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Delayed ischemic preconditioning contributes to renal protection by upregulation of miR-21

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Abstract

Delayed ischemic preconditioning effectively protects kidneys from ischemia-reperfusion injury but the mechanism underlying renal protection remains poorly understood. Here we examined the in vivo role of microRNA miR-21 in the renal protection conferred by delayed ischemic preconditioning in mice. A 15 minute renal ischemic preconditioning significantly increased the expression of miR-21 by 4 hours and substantially attenuated ischemia-reperfusion injury induced 4 days later. A locked nucleic acid-modified anti-miR-21 given at the time of ischemic preconditioning knocked down miR-21 and significantly exacerbated subsequent ischemia-reperfusion injury in the mouse kidney. Knockdown of miR-21 resulted in significant upregulation of programmed cell death protein 4, a pro-apoptotic target gene of miR-21, and substantially increased tubular cell apoptosis. Hypoxia inducible factor-1α in the kidney was activated after ischemic preconditioning and blockade of its activity with a decoy abolished the up-regulation of miR-21 in cultured human renal epithelial cells treated with the inducer cobalt chloride. In the absence of ischemic preconditioning, knockdown of miR-21 alone did not significantly affect ischemia-reperfusion injury in the mouse kidney. Thus, upregulation of miR-21 contributes to the protective effect of delayed ischemic preconditioning against subsequent renal ischemia-reperfusion injury.

Introduction

MicroRNAs (miRNA) are endogenous, small (18-22 nucleotides) RNA molecules that play an important and ubiquitous role in regulating gene expression. miRNAs typically bind to the 3′ untranslated region of their mRNA targets and downregulate gene expression via mRNA degradation or translational inhibition.[1-3] miRNAs are known to play a significant role in cell physiological processes such as cell differentiation[4], proliferation[5] and

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apoptosis[6]. Emerging evidence suggests that miRNAs could also be involved in pathological processes in the kidney.[7-9]

Acute kidney injury is a common complication of major surgical operations and sepsis resulting in adverse outcome.[10] Ischemia-reperfusion (I/R) injury is the major cause of acute kidney injury.[11] However, a short period of ischemia followed by reperfusion can activate endogenous defense mechanisms that protect against a subsequent, sustained ischemic insult, a phenomenon known as ischemic preconditioning (IPC).[12] Recent data from us and other investigators indicate that in kidney I/R injury, protective effects of IPC appear quickly after IPC and dissipate over several hours but reappear several days later.[13-16] The latter phenomenon is defined as delayed IPC. The protective mechanism of delayed IPC in the heart and brain appears to involve a series of protective mediators and/or effectors such as reactive oxygen species, protein kinase C, hypoxia-inducible factor (HIF), inducible nitric oxide synthase, heat shock protein70 and so on.[17, 18] The beneficial effects of delayed IPC require new protein synthesis and are sustained for days to weeks.[19] However, the mechanisms underlying delayed IPC in the kidney are poorly understood.

Particularly, the role of miRNAs in renal IPC is not known. Several miRNAs such as miR-200, miR-24 and miR-192 have been reported to be involved in cardiac, brain and hepatic IPC.[20-22] These miRNAs were involved in the early IPC. Yin et al reported a possible role of miR-1, miR-21, and miR-24 in an ex vivo model of late IPC in the mouse heart.[23]

MiRNA miR-21 has been shown to be a strong anti-apoptotic factor at least in part by targeting pro-apoptotic genes including programmed cell death protein 4 (PDCD4).[24-26] Tubular cell apoptosis contributes importantly to acute renal I/R injury.[27] We, therefore, hypothesized that miR-21 might play an important role in the renal protective effect of delayed IPC. We utilized a mouse model of renal delayed IPC. With highly effective in vivo knockdown of miR-21, we were able to determine the role of miR-21 in the renal protection against I/R injury conferred by delayed IPC.

Results

Delayed IPC protected mouse kidneys from I/R injury and was associated with up-regulation of miR-21

Mice were divided into two groups: an IPC+I/R group and a Sham+I/R group. The interval between IPC and I/R was 4 days. Mice in the IPC+I/R group showed marked improvement of renal function and histology compared to the Sham+I/R group. Plasma creatinine levels at 24 h after reperfusion were nearly 50% lower in the IPC+I/R group ($P<0.05$, Figure 1A). Histological examination in the Sham+I/R group revealed characteristics of acute tubulointerstitial damage, including massive tubular epithelial cell necrosis or swelling, tubular casts, interstitial edema and inflammatory cell infiltration. Morphological damage was most prominent in the outer medullary stripe, but also with patchy involvement of the cortical proximal segments. In contrast, renal morphology of preconditioned mice only showed a mild to moderate degree of cell swelling (Figure 1B, 1C). Apoptosis is

characteristic of renal I/R injury. Delayed IPC attenuated renal tubular cell apoptosis 24 h after I/R (Figure 1D, 1E).

It is important to note that the I/R injury observed in the present study was mild compared to typical I/R injury reported by others using the 30 min bilateral occlusion method[28] and by our own group using a related method[29]. We suspect this was because of a combination of several technical factors such as the specific clamps and other instrument used and the operator. To ensure the robustness of the study, we always studied control and experimental groups in parallel, kept technical factors as consistent as possible, and used multiple indices of injury (plasma creatinine, histology score, and apoptosis).

Expression levels of miR-21 were significantly higher in the kidneys from the IPC+I/R group than the Sham+I/R group (Figure 1F). Time-course analysis indicated that miR-21 was upregulated at 4 h after IPC compared to the Sham mice, and remained significantly higher 4 d after IPC (Figure 1G). Expression levels of other injury-related miRNAs such as miR-320, miR-214 and let-7e were not statistically significantly up-regulated at day 4 after IPC (data not shown).

Knock-down of miR-21 exacerbated I/R injury in mouse kidneys following delayed IPC

To determine the functional role of miR-21 in the renal protection conferred by delayed IPC, locked nucleic acid (LNA)-modified anti-scrambled or anti-miR-21 oligonucleotides were administered to mice through the tail vein just prior to the IPC surgery. All mice then underwent the IPC and, four days later, I/R procedures. As shown in Figure 2A, renal levels of miR-21 expression, measured at 24 h after the I/R injury (i.e., 5 days after the administration of LNA anti-miR), were substantially reduced in mice receiving LNA anti-miR-21. Plasma creatinine was significantly higher in mice receiving anti-miR-21 compared to mice receiving the scrambled anti-miR, suggesting that knockdown of miR-21 attenuated renal protection conferred by IPC (Figure 2B). As shown in Figure 2C and 2D, kidneys from IPC+I/R mice receiving anti-miR-21 exhibited significant histological damage including tubular casts, moderate inflammatory infiltration and cellular swelling. Renal histology in IPC+I/R mice receiving the scrambled anti-miR only showed mild to moderate cellular swelling.

The increase of plasma creatinine shown in Figure 2B was modest. To confirm the robustness of the observed effect of anti-miR-21, we treated additional groups of mice with IPC and anti-miR and measured plasma creatinine levels at 0, 1, 2, or 5 days following the I/R injury. Mice treated with delayed IPC and the anti-scrambled oligonucleotides were largely protected from the I/R injury (Figure 2E). Mice treated with delayed IPC and anti-miR-21 showed clear increases in plasma creatinine levels 1 and 2 days after the I/R injury (Figure 2E). Note that experiments shown in Figure 2B and 2E were done using different sets of clamps and other instrument and that separate groups of mice were used for each time point shown in Figure 2E (instead of repeated blood sampling from the same mice), both of which further supported the reproducibility of the observed effect of anti-miR-21.

Knock-down of miR-21 up-regulated pro-apoptotic target gene PDCD4 and exacerbated apoptosis in mouse kidneys following delayed IPC and I/R

We examined possible mechanisms mediating the protective role of miR-21 in delayed IPC. It has been well-established that PDCD4 is a target gene of miR-21 and has powerful pro-apoptotic effects.[30-32] We examined PDCD4 protein expression in IPC+I/R mice receiving LNA anti-miR-21 or the scrambled anti-miR. As shown in Figure 3A, PDCD4 protein expression was up-regulated by the anti-miR-21 treatment, consistent with targeting of PDCD4 by miR-21. Concomitantly, IPC+I/R mice receiving the anti-miR-21 treatment exhibited a substantially increased number of apoptotic tubular cells in the kidney compared to IPC+I/R mice receiving the scrambled anti-miR (Figure 3B, 3C). The result suggests that the protective effect of miR-21 might be in part mediated by miR-21 targeting of PDCD4 and the resulting attenuation of tubular cell apoptosis.

Several pathways, such as reduced phosphorylation of JNK[16] and upregulation of iNOS and HSP27[14], have been established by others as playing a role in delayed IPC. JNK activation has been shown to be an important inducer of renal tubular apoptosis after ischemia[33]. We examined the levels of total JNK and phosphorylated JNK in the kidneys following 24 hours of I/R in the presence or absence of IPC and with or without the anti-miR-21 treatment. We did not find any significant effects of IPC or the anti-miR-21 treatment on the level of JNK phosphorylation. However, we cannot rule out the possibility that JNK activation was altered at earlier time points after I/R as shown by Park et al[16].

miR-21 was upregulated by activation of HIF

We went on to examine possible upstream mechanisms leading to the up-regulation of miR-21 following ischemia. miR-21 has been shown to be responsive to hypoxia in cancer cell lines,[34] but it is not known if miR-21 is actually under the control of HIF. HIF-1α was activated in mouse kidneys following IPC (Figure 4A). The accumulation of nuclear HIF-1α was significantly increased 4 h after IPC and persisted 4 d after IPC, compared to the Sham mice.

Treatment of primary cultures of human renal epithelial (HRE) cells with cobalt chloride or hypoxia, which are classic inducers of HIF-1α activation, caused significant upregulation of miR-21 (Figure 4B, 4C). Activation of HIF-1a by the cobalt chloride treatment was confirmed by Western blot in nuclear extracts (Figure 4D). Double-stranded oligonucleotides containing a HIF hypoxia-responsive element (HIF decoy) were used to block the action of HIF. The HIF decoy, compared to scrambled oligonucleotides, significantly reduced miR-21 levels by 42% in HRE cells treated with cobalt chloride (Figure 4E). HIF decoy significantly reduced mRNA levels of erythropoietin, a prototypic HIF target gene, to 64% ± 8% of control ($n=6$, $P<0.05$), confirming the effectiveness of HIF blockade.

Knock-down of miR-21 did not affect I/R injury in the absence of IPC

To examine if miR-21 was protective in the absence of IPC, we performed two sets of experiments. First, we administered LNA anti-miR-21 or the scrambled anti-miR to mice just prior to I/R. The mice were not exposed to IPC. The anti-miR-21, again, substantially

reduced the level of miR-21 in the kidney measured 24 h after I/R (Figure 5A). However, plasma creatinine levels increased after 24 h reperfusion similarly in both the anti-miR-21 group and the scrambled anti-miR group (Figure 5B). Without IPC, I/R induced significant renal histological damage including tubular casts and inflammatory infiltration as well as tubular apoptosis. No significant difference in histology or apoptosis was observed in mice receiving anti-miR-21 or the scrambled anti-miR (Figure 5C to 5F).

In the second set of experiments, we administered LNA anti-miR-21 or the scrambled anti-miR 4 days prior to the I/R injury. The experiment mimicked the time course of the IPC study without actually applying IPC. The anti-miR-21 treatment substantially reduced the level of miR-21 in the kidney measured 24 h after I/R (Figure 5G). The treatment, however, did not exacerbate renal I/R injury (Figure 5H) or significantly increase apoptosis (Figure 5I, 5J), in contrast to the exacerbation of injury by anti-miR-21 that we observed in the presence of IPC.

These experiments suggested that up-regulation of miR-21 prior to I/R, such as that induced by IPC, might be required for the manifestation of the protective effect of miR-21.

Discussion

The present study has revealed a novel in vivo role of a specific miRNA, miR-21, in the protection against acute kidney injury conferred by preconditioning. The role of miRNAs in acute kidney injury is not well understood. Wei, et al, demonstrated that mice with broad reductions of mature miRNAs in the proximal tubule due to Dicer knockout were more resistant to renal I/R injury. These mice exhibited better renal function, less renal damage, fewer apoptotic cells in the kidney, and improved survival following bilateral I/R.[35] Godwin, et al, reported differential expression of several miRNAs following unilateral renal I/R. They went on to show that, in cultured mouse tubular epithelial cells, knockdown of miR-21 increased cell death, but overexpression of miR-21 in these cells did not prevent cell death following simulated ischemia.[36] We have now shown that knockdown of miR-21 in mice in vivo at the time of IPC worsens renal I/R injury induced 4 days later, indicating that miR-21 contributes to the protection conferred by delayed IPC. Knockdown of miR-21 in the absence of IPC, however, did not further exacerbate renal injury. Taken together, these studies support an important role for miRNAs in the development of acute kidney injury or the protection from it. While Dicer and perhaps certain dominating miRNAs in the proximal tubule might be pro-injury, specific miRNAs such as miR-21 are protective. Moreover, the protective effect of miR-21 may depend on the timing and context of injury.

Additional miRNAs may be involved in I/R injury and IPC, but their functional roles in renal I/R injury or IPC remain to be examined. In studies of renal I/R models by Godwin, et al, and Wei, et al,[35, 36] several miRNAs such as miR-214, miR-7 and miR-192 were shown to be upregulated, while others such as miR-322 were down-regulated. Ren, et al, reported decreases of miR-320 expression and increases of miR-7, miR-21, and miR-491 expression in the mouse heart 24 h after 30min ischemia.[37] Several miRNAs were differentially regulated in hippocampi following global ischemia[38] and gracilis muscles in ischemic injury.[39] Yin et al showed that IPC in the heart resulted in upregulated miR-1, miR-21, and

miR-24 expression.[23] Abundance of miR-23a, miR-326, miR-346, and miR-370 was altered in hepatic IPC.[20] miR-200 and miR-182 were upregulated after cerebral IPC.[21] In the present study, the expression of miR-214, miR-320 and let-7e was examined. These miRNAs tended to be up-regulated by renal IPC, but did not reach statistical significance.

Several studies in cancer and the heart support a strong pro-survival and anti-apoptotic role of miR-21. miR-21 is upregulated in several types of solid tumors, including breast tumors, colon tumors and gliomas.[6, 24, 40] miR-21 was found to be up-regulated early after cardiac IPC and protect against cardiac I/R injury.[32] miR-21 may exert its protective effect by targeting pro-apoptotic genes. For example, miR-21 was shown to reduce the death of ischemic cortical neurons by downregulating cell death inducing Fas ligand (FasL) gene.[41] Overexpression of miR-21 in mouse heart inhibited ischemia-induced upregulation of PTEN and FasL, limited infarct size, and attenuated apoptosis.[42] PDCD4 is a confirmed, direct target of miR-21.[31, 32] The tumor suppressor PDCD4 was originally characterized as an inhibitor of neoplastic transformation.[43] It has been reported that the activity of activator protein 1, a key signaling molecule that affects cell apoptosis in response to extracellular stress, was inhibited by PDCD4.[44] miR-21 expression has been shown to be inversely correlated with PDCD4 expression and/or cellular apoptosis in the heart[32], in cultured mouse tubular epithelial cells[36], and now in mouse kidneys in vivo in the present study.

miRNA expression could be regulated by transcriptional factors,[45,46] similar to the regulation of protein-coding genes. HIF is known as an important transcriptional regulator in cellular response to hypoxia.[47] miR-21 has been reported as one of the hypoxia-regulated miRNAs in cancer cells, and a predicted HIF binding site was found approximately 2 kb upstream from miR-21 transcription start site.[34] We found in the present study that HIF-1α was up-regulated in parallel with miR-21 in the mouse kidney following IPC, and that blockade of HIF abolished miR-21 upregulation in vitro, demonstrating a significant role for HIF in the upregulation of miR-21. We also found that knock-down of miR-21 did not affect I/R injury in the absence of IPC, suggesting that the protective effect of miR-21 might depend on HIF-1 induction by IPC. Additional mechanisms may participate in the regulation of miR-21. For example, Polytarchou et al found that under hypoxia, the binding of NF-κB, CREB and CBP/p300 to the miR-21 promoter induce miR-21 expression after the activation of protein kinase Akt2.[46]

Although the protective effect of delayed IPC on cardiac or brain I/R injury has been extensively studied,[17-19] renal delayed IPC has only been examined in a small number of studies. Park, et al, characterized a mouse model in which prior exposure to 30 min ischemia protected against a second ischemic insult imposed 8 or 15 days later. A shorter period of prior ischemia (15 min) was partially protective against subsequent ischemic injury 8 days later.[16] We previously reported that in rats, ischemic pretreatment for 20 min significantly attenuated I/R injury caused by 40 min of bilateral renal ischemia 4 days later.[13] In the current study, we found that 15min IPC had a profound protective effect on mouse kidneys exposed to I/R injury 4 days later. The renal protection conferred by delayed IPC is likely mediated by several mechanisms. The current study indicates that miR-21 represents one of the mechanisms involved.

Methods

Mouse models of delayed renal IPC and I/R

Ischemic preconditioning (IPC) was induced in 5 to 6 week-old male C57BL/6J mouse kidneys (The Jackson Laboratory, Bar Harbor, ME). Briefly, mice were anesthetized with intraperitoneal xylazine (15mg/kg) and ketamine (120mg/kg) mixture. After performing a midline laparotomy, bilateral renal pedicles were clamped for 15 min by microserrefine clips (F.S.T). Mice were maintained at 37 °C, and the abdominal cavity was hydrated with saline-moistened gauze. The kidneys in separate groups of mice were harvested at 4 h, 24 h and 4 d after the surgery. Sham mice underwent the same surgical procedures except the renal pedicles were not clamped.

For the delayed IPC and I/R model, 4 d after IPC or sham surgeries, preconditioned mice were subjected to 30 min occlusion of bilateral renal pedicles, followed by reperfusion for 24 hours or for longer periods as indicated in Results. The renal clamps were removed after the 30 min occlusion and the kidneys were observed for another min to ensure reflow, after which the incision was closed with a 6.0 suture. Additional groups of mice underwent 30 min ischemia and 24 hours reperfusion without IPC.

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

In vivo miRNA knockdown using LNA-modified anti-miR

Locked nucleic acid (LNA) modified anti-scrambled or anti-miR-21 oligonucleotides (Exiqon) were diluted in saline (5 mg/ml),[46] and delivered into the tail vein (10 mg/kg) less than 1 hour prior to ischemia surgery.

Analysis of plasma creatinine

Blood samples were taken through cardiac puncture at the indicated times. Plasma creatinine was measured using the improved Jaffe method (Quantichrom™ creatinine Assay Kit, BioAssay Systems).

Histological analysis of renal injury

Kidneys were fixed in 10% formalin and embedded in paraffin. Histopathological changes were assessed on PAS stained 4 μm-thick sections by scoring tubular cell necrosis or swelling, interstitial infiltration by multi-nucleated cells, tubular casts and brush border loss in 10 non-overlapping fields (20× magnification) of the corticomedullary junction and outer medulla. Tissue damage was examined in a blinded manner and scored according to the severity of changes on a semi-quantitative scale, where 0 was no injury, 1 mild, 2 moderate, 3 severe, and 4 very severe.[13]

TUNEL assay

Kidneys were fixed in 10% formalin, embedded in paraffin and cut into thin (4μm) sections. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to assess DNA fragmentation (In situ Cell Death Detection kit, Roche) according

to the manufacturer's protocol. The number of TUNEL-positive cells and total cell number in kidney sections were counted under a light microscope. TUNEL positive cells were expressed as percentage of total cells.

Taqman real-time PCR

Total RNA from kidney tissue was isolated using Trizol (Invitrogen). Expression levels of several miRNAs and mRNAs were quantified in total RNA using real-time PCR with the Taqman chemistry (Applied Biosystems) as described previously.[48-50] 5S and 18S rRNAs were used as internal normalizer for miRNAs and mRNAs, respectively.

Nuclear protein extraction

The preparation of nuclear extracts from mouse kidney tissue was performed using the Nuclear Extract Kit from Active Motif. Briefly, the tissue was homogenized in a hypotonic buffer containing protease and phosphatase inhibitors, DTT and detergent, and centrifuged at 14,000g for 30s at 4°C. The nuclear pellet was resuspended in 50μl complete lysis buffer, incubated on ice for 30min and centrifuged at 14,000g for 10 min at 4 °C. The supernatant contained nuclear protein.

Western blot analysis

The relative abundance of PDCD4, HIF-1α, JNK, and phosphorylated JNK was analyzed using western blot similar to what we described previously.[48-50] The primary antibody anti-PDCD4, anti-HIF-1α, anti-JNK, and anti-phosphorylated JNK was from Sigma (P0071, rabbit polyclonal, 1:500 dilution), Novus Biologicals (NB100-105, mouse monoclonal, 1:500 dilution), and Santa Cruz (sc-571, rabbit polyclonal IgG,1:200 dilution; sc-6254, mouse monoclonal IgG, 1:100 dilution) respectively. The secondary antibody was HRP-conjugated anti-rabbit or anti-mouse IgG from Santa Cruz. Coomassie blue staining of the entire membrane was used to confirm equal loading on the gel.

Cell culture and hypoxia treatment

Primary human renal epithelial (HRE) cells (Cambrex) were cultured in renal epithelial growth medium (Cambrex).[51] HRE cells at 60% to 70% confluency were exposed to 300μM cobalt chloride for 4 h or a steady flow of low-oxygen gas mixture (2% O2, 5% CO2, 94% N2) in a modular incubator chamber (Thermo Scientific) for 24 h.

HIF decoy

Double-stranded oligodeoxynucleotides carrying a hypoxia-responsive element were used as a decoy to block the activity of endogenous HIF transcriptional factor. The HIF decoy sequences were 5′-GCCCTACGTGCTGTCTCA-3′ (sense) and 5′-TGAGACAGCACGTAGGGC-3′ (antisense). The scrambled oligonucleotides were 5′-GCCCTTACAACTGTCTCA-3′ (sense) and 5′-GAGACAGTTGTAAGGGC-3′ (antisense). Sense and antisense oligonucleotides were heated at 95°C for 5 min and then cooled down slowly to room temperature.[52, 53] The double-stranded oligonucleotides were transfected into HRE cells at the final concentration of 40 nM for 4 h using Lipofectamine 2000 (Invitrogen). The cells were then exposed to 300μM cobalt chloride for another 4 h.

Statistics

Data were analyzed using the Student's t-test when comparing two groups and two-way ANOVA followed by Bonferroni t-test for the time course data. Real-time PCR data were shown as % of control because data from multiple PCR plates were combined. For these data, statistical analysis was performed on the original data before conversion to % values. A $P<0.05$ was considered significant. Data are shown as mean ± SEM.

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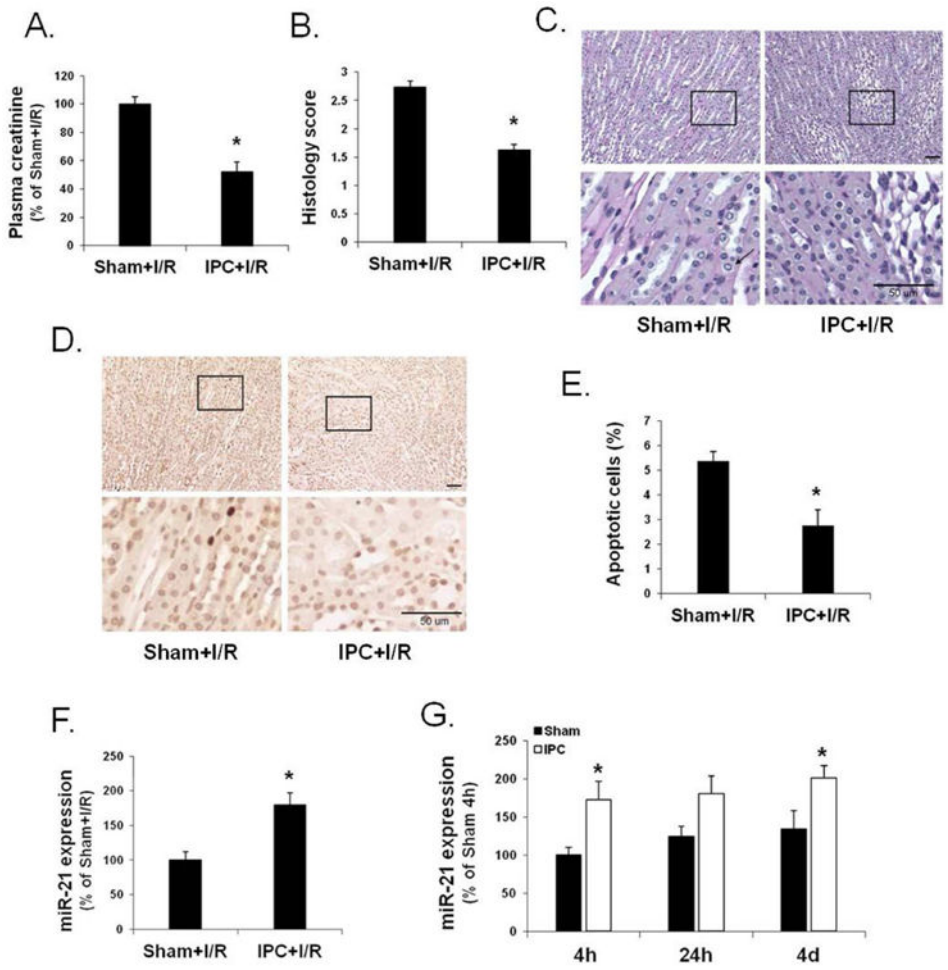

Figure 1. Delayed IPC protected mouse kidneys from I/R injury and was associated with up-regulation of miR-21

(A) Plasma creatinine and (B) renal acute tubulointerstitial injury score 24 h after I/R. (C) Kidney sections were stained with PAS and photographed at 20× magnification in the outer medulla. Calibration bar = 50 μm. The black arrow indicates swelling of tubular epithelial cells. (D, E) Delayed IPC attenuated renal tubular cell apoptosis 24 h after I/R. Cell apoptosis was determined by TUNEL staining and photographed at 20× magnification. Calibration bar = 50 μm. (F) miR-21 abundance 24h after I/R was higher in kidneys exposed to delayed IPC compared to sham. n=6/group; *P<0.05 vs. Sham+I/R. (G) miR-21 was up-regulated at several time points following IPC. n=6/group each time point; *P<0.05 vs. Sham.

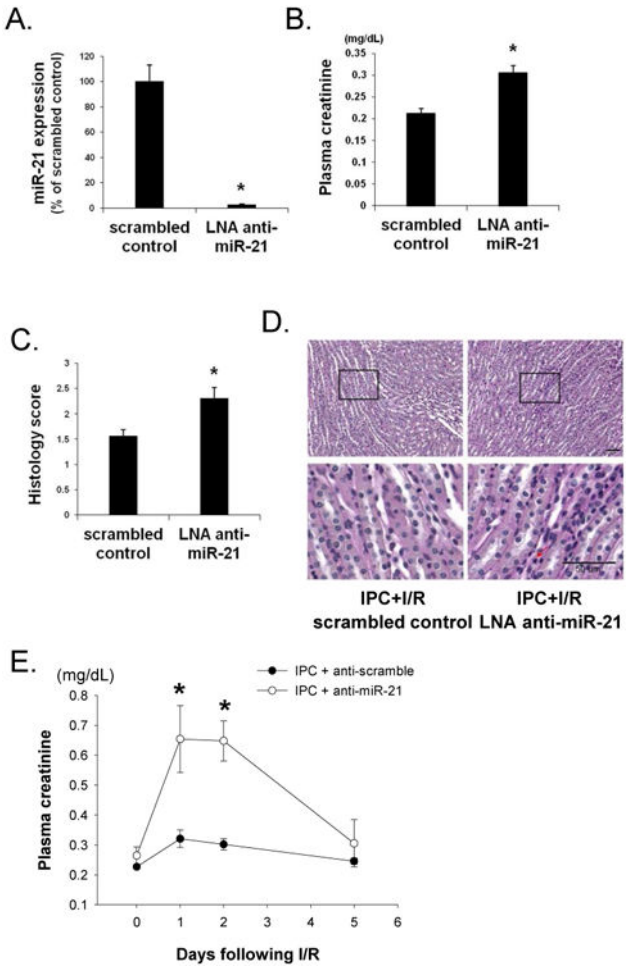

Figure 2. Knock-down of miR-21 exacerbated I/R injury in mouse kidneys following delayed IPC

(A) LNA anti-miR-21 (10mg/kg), administered at the time of IPC, decreased miR-21 expression effectively in the renal tissue examined 24 h after I/R (5 days after IPC). (B) Knockdown of miR-21 in mice exposed to delayed IPC significantly increased plasma creatinine 24 h after I/R. (C) Knockdown of miR-21 in mice exposed to delayed IPC significant worsened renal injury 24 h after I/R. (D) Kidney sections were stained with PAS and photographed at 20× magnification in the outer medulla. Calibration bar = 50 μm. The red arrow indicates infiltration of inflammatory cells. n=6 for the anti-scramble treated group; n=5 for the anti-miR-21 treated group; *significantly different from anti-scrambled treated mice ($P<0.05$). (E) Time course of plasma creatinine levels after I/R in mice exposed to delayed IPC with or without miR-21 knockdown. Separate groups of mice were used for each time point. n=3 for all time points except n=2 for the 5 day time point for anti-miR-21 and 0 and 2 day time points for anti-scrambled due to unexpected loss of mice; *significantly different from anti-scrambled treated mice ($P<0.05$).

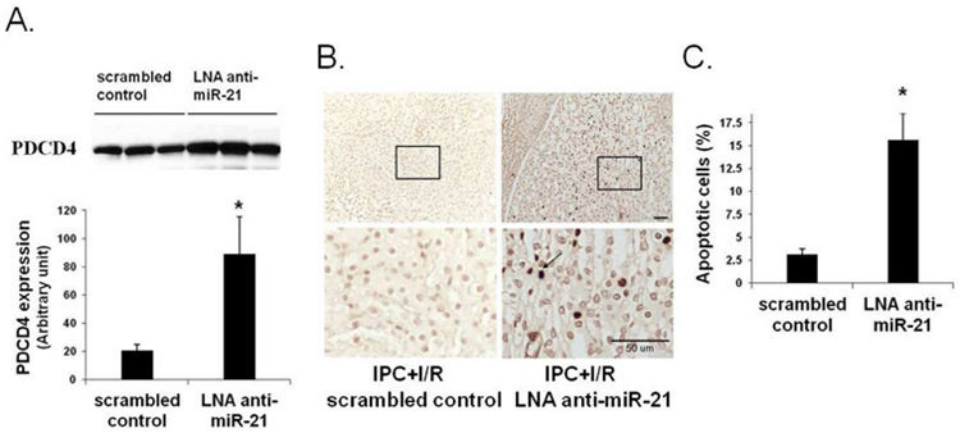

Figure 3. Knock-down of miR-21 resulted in up-regulation of pro-apoptotic target gene PDCD4 and exacerbated apoptosis in mouse kidneys following delayed IPC and I/R

(A) PDCD4 Western blot showed that LNA anti-miR-21 (10mg/kg), administered at the time of IPC, increased PDCD4 expression in mouse renal tissue 24 h after I/R. (B) LNA anti-miR-21, administered at the time of IPC, exacerbated renal tubular cell apoptosis 24 h after I/R. Cell apoptosis was determined by TUNEL staining and photographed at 20× magnification. Calibration bar = 50 μm. The arrow indicates apoptotic tubular cell. n=6 for the anti-scramble treated group; n=5 for the anti-miR-21 treated group; *significantly different from anti-scrambled treated mice ($P<0.05$).

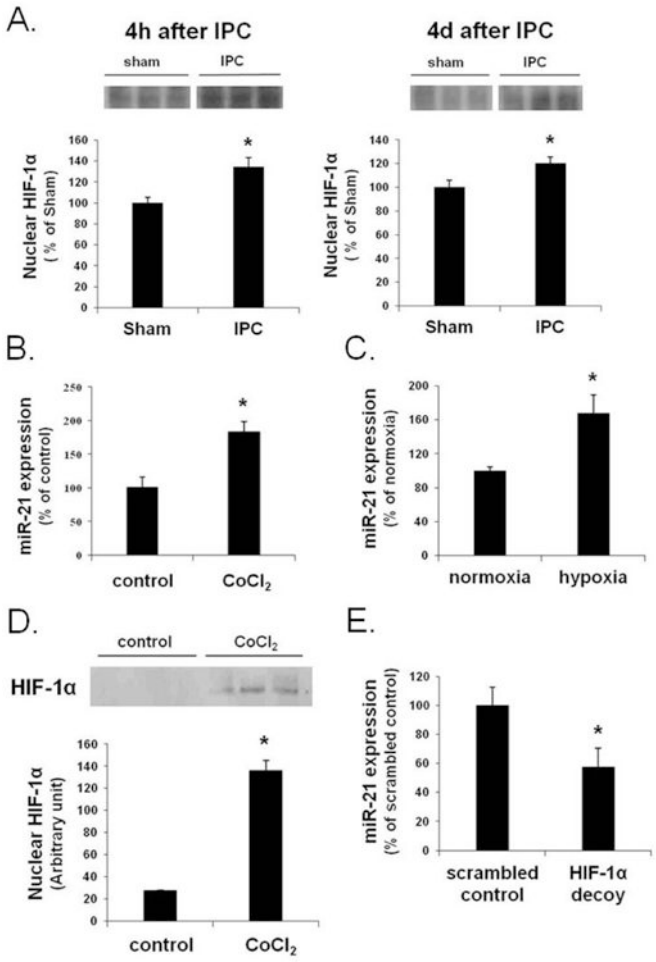

Figure 4. Role of HIF-1α in miR-21 up-regulation

(A) Nuclear levels of HIF-1α in mouse kidneys were increased 4 h after IPC and persisted 4 d after IPC, compared to Sham mice. n=6/group at each time point; *significantly different from Sham mice ($P<0.05$). (B) $CoCl_2$ and (C) hypoxia, both classical inducers of HIF-1α, up-regulated miR-21 in primary cultures of human renal epithelial (HRE) cells. HRE cells were treated with $CoCl_2$ at 300uM for 4 h or incubated in 2% O_2 (hypoxia) for 24 h. n=4/group, * significantly different from control or normoxia group ($P<0.05$). (D) HIF-1α is activated by the $CoCl_2$ treatment. n=3/group, * significantly different from control group ($P<0.05$). (E) CoCl2 induced up-regulation of miR-21 was abolished by HIF blockade with a decoy. n=6/group, * significantly different from cells treated with the scrambled control ($P<0.05$).

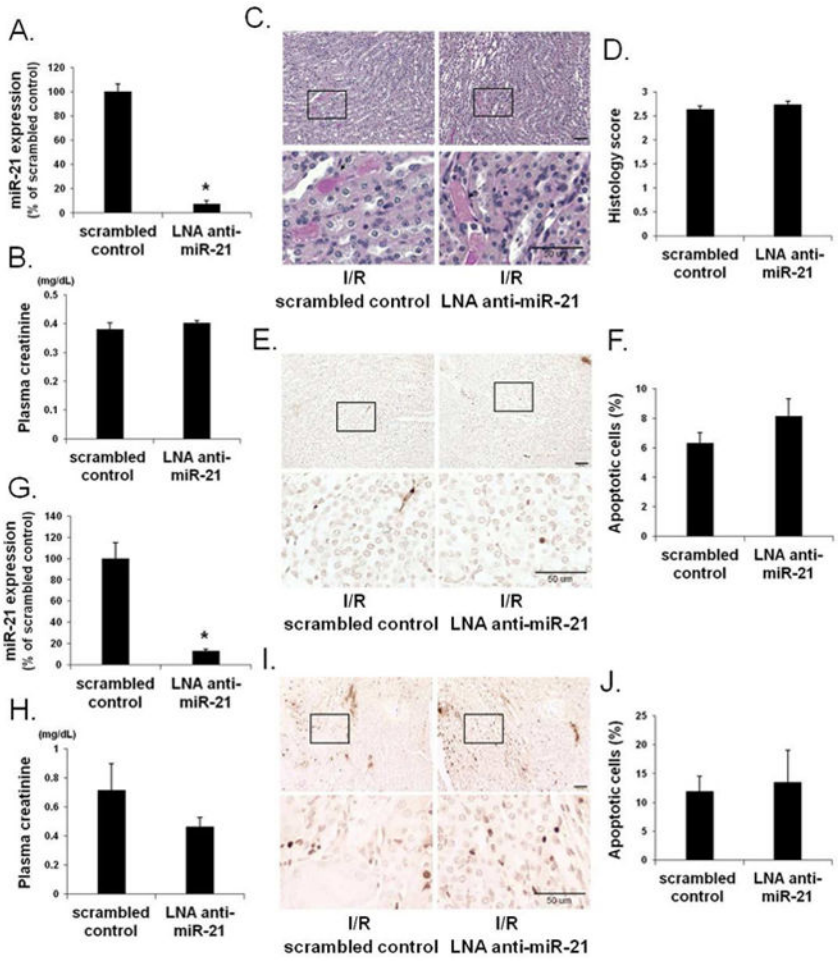

Figure 5. Knockdown of miR-21 in the absence of IPC did not affect I/R injury

(A) LNA anti-miR-21 (10mg/kg), administered at the time of I/R, in mice not exposed to IPC decreased miR-21 expression effectively in renal tissue 24 h after I/R. Knockdown of miR-21 at the time of I/R and in the absence of IPC did not significantly alter plasma creatinine (B) or renal histological injury (C, D) 24 h after I/R. Calibration bar = 50 μm. The dash arrow indicates tubular cast formation. (E, F) Renal tubular cell apoptosis 24 h after I/R was not significantly altered by knockdown of miR-21 at the time of I/R and in the absence of IPC. Cell apoptosis was determined by TUNEL staining and photographed at 20× magnification. n=5 for the anti-scrambled treated group; n=6 for the anti-miR-21 treated group; *significantly different from anti-scrambled treated mice ($P<0.05$). (G) LNA anti-miR-21 (10mg/kg), administered 4 days prior to the I/R injury in mice without IPC, decreased miR-21 expression effectively in renal tissue 24 h after I/R. Knockdown of miR-21 4 days prior to the I/R injury and in the absence of IPC did not significantly alter plasma creatinine (H) or renal tubular cell apoptosis (I, J) 24 h after I/R. Cell apoptosis was determined by TUNEL staining and photographed at 20× magnification. n=4 for the anti-scrambled treated group; n=3 for the anti-miR-21 treated group; *significantly different from anti-scrambled treated mice ($P<0.05$).