Research Paper

Limb ischemic preconditioning protects against contrast-induced nephropathy via renalase

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ARTICLE INFO

ABSTRACT

Clinical trials shows that remote ischemic preconditioning (IPC) can protect against contrast induced nephropathy (CIN) in risky patients, however, the exact mechanism is unclear. In this study, we explored whether renalase, an amine oxidase that has been previously shown to mediate reno-protection by local IPC, would also mediate the same effect elicited by remote IPC in animal model. Limb IPC was performed for 24 h followed by induction of CIN. Our results indicated that limb IPC prevented renal function decline, attenuated tubular damage and reduced oxidative stress and inflammation in the kidney. All those beneficial effects were abolished by silencing of renalase with siRNA. This suggests that similar to local IPC, renalase is also critically involved in limb IPC-elicited reno-protection. Mechanistic studies showed that limb IPC increased TNFα levels in the muscle and blood, and up-regulated renalase and phosphorylated IκBα expression in the kidney. Pretreatment with TNFα antagonist or NF-κB inhibitor, largely blocked renalase expression. Besides, TNFα preconditioning increased expression of renal renalase in vivo and in vitro, and attenuated H_2O_2 induced apoptosis in renal tubular cells. Collectively, our results suggest that limb IPC-induced reno-protection in CIN is dependent on increased renalase expression via activation of the TNFα/NF-κB pathway.

1. Introduction

Contrast induced nephropathy (CIN) has become one of the leading causes of hospital-acquired acute kidney injury (AKI) (Nash et al., 2002) as contrast agents are widely utilized in clinical diagnostic and interventional procedures (Asif and Epstein, 2004; Wang et al., 2013). CIN following cardiac angiogram or intervention is associated with significant morbidity and mortality, with an in-hospital mortality rate of 20% in unselected patients and a 1-year mortality rate of up to 66% (Rihal et al., 2002; Shlipak et al., 2002; Best et al., 2002). However, there is still no effective prophylactic regimen available to prevent occurrence of CIN (Gassanov et al., 2014). Therefore, it is urgent to develop novel strategies to decrease CIN incidence and to improve clinical outcomes.

Accumulated evidence indicates that CIN is the result of combined direct toxic effects of contrast media and hypoxic renal injury (Sendeski, 2011). Renal ischemia-reperfusion (I/R) injury due to contrast induced

hemodynamic alteration of renal blood flow plays a pivotal role in the pathogenesis of CIN (Evans et al., 2013; Persson et al., 2005). Besides, contrast media induced production of free oxygen radicals also contributes to renal tubular cell injury (Pisani et al., 2013). Thus, the strategies for protecting against hypoxic injury may also be effective for prevention and treatment of CIN.

Remote ischemic preconditioning (IPC) is defined as temporal episodes of ischemia of remote organ before a subsequent prolonged I/R injury. It can facilitate other organs to tolerate a more severe injury and to reduce the extent of distant organ damage. The tissue protective effects of this approach have been reported in myocardial infarction and AKI (Przyklenk and Whittaker, 2011; Wever et al., 2011). Limb IPC, one of remote IPC represents a promising approach for clinical intervention, has been tested in the prevention of CIN in clinic trials and gained encouraging results, especially in patients with a high risk of CIN (Er et al., 2012, Menting et al., 2015). However, its reno-protective mechanism remains elusive.

Previous studies have shown that remote IPC is involved in activation of multifactorial anti-inflammatory, neuronal, and humoral signaling pathways (Gassanov et al., 2014). For example, remote IPC could confer cardio-protection against ischemia through up-regulating erythropoietin

(EPO) and hypoxia-inducible factor-1α (HIF1α) in the kidney (Oba et al., 2015). EPO preconditioning is also able to protect against cardiac and kidney I/R injury (Gardner et al., 2014). As such, some factors/molecules produced in the kidney may play a role in the defense mechanism of remote IPC. Thus, it will be interesting to identify novel kidney-secreted proteins contributing to renal protection under remote IPC.

Among numerous candidates that exert a reno-protective effects, renalase is not only a kidney-originated amine oxidase, but also a molecule that is subjected to HIF-1 regulation at transcriptional level (Wang et al., 2015c, Du et al., 2015). It has been documented that renalase can regulate blood pressure and protect against both heart and kidney I/R injury (Lee et al., 2013; Wang et al., 2014a, 2014b, 2015a, 2015c; Du et al., 2015, Guo et al., 2014). Our previous study also demonstrated that exogenous administration of renalase was effective in alleviating CIN in rats (Zhao et al., 2015) and that local IPC induced cortex renalase upregulation via HIF-1α dependent mechanism. Furthermore, renalase plays a vital role in reno-protection of local IPC against I/R induced AKI (Wang et al., 2015c). In line with our observations, other studies showed that renalase expression was elevated and attenuated cardiac injury in mice challenged with cardiac I/R (Du et al., 2015). Recently, it has been reported that renalase may function as a pro-survival/growth factor and signals via the receptor plasma membrane calcium ATPase subtype 4b (PMCA4b) (Wang et al., 2015d, Guo et al., 2016). Nevertheless, there is no study to address the role of renalase in remote IPC against kidney I/R injury so far.

In the present study, we examined whether renal renalase would be up-regulated in rats subjected to limb IPC and whether such a remote IPC-induced increase of renalase expression would contribute to reno-protective effects in CIN. In addition, we also investigated the potential mechanisms by which limb IPC regulated renalase expression in the kidney.

2. Materials and methods

This study was approved by the Animal Care and Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

2.1. Reagents, animals and cells

Ioversol was purchased from Hengrui Corp. (Jiangsu, China). N-nitro-L-arginine methyl ester (L-NAME), indomethacin, tosyl phenylalanyl chloromethyl ketone (TPCK, a nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB inhibitor), pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor), 3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1, a HIF-1α inhibitor), and tumor necrosis factor α (TNFα) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Humira, a TNFα antagonist, was provided by AbbVie (North Chicago, IN, USA). The cholesterol-conjugated specific renalase siRNA were provided by Shanghai Biotend Corp. (Shanghai, China). Male Sprague-Dawley rats, weighing 200 ± 20 g, were provided by Shanghai Science Academy animal center (Shanghai, China). All the rats were housed under controlled conditions of light (12 h dark/12 h light cycle) and temperature (20–23 °C), and fed with standard diet and tap water. HK2 cells from ATCC (Rockefeller, MD, USA) were used as a proximal renal tubular epithelial cell model.

2.2. Rat models of delayed remote IPC and CIN

All the animal experiment protocols were demonstrated in Fig. 1.
Study 1: Limb IPC protects against CIN via renalase (Fig. 1A).
The rats were divided into a sham-operated group (Sham), a remote IPC group (IPC), a CIN group, an IPC + CIN group, a CIN + Scramble siRNA group, a CIN + Renalase siRNA group, an IPC + CIN + Scramble siRNA group and an IPC + CIN + Renalase siRNA group, N = 6 in each group. Remote IPC was delivered by occluding hind limb blood flow with a tightened tourniquet around the upper thigh for 6 cycles of 10-min occlusion followed by 10-min release under anesthesia with 50 mg/kg pentobarbital. The limb ischemia was confirmed by pallor and cyanosis of the lower limb below the tourniquet. CIN was induced similarly to that described previously (Zhao et al., 2015). Briefly, rats were given a tail vein injection of indomethacin (10 mg/kg), followed by Ioversol (3 g/kg organically bound iodine) and L-NAME.

In the IPC + CIN group, CIN were induced at 24 h after limb IPC. In rats of the CIN + Scramble siRNA group, CIN + Renalase siRNA group, IPC + CIN + Scramble siRNA group and IPC + CIN + Renalase siRNA group, a midline laparotomy was performed after anesthetizing with 50 mg/kg pentobarbital. Then, scramble siRNA or renalase siRNA was injected into the bilateral renal cortex at 12 h before IPC or 38 h before CIN induction. Rats in the CIN or IPC + CIN groups were treated with the vehicle of the same volume at the same time points.

Study 2: limb IPC induces renalase expression (Fig. 1B).

Limb IPC was induced in rats first, followed by blood and tissues harvested at 6 h, 12 h, and 24 h (N = 6 in each group).
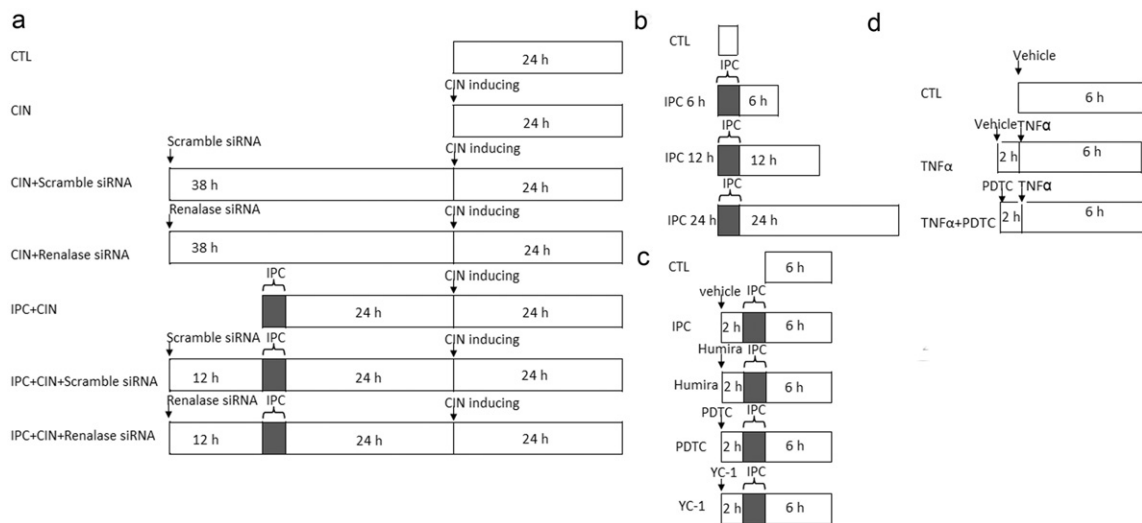
Fig. 1. The schema for animal study protocols. a. Limb ischemic preconditioning followed (IPC) by contrast-induced renal injury inducing and renalase blocking protocol. b. Renalase expression after limb IPC. c. Limb IPC induced renalase expression with TNFα blocking or NF-κB or HIF-1 blocking. d. TNFα preconditioning with NF-κB blocking. The rats were randomly divided into desired groups (as showed in the schema) with 6 animals in each group.

Study 3: limb IPC induces renalase expression via NF-κB (Fig. 1C).

Rats were divided into Sham group, IPC group, IPC + Humira group, IPC + PDTC group and IPC + YC-1 group (N = 6 in each group). Limb IPC was induced in IPC groups similarly to Study 1. Rats in IPC + Humira group, IPC + PDTC group, and IPC + YC-1 group were given an intraperitoneal injection of Humira (1 mg/kg), PDTC (100 mg/kg) or YC-1 (2 mg/kg) 2 h before IPC, respectively.

Study 4: TNFα induces renalase expression via NF-κB (Fig. 1C).

Rats were divided into Sham group, TNFα group, and TNFα + PDTC group (N = 6 in each group). Rats in IPC group received limb IPC induction. Rats in the TNFα group were given an intravenous injection of TNFα (0.1 μg/kg) or an intraperitoneal injection of PDTC 2 h before TNFα administration (Lecour et al., 2005). The tissues were harvested at 6 h after remote IPC.

2.3. Experiments in vitro

HK2 cells were cultured in K-SFM at 37 °C 5% CO2, supplemented with 5 ng/ml human recombinant EGF and 0.05 μg/ml bovine pituitary extract. HK2 cells were treated with TNFα to investigate renalase expression. TPCK was used as an NF-κB inhibitor in vitro. Besides, to determine whether TNFα preconditioning could protect against oxidative injury, HK-2 cells were pretreated with vehicle or TNFα (20 ng/ml) for 6 h and then were exposed to H_2O_2 (300 μmol/l) for an additional 12 h.

2.4. Assessment of renal function and oxidative injury

An automatic biochemical analyzer (7600, Hitachi, Japan) was employed to measure serum creatinine (Scr) and blood urea nitrogen (BUN) to assess the changes in renal function. Renal levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured using commercial kits following the manufacturer's protocol (Nanjing Jiancheng, Nanjing, China).

2.5. Histological injury assessment

The fixed left kidney was embedded in paraffin and then was cut into 3-μm sections. Histological alterations were evaluated by Periodic acid–Schiff (PAS) staining. The renal injury was scored using grading tubular necrosis, loss of brush border, cast formation, and tubular dilation in 10 randomly chosen, non-overlapping fields. The renal injury degree was estimated by the following criteria: 0, none; 1, 0–10%; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, 76–100%, as described previously (Melnikov et al., 2002).

TUNEL staining was performed using an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Numbers of TUNEL-positive tubular cells were quantified by counting 10 randomly chosen, non-overlapping fields per slide.

2.6. Macrophage infiltration in renal tissues

Immunohistochemistry was performed with an anti-CD68 antibody (Abcam, Cambridge, MA, USA) to identify infiltrated macrophages in renal tissue as described previously (Wang et al., 2015b).

2.7. Quantitative real-time PCR

Total RNA from HK2 cells or kidney tissues was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). mRNA levels were quantified using one-step real-time PCR with Taqman chemistry (Applied Biosystems, Foster City, CA, USA) as described previously (Wang et al., 2014a). 18S rRNAs were used as internal normalizer for mRNAs. The primers for rat MCP-1 were 5′ ATGCAGGTCTCTGTCACG3′ (forward) and 5′-CTAGTTCTCTGTCATA CT-3′ (reverse); the primers of rat renalase and human renalase were 5′-AAAGAGGGAGATGGGTTAGTAGTGG-3′ (forward), 5′-TCGG TTCTGAGGAGGATGGAG-3′ (reverse), and 5′-GAAAAATCATTGCAGC CTCTCA-3′ (forward), 5′-AAGTTCTGCCTGTGCCTGTGTA-3′ (reverse), respectively.

2.8. Western blot analysis

Renalase and NF-κB levels were analyzed using western blot similar to that described previously (Wang et al., 2014a). The primary antibodies, anti-renalase, anti-caspase3, anti-Phospho-IκBα (Ser32) and anti-GAPDH were from Abcam (Cambridge, MA, USA) (rabbit anti-renalase monoclonal antibody, 1:500 dilution), Cell Signaling Technology (Danvers, MA, USA) (rabbit anti-Phospho-IκBα (Ser32) monoclonal antibody, 1:500 dilution; rabbit anti-caspase3 polyclonal antibodies, 1:1000 dilution) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) (goat anti-GAPDH polyclonal antibody, 1:5000 dilution), respectively. The secondary antibodies were from Santa Cruz (horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit IgG).

2.9. Statistical analysis

The statistical software SPSS (Ver. 18.0) (USA) was used for data analysis. All the data were expressed as mean ± standard error (SEM). One-way ANOVA with Sidak compensation was employed for parametric data and Kruskal-Wallis with Dunn' compensation for non-parametric data comparison. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Limb IPC protects against CIN

As shown in Fig. 2, the rats exhibited significant exacerbation of renal function 24 h in CIN groups after administration of contrast agent, as indicated by remarkable increase of Scr and BUN. Limb IPC significantly attenuated the renal impairment (Scr, limb IPC + CIN vs. CIN, $P < 0.05$; BUN, limb IPC + CIN vs. CIN, $P < 0.05$). Histological analysis further demonstrated that the renal tubular detachment, foamy degeneration, and necrosis of tubular cells were less severe in limb IPC + CIN group compared with CIN group (Fig. 3a). Meanwhile, limb IPC reduced the pathological tubular injury score in CIN (Fig. 3b). Furthermore, limb IPC decreased renal MDA levels and increased SOD levels after Ioversol injection (limb IPC + CIN vs. CIN, $P < 0.05$) (Fig. 4). Finally, limb IPC also significantly reduced tubular cell apoptosis and CD68-positive macrophage infiltration to the injured kidney (limb IPC + CIN vs. CIN, $P < 0.05$) as shown in Figs. 5 and 6. These data indicated that limb IPC was able to protect against Ioversol-induced kidney injury via mitigating apoptosis, reducing inflammation and attenuating oxidative stress.

3.2. Limb IPC-mediated reno-protection against CIN is dependent on renalase upregulation

To determine whether renalase would be involved in the renoprotective effects of limb IPC, siRNA was administrated to knock down renal renalase prior to CIN induction. As shown in Fig. S1, siRNA injection led to significant decrease of renalase mRNA and protein expression. We observed that all the aforementioned renal protective effects offered by limb IPC including improvement of renal function and histological injury, were inhibited by renalase downregulation (Figs. 2–6). This suggests that limb IPC induced
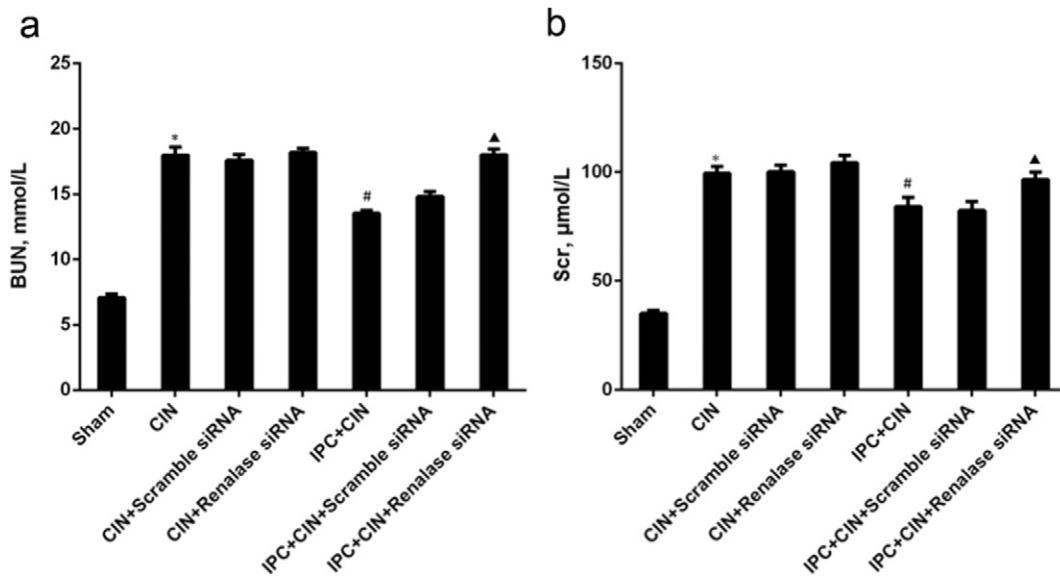
Fig. 2. Knocking down renalase exacerbates contrast induced renal injury following limb ischemic preconditioning. Contrast-induced nephropathy (CIN) was induced in rats 24 h after limb ischemic preconditioning and at 24 h after CIN, blood and tissues were collected for further analysis. a. Blood urea nitrogen. b. Serum creatinine. Sham, sham-operated control group; CIN, contrast induced nephropathy group without limb IPC; CIN + Scramble siRNA, contrast induced nephropathy group with scramble siRNA injection; CIN + Renalase siRNA, contrast induced nephropathy group with renalase siRNA injection; IPC + CIN, contrast induced nephropathy group following limb IPC; IPC + CIN + Scramble siRNA, contrast induced nephropathy group following limb IPC with scramble siRNA injection; IPC + CIN + Renalase siRNA, contrast induced nephropathy group following limb IPC with renalase siRNA injection. $N = 6$, [*]$P < 0.01$ versus Sham; [#]$P < 0.05$ versus CIN; [▲]$P < 0.05$ versus IPC + CIN + Scramble siRNA.

upregulation of renalase plays a critical role in the reno-protection of limb IPC against CIN.

3.3. Limb IPC leads to increased TNFα levels in the muscle and blood, and elevated expression of renal cortical renalase

Both renalase mRNA and protein levels were increased at 6 h (limb IPC vs. Sham, $P < 0.05$) and 12 h (limb IPC vs. Sham, $P < 0.05$) and renalase protein also increased at 24 h (limb IPC vs. Sham, $P < 0.05$) after limb ischemia (Fig. 7a–b). The maximum levels of renalase mRNA and protein were observed at 6 h and 24 h, respectively. Additionally, both muscular TNFα mRNA and serum TNFα were up-regulated at 6 h (limb IPC vs. Sham, $P < 0.05$) after limb ischemia (Fig. 7c–d).
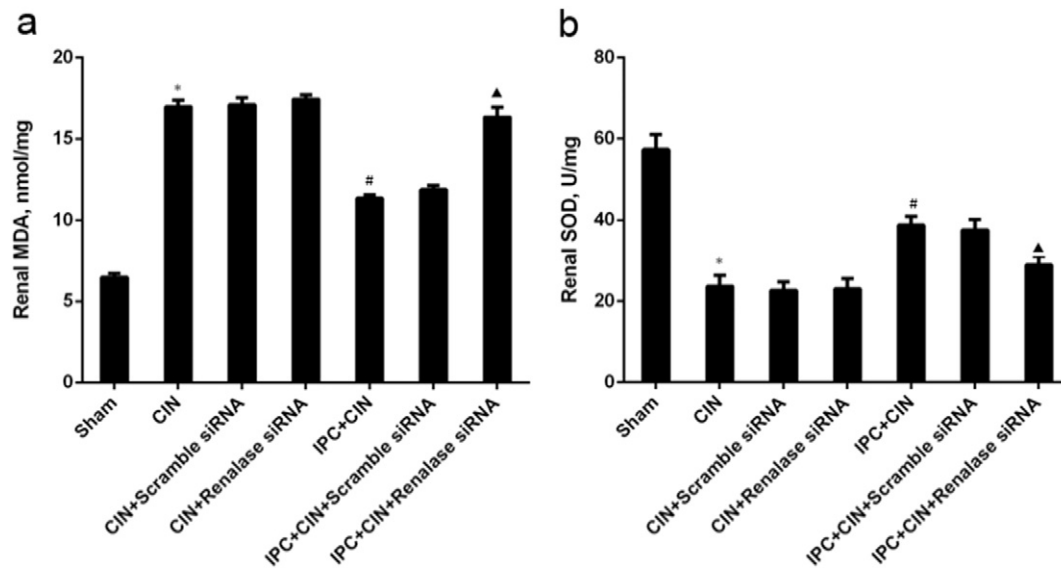
3.4. Limb IPC induces renalase up-regulation through activation of the TNFα/NF-κB pathway

Since the maximum expression of renalase mRNA was observed at 6 h after limb ischemia, we carried out a set of experiments at this time point to investigate whether NF-κB and HIF-1 pathways were involved in limb IPC-induced renalase upregulation in the kidney. As shown in Fig. 7f, expression of cortical renalase protein was increased dramatically 6 h after limb ischemia compared with sham-operated animals. Coincidentally, phospho-inhibitor of kappa B (IκBα), as the indicator of activation of NF-κB, was also increased in renal cortex after limb IPC (Fig. 7e). Pretreatment with either Humira (Scheinfeld, 2003), an antagonist of TNFα, or pyrrolidinedithiocarbamate (PDTC) (Zhou et al., 1999), a specific NF-κB inhibitor, abolished elevation of
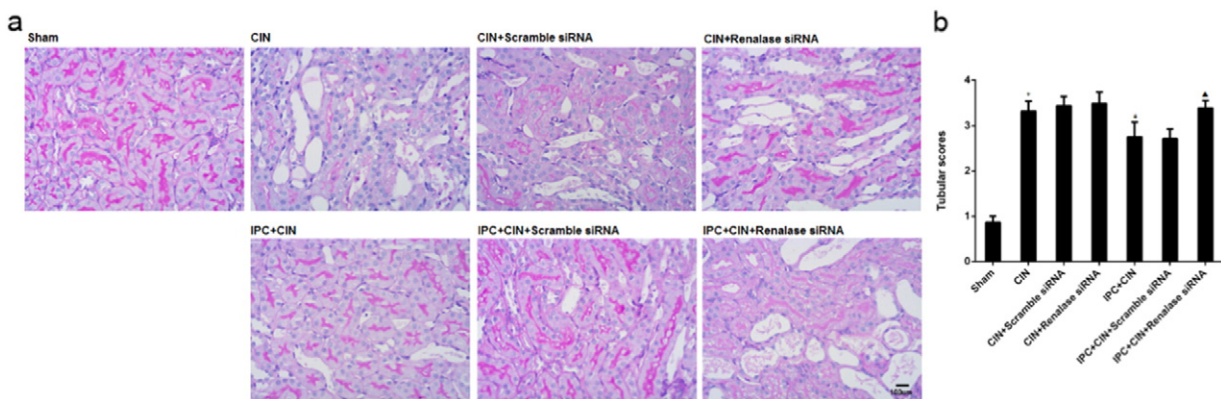
Fig. 3. Renal morphological changes are worsened after renalase inhibition in CIN rats following limb ischemic preconditioning. Contrast induced nephropathy was induced in rats 24 h after limb ischemic preconditioning and at 24 h after CIN, tissues were collected for further analysis. a. Renal histological alterations by periodic acid–Schiff (PAS) staining (representative pictures, 400×, PAS). b. Tubular injury scoring. Sham, sham-operated control group; CIN, contrast induced nephropathy group without limb IPC; CIN + Scramble siRNA, contrast induced nephropathy group with scramble siRNA injection; CIN + Renalase siRNA, contrast induced nephropathy group with renalase siRNA injection; IPC + CIN, contrast induced nephropathy group following limb IPC; IPC + CIN + Scramble siRNA, contrast induced nephropathy group following limb IPC with scramble siRNA injection; IPC + CIN + Renalase siRNA, contrast induced nephropathy group following limb IPC with renalase siRNA injection. All values are presented as means ± SEM ($N = 6$). [*]$P < 0.01$ versus Sham; [#]$P < 0.05$ versus CIN; [▲]$P < 0.05$ versus IPC + CIN + Scramble siRNA.

Fig. 4. Alterations of renal malondialdehyde and superoxide dismutase levels in CIN rats following limb ischemic preconditioning. Contrast induced nephropathy was induced in rats 24 h after limb ischemic preconditioning and at 24 h after CIN, tissues were collected for further analysis. a. Renal malondialdehyde (MDA). b. Renal superoxide dismutase (SOD). Sham, sham-operated control group; CIN, contrast induced nephropathy group without limb IPC; CIN + Scramble siRNA, contrast induced nephropathy group with scramble siRNA injection; CIN + Renalase siRNA, contrast induced nephropathy group with renalase siRNA injection; IPC + CIN, contrast induced nephropathy group following limb IPC; IPC + CIN + Scramble siRNA, contrast induced nephropathy group following limb IPC with scramble siRNA injection; IPC + CIN + Renalase siRNA, contrast induced nephropathy group following limb IPC with renalase siRNA injection. All values are presented as means ± SEM ($N = 6$). $^*P < 0.01$ versus Sham; $^#P < 0.05$ versus CIN; $^▲P < 0.05$ versus IPC + CIN + Scramble siRNA.

phospho-IκBα and renalase expression (Fig. 7e and f). In contrast, there was no significant difference in renalase and phospho-IκBα expression in CIN rats subjected to YC-1, a specific HIF-1α inhibitor (Fig. 7e–f). Taken together, these data indicated that activation of NF-κB, but not HIF-1α, was responsible for renalase expression in the kidney after limb IPC. Since limb muscle ischemia can result in the release of TNFα from injured tissue to the blood and this cytokine is able to induce activation of NF-κB and renal renalase synthesis, our data thus suggest that the TNFα/NF-κB pathway may be involved in limb IPC induced renalase upregulation.

3.5. TNFα preconditioning up-regulates renalase in the kidney via activation of the NF-κB pathway

To directly elucidate the possible role of TNFα in renalase expression and the NF-κB pathway in this process, we further assessed the effect of exogenous TNFα on renal renalase and phospho-IκBα expression in rats and the effect of NF-κB inhibition. Similar to the role of limb IPC, administration of exogenous TNFα also increased expression levels of renalase and phospho-IκBα in the kidney at 6 h. Interestingly, TNFα induced renalase and phospho-IκBα expression were also abolished by PDTC whereas YC-1 treatment did not show any inhibitory effect on expression of these two proteins (Fig. 8a–b). Thus, these data enforces the important role of the TNFα/NF-κB pathway in regulating expression of renal renalase and further suggests TNFα as the intermediator in linking limb IPC to renalase upregulation and subsequent renal protection.

3.6. TNFα induces renalase expression in proximal renal tubular epithelial cells via activation of NF-κB signaling pathway

To further validate whether TNFα would act on renal tubular cells to provoke renalase expression, we examined the effect of TNFα on renalase and phospho-IκBα expression in cultured HK2 cells, a human renal proximal tubular cell line. HK2 cells were exposed to different concentrations of TNFα for 6 h, and renalase and phospho-IκBα expression were then assessed. In agreement with our animal study, the expression level of renalase and phospho-IκBα in HK2 cells exposed to a low dose of TNFα was higher than those without incubation with this cytokine. Consistently, treatment with tosyl phenylalanyl chloromethyl ketone

(TPCK) (Wang et al., 2014a; Zhou et al., 1999), an inhibitor of NF-κB, also effectively reduced TNFα induced IκBα phosphorylation and renalase expression in this cell type (Fig. 8c–e). As such, we suggest that TNFα can stimulate renal tubular cells to produce renalase though activation of the NF-κB pathway.

*3.7. TNFα preconditioning protects against H_2O_2 cytotoxicity and apoptosis in HK-2 cells*

If TNFα-induced renalase expression is required for renal protection, preincubaton of renal tubular cells with this cytokine should protect against cell death in response to acute injury. To test this hypothesis, we investigated the TNFα preconditioning on H_2O_2 cytotoxicity in HK-2 cells. HK2 cells were treated with a low dose of TNFα (10 ng/ml) for 6 h followed by exposure of them to H_2O_2 for an additional 12 h. As shown in Fig. 9, H_2O_2 exposure led to a remarkable cell death as indicated by increases of LDH release and apoptotic cells in cultures without TNFα preconditioning, whereas TNFα pretreatment significantly decreased LDH release and caspase-3 activity.

4. Discussion

In the present study, we demonstrated that remote IPC (limb IPC) alleviates the subsequent renal injury induced by contrast media through the mechanisms associated with anti-apoptosis, anti-inflammation and anti-oxidation. We also found that the reno-protective effects of limb IPC against CIN are mediated by renalase upregulation. Furthermore, our results indicate that limb IPC induced renalase expression is regulated via the TNFα/NF-κB, but not HIF-1 pathway. Thus, we suggest that remote IPC induced renalase upregulation is critically involved in the reno-protective effect in CIN.

Remote IPC, especially limb IPC, is a harmless, nonpharmacological and effective prevention and treatment strategy for I/R injury in many organs and has been widely used in clinical settings. Recent studies suggest that this approach is also effective for prevention of AKI (Wever et al., 2011) and CIN (Liu et al., 2015). In agreement with previous animal studies (Liu et al., 2015), we found that limb IPC attenuated deterioration of renal function after Ioversol-induced CIN, which was accompanied with reduced cell apoptosis, inflammation and oxidative
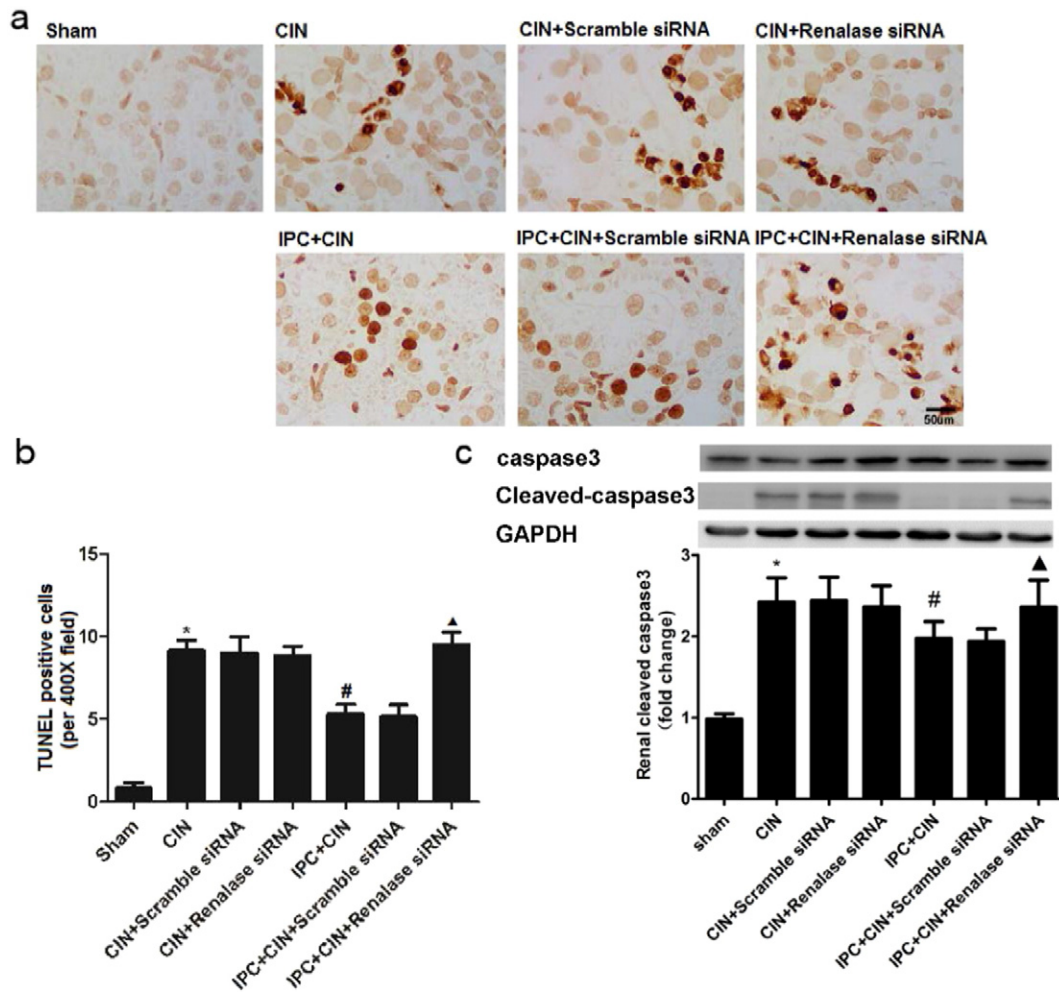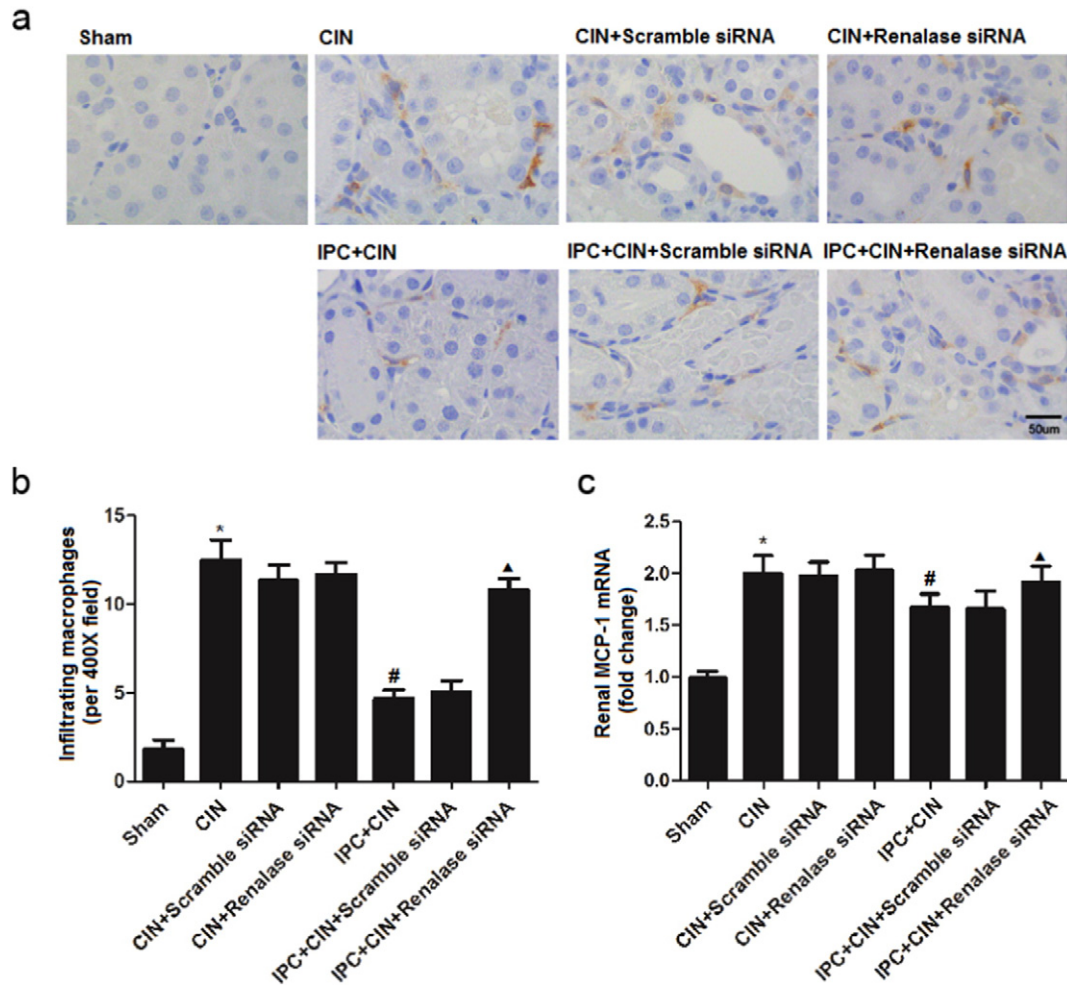
Fig. 5. Renal apoptosis is exacerbated after renalase inhibition in CIN rats following limb ischemic preconditioning. Contrast induced nephropathy was induced in rats 24 h after limb ischemic preconditioning and at 24 h after CIN, tissues were collected for further analysis. a. Renal histological apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining (representative pictures, 400×, TUNEL staining). b. Semi-quantitative analysis of number of apoptotic cells per field. c. Renal cleaved-caspase3 expression. Sham, sham-operated control group; CIN, contrast induced nephropathy group without limb IPC; CIN + Scramble siRNA, contrast induced nephropathy group with scramble siRNA injection; CIN + Renalase siRNA, contrast induced nephropathy group with renalase siRNA injection; IPC + CIN, contrast induced nephropathy group following limb IPC; IPC + CIN + Scramble siRNA, contrast induced nephropathy group following limb IPC with scramble siRNA injection; IPC + CIN + Renalase siRNA, contrast induced nephropathy group following limb IPC with renalase siRNA injection. All values are presented as means ± SEM ($N = 6$). *$P < 0.01$ versus Sham; #$P < 0.05$ versus CIN; ▲$P < 0.05$ versus IPC + CIN + Scramble siRNA.

stress. In addition, renalase, a kidney-derived protein was up-regulated after limb IPC whereas renalase knockdown eliminated the reno-protection of limb IPC as evidenced by our observations that rats pretreated with renalase siRNA exhibited more severe tubular injury and worsen renal function. These results are consistent with our previous study showing that exogenous renalase administration protected CIN through anti-oxidation, anti-inflammation and anti-apoptosis mechanisms (Zhao et al., 2015) and suggest that renalase plays a pivotal role in mediating the reno-protective effect of limb IPC.

Mounting evidence has proved that renalase can mediates cytoprotection via activating survival-associated signaling such as Mitogen-activated protein kinase (MAPK) and Protein kinase B (AKT) (Wang et al., 2014b, 2015d). Recently, it has been reported that inhibition of renalase signaling has antitumor activity in pancreatic cancer and melanoma (Guo et al., 2016). Our previous study also demonstrated that local IPC-elicited beneficial effects on renal I/R injury was partly dependent renalase upregulation, which was associated with decreased apoptosis and oxidative stress. In this study, our findings confirmed that renalase plays an essential role in in remote IPC-elicited reno-protection against CIN, which contributes to a better understanding of the biological properties of renalase. Renalase, up-regulated under both local hypoxia and remote IPC, can promote cell survival and protect against I/R injury.

Currently, the mechanism by which limb IPC induces upregulation of renal renalase is incomplete clear. Given that remote IPC-activated humoral signaling pathways is involved in the remote organ protection (Gassanov et al., 2014), the circulating cytokines released from the preconditioned limb muscle may increase the resistance to hypoxic injury in distant organ (Bonventre, 2012). In this context, several studies have revealed that low dose of TNFα has protective biological properties against ischemic injury and preconditioning with TNFα can mimic classic IPC in a time and dose dependent manner (Lecour et al., 2002; Smith et al., 2002), suggesting that TNFα may serve as a key mediator for limb IPC to induce reno-protection. This hypothesis was supported by the following observations: 1) circulating TNFα increased 6 h after limb IPC, which preceded the expression of renalase in the kidney that occurred at 24 h after limb IPC; 2) Renalase expression was up-regulated in the kidney when exogenous TNFα was administered in the animal; 3) low dose of TNFα treatment increased renalase expression in cultured renal epithelial cells, which is required for cell survival in response to oxidant injury; and 4) administration of Humira, a TNFα antagonist, blocked limb IPC induced renalase upregulation. TNFα may be released from ischemic limb into the systemic circulation and then promotes renalase synthesis in the remote kidney. In line with our observations, other studies have also indicated that low dose of TNFα can protect against I/R injury in heart, liver and neural system (Kleinbongard

Fig. 6. Renal infiltrating macrophages are increased after renalase inhibition in CIN rats following limb ischemic preconditioning. Contrast induced nephropathy was induced in rats 24 h after limb ischemic preconditioning and at 24 h after CIN, tissues were collected for further analysis. a. Renal infiltrated macrophages by CD68 staining (representative pictures, 400×, IHC). b. Semi-quantitative analysis of number of CD68-positive cells per field. c. Renal MCP-1 mRNA expression. Sham, sham-operated control group; CIN, contrast induced nephropathy group without limb IPC; CIN + Scramble siRNA, contrast induced nephropathy group with scramble siRNA injection; CIN + Renalase siRNA, contrast induced nephropathy group with renalase siRNA injection; IPC + CIN, contrast induced nephropathy group following limb IPC; IPC + CIN + Scramble siRNA, contrast induced nephropathy group following limb IPC with scramble siRNA injection; IPC + CIN + Renalase siRNA, contrast induced nephropathy group following limb IPC with renalase siRNA injection. All values are presented as means ± SEM ($N = 6$). [*]$P < 0.01$ versus Sham; [#]$P < 0.05$ versus CIN; [▲]$P < 0.05$ versus IPC + CIN + Scramble siRNA.

et al., 2011; Perry et al., 2011; Watters and O'connor, 2011; Feng et al., 2013).

Nevertheless, how TNFα mediates renal protection has not been well defined. It is well known that TNFα is a pleiotropic cytokine involved in the regulation of infection, inflammation, autoimmune, and apoptosis (Pasparakis et al., 1996; Karatzas et al., 2014), thus, this cytokine may play a detrimental role through its pro-inflammatory effects in certain tissues/organs after acute injury. However, since low dose of TNFα can induce activation of multiple signaling pathways that regulate expression/activation of different molecule with diverse biological functions, it is possible that muscular TNFα released during limb IPC may induce activation of a signaling pathway(s) that triggers expression of a protective molecule such as renalase. In this context, we observed that limb IPC-induced renalase upregulation was accompanied by activation of NF-κB signaling in the kidney and pretreatment with either TNFα antagonist (Humira), or NF-κB inhibitor (PDTC), blocked renalase expression. Therefore, the NF-κB signaling pathway may play an essential role in transmitting the renal protective signal from TNFα to expression of some molecules (i.e., renalase) required for renal protection. In support of this hypothesis, activation of the TNFα/NF-κB signaling pathway has been reported to mediate survival of several cell types including cardiomyocytes (Lecour et al., 2005), hepatocytes (Feng et al., 2013) and neuron (Watters et al., 2011) as well as renal tubular cells as

indicated in this study. Interesting, it was been reported that low dose of TNFα increased cardiomyocytes' tolerance to toxicity of "high TNFα" (Cacciapaglia et al., 2014). Although we can no rule out the possibility that limb IPC -induced activation of TNFα signaling pathway may also causes a deleterious effect, the protective effect of TNFα/NF-κB signaling observed in the current study suggests that, at least in the case of CIN, the beneficial effects mediated by this pathway overrides its deleterious effects to the kidney. Further investigations are needed to address this issue.

Other molecules and signaling pathways may also be involved in the renal protection after limb IPC. Animal and human studies have revealed that transient limb IPC can lead to a rapid increase of serum EPO (Oba et al., 2015). Increased expression of endogenous EPO or administration of exogenous EPO has been reported to play a protective effect against renal injury induced by diverse stimuli. As such, remote IPC may also induced defense against ischemic injury in kidney through release of EPO from the kidney (Gardner et al., 2014; Diwan et al., 2008). Moreover, it is possible that catecholamines may also play a role in limb IPC-induced renalase upregulation. It has been reported that remote IPC can lead to increased catecholamines levels in myocardial tissue and catecholamines can evoke renalase synthesis (Wang et al., 2014a; Li et al., 2008). In addition, pretreatment with catecholamines can mimic the effect of preconditioning (Bankwala et al., 1994; De Zeeuw et al.,
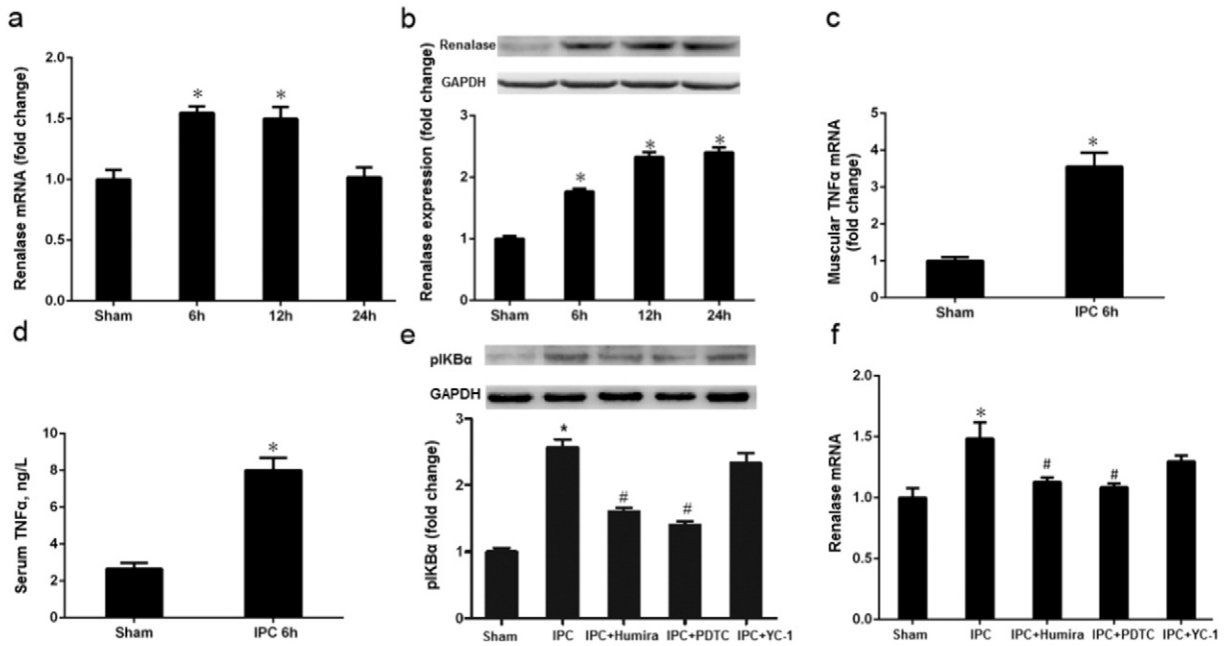
Fig. 7. Limb ischemic preconditioning induces renalase via TNFα/NF-κB pathways. Limb ischemic preconditioning (IPC) was delivered by occluding hind limb blood flow with a tightened tourniquet around the upper thigh for 6 cycles of 10-min occlusion followed by 10-min release. a. Kidney renalase mRNA changes at different time points after limb IPC. b. Kidney renalase protein levels at different time points after limb IPC. c. TNFα mRNA at 6 h after limb IPC. d. Serum TNFα level at 6 h after limb IPC. e. Kidney phospho-IκBα (pIκBα) expression at 6 h after limb IPC with TNFα blocking (Humira), NF-κB blocking (PDTC) or HIF-1α blocking (YC-1). f. Kidney renalase expression 6 h after limb IPC with TNFα blocking (Humira), NF-κB blocking (PDTC) or HIF-1α blocking (YC-1). All values are presented as means ± SEM ($N = 6$).*$P < 0.05$ versus Sham; #$P < 0.05$ versus IPC.

2001). Thus, it will be interesting to further examine, to what extent, various molecules/signaling pathways contribute to the reno-protection after limb IPC and whether they act coordinately to regulate this response.

In summary, this study reveals an important role of endogenous renalase in limb IPC-mediated reno-protection against CIN. Limb IPC may exert its beneficial effects through a humoral signaling pathway by secreting TNFα into the circulation, subsequently activating NF-κB survival signaling in the kidney. This work provides an insight into the mechanisms of limb IPC as well as renalase biogenesis, and lays the groundwork for clinical CIN prevention and treatment with renalase therapy.
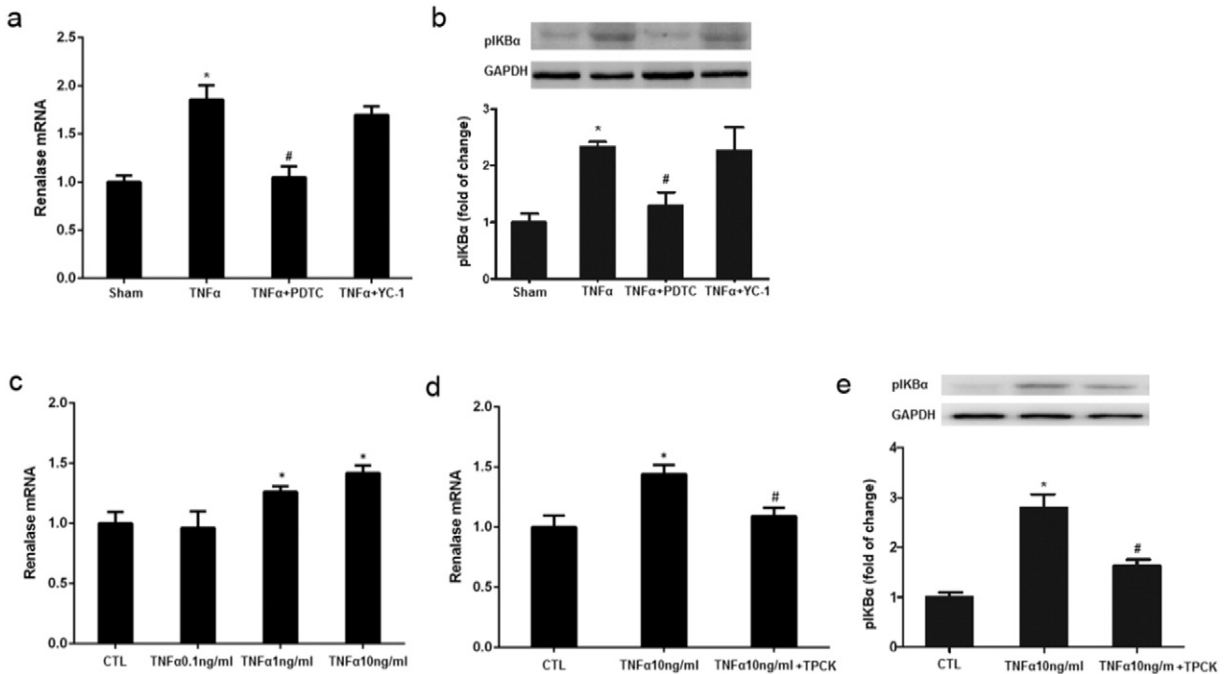
Fig. 8. TNFα preconditioning induces renalase upregulation via NF-κB pathway. TNFα preconditioning was induced by intravenous injection of TNFα (0.1 μg/kg) in rats. The kidneys were harvested at 6 h after TNFα preconditioning. HK2 cells were exposed to TNFα for 6 h, and renalase expression was assessed. a. Renalase expression induced by limb IPC with TNFα blocking (Humira), NF-κB blocking (PDTC) or HIF-1α blocking (YC-1), *$P < 0.05$ versus Sham; #$P < 0.05$ versus IPC; b. Phospho-IκBα (pIκBα) expression at 6 h after TNFα preconditioning. c. Renalase expression induced by TNFα with NF-κB blocking (PDTC) or HIF-1α blocking (YC-1). *$P < 0.05$ versus Sham; #$P < 0.05$ versus TNFα; d. Renalase expression in HK2 under different TNFα concentration, *$P < 0.05$ versus Control (CTL). e. TPCK blocked TNFα induced renalase upregulation in HK2 cells. *$P < 0.05$ versus Control (CTL); #$P < 0.05$ versus TNFα 10 ng/l. All the values depicted are mean ± SEM from 3 independent experiments.
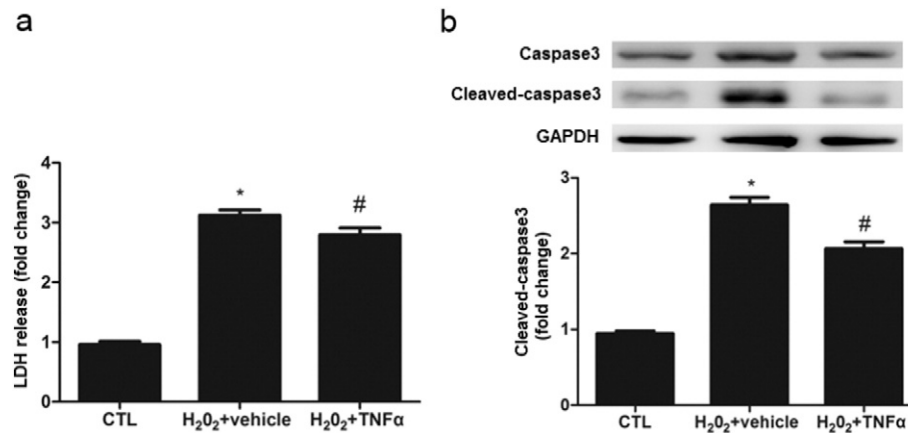
Fig. 9. Low dose of TNFα pretreatment protects against H_2O_2-induced oxidative injury. HK-2 cells were pretreated with vehicle or TNFα (10 ng/ml) for 6 h and then were exposed to H_2O_2 (300 μm/l) for an additional 12 h. Cells were harvested for measuring LDH release and immunoblot analysis of cleaved caspase-3. a. LDH release; b. cleaved caspase3 expression. $^*P < 0.05$ versus Control (CTL); $^{\#}P < 0.05$ versus TNFα 10 ng/ml. All the value depicted are mean ± SEM from 3 independent experiments.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.05.017.

Authors' Contributions

W.F., W.N., and Z.S. conceived of and designed the experiments. W.F., Y.J., L.Z., and Z.G. performed the experiments. W. F. and T.X analyzed and interpreted the data. W.F., Y.J., and Z.S. wrote the manuscript. All authors read and approved the final version of manuscript.

Conflicts of Interests

The authors declare no conflict of interests.

Acknowledgements

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