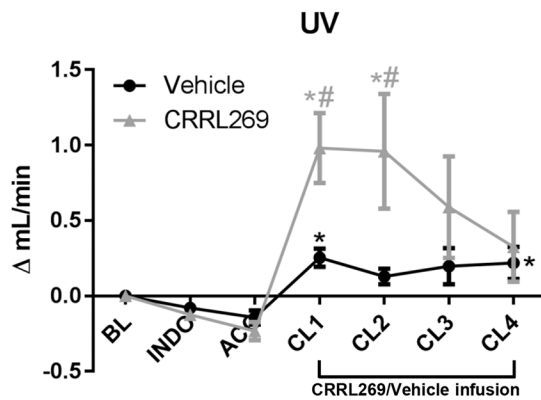


Fig.2D

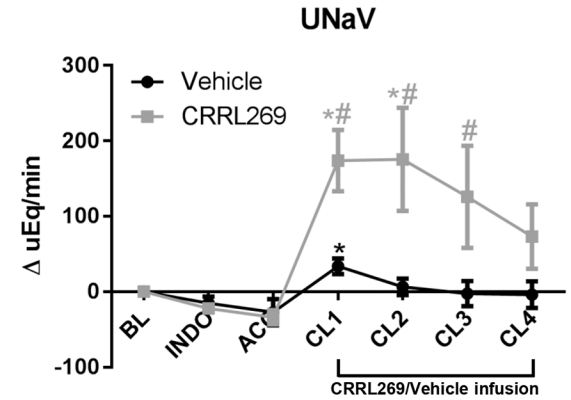


Figure 2. Renal function parameters by CRRL269 in AKI.

(A) glomerular filtration rate (GFR), (B) renal blood flow (RBF), (C) urinary output (UV) and (D) urinary sodium excretion rate (UNaV) in CRRL269 or vehicle infusion group. Data were presented as absolute changes from baseline. Experimental schedule was described in Figure 1 legend. n=5 each group, # p<0.05 vs vehicle using two-way ANOVA followed by Bonferroni's *post-hoc* test, * p<0.05 vs BL using one-way ANOVA followed by Dunnett's *post-hoc* test.

Fig.3A

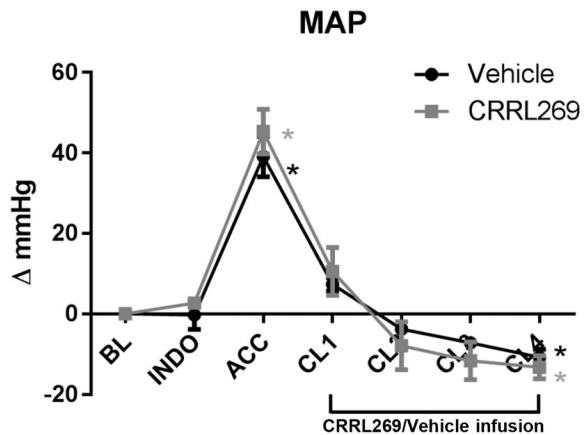


Fig.3B

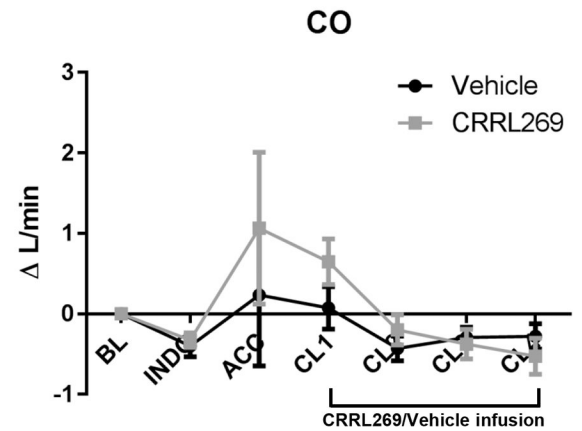


Fig.3C

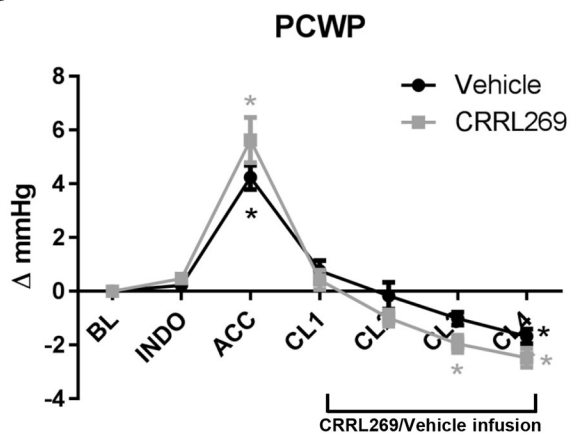


Fig.3D

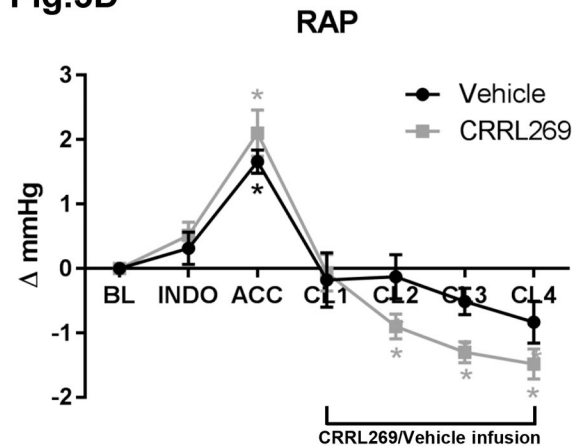


Figure 3. Cardiovascular function parameters by CRRL269 in AKI.

(A) mean arterial pressure (MAP), (B) cardiac output (CO), (C) right atrial pressure (RAP) and (D) pulmonary capillary wedge pressure (PCWP) in CRRL269 or vehicle infusion group. Data were presented as absolute changes from baseline. Experimental schedule was described in Figure 1 legend. n=5 each group, * p<0.05 vs BL using one-way ANOVA followed by Dunnett's *post-hoc* test.

Fig.4A

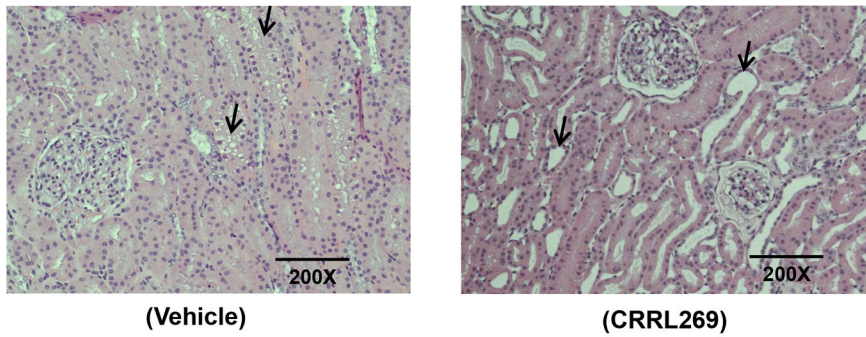


Fig.4B

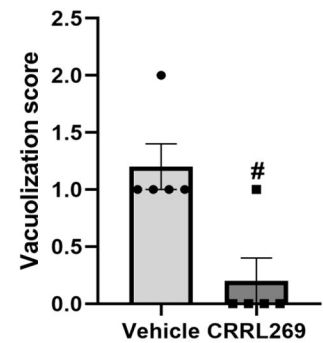


Fig.4C

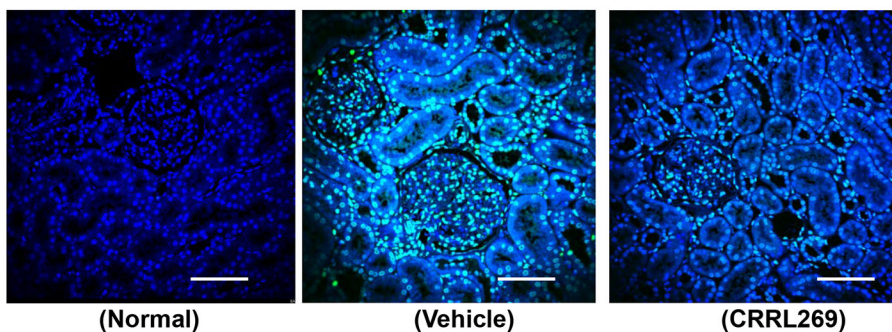


Fig.4D

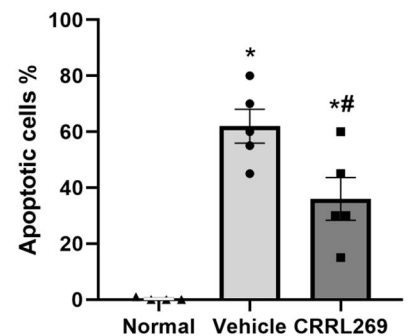


Figure 4. H&E staining and TUNEL apoptosis results ex vivo from AKI canine kidneys. (A) Representative images of H&E staining of kidney cortex in vehicle and CRRL269 groups. Arrow heads indicate vacuolization or tubular dilation. (B) Quantification of renal cortex vacuolization score. n=5 each group, # p<0.05 vs vehicle with unpaired t-test (C) Representative images and (D) quantification of kidney cortex apoptosis assessed by TUNEL assay in normal, vehicle and CRRL269 groups. n=4 normal, n=5 vehicle and n=5 CRRL269, * p<0.05 vs normal, # p<0.05 vs vehicle, with unpaired t-test. TUNEL results were analyzed by confocal fluorescence microscopy. Green fluorescence indicates DNA fragmentation and blue fluorescence labels the nucleus. Scale bars=100um.

Fig.5A

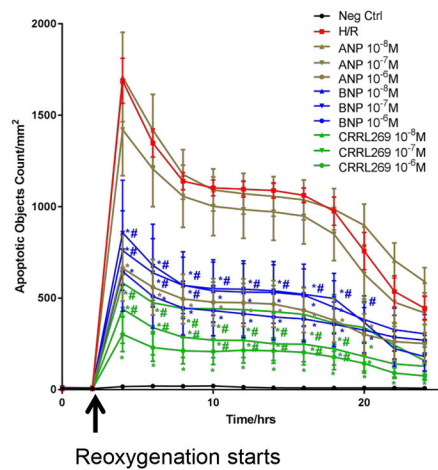


Fig.5B

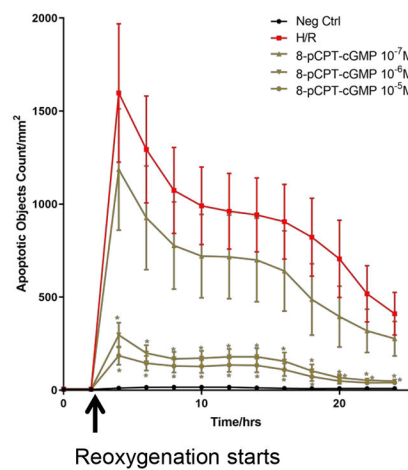


Fig.5C

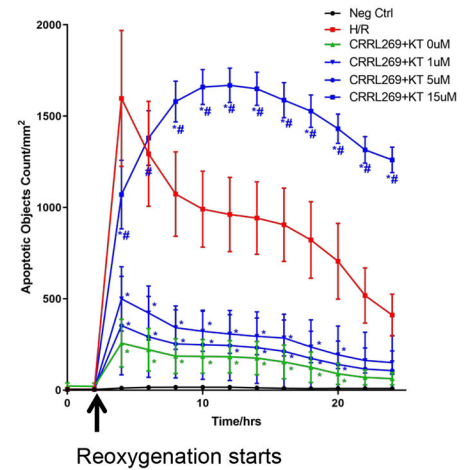


Fig.5D

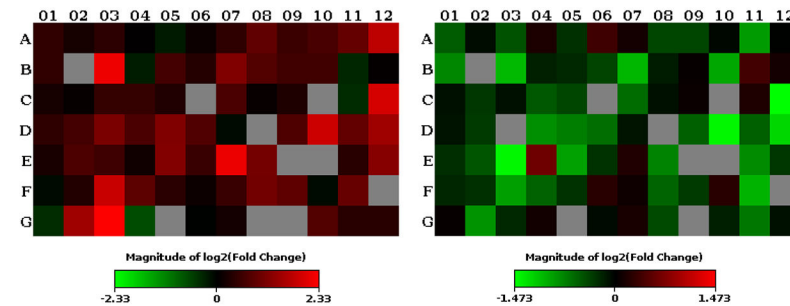


Fig.5E

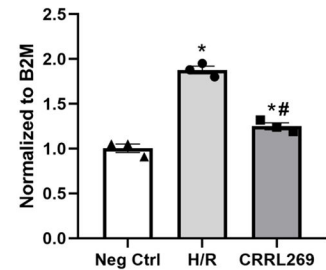


Figure 5. CRRL269 inhibits apoptosis induced by H/R in human primary proximal tubular epithelial cells (HRPTC).

(A) Apoptotic signals were quantified by IncuCyte software and comparisons were made between negative control, H/R and H/R with 3 concentrations (10^{-8} , 10^{-7} , or 10^{-6} M) of ANP, BNP or CRRL269. $n=8$, * $p<0.05$ vs H/R, # $p<0.05$ vs ANP using two-way ANOVA followed by Dunnett's *post-hoc* test. (B) Apoptosis by control, H/R and H/R with 3 concentrations (10^{-7} , 10^{-6} , or 10^{-5} M) of PKG-specific (low affinity to phosphodiesterases) cGMP analogue 8-pCPT-cGMP. $n=8$, * $p<0.05$ vs H/R, two-way ANOVA followed by Dunnett's *post-hoc* test. (C) Apoptosis by control, H/R and H/R with 4 concentrations (0, 1, 5, 15 μ M) of PKG inhibitor KT-5823 (KT) with 10^{-6} M CRRL269. $n=8$, * $p<0.05$ vs H/R, # $p<0.05$ vs 0 μ M KT-5823, two-way ANOVA followed by Dunnett's *post-hoc* test. (D) Apoptosis pathway genes expression were compared between H/R and negative control (left panel), 10^{-6} M CRRL269 and H/R (right panel). (E) Caspase7 gene was verified with RT-PCR in negative control, H/R and H/R with 10^{-6} M CRRL269. $n=3$, * $p<0.05$ vs negative control, # $p<0.05$ vs H/R, unpaired t-test.

Fig.6A

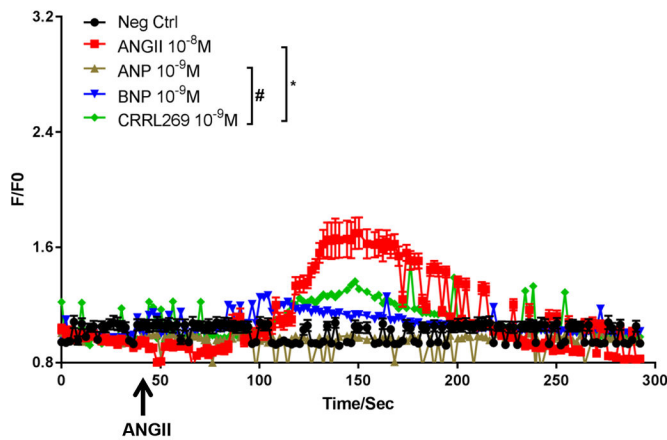


Fig.6B

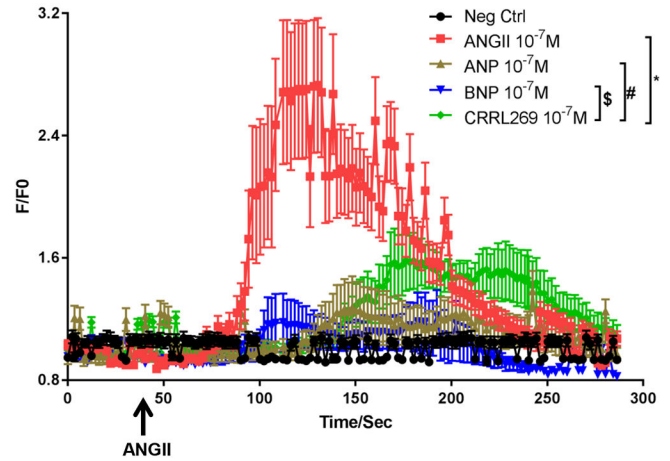


Figure 6. Intracellular Ca^{2+} levels in human primary aortic smooth muscle cells (HASMC). (A) Relative Ca^{2+} levels were calculated based upon recorded fluorescence signals in Fura-2 loaded HASMC cells. Fold difference F/F_0 was used as relative Ca^{2+} concentration values. Cells were pretreated with 10^{-9} M ANP, BNP or CRRL269 for 5 min before 10^{-8} M ANGII was perfused. Perfusion lasted for about 4 min. (B) Cells were pretreated with 10^{-7} M ANP, BNP or CRRL269 for 5 min before 10^{-7} M ANGII was perfused. $n=9-11$, * $p<0.05$ vs ANGII, # $p<0.05$ vs ANP, \$ $p<0.05$ vs BNP, two-way ANOVA followed by Dunnett's *post-hoc* test.

Figure 7A

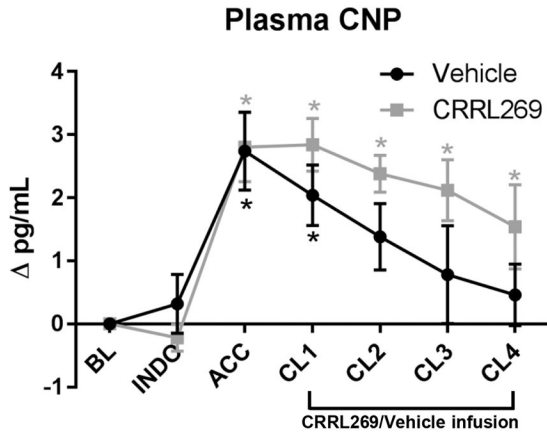


Figure 7B

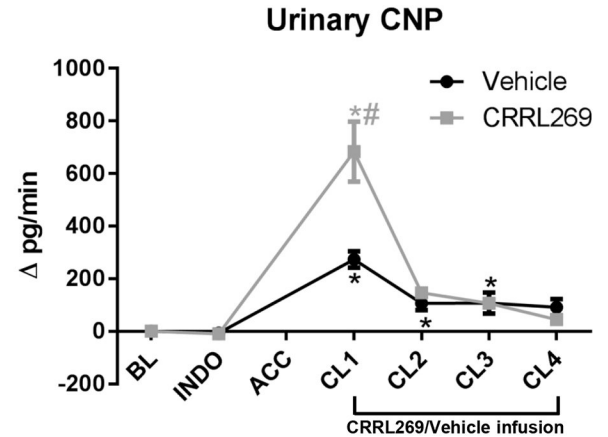


Figure 7. C-type natriuretic peptide (CNP) changes by CRRL269 in AKI.

(A) plasma CNP, $n=5$ each group, # $p<0.05$ vs vehicle using two-way ANOVA followed by Bonferroni's *post-hoc* test, * $p<0.05$ vs BL using one-way ANOVA followed by Dunnett's *post-hoc* test (B) urinary CNP, statistics same as plasma CNP. Data were presented as absolute changes from baseline. Experimental schedule was described in Figure 1 legend.