INTRODUCTION

Aristolochic acid (AA) nephropathy (AAN) was reported for the first time as Chinese herb nephropathy in Belgium in the 1990s and is now a worldwide disease as Chinese herbs have gained popularity worldwide. Renal tubulointerstitial fibrosis (TIF) is the pathological feature of AAN, but the precise mechanism that modulates renal fibrosis in AAN is still unknown.

It is known that proximal tubular cells (PTCs) are the most sensitive cells to kidney injury factors. When the damage is mild, PTCs will regenerate to restore normal kidney structure, while severe or persistent damage will lead to renal fibrosis.

Aristolochic acid induces renal fibrosis by arresting proximal tubular cells in G2/M phase mediated by HIF-1α

Hao Zhao | Na Jiang | Yachun Han | Ming Yang | Peng Gao | Xiaofen Xiong |
Shan Xiong | Lingfeng Zeng | Ying Xiao | Ling Wei | Li Li | Chenrui Li |
Jinfei Yang | Chengyuan Tang | Li Xiao | Fuyou Liu | Yu Liu | Lin Sun

Hunan Key Laboratory of Kidney Disease and Blood Purification, Department of Nephrology, The Second Xiangya Hospital, Central South University, Changsha, China

Correspondence
Lin Sun and Yu Liu, Hunan Key Laboratory of Kidney Disease and Blood Purification, Department of Nephrology, The Second Xiangya Hospital, Central South University, No.139 Renmin Middle Road, Changsha, 410011, Hunan, China.
Email: sunlin@csu.edu.cn (L. S.) and rory0423@163.com (Y. L.)

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Abstract
Renal tubulointerstitial fibrosis (TIF) is a common pathological feature of aristolochic acid (AA) nephropathy (AAN). G2/M arrest of proximal tubular cells (PTCs) is implicated in renal fibrosis of AAN, but the upstream regulatory molecule remains unknown. Hypoxia inducible factor-1α (HIF-1α) promotes renal fibrosis in kidney disease, but the role of HIF-1α in AAN is unclear. Evidence shows that HIF-1α and p21, a known inducer of cellular G2/M arrest, are closely related to each other. To investigate the role of HIF-1α in renal fibrosis of AAN and its effects on p21 expression and PTCs G2/M arrest, mice with HIF-1α gene knockout PTCs (PT-HIF-1α-KO) were generated, and AAN was induced by AA. In vitro tests were conducted on the human PTCs line HK-2 and primary mouse PTCs. HIF-1α and p21 expression, fibrogenesis, and G2/M arrest of PTCs were determined. Results showed that HIF-1α was upregulated in the kidneys of wild-type (WT) AAN mice, accompanied by p21 upregulation, PTCs G2/M arrest and renal fibrosis, and these alterations were reversed in PT-HIF-1α-KO AAN mice. Similar results were observed in HK-2 cells and were further confirmed in primary PTCs from PT-HIF-1α-KO and WT mice. Inhibiting p21 in HK-2 cells and primary PTCs did not change the expression of HIF-1α, but G2/M arrest and fibrogenesis were reduced. These data indicate that HIF-1α plays a key role in renal fibrosis in AAN by inducing PTCs G2/M arrest modulated through p21. HIF-1α may serve as a potential therapeutic target for AAN.

KEYWORDS
aristolochic acid nephropathy, G2/M arrest, HIF-1α, p21, renal fibrosis

1 INTRODUCTION

Aristolochic acid (AA) nephropathy (AAN) was reported for the first time as Chinese herb nephropathy in Belgium in the 1990s and is now a worldwide disease as Chinese herbs have gained popularity worldwide. Renal tubulointerstitial fibrosis (TIF) is the pathological feature of AAN, but the precise mechanism that modulates renal fibrosis in AAN is still unknown.

It is known that proximal tubular cells (PTCs) are the most sensitive cells to kidney injury factors. When the damage is mild, PTCs will regenerate to restore normal kidney structure, while severe or persistent damage will lead to renal fibrosis.
maladaptive repair. During the maladaptive repair process, PTCs are arrested in the G2/M phase of the cell cycle. A recent study demonstrated that G2/M-arrested PTCs could promote renal fibrosis in AAN. However, the upstream regulation of the G2/M arrest of PTCs in AAN remains to be elucidated.

Recent studies have demonstrated that renal hypoxia is a common pathophysiological feature in kidney diseases, and hypoxia inducible factor-1α (HIF-1α) is the key transcriptional regulator under hypoxic conditions. Overexpression of HIF-1α in the kidney could promote renal fibrosis in some kidney diseases, but the role of HIF-1α in AAN has not been reported. As a member of the cyclin-dependent kinase inhibitor (CDKI) family, p21 is a well-known important cytokine that induces cellular G2/M arrest. It has been reported that HIF-1α can induce the expression of p21 and can bind to the promoter region of p21. However, a different study showed that p21 could also induce the expression of HIF-1α. In summary, HIF-1α and p21 are closely related to each other in different cells, but the exact role of HIF-1α and p21 in renal fibrosis of AAN and the related mechanism still need to be further investigated. With the literature review in mind, we hypothesized that HIF-1α could induce PTCs G2/M arrest and promote renal fibrosis by p21 in mice with AAN.

To verify our hypothesis, mice with HIF-1α gene deficiency in PTCs (PT-HIF-1α-KO) were generated and treated with AA to induce AAN. The results showed that HIF-1α expression was upregulated in the kidney cortex of AAN mice, accompanied by increased p21 expression, PTCs G2/M arrest, and renal fibrosis compared to the control, while these alterations were reversed by HIF-1α gene knockout in mouse PTCs. Similar results were observed in HK-2 cells cultured with AA. In addition, overexpression of HIF-1α in HK-2 cells cultured with AA further increased p21 expression, G2/M arrest and fibrogenesis, while the effects were partially blocked by cotreatment with HIF-1α siRNA. Furthermore, PTCs isolated from PT-HIF-1α-KO mice showed significantly lower p21 expression and cellular G2/M arrest as well as reduced fibrogenesis compared to PTCs from WT mice cultured with AA. In addition, inhibiting p21 in HK-2 cells and primary PTCs decreased cellular G2/M arrest and fibrogenesis but had no obvious effects on HIF-1α expression. These data indicated that HIF-1α could promote renal fibrosis by arresting PTCs in G2/M phase mediated by p21 in AAN.

2 | MATERIALS AND METHODS

2.1 | Generation and identification of mice with HIF-1α knockout PTCs

HIF-1α^flo×flo× mice were purchased from the Jackson Laboratory, USA. Phosphoenolpyruvate carboxykinase-cAMP-response element (PEPCK-Cre) mice were provided by Dr Tang (Second Xiangya Hospital, Central South University, Hunan, China). HIF-1α^flo×flo× mice were crossed with PEPCK-Cre mice to produce mice with HIF-1α conditional knockout in PTCs (PT-HIF-1α-KO) and littermate wild-type (WT) mice, which is described in Figure 1A. The mice were genotyped by PCR analysis of genomic DNA from tail tissues using a Mouse Direct PCR kit (Bimake, USA) according to the manufacturer’s instructions. The following sequences of the primers (Sangon Biotech Corporation, China) were used to identify the genotype: HIF-1α, 5′-TGCATGTGTATGGGTGTTTTG-3′ (sense), 5′-GAAAAACTGCTGTAACTTCATTCC-3′ (antisense); Cre, 5′-ACCTGAAGATGTTCGCGATTATCT-3′ (sense), and 5′-ACCGTCAGTGAGATATCTT-3′ (antisense). The mice were housed in the animal facility at Second Xiangya Hospital of Central South University under a 12-hour light/dark cycle with free access to water and food. All procedures were conducted in accordance with the relevant institutional guidelines of the Animal Experimentation Ethics Committee of Second Xiangya Hospital of Central South University.

2.2 | Inducing AAN in mice with AA

Type I AA (Sigma-Aldrich, USA) was used in the current study to induce AAN in mice. Male mice aged 8 weeks weighing 24-25 g were selected. AAN mice were induced by a single intraperitoneal injection of AA (5 mg/kg) in sterile PBS as previously described. The control group was intraperitoneally injected with the same amount of sterile PBS. After 1 month of AA or PBS injection, the serum and kidney cortex tissues were harvested for further study.

2.3 | Isolation and culture of primary PTCs

Primary PTCs were isolated from mouse kidneys as described previously. Briefly, the kidneys from mice were flushed with sterile saline in vivo to clear the blood cells. Kidney cortices were cut into pieces of approximately 1 mm³ and washed with sterile saline three times. Hank’s balanced salt solution (HBSS) containing 2 mg/mL of collagenase II (Sigma-Aldrich, USA) was used to digest the tissue pellet at 37°C for 50 minutes. Then, the digested cortical tissue was centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in K1 medium containing 5% of fetal bovine serum (FBS). Then, the cell suspension was placed on collagen-coated Petri dishes (Corning BioCoat, USA) and incubated in a cell incubator at 37°C with 5% of CO₂ and 95% of air. The phenotype was identified by staining with anti-megalin antibody (1:500, Abcam, ab76969, USA).
HK-2 cells were cultured in DMEM supplemented with 10% of FBS, and primary PTCs were cultured in K1 medium containing 5% of FBS until the cells were 70% confluent. Cells were then treated with AA (5 µg/mL) for 48 hours as described previously.6 In the cell assays with siRNA or plasmid intervention, cells were first transfected with siRNA or plasmid by Lipofectamine 3000 (Invitrogen, USA) in accordance with the manufacturer's instructions. HIF-1α siRNA and p21 siRNA were purchased from RiboBio, China. The human HIF-1α plasmid was kindly provided by Dr Nicolas (Hospital Saint Louis, Paris, France). The murine HIF-1α plasmid was purchased from Sino Biological, China.

2.5 | Physiologic parameters assessment

The body weight and serum creatine were measured at the 28th day after AA injection as indicated. To assess the serum creatine, blood samples were collected when the mice were sacrificed after 28 days of AA or sterile PBS injection, and...
the serum creatine level was assessed by a QuantiChrom Creatinine Assay Kit according to the instructions (BioAssay Systems, USA).25

2.6 | Morphological analysis

As previously described,26 kidney tissues were fixed with 4% of buffered paraformaldehyde and embedded in paraffin, and four-micrometer sections of formalin-fixed paraffin-embedded kidneys sections were stained with hematoxylin and eosin staining (HE) and Masson trichrome staining according to the HE and Masson trichrome staining Kit instructions (Servicebio, China). Tissue damage was scored according to the percentage of damaged tubules observed by HE staining as previously described27: 0, no damage; 1, less than 25% damage; 2, 25%-50% damage; 3, 50%-75% damage; and 4, more than 75% damage. The degree of TIF was scored according to the stained tubulointerstitial area in Masson trichrome stained sections as previously described27: 0, no staining; 1, less than 25% staining; 2, 25%-50% staining; 3, 50%-75% staining; and 4, more than 75% staining.

2.7 | Immunofluorescence (IF) staining

IF staining of the kidney was performed in a similar manner on kidney sections as previously described.6 Briefly, the tissue sections were rehydrated and labeled with target antibodies against HIF-1α (1:50, Abcam, ab179483, USA), p21 (1:400, Abcam, ab109199, USA), Ki-67 (1:100, Abcam, ab15580, USA), p-H3 (1:1000, Abcam, ab14955, USA), fibronectin (FN) (1:200, Abcam, ab2143, USA), α-SMA (1:300, Abcam, ab124964, USA), collagen-I (COL-I) (1:500, Abcam, ab34710, USA), and collagen-IV (COL-IV) (1:250, Abcam, ab6586, USA). Then, the slides were incubated with Alexa Fluor 594-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated goat anti-rabbit antibodies or Alexa Fluor 594-conjugated goat anti-mouse antibodies, respectively.

2.8 | Flow cytometry analysis of the cell cycle

Flow cytometry technology was used to analyze the distribution of the cell cycle as previously described.6 Briefly, HK-2 cells and primary PTCs were harvested after incubation and fixed with 70% of ethanol for 4 hours at 4°C. Fixed cells were then washed in 4°C PBS and incubated with 1-2 mL of FxCycle PI/RNase Staining Solution (Invitrogen, USA) for 30 minutes at 4°C in the dark. The cell cycle distribution was measured using a flow cytometer (BD, FACS Jazz, USA) and analyzed with the ModFit LT program (Verity Software House, USA).

2.9 | Cell immunofluorescence

Treated HK-2 cells and primary PTCs were fixed with 4% of PFA for 5 minutes, permeabilized with ice-cold methanol for 10 minutes at −20°C, blocked with blocking buffer (PBS + 1% BSA + 0.3% TritonX-100) for 1 hour at 22°C, and then, incubated with primary antibodies against p-H3 (1:200, Abcam, ab5176) for 2 hours at 22°C. After washing with PBS, Alexa Fluor 488-conjugated goat anti-rabbit antibodies were applied for 1 hour at 37°C, followed by counterstaining with Hoechst to delineate the nuclei. Cells were then examined by LSM 780 META laser scanning microscopy.

2.10 | qPCR analysis

RNA was extracted from the kidney superficial cortex or cultured cells and was reverse transcribed into cDNA using a cDNA Synthesis Kit (TaKaRa, Japan) as described in the instructions. qPCR was performed using SYBR premix EXTaq reagents (TaKaRa, Japan) according to the instructions. The sequences of the primers (Sangon Biotech Corporation, China) were as follows: HIF-1α (human), 5′-GAACGTGAAAGAAAAGTCTCG-3′ (sense) and 5′-CCTTATCAAGATGCGAACTCACA-3′ (antisense), HIF-1α (mouse), 5′-ACCTTCATCGGAACCTCACAAGAG-3′ (sense) and 5′-CTGTTAGGCTGGAAAAAGTTAGG-3′ (antisense), p21 (human), 5′-TGGCAGAACCACATGC-3′ (sense) and 5′-AAAGTCAAGAGTTCGAGTCTCG-3′ (antisense), p21 (mouse), 5′-AACGAGCTCACAAGAGTTCGAGTCTCG-3′ (sense) and 5′-CCATGAGGCCATCGACCAC-3′ (antisense), FN (human), 5′-CGGTGGCTGTCAAGATGAGG-3′ (sense) and 5′-GCCCAGTGTATTCAGCAAAAGG-3′ (antisense), FN (mouse), 5′-CCATGACGGCCATCGACCAC-3′ (sense) and 5′-TACATGCGGGAGGCTACATG-3′ (antisense), α-SMA (human), 5′-AAAAGGACAGCTACGTGGGATG-3′ (sense) and 5′-GCCATGTTCTTATCAGGTCATCC-3′ (antisense), α-SMA (mouse), 5′-TGGCAGTGTATTCAGCAAAAGG-3′ (sense) and 5′-GCCATGTTCTTATCAGGTCATCC-3′ (antisense), α-SMA (mouse), 5′-CCATGACGGCCATCGACCAC-3′ (sense) and 5′-TACATGCGGGAGGCTACATG-3′ (antisense), COL-I (human), 5′-GTGACGCGGATGACGGTATC-3′ (sense) and 5′-GGTGTCGCTCTTCTGGTG-3′ (antisense), COL-I (mouse), 5′-TCCGAGCGGGGATGACGGTATC-3′ (sense) and 5′-GGTGTCGCTCTTCTGGTG-3′ (antisense), COL-IV (human), 5′-GGTGTCGCTCTTCTGGTG-3′ (antisense), COL-IV (mouse), 5′-TCCGAGCGGGGATGACGGTATC-3′ (sense) and 5′-GGTGTCGCTCTTCTGGTG-3′ (antisense). The data are presented as fold changes (2−ΔΔCt).
2.11 | Western blot analysis

Western blot was performed in a common way, briefly, cells and mouse kidney tissues lysates were prepared in radioimmunoprecipitation assay buffer (Beyotime, China) supplemented with protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, USA) on ice for 30 minutes. Protein concentration was measured and equal amounts of protein were electrophoresed on SDS polyacrylamide gels and then the separated proteins from gel were electro-transferred on to nitrocellulose membrane. The membrane was blocked with 5% of milk in Tris-buffered saline and incubated with primary antibody, HIF-1α (1:1000, Abcam, ab21855, USA), p21 (1:1000, Abcam, ab109199, USA), CyclinB1 (1:2000, Proteintech, 55004-1-AP, China), CyclinD1 (1:4000, Proteintech, 60186-1-lg, China), FN (1:2000, Abcam, ab2413, USA), α-SMA (1:3000, Abcam, ab124964, USA), and β-actin (1:5000, Abcam, ab8226, USA) at 4°C overnight. The membrane was washed and then, incubated for 1 hour with rabbit or mouse horseradish peroxidase-conjugated secondary antibody. The membrane was washed and then developed to visualize protein bands using enhanced chemiluminescence (ECL) reagent (Merck Millipore, USA).

2.12 | Statistical analysis

SPSS 13.0 software was used to analyze the experimental data, and the results are presented as the mean ± SD and analyzed by one-way ANOVA. The correlations between two variables were assessed using Pearson’s correlation analysis. P values less than .05 indicated that the difference was statistically significant.

3 | RESULTS

3.1 | Breeding and identification of mice with HIF-1α knockout PTCs

To determine the role of HIF-1α in renal fibrosis in mice with AAN, we initially established conditional knockout mice in which HIF-1α was deleted specifically from PTCs. The breeding protocol is shown in Figure 1A. Briefly, male mice bearing floxed HIF-1α alleles (HIF-1α^flx/flx^XCreXY) crossed with female PEPCK-Cre mice (HIF-1α^+/+^X^Cre^X^Cre^) that express Cre recombinase under the control of a modified PEPCK promoter that controls the expression of Cre mainly in kidney PTCs. After the first round of breeding, heterozygous female mice (HIF-1α^flx/+^X^Cre^X) were selected to cross with male HIF-1α^flx/flx^X^Cre^X mice to generate PT-HIF-1α-KO and littermate WT mice for further experiments. The genotype of PT-HIF-1α-KO mice was identified by PCR as the amplification of 120-bp fragment of the floxed allele and no amplification of 100-bp fragment of the WT allele, as well as amplification of a 370-bp fragment of the Cre gene at the same time (Figure 1B, lanes 2, 6, and 10). The mice without Cre amplification were used as littermate WT controls (Figure 1B, lanes 1, 4, 5, 7, and 9). Since the protein expression of HIF-1α in mouse kidneys is very low under normal conditions, to further verify the genotypes at the protein level, the mice were treated with AA to induce AAN, and HIF-1α expression was detected by WB and IF staining. By IF staining, a notable increase in the expression of HIF-1α was observed in the PTCs of WT mice, but no obvious expression was observed in the PT-HIF-1α-KO mice (Figure 1C,D). The results were confirmed by WB analysis, and the expression of HIF-1α in the extracted protein from medullary tissues was not significantly different between the WT and PT-HIF-1α-KO groups (Figure 1E,F).

3.2 | HIF-1α gene knockout in PTCs has renoprotective effects in AAN mice

PT-HIF-1α-KO and WT mice without AA treatment showed similar levels of body weight and serum creatinine, while a higher serum creatinine level and lower body weight were found in WT mice compared to PT-HIF-1α-KO mice after treated with AA at the 28th day (Figure 2A,B). Histologic analysis showed a significantly reduced pathological change in the kidneys of PT-HIF-1α-KO mice treated with AA compared to WT mice treated with AA (Figure 2C). Further semiquantitative analysis showed that the mean tubular damage score and TIF score were 2.82 and 2.77 in WT mice treated with AA, respectively, whereas the scores were markedly decreased to 1.65 and 1.75 in PT-HIF-1α-KO mice treated with AA, respectively (Figure 2D). By qPCR, the mRNA expression levels of FN, α-SMA, COL-I, and COL-IV in the kidney cortex of WT mice treated with AA were higher than those of PT-HIF-1α-KO mice treated with AA. No difference was observed between WT and PT-HIF-1α-KO mice without AA treatment (Figure 2E). WB analysis of FN and α-SMA expression showed similar results to qPCR (Figure 2F,G), as well as IF staining analysis of FN, α-SMA, COL-I, and COL-IV expression (Figure 2H). These results indicate that HIF-1α can promote renal fibrosis and that inhibition of HIF-1α has renoprotective effects in mice with AAN.

3.3 | HIF-1α absence in PTCs-reduced p21 expression and G2/M arrest in AAN mice

By qPCR, the mRNA expression of HIF-1α was slightly higher in the kidney cortex of WT mice without AA treatment than PT-HIF-1α-KO mice without AA treatment, but p21 mRNA expression was not significantly different...
FIGURE 2  HIF-1α knockout in PTCs has renal protective effects in AAN mice. A, Body weight changes of mice in each group as indicated. B, Serum creatinine level of mice in each group after 1 month of intervention as indicated. C, HE and Masson trichrome staining of kidney sections from mice in each group, scale bar: 100 μm. D, Renal tubular damage score and tubulointerstitial fibrosis (TIF) score analysis of mice in each group. E, qPCR analysis of mRNA expression of FN, α-SMA, COL-I, and COL-IV in the kidney cortex of mice in each group. F and G, WB analysis (F) and quantification (G) of FN and α-SMA expression in the kidney cortex of mice from each group. H, IF staining and quantitative analysis of FN, α-SMA, COL-I, and COL-IV in kidney sections from mice in each group, scale bar: 100 μm. In all the panels, data are represented as the mean ± SD, n = 6, *P < .05, NS: nonsignificant.
FIGURE 3  HIF-1α absence in PTCs reduces p21 expression and G2/M arrest in the kidneys of mice with AAN. A, qPCR analysis of HIF-1α and p21 mRNA expression in the kidney cortex of mice from each group as indicated. B and C, WB analysis (B) and quantification (C) of HIF-1α and p21 expression in the kidney cortex of mice from each group. D and E, IF staining (D) and quantitative analysis (E) of HIF-1α and p21 expression in the kidney sections of mice from each group, scale bar: 100 μm. F and G, Co-IF staining (F) of Ki-67 (green) and p-H3 (red) and the analysis (G) of the p-H3+/Ki-67+ ratio in PTCs in kidney sections of mice from each group, scale bar: 100 μm (scale bar = 30 μm in the enlarged images. H, Co-relationship analysis of HIF-1α and p21, HIF-1α and the percentage of proliferating PTCs in G2/M phase, HIF-1α and renal fibrosis, p21 and the percentage of proliferating PTCs in G2/M phase, p21 and renal fibrosis, and the percentage of proliferating PTCs in G2/M phase and renal fibrosis in the kidney cortex of mice from each group. Data represent the mean ± SD, n = 6, *P < .05, NS: nonsignificant.
between the two groups. However, after AA treatment, more robust amplification of HIF-1α and p21 mRNA was observed in the kidney cortex of WT mice than in PT-HIF-1α-KO mice (Figure 3A). The WB results showed no difference in the expression of HIF-1α and p21 between the WT and PT-HIF-1α-KO mice without AA treatment, while the expression was much higher in the kidney cortex of WT mice than PT-HIF-1α-KO mice after AA treatment (Figure 3B,C). Similar results were observed in the IF staining of kidney sections (Figure 3D,E). Since Ki-67 expression marks proliferating cells (G1, S, G2, and M phases), phosphorylation of histone H3 at Ser10 (p-H3) expression represents only the cells in G2/M phase, and the ratio of p-H3+ cells to Ki-67+ cells has been used to calculate the percentage of G2/M phase of the proliferating PTCs.6 IF double staining with p-H3- and Ki-67-specific antibodies was conducted. As shown in Figure 3F,G, the percentage of proliferating PTCs in G2/M phase in WT/Sham mice was 12.00% ± 4.68%, in PT-HIF-1α-KO/Sham mice it was 11.60% ± 3.85%, in WT/AA mice it was 47.90% ± 8.30% and in PT-HIF-1α-KO/AA mice it was 28.88% ± 6.36%. The results indicated that the percentage of proliferating PTCs in G2/M phase was obviously decreased in PT-HIF-1α-KO mice treated with AA compared to WT mice treated with AA. Further univariate correlation analysis (Figure 3H) showed that there were positive associations between the protein expression of HIF-1α and p21 (r = 0.89), HIF-1α expression and G2/M percentage (r = 0.87), HIF-1α expression and renal fibrosis (r = 0.87), p21 expression and G2/M percentage (r = 0.85), p21 expression and renal fibrosis (r = 0.89), and G2/M percentage and renal fibrosis score (r = 0.91).

3.4 | HIF-1α upregulates p21 expression and induces G2/M arrest and fibrogenesis in HK-2 cells treated with AA

qPCR showed significantly increased mRNA expression of HIF-1α and p21 in HK-2 cells treated with AA compared to the control, and the expression was further increased in cells transfected with HIF-1α plasmid or silenced with HIF-1α siRNA transfection (Figure 4A). The WB showed consistent results with qPCR (Figure 4B,C). Flow cytometry analysis showed that the proportion of HK-2 cells in G2/M phase was significantly increased by AA treatment compared to the control (24.65% ± 0.72% vs 9.97% ± 1.24%), and the effects were further increased in cells transfected with HIF-1α plasmid (30.27% ± 1.04%) and decreased in cells treated with HIF-1α siRNA (19.25% ± 0.91%) (Figure 4D,E). In addition, by qPCR analysis, the mRNA expression of FN, α-SMA, COL-I, and COL-IV was significantly increased in HK-2 cells treated with AA compared to the control, while the expression was further increased in HK-2 cells transfected with HIF-1α plasmid but decreased when the cells were treated with HIF-1α siRNA (Figure 4F). WB analysis of FN, α-SMA, and cyclinB1/cyclinD1 expression showed similar results (Figure 4G,H).

3.5 | Downregulation of p21 expression, cellular G2/M arrest, and fibrogenesis in AA-treated primary PTCs isolated from PT-HIF-1α-KO mice

qPCR results showed that, when without AA treatment, the mRNA expression of HIF-1α in primary PTCs from PT-HIF-1α-KO mice was less compared to that from WT mice, confirming that the HIF-1α knockout in PTCs of mice is effective, the mRNA expression of p21 between the two groups has no difference. However, after cultured with AA, notably more HIF-1α and p21 mRNA expression was observed in primary PTCs from WT mice compared to that from PT-HIF-1α-KO mice (Figure 5A). By WB analysis, the expression levels of HIF-1α and p21 were much higher in primary PTCs from WT mice than in those from PT-HIF-1α-KO mice after cultured with AA (Figure 5B,C). On the contrary, flow cytometry analysis showed that the percentage of G2/M phase primary PTCs from WT mice was similar to that from PT-HIF-1α-KO mice without AA treatment (10.65% ± 1.19% vs 10.61% ± 0.70%), while the percentage was significantly higher in PTCs from WT mice compared to those from PT-HIF-1α-KO mice after cultured with AA (25.34% ± 1.34% vs 18.09% ± 0.37%) (Figure 5D,E). In addition, qPCR showed that the mRNA expression levels of FN, α-SMA, COL-I, and COL-IV were not different between the primary PTCs from WT and PT-HIF-1α-KO mice without AA but were notably higher in the primary PTCs from WT mice after cultured with AA and reduced in those from PT-HIF-1α-KO mice (Figure 5F). WB analysis of FN, α-SMA, and cyclinB1/cyclinD1 expression showed similar results to qPCR (Figure 5G,H).

3.6 | Inhibition of p21 prevents G2/M arrest and fibrogenesis in HK-2 and primary PTCs but has no obvious effects on HIF-1α expression

WB analysis showed that the expression of HIF-1α and p21 was notably increased in HK-2 cells treated with AA compared to the control and was further increased by transfection with HIF-1α plasmid. When cells were cotreated with p21 siRNA, p21 expression was significantly reduced, but there were no obvious effects on HIF-1α expression (Figure 6A,B). Cell cycle analysis by flow cytometry showed that the percentage of G2/M phase of HK-2 cells was significantly increased.
FIGURE 4 HIF-1α upregulates p21 expression and induces G2/M arrest and fibrogenesis in HK-2 cells. A, qPCR analysis of the mRNA expression of HIF-1α and p21 in HK-2 cells treated as indicated. B and C, WB analysis (B) and quantification (C) of HIF-1α and p21 expression in HK-2 cells. D and E, Flow cytometry (D) and analysis (E) of cell cycle distribution of HK-2 cells with different treatments as indicated. F, qPCR analysis of the mRNA expression of FN, α-SMA, COL-I, and COL-IV in HK-2 cells with different treatments. G, WB analysis of the expression of FN, α-SMA, CyclinB1, and CyclinD1 in HK-2 cells. H, Quantification analysis of the protein expression of FN and α-SMA and the ratio of CyclinB1/CyclinD1 in HK-2 cells. Data are shown as the mean ± SD, n = 3, *p < .05, NS: nonsignificant.
FIGURE 5  HIF-1α upregulates p21 expression and induces G2/M arrest and fibrogenesis in primary PTCs. A, qPCR analysis of HIF-1α and p21 mRNA expression levels in primary PTCs isolated from WT and PT-HIF-1α-KO mice treated as indicated. B and C, WB analysis (B) and quantification (C) of HIF-1α and p21 expression in primary PTCs. D and E, Flow cytometry (D) and analysis (E) of the cell cycle distribution of primary PTCs treated as indicated. F, qPCR analysis of the mRNA expression of FN, α-SMA, COL-I, and COL-IV in primary PTCs with different treatments. G, WB analysis of the expression of FN, α-SMA, CyclinB1, and CyclinD1 in primary PTCs with different treatments. H, Quantification analysis of the protein expression of FN and α-SMA and the ratio of CyclinB1/CyclinD1 in primary PTCs. Data are shown as the mean ± SD, n = 3, *P < .05, NS: nonsignificant.
FIGURE 6  Inhibition of p21 prevents G2/M arrest and fibrogenesis but has no obvious effects on HIF-1α expression in HK-2 cells. A and B, WB analysis (A) and quantification (B) of HIF-1α and p21 expression in HK-2 cells treated as indicated. C and D, Cell cycle distribution analysis by flow cytometry of primary PTECs with different treatments. E, qPCR analysis of the mRNA expression of FN, α-SMA, COL-I, and COL-IV in HK-2 cells. F, WB analysis of the expression of FN, α-SMA, CyclinB1, and CyclinD1 in HK-2 cells. G, Quantification analysis of the protein expression of FN and α-SMA and the ratio of CyclinB1/CyclinD1 in HK-2 cells. Data are presented as the mean ± SD, n = 3, *P < .05, NS: nonsignificant.
FIGURE 7  Inhibition of p21 prevents G2/M arrest and fibrogenesis but has no obvious effects on HIF-1α expression in primary PTCs. A and B, WB analysis (A) and quantification (B) of HIF-1α and p21 expression in primary PTCs. C and D, Cell cycle distribution analysis by flow cytometry of primary PTCs with different treatments. E, qPCR analysis of the mRNA expression of FN, α-SMA, COL-I, and COL-IV in primary PTCs with different treatments. F, WB analysis of the expression of FN, α-SMA, CyclinB1, and CyclinD1 in primary PTCs. G, Quantification analysis of the protein expression of FN and α-SMA and the ratio of CyclinB1/CyclinD1 in primary PTCs. Data are presented as mean ± SD, n = 3, *P < .05, NS: nonsignificant
by AA treatment compared to the control (9.65% ± 1.24% vs 25.44% ± 0.40%), and the effects were further increased in cells transfected with HIF-1α plasmid (30.73% ± 1.38%) but were decreased by p21 siRNA (13.92% ± 1.62%), furthermore, the percentage of G2/M cells was 21.60% ± 1.27% in cells cocultured with AA + HIF-1α plasmid + p21 siRNA (Figure 6C,D), indicating that overexpression of HIF-1α further enhanced AA-induced G2/M arrest in HK-2 cells, but the effect was blocked by p21 siRNA. By qPCR analysis, significantly increased mRNA expression of FN, α-SMA, COL-I, and COL-IV was found in HK-2 cells with AA and was further enhanced in those transfected with HIF-1α plasmid but notably reduced by p21 siRNA (Figure 6E). WB analysis of FN, α-SMA, and cyclinB1/cyclinD1 expression showed similar results to qPCR (Figure 6F,G). To confirm these results, experiments on primary PTCs isolated from WT mice were performed, as shown in Figure 7, WB analysis of HIF-1α and p21 expression, flow cytometry cell cycle analysis, qPCR analysis of FN, α-SMA, COL-I, and COL-IV mRNA levels and WB analysis of FN, α-SMA, and cyclinB1/cyclinD1 expressions in primary PTCs treated as indicated showed similar results with the experiments conducted in HK-2 cells as showed in Figure 6. In this part, the percentages of G2/M phase primary PTCs in the control, AA, AA + HIF-1α plasmid, AA + p21 siRNA, and AA + HIF-1α plasmid + p21 siRNA groups were 9.28% ± 0.82%, 23.98% ± 0.89%, 33.49% ± 3.04%, 16.65% ± 1.07%, and 19.17% ± 0.96%, respectively. The results show that HIF-1α induces G2/M arrest and promotes renal fibrosis through p21.

3.7 | G2 phase arrest was more characteristic for the fibrotic effects in HK-2 cells and primary PTCs treated with AA

We further investigated the proportion of G2 and M phase of the cells which were arrested in G2/M phase after cultured with AA. We found that about 90% of the G2/M arrested HK-2 cells were in G2 phase after treated with AA, as observed by a typical G2 phase foci-like staining of p-H3 (Figure 8A,C). Similar results were observed in AA cultured primary PTCs isolated from WT mice, but the proportion of G2 phase was significantly decreased in the AA treated primary PTCs isolated from PT-HIF-1α-KO mice (Figure 8B,D).

4 | DISCUSSION

AAs are a family of nitrophenanthrene carboxylic acids found in certain plants. Products containing AA have been medicinally used worldwide, but it is well known that AA has strong renal toxicity, and exposure to AA will cause AAN in animals and humans. AAN is characterized by rapid progress toward end-stage renal disease (ESRD). It has been reported that high-dose AA exposure may lead to ESRD in as little as 1 month in patients, and the renal survival rate of AAN was only 17% in 2 years. In addition, there is no specific therapy for AAN so far. Thus, AAN is still a global health problem. The main kidney pathological feature of AAN is renal TIF with tubule atrophy and loss of the tubules predominantly located in the superficial cortex, but the precise mechanism by which AA induces renal fibrosis in AAN is not fully understood. It was demonstrated that AA could induce the TGF-β-associated fibrosis pathway, and macrophage accumulation in the kidney also participates in kidney fibrosis in AAN. However, the underlying mechanism of these phenomena still needs to be identified.

It is known that HIF-1α promotes G2/M arrest and renal fibrosis in a mouse unilateral ureteral obstruction (UUO) model, but whether HIF-1α participates in the process of renal fibrosis in AAN is unclear. In this study, we constructed mice with HIF-1α gene knockout in PTCs and identified the mice by PCR, WB, and IF staining (Figure 1). After treating the mice with AA to induce AAN, the expression of HIF-1α was notably upregulated in the kidney cortex of WT mice but reduced in that of PT-HIF-1α-KO mice. Furthermore, we found that renal fibrosis was much more serious in WT mice treated with AA than in PT-HIF-1α-KO mice treated with AA (Figure 2). These data indicate that HIF-1α plays an important role in promoting renal fibrosis in AAN and that inhibiting HIF-1α has renoprotective and antifibrotic effects.

PTCs arrested in the G2/M phase of the cell cycle can promote renal fibrosis in AA-induced kidney injury. However, the upstream regulatory molecule mediating G2/M arrest in AAN is still unknown. HIF-1α is involved in cellular G2/M arrest, and HIF-1α and p21, a known inducer of cellular G2/M arrest, are tightly related to each other in cell processes. As a transcriptional regulator, HIF-1α may induce the upregulation of p21 by directly binding to the promoter region of p21 and promoting p21 expression. However, some studies reported that p21 could also induce HIF-1α expression. The opposite results may be dependent on different cell models or diseases. Here, we demonstrated for the first time the exact relationship between HIF-1α and p21 and their effects on PTCs G2/M arrest and renal fibrosis in an AAN mouse model. As shown in Figure 3, a significantly reduced p21 expression and G2/M phase of PTCs as well as renal fibrosis was observed in PT-HIF-1α-KO AAN mice compared to WT AAN mice. Further correlation analysis showed that the expression of HIF-1α was positively correlated with p21 levels, G2/M arrest, and renal fibrosis. The results were confirmed by the in vitro study of HK-2 cells. In addition, we found that the expression of p21, cellular G2/M arrest, and fibrogenesis were reduced in primary PTCs isolated from PT-HIF-1α-KO mice compared to those from WT mice after cultured with AA (Figures 4 and 5).
To confirm the relationship between HIF-1α and p21 in the renal fibrosis of AAN, p21 siRNA was applied to HK-2 cells and primary PTCs from WT mice. The results showed that inhibition of p21 caused no obvious effect on HIF-1α expression but reduced cellular G2/M arrest and fibrogenesis in HK-2 cells and primary PTCs cultured with AA. These data suggest that HIF-1α is the upstream molecule of p21, and HIF-1α could promote fibrogenesis by inducing

**FIGURE 8** G2 phase arrest was more characteristic for the fibrotic effects in HK-2 cells and primary PTCs treated with AA. A and B, IF staining of p-H3 in HK-2 cells (A) and primary PTCs (B) with or without AA treatment, scale bar: 10 μm. C and D, Analysis of the proportion of G2 and M phase in HK-2 cells (C) and primary PTCs (D) treated as indicated. Data are presented as the mean ± SD, n = 3, *P < .05, NS: nonsignificant
cellular G2/M arrest modulated by p21 in the AAN cell model (Figures 6 and 7).

In order to verify whether G2 or M phase arrest was more characteristic for the observed fibrosis effects, we further distinguished the G2 phase and M phase of the G2/M arrested cells in vitro by p-H3 staining as previously described. Results showed that about 90% of the G2/M arrested HK-2 cells were in G2 phase after treated with AA, similar results were observed in primary PTCs isolated from WT mice, but the proportion of G2 phase was significantly decreased in the primary PTCs isolated from PT-HIF-1α-KO mice (Figure 8). The results are consistent with Yang et al described previously, suggesting that G2 phase arrest is more important for the fibrotic phenotype of the AA-treated HK-2 and primary PTCs.

The next question is why the expression of HIF-1α is upregulated in tubular cells treated with AA without hypoxic stimulation. The following findings may provide some clues. First, AA can increase the production of reactive oxygen species (ROS) in tubular cells and trigger oxidative DNA damage in tubular cells, indicating that AA may destroy intracellular redox homeostasis and lead to intracellular hypoxia, which further induces HIF-1α expression. Second, in addition to hypoxia, HIF-1α has been reported to be up-regulated by other factors, such as nitric oxide, TNF-α, angiotensin II, IL-1, and insulin. Some of these factor(s), such as TNF-α and IL-1, have been reported to be increased in AAN. These factors may induce the upregulation of HIF-1α, but the precise mechanism needs to be further investigated in the future.

In conclusion, the current study demonstrated for the first time that HIF-1α plays a key role in renal fibrosis in mice with AAN. AA induces renal fibrosis by upregulating the expression of HIF-1α, and then, enhancing p21 expression, further leading to PTCs arrest in the G2/M phase of the cell cycle. These data suggest that HIF-1α may serve as a potential therapeutic target to prevent renal fibrosis in AAN.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests and have nothing to disclose.

AUTHOR CONTRIBUTIONS
H. Zhao performed the experiments and wrote the manuscript. N. Jiang, Y. Han, M. Yang, P. Gao, X. Xiong, Shan Xiong, L. Zeng, L. Li, C. Li, and J. Yang provided technical support for this study. C. Tang, L. Xiao, and Y. Liu participated in the discussion about this study. L. Sun and Y. Liu designed the study and discussed the manuscript. All the authors approved the final version to be published.

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