Original Paper

Hypoxia-Inducible Factor-1 α Dependent Pathways Mediate the Renoprotective Role of Acetazolamide Against Renal Ischemia-Reperfusion Injury

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Key Words

Acute kidney injury • Kidney transplantation • Ischemia reperfusion • Acetazolamide • Hypoxia-inducible factor-1 α

Abstract

Background/Aims: Acute kidney injury (AKI) is a major complication of kidney transplantation, resulting in early graft dysfunction. Since diuretic acetazolamide (AZA) has been shown to improve contrast induced AKI, we hypothesized that AZA also protected against ischemia-reperfusion (I/R) caused AKI. **Methods:** An *in vivo* mouse renal I/R injury model and an *in vitro* H₂O₂ stimulated HK-2 cell injury model were utilized to examine the renoprotective effect of AZA. Renal injury and blood flow were measured. Nitric oxide synthase (eNOS)/Nitric oxide (NO), cell apoptosis and hypoxia-inducible factor-1 α (HIF-1 α) changes were analyzed. **Results:** AZA reduced kidney injury scores and improved renal function by decreasing serum creatinine and BUN levels after I/R. Impaired renal blood flow was restored by increasing eNOS activities and NO production, as indicated by Laser Doppler imaging. TUNEL staining presented that AZA reduced apoptotic cells due to attenuated caspase activation and increased Bcl-2/Bax ratio. Furthermore, HIF-1 α induction by AZA was demonstrated. AZA also enhanced *in vitro* NO production, reduced cell apoptosis and increased HIF-1 α expression. Knockdown of HIF-1 α by RNAi confirmed that AZA exerted its protective role depending on HIF-1 α . AZA's effects were significantly reduced by Akt inhibitor LY294002. **Conclusions:** The present study demonstrated that AZA exerted a renoprotective role against I/R induced AKI through activating HIF-1 α and downstream pathways.

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Introduction

Acute kidney injury (AKI) has long been a severe threat to kidney disease patients [1-4]. Among various factors inducing the disorder, renal ischemia-reperfusion (I/R) injury is considered the leading cause [5, 6]. With respect to the inevitable association between kidney transplantation and ischemia followed by reperfusion, patients receiving kidney transplantation tend to be diagnosed with early graft dysfunction due to I/R induced AKI [7, 8]. After ischemic insult and during reperfusion, renal tubular cells sustain oxidative injury resulting in cell apoptosis and necrosis, partially due to impairment of renal blood flow [9]. Therefore, pharmacologically restoring renal blood flow and interfering apoptotic cell death are regarded as effective strategies to improve renal I/R injury.

There have been reports about the improvement of renal I/R injury through regulating the hypoxia-inducible factor-1 α (HIF-1 α), which is a key transcriptional factor mediating cellular adaptation under stress conditions including I/R injury via an array of protective genes involved in the process of cell metabolism, angiogenesis, erythropoiesis and anti-apoptosis [10]. It has been well documented that HIF-1 α plays a crucial role in ameliorating I/R injury in different organs, such as brain [11], heart [12], liver [13] and kidney [14]. Therefore, pharmacological methods to regulate HIF-1 α are intensively pursued.

Acetazolamide (AZA) is prescribed to treat edema related diseases such as glaucoma and high mountain sickness. AZA has been reported to effectively induce vessel dilation including the renal circulating system [15]. Moreover, administration of AZA could exert a renoprotective role against contrast induced AKI clinically [16, 17]. The possible protective mechanism has been linked to alkalinizing the renal tubular fluid by AZA and then increased scavange of contrast induced reactive oxygen species. The other explanation is vasodilation effect of AZA may ameliorate contrast caused vasoconstriction and regional hypoxia [15, 18]. However, a renoprotective role of AZA against I/R injury has not been tested.

In the present study, AZA was found to improve renal function in a murine I/R model *in vivo* and prevent human kidney-2 (HK-2) cell death *in vitro*. AZA also restored renal blood flow by activating the eNOS/NO production and reduced cell apoptosis via up-regulating the ratio of Bcl-2/Bax, both in an HIF-1 α dependent manner.

Materials and Methods

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Association for Laboratory Animal Science. All animal care and experimental protocols were approved by the Animal Care Committee of Peking University Health Science Center. All sacrifice was performed under pentobarbitone anesthesia, and every effort was made to minimize suffering.

Agents

Acetazolamide and LY294002 were from Sigma-Aldrich Corporation (St. Louis, MO, USA). Anti HIF-1 α (BS3514), Bcl-2 (BS1511), Bax (BS2538), Activated-Caspase-3 (BS7004) and PTEN (BS1305) primary antibodies were from Bioworld Technology, Inc. (Louis Park, MN, USA). Primary antibodies to PI3K p85 (#4257), Akt (#9272), p-Akt (Ser473, #4060), mTOR (#2983), p-mTOR (Ser2448, #5536), ERK1/2 (#4695), p-ERK1/2 (Thr202/Tyr204, #4370) and p-eNOS (Ser1177, #9570) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti eNOS (sc-654) and anti PI3K p-p85 (sc-12929) primary antibodies were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Unless specifically indicated, all the other biochemical reagents were obtained from Amresco (Solon, OH, USA).

Experimental animals and renal ischemia-reperfusion model

Male C57BL/6j mice at ages of 12 weeks weighing 20-22g were obtained from Animal Center at Peking University Health Science Center (Beijing, China). Renal ischemia-reperfusion (I/R) injury was induced

under pentobarbital (80mg/kg) anesthesia as previously described [19]. Briefly, mice were divided into 3 groups: sham, I/R model and AZA treated group with a dose of 60mg/kg/day, with 6 mice per group. For I/R insult, left renal artery and vein underwent ligation with a non-traumatic clamp for 30 minutes, then the clamp was released to cause reperfusion, which lasted for 48 hours. In sham group, the same operation was performed without renal vessels occlusion. Mice in AZA treated group was administrated orally with 60mg/kg/day acetazolamide solution for two consecutive days before ischemia insult and during 48h of reperfusion, while corresponding vehicle was given to mice in either sham group or I/R model group. In addition, all underwent right nephrectomy operation and during the operations body temperature was maintained at 37°C by a homoeothermic blanket. Systemic blood pressure, the main electrolytes Na⁺ and K⁺ concentrations in plasma and urine were also monitored by a noninvasively operating blood pressure analyzer (BP-2000, Visitech Systems Inc., USA) and an automatic analyzer (Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA), respectively.

Renal function test

Renal function was measured by 2 common parameters: serum creatinine and blood urea nitrogen (BUN) [20], which were both determined by commercial kits (Cayman Chemical Company, Ann Arbor, USA). Serum collected from mice was utilized to measure both 2 parameters according to the manufacturer's instructions.

Hematoxylin-eosin and TUNEL staining

Mice kidney paraffin-embedded sections at 4 μ m were stained with hematoxylin and eosin. After images (200 $\times$ magnification) were recorded, histomorphological injury scoring of renal tissue damage was analyzed by observing loss of epithelial cells brush border, dilation of tubules, casts production and tubular necrosis as described previously [21, 22]. The scoring grades were determined by a double-blind observer as follows: 0, normal; 1, damage less than 25%; 2, damage between 25% and 50%; 3, damage between 50% and 75%; 4, damage more than 75%. For each animal, at least 6 independent images were collected for observation and statistical analysis. At least 3 fields in each image were analyzed for histomorphological injury scoring.

The kidney sections were also assayed for apoptosis measurement by using a commercial *in situ* cell-death detection kit (G3250, Promega, Madison, USA), based on terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining according to the manufacturer's instructions. TUNEL-positive cells, which were counted for quantification, were indicated in green and/or orange (overlay) fluorescence in Figures 3A, 3E and 4K.

Laser Doppler perfusion imaging

Renal blood flow was measured by using a Laser Doppler perfusion imager (MoorLDI, Moor Instruments Ltd, Devon, UK) before ischemia insult (0), after 30 min of ischemia (30 min) and after 48 h of reperfusion (48h). Relative measurement of blood flow was expressed in arbitrary perfusion units (PU). The recorded images were analyzed using corresponding MoorLDI v2.1 software (Moor Instruments Ltd, Devon, UK) and the perfusion value was taken and divided by the baseline measurement to give a percentage representing the relative blood flow changes.

Cell culture and H₂O₂ stimulation

Human kidney-2 (HK-2) cell line was from Cell Culture Center, Institute of Basic Medical Science, Chinese Academy of Medical Sciences (Beijing, China). Briefly, HK-2 cells were cultured in DMEM/F12 medium supplemented with antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (FBS; Hyclone, Logan, Utah, USA) in a humid atmosphere incubator with 5% CO₂ at 37°C. The cells were treated with drugs or vehicle after growing to 70% to 80% confluence. For H₂O₂ stimulation, HK-2 cells were stimulated with 500 μ mol/L for 8 hours to induce *in vitro* proximal tubular epithelial cells injury as described before [19].

Nitric oxide measurement

The nitric oxide level was measured via a commercial NO (nitric oxide) assay kit (Beyotime, Haimen, China) as described previously [23]. Briefly, after homogenizing frozen mice kidneys in a buffer containing protease inhibitors and centrifugation, the supernatant was used for NO measurement. For HK-2 cells, the

culture medium was collected for testing NO levels. After incubation with reagents in the assay kit according to the manufacturer's instructions, the absorbance at 540 nm was measured with a microplate reader (Thermo Scientific, Menlo Park, CA, USA). The concentrations of NO were calculated following instructions of the kit.

Cell viability assay

Cell viability was measured through a commercial MTS assay kit (Promega, Fitchburg, Wisconsin, USA). After reagent in this assay kit was added for 4 hours in each well, the absorbance at 490 nm was determined by a microplate reader (Thermo Scientific, Menlo Park, CA, USA).

HIF-1 α RNAi assay

Commercial HIF-1 α siRNA (sc-35561) and negative control scramble siRNA (sc-37007) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Briefly, HIF-1 α or scramble siRNA at a concentration of 100 nmol/L was transiently transfected into HK-2 cells through Lipofectamine-2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, respectively. After incubation with HIF-1 α or scramble siRNA for 72 hours, HK-2 cells were harvested for further examination.

Immunoblot analysis

After frozen mice kidney tissues were homogenized and HK-2 cells were harvested, they were lysed in RIPA buffer containing PMSF (phenylmethylsulfonyl fluoride) and protease inhibitor cocktail (Calbiochem, San Diego, CA, USA). Following centrifugation at 12,000g for 15 min at 4°C, the supernatants were collected and protein content was measured by the bicinchoninic acid (BCA) assay kit (Thermo Scientific Pierce, Rockford, IL, USA). Briefly, samples (80 μ g) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Upon completion of the electrophoresis, the proteins were transferred to PVDF membrane. Non-specific binding sites were blocked by pre-incubating the membrane in 5% skimmed milk in Tris-buffered saline Tween (TBS-T, 20mmol/L Tris, 137 mmol/L NaCl, pH 7.6). PVDF membranes were then incubated overnight at 4°C with specific primary antibodies. After washing three times with TBS-T buffer, the membranes were incubated with the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), blots were developed by using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Then the western blot bands were scanned, and bands intensity was analyzed by Bio-Rad Quantity One software (version 4.4.0; Bio-Rad, Hercules, CA, USA) for quantification.

Data and statistical Analysis

All results are expressed as means $\pm$ S.E.M. For multiple comparisons, the statistical analysis was performed by using one-way analysis of variance (ANOVA) followed by the Newman-Keuls multiple comparison tests. $P < 0.05$ was considered statistically significant.

Results

Effects of acetazolamide on renal histomorphology and function

To investigate whether AZA could exert an *in vivo* renoprotective effect, C57BL/6j mice were subjected to sham operation (sham group), or ischemia/reperfusion with treatment of saline (I/R group) or 60mg/kg/day AZA (AZA group). Kidney sections in the I/R group stained with hematoxylin-eosin presented plenty of injuries like loss of epithelial brush border, dilation of tubules, tubular necrosis and cast production (black arrows), while renal injury area was markedly reduced in the AZA group (Fig. 1A and 1B). Then we detected the renal function by measuring serum creatinine and BUN, and the basal levels of serum creatinine and BUN were 3.87 ± 0.38 mg/L and 37.32 ± 1.98 mg/100mL, respectively. Furthermore, impaired renal function after I/R injury was improved by AZA treatment through bringing down the levels of both serum creatinine (from 17.14 ± 1.96 mg/L to 9.47 ± 1.31 mg/L) and BUN (from 72.28 ± 14.57 mg/100mL to 46.18 ± 3.16 mg/100mL). Other basic parameters were shown in Table 1.

Fig. 1. AZA treatment improved mouse renal function after ischemia-reperfusion injury. (A) Representative light microscopy images of the renal cortex in mice subjected to sham operation (Sham, left panel), 30 min of left warm renal ischemia followed by 48 h of reperfusion with vehicle (I/R Model, middle panel) or acetazolamide treatment (AZA, right panel) (hematoxylin-eosin staining, original magnification $\times 400$). In the AZA group, 60mg/kg/day of acetazolamide were administered (i.g.) for two consecutive days before ischemia insult and during 48 h of reperfusion. Black arrows indicated renal injuries including: a, loss of epithelial cell brush border; b, dilation of tubules; c, tubular necrosis; and d, cast formation. (B) Histogram of histomorphological injury scores of renal tissue damage indicated by hematoxylin-eosin staining. N = 6 per group. Serum creatinine levels (C) and blood urea nitrogen levels (D) in mice from three different groups, n = 6 mice per group. For all graphs: data were shown as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. I/R group.

Table 1. Body weight, systemic blood pressure, serum and urine analysis of sham, I/R and AZA treated mice. N: number of mice in each group. [Na] and [K]: sodium and potassium concentration, respectively. FE Na and FE K: fractional excretion of sodium and potassium, respectively. Values are shown as mean $\pm$ s.e.m. * $p < 0.05$ vs. sham group. # $p < 0.05$ vs. I/R group.

	Sham	I/R	AZA
N	7	8	8
Body weight (g)	20.2 $\pm$ 0.2	20.7 $\pm$ 0.5	20.1 $\pm$ 0.6
SBP, baseline (mmHg)	94 $\pm$ 2.8	94 $\pm$ 3.1	94 $\pm$ 2.6
SBP, ischemia 30min (mmHg)	92 $\pm$ 4.8	84 $\pm$ 6.1	88 $\pm$ 5.7
SBP, reperfusion 48h (mmHg)	94 $\pm$ 1.4	92 $\pm$ 2.2	92 $\pm$ 3.6
Urine volume (mL/24h)	0.85 $\pm$ 0.12	0.34 $\pm$ 0.14 *	0.47 $\pm$ 0.12
Urine creatinine (mg/L)	1502 $\pm$ 125.1	934 $\pm$ 51.4 *	1209 $\pm$ 156.8 #
Creatinine excretion (mg/24h)	1.23 $\pm$ 0.24	0.28 $\pm$ 0.14 *	0.70 $\pm$ 0.31 #
Clearance of creatinine (mL/min)	0.23 $\pm$ 0.03	0.03 $\pm$ 0.01 *	0.13 $\pm$ 0.03 #
Serum [Na] (mmol/L)	135 $\pm$ 0.7	124 $\pm$ 0.4 *	132 $\pm$ 0.5 #
Urine [Na] (mmol/L)	161 $\pm$ 3.4	243 $\pm$ 2.7 *	182 $\pm$ 4.4 #
Na excretion (μ mol/24h)	102 $\pm$ 3.8	124 $\pm$ 4.3 *	105 $\pm$ 2.6 #
FE Na (%)	0.13 $\pm$ 0.02	2.63 $\pm$ 0.49 *	0.79 $\pm$ 0.23 #
Serum [K] (mmol/L)	4.9 $\pm$ 0.12	4.7 $\pm$ 0.14	4.7 $\pm$ 0.05
Urine [K] (mmol/L)	318.1 $\pm$ 10.2	339.9 $\pm$ 14.0	327.8 $\pm$ 5.7
K excretion (μ mol/24h)	201.0 $\pm$ 25.7	267.9 $\pm$ 38.1	216.7 $\pm$ 41.1
FE K (%)	16.3 $\pm$ 2.0	42.5 $\pm$ 9.0 *	25.3 $\pm$ 6.3 #

Fig. 2. AZA treatment restored impaired renal blood flow and increased nitric oxide (NO) concentrations by elevating the expression and activities of endothelial nitric oxide synthase (eNOS) after I/R injury both *in vivo* and *in vitro*. (A) Representative Laser Doppler images by scanning mouse kidneys in three different groups. For each group, images before ischemia insult (0), after 30 min of ischemia (30 min) and after 48 h of reperfusion (48h) were shown, respectively. For each group, both colourful laser Doppler images (left) indicating renal blood flow and phase-contrast images (right) indicating morphology of kidneys at each time point were shown respectively. A colourful scale illustrated variations in renal blood flow from minimal flow (dark blue) to high flow (red). (B) Statistics of renal perfusion rates from laser Doppler images at the beginning, after ischemia for 30 min and reperfusion for 48 hours. Red dots and lines: the sham group; black squares and lines: the I/R model group; yellow triangles and lines: the AZA treatment group. From 0

min to 30 min of ischemia, the yellow line of the AZA group coincided with the black line of the I/R group. N = 6 mice per group. (C) Relative NO levels ($\mu\text{mol/g}$) in mouse kidneys in the sham, I/R Model and AZA groups, normalised to the protein concentrations of kidney extracts. N = 6 mice per group. Data were shown as mean $\pm$ s.e.m. * $p < 0.05$ vs. sham group; # $p < 0.05$ vs. I/R group. (D) Relative NO levels (% CTRL) normalised to the levels of the control group (AZA- and H_2O_2 -) in HK-2 cells pretreated by 3×10^{-7} mol/L AZA (AZA+) or vehicle (AZA-) for 24 hours followed by stimulation of 500 $\mu\text{mol/L}$ H_2O_2 (H_2O_2 +) or a corresponding vehicle (H_2O_2 -) for eight hours, and culture supernatant was collected for NO measurement. N = 3 independent experiments per group. Data were shown as mean $\pm$ s.e.m. ** $p < 0.01$ vs. AZA- and H_2O_2 - group; ## $p < 0.01$ vs. AZA- and H_2O_2 + group. (E) Representative immunoblotting images of phosphorylated eNOS (p-eNOS), eNOS and β -actin in mouse kidneys from the sham, I/R Model and AZA treatment groups from six independent western blotting experiments. (F) Densitometric quantification of relative band intensities normalised to β -actin from the immunoblotting images of p-eNOS, eNOS and β -actin in mouse kidneys from three groups. N = 6 independent Western Blot experiments per group. Data were shown as mean $\pm$ s.e.m. ** $p < 0.01$ vs. sham group; # $p < 0.05$ vs. I/R group. (G) Representative immunoblotting images of p-eNOS, eNOS and β -actin in HK-2 cells pretreated with AZA or vehicle for 24 hours followed by stimulation of H_2O_2 or vehicle for eight hours from three independent western blotting experiments. (H) Densitometric quantification of relative band intensities normalised to β -actin from immunoblotting images of p-eNOS, eNOS and β -actin in HK-2 cells from four groups. N = 3 independent Western Blot experiments per group. Data were shown as mean $\pm$ s.e.m. *** $p < 0.001$ vs. AZA- and H_2O_2 - group; ### $p < 0.001$ vs. AZA- and H_2O_2 + group.

Effects of acetazolamide on H_2O_2 stimulated human kidney-2 (HK-2) cells

To confirm the above *in vivo* findings, an *in vitro* H_2O_2 stimulated HK-2 cell model was used to mimic I/R injury [19]. Based on the findings of optimizing conditions, stimulation with 500 $\mu\text{mol/L}$ H_2O_2 for eight hours was utilised in subsequent experiments. H_2O_2 injury caused remarkable reduction of cell viability ($42.8 \pm 3.8\%$ of control), while pretreatment of 3×10^{-7} mol/L AZA for 24 hours substantially improved HK-2 cell viability to $69.2 \pm 4.5\%$ of control.

Effects of acetazolamide on renal blood flow and nitric oxide production

AZA has been reported to exhibit a vasodilatation effect on the kidneys in the clinical setting [15, 18], however no report is found about the association between such effect and renal protection. In light of our observation of AZA's protective role against renal I/R injury, we hypothesised that AZA could affect renal blood flow due to its vasodilatation effect. Laser Doppler images (Fig. 2A and 2B) demonstrated that impaired renal blood flow after I/R injury was restored after AZA treatment, and the perfusion rate recovered from $19.1 \pm 4.1\%$ (I/R) to $62.3 \pm 8.2\%$ (AZA) normalised to levels in the sham group.

Next, the involvement of NO, a key vasodilator to increase renal blood flow was explored in AZA's vasodilatation effect [24]. The levels of NO were examined in both mouse kidneys and cultured cells, and marked increase of NO production by AZA was seen (Fig. 2C and 2D). With respect to the crucial role of nitric oxide synthase (NOS) in catalyzing NO production [23], it was possible that AZA elevated NO levels by regulating NOS expression and/or activity. We found that constitutive NOS (cNOS) and total NOS (tNOS) activities were significantly increased after AZA treatment. The levels of activated cNOS (mainly phosphorylated eNOS (p-eNOS)) were detected by immunoblotting. I/R injury resulted in the reduction of p-eNOS level which was then reversed by AZA *in vivo* (Fig. 2E and 2F) and *in vitro* (Fig. 2G and 2H). These results suggested that AZA could increase NO levels through promoting eNOS activation, leading to its protective effect on renal blood flow.

Effects of acetazolamide on cell apoptosis both in vivo and in vitro

In addition to impaired renal blood perfusion, apoptosis also mediates acute kidney injury after I/R insult [9]. Meanwhile, *in vitro* studies indicated that AZA treatment could improve renal cell survival after injury. In the present study, we confirmed that AZA treatment

Fig. 3. AZA treatment exerted anti-apoptotic effect in mouse kidneys and HK-2 cells after renal I/R injury. (A) Quantification of the amounts of relative apoptotic cells illustrated by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining (representative images see left panel) in mouse kidneys from the sham, I/R Model and AZA treatment groups. The number of TUNEL-positive cells per high-power field (HPF) was counted for statistics (right panel). N = 6 independent TUNEL experiments per group. Representative immunoblotting images (B) of activated caspase-3, Bax, Bcl-2 and β -actin in mouse kidneys in three groups from six independent western blotting experiments and densitometric quantification of relative band intensities of (C) activated caspase-3 and (D) relative ratio of Bcl-2/Bax normalised to β -actin.

For all graphs: data were presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. I/R group. (E) Quantification of the number of relative apoptotic cells illustrated by TUNEL staining (representative images see left panel) in HK-2 cells pretreated with AZA or vehicle for 24 hours followed by stimulation of H₂O₂ or vehicle for eight hours. The percentage of each group was calculated by comparing the number of TUNEL-positive cell to that of total cells per HPF (right panel). N = 3 independent TUNEL experiments per group. Representative immunoblotting images (F) of activated caspase-3, Bax, Bcl-2 and β -actin in HK-2 cells from four groups from three independent western blotting experiments and densitometric quantification of relative band intensities of (G) activated caspase-3 and (H) relative ratio of Bcl-2/Bax normalised to β -actin. For all graphs: data were shown as mean $\pm$ s.e.m. ** $p < 0.01$, *** $p < 0.001$ vs. AZA- and H₂O₂- group; # $p < 0.05$, ## $p < 0.01$ vs. AZA- and H₂O₂+ group.

could significantly decrease the number of apoptotic cells both *in vivo* (Fig. 3A) and *in vitro* (Fig. 3E).

Then, the levels of activated caspase-3, Bcl-2 and Bax, three key apoptosis related proteins [25] were measured by immunoblotting. AZA reduced the level of activated caspase-3 after I/R injury but elevated the ratio of Bcl-2/Bax in comparison with the injury group (Fig. 3B-3D). This finding was further supported by the data from HK-2 cells. As shown in Figures 3F-3H, AZA treatment prevented caspase-3 activation and increased Bcl-2/Bax ratio.

Role of hypoxia-inducible factor-1 α in the renoprotective effect of acetazolamide

HIF-1 α plays an important protective role against AKI [14, 26, 27], which is related to its downstream proteins including the vascular endothelial growth factor (VEGF) [28], NOS [12, 29] and Bcl-2 [30, 31]. Based on our previous findings, we hypothesised that AZA performed its renoprotective function by modulating eNOS and Bcl-2 which could be connected with HIF-1 α . To test this hypothesis, HIF-1 α protein levels were detected *in vivo* and *in vitro*. As shown in Figures 4A-4D, I/R injury or H₂O₂ stimulation substantially attenuated HIF-1 α expression which was then markedly increased after AZA treatment.

To further investigate that modulation of eNOS and Bcl-2 by AZA was dependent on HIF-1 α up-regulation, loss-of-function on HIF-1 α by RNA interference was conducted in our present study. The expression of HIF-1 α was knocked down by 50% in HK-2 cells by specific siRNA. Immunoblotting results demonstrated that knockdown of HIF-1 α neutralised AZA's ability to elevate eNOS phosphorylation or Bcl-2/Bax ratio (Fig. 4E-4H). Similarly, HIF-1 α siRNA knockdown blocked AZA mediated NO production and cell viability improvement (Fig. 4I-4J). As indicated in the TUNEL assay, AZA could significantly reduce apoptosis in the wild-type HK-2 cells, which was then cancelled in HIF-1 α siRNA knockdown cells (Fig. 4K and 4L).

Role of the PI3K/Akt signaling pathway in the regulating effects of acetazolamide upon hypoxia-inducible factor-1 α

Both phosphoinositide 3-kinase (PI3K)/Akt and ERK are key signaling pathways regulating HIF-1 α expression [32, 33]. PI3K/Akt is also an important survival pathway against AKI [34]. To investigate the role of the PI3K/Akt signaling pathway in the regulating effect of AZA on HIF-1 α , the phosphorylation levels of PI3K, Akt, mTOR and endogenous PI3K inhibitor PTEN were examined in HK-2 cells treated with AZA for 0 to 4 hours. As a result, the PI3K/Akt/mTOR pathway was sequentially activated (PI3K p-p85 peaked at 5 min; p-Akt at 15 min; and p-mTOR at 1 hour) which then returned to control levels. The decrease of PTEN expression was in a time-dependent manner (Fig. 5A). In addition, the up-regulation of HIF-1 α , eNOS and Bcl-2 by AZA was reversed after treatment with the PI3K/Akt specific inhibitor LY294002 (Fig. 5B). Inhibition of PI3K/Akt by LY294002 also blocked the NO production and the cellular protection of AZA in HK-2 cells (Fig. 5C and 5D). These results suggested the up-regulation of HIF-1 α by AZA through activating the PI3K/Akt pathway.

Fig. 4. AZA treatment increased hypoxia-inducible factor-1 α (HIF-1 α) both *in vivo* and *in vitro* and HIF-1 α siRNA knockdown reversed the effect of AZA. (A) Representative immunoblotting images of HIF-1 α and β -actin in mouse kidneys from the sham, I/R Model and AZA treatment groups from six independent

western blotting experiments. (B) Densitometric quantification of relative band intensities normalised to β -actin from the immunoblotting images of HIF-1 α and β -actin in mouse kidneys from three groups. N = 6 independent Western Blot experiments per group. Data were shown as mean $\pm$ s.e.m. * $p < 0.05$ vs. sham group; ## $p < 0.01$ vs. I/R group. (C) Representative immunoblotting images of HIF-1 α and β -actin in HK-2 cells pretreated with AZA or vehicle for 24 hours followed by stimulation of H₂O₂ or vehicle for eight hours from three independent western blotting experiments. (D) Densitometric quantification of relative band intensities normalised to β -actin from the immunoblotting images of HIF-1 α and β -actin in HK-2 cells from four groups. N = 3 independent Western Blot experiments per group. Data were shown as mean $\pm$ s.e.m. ** $p < 0.01$ vs. AZA- and H₂O₂- group; ## $p < 0.01$ vs. AZA- and H₂O₂+ group. (E) Representative immunoblotting images of HIF-1 α , p-eNOS, eNOS, Bcl-2, Bax and β -actin in HK-2 cells incubated with HIF-1 α siRNA or scramble siRNA for 72 hours, while being pretreated with AZA or vehicle for 24 hours followed by stimulation of H₂O₂ for eight hours from three independent western blotting experiments. Densitometric quantification of relative (F) HIF-1 α , (G) eNOS and (H) Bcl-2 band intensities normalised to β -actin from the immunoblotting images in HK-2 cells from eight groups. N = 3 independent Western Blot experiments per group. For all graphs: data were shown as mean $\pm$ s.e.m. In Figure 4F, *** $p < 0.001$ vs. AZA-, H₂O₂- and HIF-1 α siRNA- group. In Figures 4G and 4H, *** $p < 0.001$; ns meant no significance. (I) Relative NO concentrations measured in HK-2 cells incubated with HIF-1 α siRNA (HIF-1 α siRNA +) or scramble siRNA (negative control, HIF-1 α siRNA -) for 72 hours, while being pretreated with AZA or vehicle for 24 hours followed by stimulation of H₂O₂ for eight hours (eight groups in total); culture supernatant was collected for NO measurement. N = 3 independent experiments per group. (J) Cell viability (% CTRL) normalised to the levels of the control group (AZA-, H₂O₂- and HIF-1 α siRNA- group) measured from HK-2 cells in eight groups mentioned as Figure 4E. N = 3 independent experiments per group. (K) Representative fluorescence photomicrographs of HK-2 cells illustrating apoptotic cells by TUNEL staining from three independent experiments, with green dots indicating TUNEL-positive cells, original magnification $\times 200$. (L) Statistical histograms of relative apoptotic cells per high-powered field (HPF) from TUNEL staining images of HK-2 cell in eight groups, and the percentage of each group was calculated by comparing the number of TUNEL-positive cell to that of total cells per HPF. N = 3 independent TUNEL experiments per group. For Figures 4I, 4J and 4L, data were presented as mean $\pm$ s.e.m. * $p < 0.05$; *** $p < 0.001$; ns meant no significance.

Discussion

Ischemia/reperfusion, the leading cause of AKI, commonly occurs in various medical conditions and surgical procedures such as kidney transplantation [35]. Regulation of AKI after I/R injury holds the potential to prevent from early graft dysfunction [7]. In the present study, the renoprotective role of AZA and its underlying mechanism were investigated. Herein, we confirmed for the first time that AZA could effectively protect against I/R induced AKI, which improved renal histomorphology and function after I/R injury while preventing HK-2 cells from H₂O₂ induced cell injury. Similarly, there have been reports about the reduced risks of contrast induced AKI by AZA [16, 17, 36].

As a carbonic anhydrase inhibitor and a diuretic agent, AZA was initially regarded to attenuate I/R induced AKI through an increase in urine output. However, surprisingly compared with the sham group no significant further increase in mouse urine volumes was seen in our experiments (Table 1). Thus, such renoprotective effect may be attributed to novel mechanisms other than carbonic anhydrase inhibition. Generally, HIF-1 α has been documented as a beneficial transcriptional factor mediating cells adaptation against I/R induced injury [26, 27, 37]. Hill et al. [38] reported that hif1 α ^{+/-} mice exhibited more severe histologic injury and more impaired renal function after kidney I/R insult. Another report proved that induction of HIF-1 α by silencing its degradation enzyme Prolyl Hydroxylase Domain-1 (PHD-1^{-/-}) attenuated myocardial I/R injury through up-regulating its target genes including eNOS and Bcl-2 [12]. Repetitive hypoxic preconditioning could ameliorate renal I/R injury through HIF-1 α -dependent Bcl-2 anti-apoptotic pathway [39]. On the contrary, a few studies suggested that HIF-1 α did not participate in the protective effect under I/R injury [40, 41]. Although there are conflicts about the protective role of HIF-1 α against I/R

Fig. 5. AZA treatment activated the PI3K/Akt/mTOR signaling pathway and the protective effect of AZA treatment upon HK-2 cells were eliminated by the PI3K/Akt inhibitor LY294002. (A) Representative immunoblotting images of phosphorylated phosphoinositide 3-kinase p85 (PI3K, p-p85), PI3K p85, phosphorylated Akt (p-Akt), Akt, phosphorylated mammalian target of rapamycin (p-mTOR), mTOR, phosphatase and tensin homolog (PTEN) and β -actin in HK-2 cells treated with 3×10^{-7} mol/L AZA from 0 to 4 hours (8 time points in total) from three independent western blotting experiments. (B) Representative immunoblotting images of HIF-1 α , p-eNOS, eNOS, Bcl-2, Bax and β -actin in HK-2 cells pre-incubated with the PI3K/Akt inhibitor LY294002 (20 μ mol/L, LY294002 +) or vehicle (LY294002 -) for 1 hour followed by treatment with AZA or vehicle for 24 hours followed by stimulation of H₂O₂ for eight hours (eight groups in total) from three independent western blotting experiments. (C) Relative NO concentrations measured in HK-2 cells pre-incubated with LY294002 or vehicle for 1 hour, and then treated with AZA or vehicle for 24 hours

followed by stimulation of H₂O₂ for eight hours; culture supernatant was collected for NO measurement. N = 3 independent experiments per group. (D) Cell viability (%CTRL) normalised to the level of the control group (AZA-, H₂O₂- and LY294002- group) measured in HK-2 cells in eight groups mentioned as Figure 5B. N = 3 independent experiments per group. For all graphs: data were shown as mean $\pm$ s.e.m. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns meant no significance.

injury due to large amount of genes regulated by HIF-1, pharmacologically activating HIF-1 α is still considered an effective way to ameliorate I/R induced AKI. In our present study, HIF-1 α activation has been linked to mediate the renoprotective role of AZA. We also showed that AZA increased eNOS-NO production and Bcl-2/Bax ratio in a HIF-1 α -dependent manner, as HIF-1 α siRNA blocked the up-regulation of both eNOS and Bcl-2 and the subsequent protective effect. However, one limitation in our present study is that we only provided

in vitro HIF-1 α siRNA evidence to elucidate HIF-1 α mediated the regulation of both eNOS and Bcl-2 by AZA, but *in vivo* experiments were still lacking. Thus, in future work, either specific inhibitor targeting HIF-1 α or hif1 α knock-out mice will be needed to further confirm our current findings.

AZA has been reported to dilate both small- and medium- caliber vessels in kidneys and other organs [15, 18, 42, 43], suggesting a possible role of AZA in increasing renal perfusion rates after I/R insult by vessel dilation. Consistently, AZA was found to restore impaired renal blood flow after I/R injury in our present study, possibly due to enhanced NO production. It was reported that AZA stimulated the production of the vasodilator nitric oxide (NO), resulting in an increase in blood flow because a NO synthase (NOS) inhibitor could reverse the effect of AZA on blood flow [44]. However, it is still controversial whether NO is the essential mediator of AZA induced vessels dilation, as one report said AZA could increase cerebral and ocular blood flow without any association with NO [45]. Given the conflicts, to verify the critical role of NO, in our future work specific eNOS inhibitor should be utilized.

How AZA restored HIF-1 α in the normoxic cell culture system is an important but not fully understood problem in our present study. There are mainly two ways in which HIF-1 α levels are regulated: transcriptional regulation and post-translational modulation (mostly degradation) [10, 26]. From our results, activation of PI3K/Akt signaling pathway by AZA can be responsible for the transcriptional up-regulation of HIF-1 α . However, the mechanism how AZA prevented HIF-1 α from oxygen-mediated degradation is still unclear. We speculated that: The first possible reason is acidosis induced by AZA treatment. It was reported that pH change during acidosis could trigger nucleolar sequestration of von Hippel-Lindau, thereby inhibiting its function to recognize hydroxylated HIF-1 α resulting in degradation [46, 47]. It is well known that AZA treatment induces metabolic acidosis via carbonic anhydrase inhibition [48], and thus, we propose that AZA stabilizes HIF-1 α through acidosis mediated von Hippel-Lindau protein nucleolar sequestration and subsequent dysfunction. Consistent with our hypothesis, Xu et al. [49] reported that AZA significantly induced cerebral HIF-1 α under normoxic condition, and acidosis played an important role. Secondly, we cannot rule out the possibility that AZA increases HIF-1 α expression through, at least in part, modulating PHDs, which hydroxylate HIF-1 α leading to its degradation [50, 51]. Pharmacologically suppressing or genetically disruption of PHDs has also been proposed as effective strategies to stabilize and activate HIF-1 α [12, 14]. Accordingly, PHD inhibitors significantly reduced serum creatinine levels and renal injury scores after I/R injury by activation of HIF-1 α [52]. However, till now there is no report to demonstrate the relationship between AZA and PHDs. Therefore, to fully elucidate the mechanism how AZA prevents HIF-1 α from degradation under normoxic condition, the role of acidosis and impact of AZA on PHDs need to be further investigated in our future work.

We should always be aware that although AZA is an ancient diuretic agent affording potential clinical benefits [17], it should be used cautiously in patients with advanced-stage renal failure because complications of AZA such as calcium phosphate kidney stones frequently occur [53]. A recent work by Betten et al. [54] supported our findings and showed that AZA administration effectively improved renal function on a patient with acute renal failure and alkalemia, possibly due to normalization of body pH. Thus, the relationship between AZA and acute kidney injury is still disputed, and various patient conditions, AZA dosages and administration periods will result in different outcomes. Although our present study showed that AZA effectively ameliorated mouse renal ischemia-reperfusion injury, question is still remaining whether longer time or higher dose of AZA administration may cause further kidney injury. In summary, given both the beneficial effects and the complications of AZA treatment, our suggestion is that AZA can be used to ameliorate contrast or ischemia induced AKI within a short period, however, longer-time usage of this agent should be cautious. In addition, in our future work, a protective role of post-transplant AZA administration against I/R induced AKI still needs to be studied, and by utilizing the animal kidney transplantation model, whether the donor or the recipient should be treated with AZA also needs to be elucidated.

Conclusion

In conclusion, our present work provided multiple lines of evidence to support that AZA protected against renal I/R injury in mice and human HK-2 cells. AZA exerted renoprotective function via up-regulating HIF-1 α through activation of the PI3K/Akt signaling pathway. The increase of HIF-1 α enhanced the downstream eNOS/NO production and Bcl-2/Bax ratio, resulting in the improvement of renal blood flow and attenuation of cell apoptosis. Finally, AZA treatment restored mouse renal function against I/R induced AKI.

Abbreviations

AKI (acute kidney injury); ARF (acute renal failure); AZA (acetazolamide); Bcl-2 (B-cell lymphoma 2); BUN (blood urea nitrogen); ERK (extracellular regulated protein kinase); HIF-1 α (hypoxia-inducible factor-1 α); HK-2 (human kidney-2); ICU (intensive care unit); I/R (ischemia-reperfusion); MAPK (mitogen activated protein kinase); NO (nitric oxide); NOS (nitric oxide synthase); eNOS (endothelial nitric oxide synthase); nNOS (neural nitric oxide synthase); PHD (prolyl hydroxylase domain); PI3K (phosphoinositide 3-kinase); PTEN (phosphatase and tensin homolog); mTOR (mammalian target of rapamycin); TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling); VEGF (vascular endothelial growth factor); pVHL (von Hippel-Lindau).

Conflict of Interest

The authors declare no conflict of interests.

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