Published in final edited form as:

Transplantation. 2015 November ; 99(11): 2274–2284. doi:10.1097/TP.0000000000000762.

Stimulation of Dopamine D₃ Receptor Attenuates Renal Ischemia/Reperfusion Injury via Increased Linkage with Gα₁₂

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Abstract

Background—Renal ischemia reperfusion (I/R) injury causes renal tubular necrosis, apoptosis, and inflammation leading to acute renal dysfunction. Recent studies have revealed that deletion of Gα₁₂ mitigates the renal damage due to I/R injury. Our previous study showed that activation of D₃R increased its linkage with Gα₁₂, and hampered Gα₁₂-mediated stimulation of renal sodium transport. In the present study, we used an *in-vivo* rat model and an *in-vitro* study of the renal epithelial cell line (NRK52E) to investigate whether or not an increased linkage between D₃R and Gα₁₂ contributes to the protective effect of D₃R on renal I/R injury.

Methods—For *in-vivo* studies, ischemia/reperfusion injury was induced in a rat renal unilateral clamping model. For *in-vitro* studies, hypoxia/reoxygenation and cold storage/rewarming injuries were performed in NRK52E cells. PD128907, a D₃R agonist, or vehicle, was administered 15 min before clamping (or hypoxia) in both the *in-vivo* or *in-vitro* studies.

Results—In the rat renal unilateral clamping model, pretreatment with PD128907 (0.2 mg/kg, IV) protected against renal I/R injury and increased survival rate during a long-term follow-up after seven days. A decrease in the generation of ROS, apoptosis and inflammation may be involved in the D₃R-mediated protection since pretreatment with PD128907 increased renal glutathione and superoxide dismutase levels and decreased malondialdehyde levels in the I/R

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All authors declare no conflicts of interest, financial or otherwise.

Authors' contributions

ZW, WWG, and YH performed most of the experiments and analyzed data and wrote the manuscript, including additional experiments. HMR, XFT, HZ, JJF and DFH performed the experiments. YKL and LZ reviewed and edited the manuscript. LDA and PAJ edited the manuscript and contributed to the discussion. LYC and CYZ designed the study and edited the manuscript. All authors read and approved the final manuscript.

group. The increase in cytokines (TNF- α , IL-1 β , and IL-10) and myeloperoxidase in I/R injured kidney was also prevented with a , simultaneous decrease in the apoptosis of the epithelial cells and expression of apoptosis biomarkers in kidney harvested one day after I/R injury. The increase in the co-immunoprecipitation between D₃R and G α 12 with D₃R stimulation paralleled the observed renal protection from I/R injury. Moreover, *in vitro* studies showed that transient overexpression of G α 12 in the NRK52E cells attenuated the protective effect of PD128907 on H/R injury. The protective effect of PD128907 might be of significance to renal transplantation, since cold storage/rewarming induced injury increased LDH release and decreased cell viability in NRK52E cells. Conversely, in the presence of PD128907, the increased LDH release and decreased cell viability were reversed.

Conclusion—These results suggest that activation of D₃R, by decreasing G α 12-induced renal damage, may exert a protective effect from I/R injury.

Keywords

ischemia/reperfusion injury; cold storage/rewarming injury; dopamine D₃ receptor; G α 12; reactive oxygen species; apoptosis; inflammation; protein-protein interaction

Introduction

Ischemia/reperfusion (I/R) injury is a common event in clinical conditions such as infarction, sepsis, and organ transplantation, and results in significant mortality and morbidity.(1) Renal proximal tubules are most susceptible to injury during I/R.(2, 3) Multiple cellular factors are involved in the post-I/R injury events that lead to acute renal failure. Restoration of blood flow to ischemic tissue paradoxically exacerbates tissue damage by initiating a cascade of reactive oxygen species (ROS)-inflammatory events that contribute to cellular dysfunction, and apoptosis/necrosis. (4–6) To date, the cellular and molecular bases of I/R injury remain to be fully understood, and effective therapies are needed to be developed to minimize its side effects.

G proteins are key molecular messengers linking extracellular stimuli to cellular biological activities. G proteins consist of α , β , and γ subunits; α subunits can be divided into four families: G α s, G α i/o, G α q, and G α 12 and are all expressed in the renal proximal tubule cells (RPTs).(7–9) Recently, activated G α 12 has been reported to be a central mediator of ROS-initiated events in renal I/R injury.(10–14) G α 12 couples to more than thirty G protein-coupled receptors (GPCRs), is activated by various stimuli, including H₂O₂, thrombin, thromboxane A₂, lysophosphatidic acid, angiotensin II, and thyroid-stimulating hormone, and is involved in many pathological roles, including disruption of junctional complexes, proliferation, apoptosis, and cell migration.(7, 10, 15–19) Activation of G α 12-coupled GPCRs, like angiotensin II receptor 1, endothelin A receptor, protease-activated receptor 1 and 4, lysophosphatidic acid receptor 3, and sphingosine 1-phosphate receptor 2, can induce renal injury associated with ischemia and I/R.(20–25). Indeed, knockout of G α 12 protects mice from renal I/R injury. (10) However, the knock-out of G α 12 in the kidney to protect against renal I/R injury may not be clinically feasible. Therefore, drugs that can hamper G α 12 action may be the best alternative choice.

Dopamine receptors are GPCRs that are classified into two subgroups, D₁-like receptors (D₁R and D₅R) that couple to G_{αs}, and D₂-like receptors (D₂R, D₃R, and D₄R) that couple to G_{αi}. Both D₁-like and D₂-like receptors are found to facilitate sodium excretion in kidney.(26, 27) In addition, dopamine receptors play an important role in I/R injury in the brain, heart, and kidney.(28–30) However, the details on how the dopamine receptor subtype(s) exerts this protective effect is not clearly understood. The D₃R, same as the other D₁-like receptors, is expressed in the renal proximal tubule. (31, 32) Our previous study showed that there is a linkage between the D₃R and G_{α12}; stimulation of D₃R increases D₃/G_{α12} linkage and impairs G_{α12}-mediated stimulation of renal sodium transport. (33) We hypothesize that decreasing G_{α12} action by increasing its interaction with D₃R, via the stimulation of D₃R, may contribute to the protection against I/R-mediated renal injury. To test this hypothesis, we used a well-established rat model of renal I/R injury and examined the renal functional, morphological, and biochemical parameters that correlate with kidney injury after I/R. Our data suggest that stimulation of the D₃R increases its interaction with G_{α12} and protects against renal I/R injury by decreasing the production of ROS and inflammation.

Results

1. Stimulation of the D₃R restores renal function after I/R injury

After being subjected to renal I/R injury, renal function was evaluated after one day and three days. Results showed that the I/R injured rats (Figure 1A) had elevated serum urea nitrogen and creatinine levels, as compared to the sham-operated control rats. These increases induced by I/R injury were significantly ameliorated by PD128907 treatment (Figures 1B and 1C), which by itself had no effect on these renal function markers. Twenty-four hours after reperfusion, the kidney had severe damage as indicated by tubular cell swelling and desquamation, and cast formation. Pretreatment with PD128907 reduced the renal damage due to the I/R injury (Figures 1D and 1E). Survival rates were examined after renal I/R injury (n = 9). None (0%) of rats in the vehicle treatment I/R group survived over five days. However, three (33.3%) of nine rats in the PD128907 treated I/R group survived for over seven days (Table 1).

2. Stimulation of the D₃R decreases renal epithelial apoptosis in I/R injured kidney

To uncover the mechanisms leading to renal dysfunction with I/R injury, we studied renal epithelial apoptosis from kidneys harvested one day after I/R injury as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. I/R increased renal epithelial apoptosis which was decreased by pretreatment with D₃R agonist, PD128907 (Figures 2A and 2B). Consistent with the TUNEL studies, I/R increased Bax levels and decreased Bcl-2 levels thus increasing the Bax/Bcl-2 ratio; I/R also increased caspase-3 expression and caspase-3 activity. Similar to the beneficial effect of PD128907 on renal pathology and function, PD128907 also ameliorated the I/R injury-induced changes in the Bax and Bcl-2 levels, and caspase-3 expression and activity (Figures 2C–2E).

3. Stimulation of the D₃R attenuates renal oxidative stress in I/R injured kidney

Because oxidative stress and inflammation participate in the development of renal I/R, (34, 35) we studied the states of oxidative stress and inflammation from kidneys harvested one day after I/R injury. We found that renal I/R injury significantly decreased the expression of glutathione (GSH) (Figure 3A). Glutathione disulfide (GSSG) is formed by the oxidation of the cysteine sulfhydryl groups of GSH by joining two GSH molecules with a disulfide bridge. GSH/GSSG represents the oxido-reduction state. (36) Compared with the sham-operated group, I/R injury significantly reduced the GSH/GSSG ratio (Figure 3B). I/R also decreased the renal expression of another antioxidant enzyme, superoxide dismutase (SOD) (Figure 3C), but increased the expression a pro-oxidant enzyme, myeloperoxidase (MPO) and malondialdehyde (MDA) levels (Figures 3D and 3E), indicating an increase in lipid peroxidation. (37) The changes in GSH, GSH/GSSG, SOD, MPO, and MDA levels due to I/R were almost completely or partially prevented by pretreatment with PD128907 (Figures 3A–3E).

Anti-inflammatory, pro-inflammatory cytokines and chemokines (38) were also investigated. The pro-inflammatory TNF- α (Figure 4A) and IL-1 β (Figure 4B) were higher, while the anti-inflammatory IL-10 (Figure 4C) was lower in the I/R injured than in the sham-control kidney. Pretreatment with PD128907 partially prevented the changes in these anti-and pro-inflammatory cytokines/chemokines (Figures 4A–4C).

4. Stimulation of the D₃R partially prevents the decrease in renal D₃R expression and D₃R/G α 12 co-immunoprecipitation and the increase in G α 12 expression caused by I/R injury

We have reported that stimulation of renal D₃R increased its linkage to G α 12 and impaired the G α 12-mediated stimulation of renal sodium transport. (33) Because G α 12 stimulates apoptosis (39) and D₃R is protective against apoptosis (40), we determined whether or not D₃R, via the inhibition of G α 12, can negatively regulate apoptosis. Laser confocal microscopy showed that the D₃R was found mostly in the subapical areas of the renal proximal tubules while G α 12 through immunostaining was found predominantly in the brush border membrane and subjacent areas (Figure 5A). There was colocalization between D₃R and G α 12, which was mainly observed at the subjacent areas in all renal proximal tubules. Using the same time-schedule as the above-mentioned studies, the kidneys were harvested one day after I/R injury. PD128907 increased the D₃R (Figure 5B) but not the G α 12 expression. (Figure 5C) and increased D₃R/G α 12 colocalization and co-immunoprecipitation (Figures 5A and 5D) in the control rat. Renal I/R injury decreased D₃R expression (Figure 5B) and increased G α 12 expression (Figure 5C) but did not affect their colocalization/co-immunoprecipitation (Figures 5A and 5D). PD128907 ameliorated the renal I/R-induced decrease in D₃R (Figures 5A and 5B) and D₃R/G α 12 colocalization/co-immunoprecipitation (Figures 5C and 5D).

5. Over-expression of Gα12 in NRK52E cells increases intracellular ROS generation and attenuates the protective effect of PD128907 in hypoxia/reoxygenation-injured NRK52E cells

The ROS generation inhibition and protective effect of PD128907 were confirmed in a renal epithelial cell line (NRK52E). Hypoxia/reoxygenation (H/R) increased total intracellular ROS generation, lactate dehydrogenase (LDH) release and decreased cell viability, effects that were accentuated by the over-expression of Gα12 but attenuated by PD128907.

Moreover, over-expression of Gα12 completely blocked the protective effect of PD128907 (Figures 6A, B, and C).

6. PD128907 pretreatment decreases LDH release and improves cell viability in NRK52E cells after cold storage/rewarming induced injury

Our above-mentioned results showed the protective effect of D₃R on I/R injury in the *in-vivo* and *in-vitro* studies. As a special model of I/R injury, renal transplantation has some special characteristics. In addition to I/R injury, transplanted kidneys also endure cold storage/rewarming induced injury. Therefore, we examined the effect of D₃R stimulation on I/R injury in NRK52E cells. Cold storage/rewarming induced injury increased LDH release and decreased cell viability, but with PD128907 pre-treatment, the increased LDH release and decreased cell viability were reversed in NRK52E cells (Figures 7A and 7B).

Discussion

Renal I/R injury is a successive cascade of cellular events that include the release of ROS, cell apoptosis, necrosis, infiltration by inflammatory cells, and the release of active mediators leading to tissue damage. (41, 42) Increased or inappropriate generation of ROS and inflammation has been suggested to be important in the initiation and maintenance of the acute tubular necrosis found in I/R injury. (34, 35, 43) Our current study demonstrated that stimulating the D₃R with PD128907 decreased the impairment of renal function caused by I/R *in vivo* and reduced intracellular ROS generation, LDH release and improved renal tubular cells viability *in vitro*. The protective effect of PD128907 on I/R injury might be of significance to renal transplantation, although transplantation associated I/R injury includes the unique condition of an alloimmune response. We examined the protective effect of PD128907 in a cold storage/rewarming induced injured cell model and the results showed that cold storage/rewarming induced injury increased LDH release and decreased cell viability, which in the presence of PD128907, the increased LDH release and decreased cell viability were reversed in NRK52E cells.

There is increasing evidence on the importance of G proteins in renal I/R injury,(10) specifically Gα12, which may play a key role in the induction of I/R injury through multiple mechanisms, including the disruption of tight cell junctions, oxidative stress, inflammation, and promotion of apoptosis. (10, 14, 44) Silencing Gα12 protected renal tubule cells from hydrogen peroxide-induced barrier disruption.and found in the knockout of Gα12 that also protected mice from renal I/R injury.(10) In our previous study, we found the D₃R exhibits a powerful ability to maintain normal renal function by multiple mechanisms.(45–47) Furthermore, we have reported a direct linkage between the dopamine receptor D₃R and

Gα12/13.(33) The fact that a D₃R agonist, PD128907, increases the interaction between the D₃R with either Gα12 or Gα13 in renal brush border membranes and renal proximal tubule cells from WKY rats suggests that the D₃R may participate in the regulation of the sodium transport/pump activity by hampering the stimulatory effect of Gα12 or Gα13 action on Na⁺-K⁺ ATPase activity in the renal proximal tubule. (33) Specifically, PD128907, a preferential D₃R agonist, has been recognized as a potentially valuable drug in the treatment of Parkinson's disease (48) and hypertension. (33) In the current study, we found that PD128907 pretreatment reduced the increase in Gα12 expression caused by I/R injury and increased the expression of D₃R and its interaction with Gα12 that was accompanied by a reduction in renal I/R injury. To further confirm that the D₃R-mediated regulation of Gα12 function as a mechanism in the protective effect of PD128907 in I/R, we studied the effect of H/R in a rat renal epithelial cell line (NRK52E) made to over-express Gα12. The increase in ROS production and LDH release and decrease in cell viability seen in NRK52E cells exposed to hypoxia and subsequently to reoxygenation were accentuated by over-expression of Gα12 but attenuated by PD128907.

There are reports showing that activation of Gα12 induces oxidative stress and increases inflammation and apoptosis. It has been shown that Gα12 can directly increase the production of ROS through several pathways which include Rho and Rac. (49, 50) Gα12 can also negatively modulate the activity of NF-E2-related factor-2, a key antioxidative transcription factor.(51) In addition, the activation or increased levels of Gα12 can be related to the up-regulation of pro-inflammatory cytokines as well as the activation of neutrophils and monocytes.(44, 52) Gα12 can induce apoptosis by increasing the degradation of the anti-apoptotic protein, Bcl2 and the upregulation of the pro-apoptotic IκBα.(14, 53) Our current study found that the increase in renal Gα12 expression as a result of renal I/R injury was accompanied by increased oxidative stress, inflammatory cytokines/chemokines and decreased anti-inflammatory cytokine. Stimulation of D₃R did not affect the renal expression of antioxidant, pro-oxidant, anti-inflammatory or pro-inflammatory cytokines/chemokines but prevented the adverse effects of renal I/R injury on these variables which may be related to the increased expression/activity of Gα12.

Dopamine has shown an anti-oxidative factor and anti-inflammation property (28, 54–56). However, contradictory results have been reported in clinical and animal studies.(57–61) Although the reasons leading to the different results are not known, the use of non-selective dopamine and its effect on all dopamine receptor subtypes might, at least in part, explain this phenomenon. Moreover, the dopamine-induced increase in blood pressure and tachycardia might worsen the protective effect of dopamine on renal function. Using agonists specific to dopamine receptor, previous studies have shown that both D₁-like and D₂-like receptors can directly and indirectly down-regulate the production of ROS in many cell types, including the renal proximal tubular cells.(62–65) Our pilot study show that, among the five dopamine receptor, the protective effect of PD128907 on the I/R injured kidney function has the strongest effect (data not shown). However, the higher specificity of PD128907, with its 120-fold selectivity over the D₂ receptor, on the D₃R, could not exclude the effect of PD128907 on the D₂ receptor.(66) To make clear the contribution of the D₃R on the protective effect of PD128907 on renal I/R injury, D₂ receptor deficient mice need to be used in the future.

Taken together, our current study indicates that some of the nephroprotective effects of D₃R may be mediated by the interaction between D₃R and Gα₁₂. Exploiting this interaction may provide insights into alternative strategies that can lead to the reduction of I/R injury and its deleterious effect on the kidney.

Materials and Methods

Animals and surgical procedures

Adult male Wistar rats, weighting 220–280 g, were obtained from the Daping Hospital Animal Center. All experimental procedures were in compliance with the institutional regulations and approved by the Daping Hospital authorities. The rats were randomly divided into four groups: sham control group; sham+PD128907 group; I/R group; and I/R +PD128907 group. PD128907 was dissolved in saline immediately before use. To avoid PD128907 induced hypothermia, the dose of 0.2 mg/kg was chosen based on our preliminary experiments (data not shown) and a previous report. (67)

Rats were anesthetized by an intraperitoneal injection of pentobarbital (50mg/ kg), and placed on a heating pad to maintain their body temperature (37°C). during the surgery. Following abdominal incisions and right kidney nephrectomy, the D₃R agonist PD128907 (Tocris, USA) (0.2) mg/kg, was administered intravenously as a bolus infusion. Fifteen minutes later, the left renal pedicle was occluded with a small microvascular clamp to induce renal ischemia for 45 min. During the procedure, animals were kept at a constant temperature (37°C). For the sham group, only an abdominal incision and right kidney nephrectomy was performed. After the experiment, the incisions were sutured, and the animals were allowed to recover with free access to food and water. Blood was collected and the left kidney was harvested for analysis 24 hrs after reperfusion.

The blood was centrifuged (3000×g for 10 min) and the serum was extracted, and stored at –80°C until studied. Rat kidneys were divided into two sagittal sections. One section was used for the detection of oxidative stress and apoptotic molecules. After weighing, part of the renal cortex was ground and homogenized in ice-cold lysis-buffer, the rest of the kidney was stored at –80 °C for further studies. The tissue homogenate was sonicated and kept on ice for 1 hr. The lysate was then centrifuged for 30 min at 12,000 rpm. Supernatant was collected and stored at –80 °C for further detection. The concentration of the protein was quantified by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. The other half of the kidneys were fixed in 4% paraformaldehyde for H&E (haematoxylin and eosin) and TUNEL staining. Renal function was estimated by plasma creatinine and urea levels using an automated method (Beckman Analyzer, Beckman Instruments GmbH, Munich, Germany).

Histological analysis and immunostaining

The fixed kidneys were dehydrated in increasing concentrations of ethanol, cleared with xylene, and then embedded in paraffin. The samples were cut into 4 μm thick sections. After deparaffination and rehydration, the sections were stained with H&E, and each sample was examined by a pathologist who was prevented from access as to the specific treatments. The

renal tubular morphological changes were scored to assess the degree of renal damage, which included tubular epithelial cell swelling and vacuolization, cast formation, and desquamation. Pathological score ranged from 0 to 4 point based on the area of injury involvement. The scale is as follows: 0, normal; 1, histological changes involving <25 % of injury area; 2, similar changes involving >25 % but < 50 % of injury area; 3, similar changes involving > 50 % but < 75 % of injury area; 4, similar changes involving > 75 % of injury area. (68, 69) All sections were provided as coded slides to the pathologist to prevent bias as to the treatment group and duration of ischemic injury. Immunostaining was performed using the following primary antibodies: D₃R was probed using a polyclonal goat anti-D₃R antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), followed by Alexa Fluor 488-donkey anti-goat IgG antibody (Beyotime, Shanghai, China), while the Gα₁₂ was visualized using an IgG affinity-purified rabbit anti-Gα₁₂ antibody (1:200, Santa Cruz Biotechnology, USA), followed by Alexa Fluor 555-donkey anti-rabbit IgG antibody (Beyotime, China). The cover slips were mounted on microscope slides using Antifade Mounting Medium (Beyotime, China). Confocal and differential interference contrast images were obtained using a confocal fluorescence imaging microscope, Leica TCS-SP5 (Bensheim, Germany) fitted with a ×40 immersion objective.

Measurement of oxidative stress

The concentration of MDA in renal cortex was measured by the thiobarbituric acid (TBA) method, following the manufacturer's protocol (Assay Kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the amounts of lipid peroxides, the production of MDA, were detected to indicate MDA concentration. Absorbance was measured at 532 nm by using a spectrometer (Bio-Rad, USA).

Total SOD activity was measured by the nitroblue tetrazolium (NBT) method, following the manufacturer's protocol (Beyotime, China). Briefly, SOD activity in renal homogenates was measured by the inhibition of NBT reduction which was produced by the xanthine-XO system as a superoxide generator. Activity was assessed in the ethanol phase of the lysate after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of samples and centrifuged. One unit of SOD was defined as the amount of enzyme causing 50% inhibition in the NBT reduction rate. SOD activity was also expressed as units per milliliter.

GSH activity was measured following the instructions of a commercial assay kit (Beyotime, China). The redox state of kidney was assessed through the determination of the GSH/GSSG ratio. The concentrations of total glutathione (T-GSH), reduced glutathione (GSH) and oxidized disulfide glutathione (GSSG) were measured by an enzymatic method. (70) Briefly, T-GSH was assayed using the 5, 5-dithio-bis (2-nitrobenzoic) acid (DTNB)-GSSG reductase recycling. GSSG was measured by measuring 5-thio-2-nitrobenzoic acid (TNB) which was produced from the reaction of reduced GSH with DTNB. The rate of TNB formation was measured at 412 nm. The concentration of reduced GSH was obtained by subtracting GSSG from T-GSH. The results were expressed in μmol GSH/mg tissue, μmol GSSG/mg tissue, and GSH/GSSG ratio.

For intracellular ROS measurement, about 1×10^5 cells per well were incubated for 30 min at 37°C with the oxidation-sensitive fluorescent probe DCFH-DA (10 μM). Fluorescence

intensity was measured using a fully automated chemiluminescent immunoanalyzer (Varioskan Flash, Thermo Scientific, Phadia GmbH, Freiburg, Germany) with ImagePro Plus software for the quantification of fluorescent changes. The intracellular probe-oxidation-induced change of immunofluorescence was measured as total intracellular ROS generation.

Measurement of inflammation in renal I/R injury

Systemic levels of IL-10, TNF- α and IL-1 β were determined by ELISA. Briefly, IL-10, TNF- α , and IL-1 β concentrations were determined on serial dilutions of plasma samples, using commercial ELISA kits (USCN Life Science Inc., Wuhan, China). Absorbance was determined at 450 nm, and values were calibrated using a standard curve.

MPO activity was determined in renal homogenates to assess leukocyte infiltration (Assay kit from Nanjing Jiancheng, China). (68)

Apoptosis in I/R injured kidney

A fluorescein *in situ* cell death detection kit was used according to the manufacturer's instructions for TUNEL assay (Roche Applied Science, Mannheim, Germany). Positive cells were counted in ten non-overlapping fields of outer medulla in each sample (magnification, $\times 400$).

The activity of caspase-3 in renal cortex was measured using a Caspase 3 Colorimetric Assay Kit, following the manufacturer's protocol (Beyotime, China). (68)

Immunoblotting and co-immunoprecipitation

Protein (40 μ g) was separated using 15 % SDS-PAGE and transferred to a nitrocellulose membrane. After the non-specific binding sites were blocked in PBS containing 5% non-fat dry milk for 1 h, the membranes were incubated with primary antibodies (anti-D3R (1: 250), anti-G α 12 (1: 300) (Santa Cruz Biotechnology, USA), anti-Bcl-2 (1: 300), anti-Bax (1: 400), or anti- β -actin (1: 1000); (Cell Signaling Technology, USA) overnight at 4 °C. β -actin was used as a housekeeping protein. The secondary antibody (goat anti-rabbit IR Dye 800 (Li-Cor Biosciences, USA) was used at a dilution of 1: 15000 to bind to the primary antibody at room temperature for 1 h. The membranes were then washed three times with TBST (Tris-Buffered Saline and 0.05 % Tween 20), and the bound complex on the membranes was detected by the Odyssey Infrared Imaging System (Li-Cor, USA). The images were analyzed using the Odyssey Application Software to obtain the integrated intensities.

Renal cortical lysates (supernatant, 500 μ g protein/ml) were incubated with anti-G α 12 antibodies (1 μ g/ml) and protein-G agarose at 4°C overnight. The immunoprecipitates were pelleted and washed four times with lysis buffer. After the sample buffer was added, the samples were boiled for 10 min and subjected to immunoblotting using the anti-D3R antibody. To determine the specificity of the bands found on the immunoblots, IgG (negative control) and D3R antibody (positive control) were used as the immunoprecipitants instead of the G α 12 antibody (data not shown).

Cell treatment

NRK52E cells were obtained as a kind gift from Professor Yu XQ (Department of Nephrology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China). (71) The cells were maintained in cell culture flasks using Dulbecco modified eagle medium (DMEM) F12 medium (Invitrogen, Beijing, China) supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. Cells were seeded in 96-well plates, grown to 70–80% confluence and subjected to serum deprivation for 24 hrs before experimental manipulation. The pCMV6- Ga12 plasmid (2 µg/ml, Origene, Rockville, MD, USA), or pCMV6-control plasmid (2 µg/ml, Origene, USA) was mixed with the Attractene transfection reagent (2 µl/ml, Qiagen, Dusseldorf, Germany) and incubated for 15 min at room temperature. The lipid-coated DNA was then added to each well containing 100 µl DMEM medium and incubated for 6 hrs. The cells were then exposed to 24 hr of hypoxia (5% CO₂, 1% O₂, 94% N₂) and followed by 3 hrs reoxygenation (5% CO₂, 21% O₂, 74% N₂). (72) After H/R injury, the treated NRK52E cells and supernatant were used in cell viability and LDH assays. The dose of PD 128907 (10⁻⁷) used *in vitro* was chosen based on our previous report. (45)

For cold storage/rewarming injury study, the cells were seeded in 96-well plates, grown to 60% confluence and divided in three groups: (1) Untreated, (2) cold storage and rewarming (CS.RW) with vehicle, (3) CS.RW treatment in the presence of different dosages of PD128907. In experimental groups, cells were washed with cold PBS twice and stored in University of Wisconsin (UW) solution containing various doses (10⁻⁸– 10⁻⁶M) PD128907 or vehicle for 24hrs at 4°C. To simulate rewarming, the UW solution was replaced with DMEM F12 containing 5% FCS for 6 hrs at 37°C after cold-storage for 24hrs. Untreated group remained in DMEM F12 containing 5% FCS for 24 hrs at 37°C. (73) Then, the cell viability and LDH assays were going like before.

Statistical analysis

The data are expressed as mean ± SEM. Comparison within groups was made by ANOVA for repeated measures, and comparison among groups was made by factorial ANOVA with Holm-Sidak test. A value of $P < 0.05$ was considered significant.

Acknowledgments

These studies were supported in part by grants from the National Natural Science Foundation of China (31430043, 81071588), National Basic Research Program of China (973Program, 2012CB517801), and the National Institutes of Health (HL0749400).

Abbreviations

I/R	Ischemia/reperfusion
D ₃ R	dopamine D ₃ receptor
ROS	reactive oxygen species
RPTs	renal proximal tubule cells
GPCRs	G protein-coupled receptors

H/R	hypoxia/reoxygenation
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
GSH	glutathione
GSSG	glutathione disulfide
SOD	superoxide dismutase
MPO	myeloperoxidase
MDA	malondialdehyde
H&E	haematoxylin and eosin
LDH	lactate dehydrogenase

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Group	Treatment	Surgical procedure
I: Sham	Saline	Sham-operated
II: PD	PD128907	Sham-operated
III: I/R	Saline	Unilateral renal ischemia 45 min
IV: I/R + PD	PD128907	Unilateral renal ischemia 45 min

B

C

E **Figure 1.**

Protective effect of D₃R stimulation by PD128907 in renal ischemia/reperfusion (IR) injury. (A) Schematic representation of induction of renal I/R injury. Renal function was assessed by measuring serum urea (B) and creatinine (C) levels in day 1 and day 3 (n=3–9). Sham and I/R kidneys pre-treated with vehicle (saline) or PD128907 were harvested one day after I/R injury, then fixed and paraffin-embedded; 4 µm sections were cut and stained with hematoxylin and eosin. The histological degree of renal I/R injury was assessed by a semi-quantitative scoring method. PD128907, by itself, did not alter renal morphology but reduced morphological (×40 and ×100 magnification) changes associated with I/R injury (D) and (E). Values are expressed as mean ± SEM. **P* < 0.01 vs. others, #*P* < 0.01 vs. I/R, sham, and PD.

B

C

D

E **Figure 2.**

PD128907 attenuates the apoptosis caused by renal I/R injury. Kidney was harvested one day after I/R injury. Renal apoptosis was determined by TUNEL staining. Apoptosis was greater in I/R than sham group. Pretreatment with PD128907, by itself, had no effect on apoptosis but significantly decreased the apoptosis caused by I/R injury (**A** and **B**). I/R increased Bax/Bcl ratio (**C**) and caspase-3 expression (**D**) and activity (**E**). PD128907, by itself, did not affect these variables but partially ameliorated the effects of I/R injury. The expressions of Bax and Bcl were normalized by β -actin. Values are expressed as mean $\pm$ SEM. * $P < 0.01$ vs. others, # $P < 0.01$ vs. I/R, sham, and PD ($n = 5$ /group).

A

B

C

D

E **Figure 3.**

PD128907 attenuates the oxidative stress caused by I/R injury. Kidney was harvested one day after I/R injury. GSH levels (A), GSH/GSSG ratio (reduced and oxidized glutathione ratio) (B) and superoxide dismutase (SOD) activity (C) were decreased by I/R injury, while myeloperoxidase (MPO, D) and malondialdehyde (MDA, E) were increased by I/R injury. PD128907, by itself, did not affect these variables but ameliorated the effects of I/R injury. Values are expressed as mean $\pm$ SEM. * $P < 0.01$ vs. sham, # $P < 0.01$ vs. I/R (n = 5/group).

A

B

C

Figure 4.

PD128907 ameliorates the increase in pro-inflammatory cytokines/chemokines and decrease in anti-inflammatory cytokine/chemokine caused by I/R injury. Plasma was taken one day after I/R injury. Plasma levels of cytokines were determined by ELISA. The levels of pro-inflammatory cytokines/chemokines, TNF- α (A) and IL-1 β s (B) were increased while the levels of anti-inflammatory cytokine IL-10 (C) were decreased in I/R injury. PD128907, by itself, did not affect these variables but ameliorated the effects of I/R injury. Values are expressed as mean $\pm$ SEM. * $P < 0.01$ vs. sham, # $P < 0.01$ vs. I/R ($n = 5$ /group).

A

B

C

D **Figure 5.**

PD128907 partially prevents the decrease in renal D₃R expression and D₃/G α 12 colocalization/co-immunoprecipitation and increase in G α 12 expression caused by I/R injury. Kidney was harvested one day after I/R injury. Immunofluorescence microscopy showed colocalization of D₃R and G α 12 (A). D₃R and G α 12 expressions were determined by immunoblotting (B and C). The linkage between D₃R and G α 12 was determined by co-immunoprecipitation (D). Values expressed as mean $\pm$ SEM. **P* < 0.01 vs. sham, &*P* < 0.05 vs. sham, #*P* < 0.01 vs. I/R (n = 5/group).

A

B

C

Figure 6. Protective effects of PD128907 against hypoxia/reoxygenation injury were attenuated by over-expression of Gα12 in NRK52E cells. The injury of hypoxia/reoxygenation to NRK52E cells was determined by ROS levels (A), LDH concentration in the culture medium (B) and cell viability (C). (n=5/group, * $P<0.05$ vs. control, # $P<0.01$ vs. vehicle, & $P<0.05$ vs. PD).

A

B **Figure 7.**

Protective effects of PD128907 against cold storage/rewarming injury were evaluated in NRK52E cells. The injury of cold storage/rewarming stress to NRK52E cells with/without PD128907 pretreatment was determined by LDH release (A) and cell viability assay (B). (n=3/group, * $P<0.05$ vs. control, # $P<0.01$ vs. vehicle).

Table 1

Effect of PD128907 on Survival Days Over a 7-Day Period

Group	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Vehicle treatment I/R group (n=9)	9	9	5	3	1	1	0	0
PD treatment I/R group (n=9)	9	9	7	5	4	4	3	3

Note: The survival days over a seven-day-period after renal I/R injury. None (0%) of rats in the vehicle treatment I/R group survived over five days. However, three (33.3%) of nine rats in the PD128907 treated I/R group survived over seven days.