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Hypoxia Reduces Mouse Urine Output via HIF1α-Mediated Upregulation of Renal AQP1

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Keywords
Hypoxia · Hypoxia-inducible factor · Aquaporin-1 · Roxadustat · Urine concentration

Abstract

Introduction: Patients with acute mountain sickness (AMS) due to hypoxia at high altitudes often exhibit abnormal water metabolism. Hypoxia-inducible factors (HIFs) are major regulators of adaptive responses to hypoxia. As transcription factors, HIFs are involved in the regulation of erythropoiesis, iron metabolism, angiogenesis, energy metabolism, and cell survival by promoting the transcriptional expression of hundreds of target genes. Roxadustat, a novel drug for the treatment of anemia associated with chronic kidney disease (CKD), acts by inhibiting the degradation of HIFs to increase their protein levels. However, the clinical use of roxadustat is frequently associated with peripheral edema, suggesting the involvement of HIFs in regulating the body's water balance possibly by modulating water reabsorption in the kidney. ***Methods:*** We first evaluated the effect of hypoxia (8% O_2) on mouse urine output. We then performed in vitro experiments using hypoxia (1% O_2) and roxadustat on mouse primary proximal tubular cells (mPTCs). The quantitative polymerase chain reaction, Western blot, and immunofluorescence were used to assess AQP1 mRNA and protein expression levels. Luciferase, Chromatin immunoprecipitation (ChIP), and electrophoretic mobility shift assay (EMSA) were used to investigate the transcriptional regulation of AQP1 by HIF1α. ***Results:*** We found that mice exposed to hypoxia (8% O_2) had significantly reduced urine volume compared to mice exposed to normoxia (21% O_2). Hypoxia significantly elevated AQP1 expression at both mRNA and protein levels. In vitro experiments using mouse primary cultured proximal tubular cells (mPTCs) revealed that both hypoxia and roxadustat increased AQP1 expression. Mechanistically, overexpression of HIF1α, but not HIF2α, markedly increased AQP1 protein expression. Furthermore, the upregulation of AQP1 by hypoxia and roxadustat can be blocked by the HIF1α inhibitor PX-478 in mPTCs. Finally, we found that the AQP1 gene promoter contains a putative hypoxia response element and confirmed that AQP1 is a target gene of HIF1α

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using Luciferase reporter, ChIP, and EMSA assays. ***Conclusion:*** This study demonstrates that hypoxia can reduce the urine volume of mice via upregulating AQP1 expression by HIF1α in the proximal tubular epithelial cells. Our findings also suggest a potential mechanism involved in water metabolism disorders in patients with AMS and in patients with CKD receiving roxadustat treatment.

Introduction

Acute mountain sickness (AMS) is a condition characterized by nonspecific symptoms that occur when individuals ascend to high-altitude areas above 2,500 meters or when long-term residents of high altitudes ascend further. AMS is caused by hypobaric hypoxia, leading to symptoms such as nausea, headaches, and fatigue [1–3]. In severe cases, pulmonary and cerebral edema may develop [4, 5]. The occurrence of edema is related to the destruction of the body's water and electrolyte balance, which makes water metabolism and plateau acclimatization become the focus of plateau medical research. High-altitude exposure serves as a valuable model for studying human responses to hypoxia, but the impact of altitude on body water metabolism is still controversial and lacks consensus.

Study have shown that individuals with severe AMS experience significant water retention within the first 3 h of hypoxia exposure, while those without AMS tend to show significant diuresis [6]. Additionally, Hackett et al. [7] found that the incidence of peripheral edema increased with the aggravation of symptoms of AMS, and paralleled with the increase of body weight, indicating that there was a strong correlation between acute altitude sickness and fluid retention. However, other studies suggested that acute hypoxia triggers a hypoxic diuretic response, possibly due to hypoxemia, hypocapnia, and a hypoxic ventilatory response [8, 9]. Although the results of these studies are not consistent, but all suggest that hypoxia can disrupt the body's water metabolism and impair urine output.

Adaptation to hypoxia in cells and tissues leads to the transcriptional induction of a series of genes involved in erythropoiesis, iron metabolism, angiogenesis, energy metabolism, and cell survival [10–12]. The key factors that mediate this response are hypoxia-inducible factor 1 (HIF1) and 2 (HIF2), which are oxygen-sensitive transcriptional factors. HIFs are heterodimeric proteins, consisting of α and β subunits. The α subunit contains an oxygen-dependent degradation domain (ODD) that is sensitive to oxygen levels during hypoxia [13]. While the β subunit lacks ODD and is unaffected by oxygen levels [14]. HIF1 and HIF2 activate the transcription of thousands of target genes, which are either shared or specific, depending on the particular target gene and the specific expression of different α subunits in cells [15]. Under normoxia, HIF1α has an extremely short half-life of less than 5 min, which is due to the rapid degradation of HIF1α protein through the ubiquitin-dependent pathway [13, 16]. The prolyl hydroxylase domain-containing proteins modify the proline residues in the ODD of HIF1α protein, enabling their interaction with von Hippel-Lindau protein. This interaction leads to ubiquitination and degradation of HIF1α by the E3 ubiquitin ligase complexes. Under hypoxic conditions prolyl hydroxylase domains are inactive, preventing hydroxylation of HIFα protein [17, 18]. Stabilized HIFα units forms a complex with HIFβ and moves into the cell nucleus to regulate transcription of its target genes [19].

Roxadustat is an orally administered HIF prolyl hydroxylase inhibitor (HIF-PHI) for the treatment of chronic kidney disease (CKD)-related anemia. This drug stabilizes the level of HIFs and subsequently increases renal erythropoietin levels in patients [20]. A meta-analysis evaluating the long-term effectiveness and safety of HIF-PHI in the treatment of CKD-related anemia noted peripheral edema as a potential side effect [21], suggesting the involvement of HIF in regulating the body's water balance and in roxadustat-related water retention.

The kidney plays a central role in maintaining the body's water homeostasis through urine production, where aquaporins promote urine concentration by reabsorbing water [22, 23]. Aquaporin-1 (AQP1) is abundantly expressed on both apical and basolateral membrane of the proximal tubular epithelial cells, as well as the thin descending limb of the loop of Henle and vasa recta, responsible for the reabsorption of about 80% of the water in primary urine [24].

In the present study, we show that hypoxia exposure reduces the urine volume of mice, and HIF1α plays a critical role in this process by increasing the transcription of the AQP1 gene. This finding provides valuable insights into the adaptation of the kidney to acute hypoxic stress and the role of HIF1α in body fluid balance at high altitudes. It also provides an attractive target for the treatment of water balance disorders related to systemic or renal hypoxia.

Materials and Methods

Animals

Mice were housed under controlled temperature and humidity with a 12:12-h light-dark cycle, and had unlimited access to tap water and standard rodent chow.

Experiments were performed with male mice aged 8 weeks. The mice were divided into two groups: a normoxic group (21% oxygen) and a hypoxic group (8% oxygen). The hypoxic environment was achieved using a normal-pressure oxygen chamber where nitrogen was injected continuously under electronic control to maintain oxygen levels at 8%.

Urine Collection and Analysis

Mice were housed in individual metabolic cages (Ttecniplast, Italy) with free access to water and food for collection of 24-h urine. Body weight, urine excretion, and water consumption were measured. Mice underwent a 24-h adaptation period in individual metabolic cages before measurement. Urine samples were centrifuged at 3,000 *g* at 4°C for 5 min before the supernatant was collected for analysis. Osmotic pressure was determined using a freezing point depression osmometer (Micro-Osmometer 3300).

Primary Culture of Mouse Proximal Tubular Epithelial Cells

The mouse kidneys were placed in Hank's solution to separate the renal cortex and the medulla. Renal cortices were sliced into pieces of ~1 mm wide and then digested in Hank's solution containing 0.75 mg/mL of collagenase IV and 0.75 mg/mL of trypsin inhibitor at 37°C for 1 h. After digestion, the supernatant was sieved through two nylon sieves (pore size 70 μm and 40 μm). Cellular debris and glomeruli were discarded. The filtrate containing proximal tubule fragments was centrifuged at 1,000 rpm for 3 min, washed, and then resuspended in DMEM/F12 containing 10% FBS. The proximal tubule fragments were seeded onto 6-well plates and left unstirred for 48 h at 37°C and 95% air-5% CO_2 in a standard humidified incubator.

Primary Culture of Rat Proximal Tubular Epithelial Cells

The primary culture method of rat proximal tubular cells was basically the same as that of mice. The difference was that the supernatant of the digested rat kidney was passed through two nylon sieves with different pore sizes (100 μm and 70 μm).

RNA Extraction and Real-Time PCR

Total RNA was extracted from kidney tissue or cell samples using Trizol reagent (Vazyme, Nanjing, Cat#.R401) following standard protocols. RNA was reverse transcribed to cDNA using the FastKing RT Kit with gDNase (TIANGEN, Beijing, Cat#.KR116). Real-time quantitative polymerase chain reaction (qPCR) was performed using the LightCycler 480 system (Roche) with reagents from Takala (Cat#.RR820A). The sequences of the primers were as follows:

Mouse AQP1 primer: forward, 5′-TACATCATC GCCCAGTGTGT-3′ and reverse, 5′-TGCAGAGTG CCAATGATCTC-3′; Mouse β-actin primer: forward, 5′-TGTTACCAACTGGGACGACA-3′ and reverse, 5′-GGGGTGTTGAAGGTCTCAAA-3′; Rat AQP1 primer: forward, 5′-TGCAGAGTGCCAATGATCTC-3′ and reverse, 5′-GGCATCACCTCCTCCCTAGT-3′; Rat β-actin primer: forward, 5′-CTTTCTACAATGAGCTGC GTG-3′ and reverse, 5′-TCATGAGGTAGTCTGTCA GG-3′.

Western Blot Analysis

Quick-frozen kidney tissue or cell samples were lysed using the RIPA buffer (Millipore, Bedford, MA, USA) supplemented with phosphatase and protease inhibitors. Following centrifugation, the supernatant containing proteins was collected for denaturation. Subsequently, electrophoresis was performed on a 10% sodium dodecyl sulfate-polyacrylamide gel, followed by transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membrane was blocked with 5% BSA and incubated overnight with primary antibodies, including anti-AQP1 (ab9566, Abcam, USA), anti-HIF1α (NB100-105, Nouvs, USA), and anti-HIF2α (NB100-122, Nouvs, USA). On the following day, the PVDF membrane was probed with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h and visualized using a chemiluminescence detection method. The density of the immunoreactive bands was quantified using ImageJ software.

Immunofluorescence Staining

Mouse kidney tissues were fixed in 4% paraformaldehyde and embedded in paraffin 3 μm thick sections were prepared from the paraffin-embedded tissues for dewaxing. Antigen retrieval involved a 15-min incubation at 95°C in citric acid-based antigen repair buffer (Servicebio, Wuhan, China). Tissue autofluorescence was quenched by applying Quencher A liquid (Servicebio, Wuhan, China), followed by a 30-min room temperature incubation and a 5-min rinse with distilled water. The sections were incubated with 5% BSA (in PBS) at room temperature for 1 h. After overnight incubation with an AQP1 antibody (Sc-25287, Santa Cruz, USA) at 4°C, sections were washed and incubated with

Alexa 488-labeled secondary antibody at room temperature for 1 h. After rinsing, tissues were treated with autofluorescence quencher B liquid (Servicebio, Wuhan, China), incubated in the dark at room temperature for 5 min then washed under running water for 3 min. A fluorescence quencher (Servicebio, Wuhan, China) containing DAPI was applied to mount the slides for microscopic examination.

Immunohistochemical Staining

Mouse kidney specimens were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 3 μm thickness were cut and prepared for antigen retrieval and peroxidase quenching. After blocking with 5% BSA, tissue sections were incubated overnight at 4°C with an AQP1 antibody (Sc-25287, Santa Cruz, CA, USA). The sections were then incubated with a secondary antibody, followed by development with DAB substrate. Finally, the nuclei were counterstained with hematoxylin, and the slides were mounted using neutral resin.

Luciferase Assay

Genomic DNA was extracted from mouse kidney tissues using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, Cat#.DP304). PCR was used to amplify a 1,580 bp fragment of the mouse AQP1 promoter using specific primers (Forward primer: 5′-CCG AGCTCTTACGCGCAGCACACTCAAACAGTAGGC A-3′, Reverse primer: 5′-GATCGCAG′ATCTCGAAG TGTGACCGCAGGGTTG-3′). The 1,580 bp fragment was cloned into the pGL3 luciferase vector to obtain an AQP1 gene promoter-driven luciferase reporter (AQP1-Luc). All plasmid constructs were confirmed by DNA sequencing. In dual luciferase assays, the AQP1-luc reporter, along with HIF1α and Renilla luciferase plasmids, was co-transfected into 293T cells using Lipofectamine™ 3000 (Thermo Scientific, USA, Cat#.l3000001), following the manufacturer's instructions. Luciferase activity was assessed using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocol.

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assay

In this experiment, 293T cells were cultured and transfected with expression plasmids. Nuclear proteins were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, USA, Cat.#78833) and verified via Western blot. Extracts were stored at −80°C for further use. Utilizing computer searches and promoter scanning software, the potential binding sites (HRE) in the AQP1 promoter for HIFs were identified. Based on these sites, an oligonucleotide probes (30bp) for electrophoretic mobility shift assay (EMSA) were designed and the sequences were as follows (Forward primer: 5′-TCCTGTTTCTGACGTGCT GTTTTTTCCTGT-3′, and Reverse primer: 5′-ACA GGAAAAAACAGCACGTCAGAAACAGGA-3′). Biotinylation was performed using the Biotin 3′ End DNA Labeling Kit (Thermo Scientific, USA, Cat.#89818). EMSA was conducted according to the manufacturer's protocol using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, USA, Cat. #20148). Each EMSA reaction (20 μL) contained 10 μg of nuclear extract protein, biotin-labeled double-stranded probes, 1 μg poly-dI/dC, and 2 μL of 10× reaction buffer. The reaction mix was incubated at room temperature for 20 min, followed by separation of the protein-DNA complexes using a 6% non-denaturing polyacrylamide gel. The complexes were then transferred to a nylon membrane. For detection, the membrane was exposed to ultraviolet light and subsequently detected using streptavidin-horseradish peroxidase conjugate. The membrane was incubated with a chemiluminescent substrate for signal detection. In competition assays, unlabeled competitive oligonucleotides were pre-incubated with the labeled probe at a 200-fold excess.

Chromatin Immunoprecipitation

The chromatin was prepared using the Activemotif commercial kit as per the manufacturer's instructions. Following enzymatic digestion, proceed with the chromatin immunoprecipitation (ChIP) assay by adding magnetic beads and antibodies (using IgG as a control antibody and HIF1α as a target antibody) to the digested chromatin and incubate overnight. Subsequently, the complex was purified to isolate DNA. The ChIP assay was performed using a AQP1 gene promoter primer set: Forward primer (5′→3′): CAGGAAACATGGTGGGTG CT; Reverse primer (5′→3′): TCAGTCACTAGCATC ACAGGAA. The resulting amplified product includes the predicted HIF binding site ACGTG (−1,047/−1,043) on the AQP1 gene promoter.

Statistical Analysis

The data were statistically analyzed using GraphPad Prism software and are presented as mean ± standard error of the mean. Statistical analysis for single comparisons was conducted using the Student's *t* test, while comparisons among multiple sample groups were performed using one-way ANOVA. Statistically, significance was set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

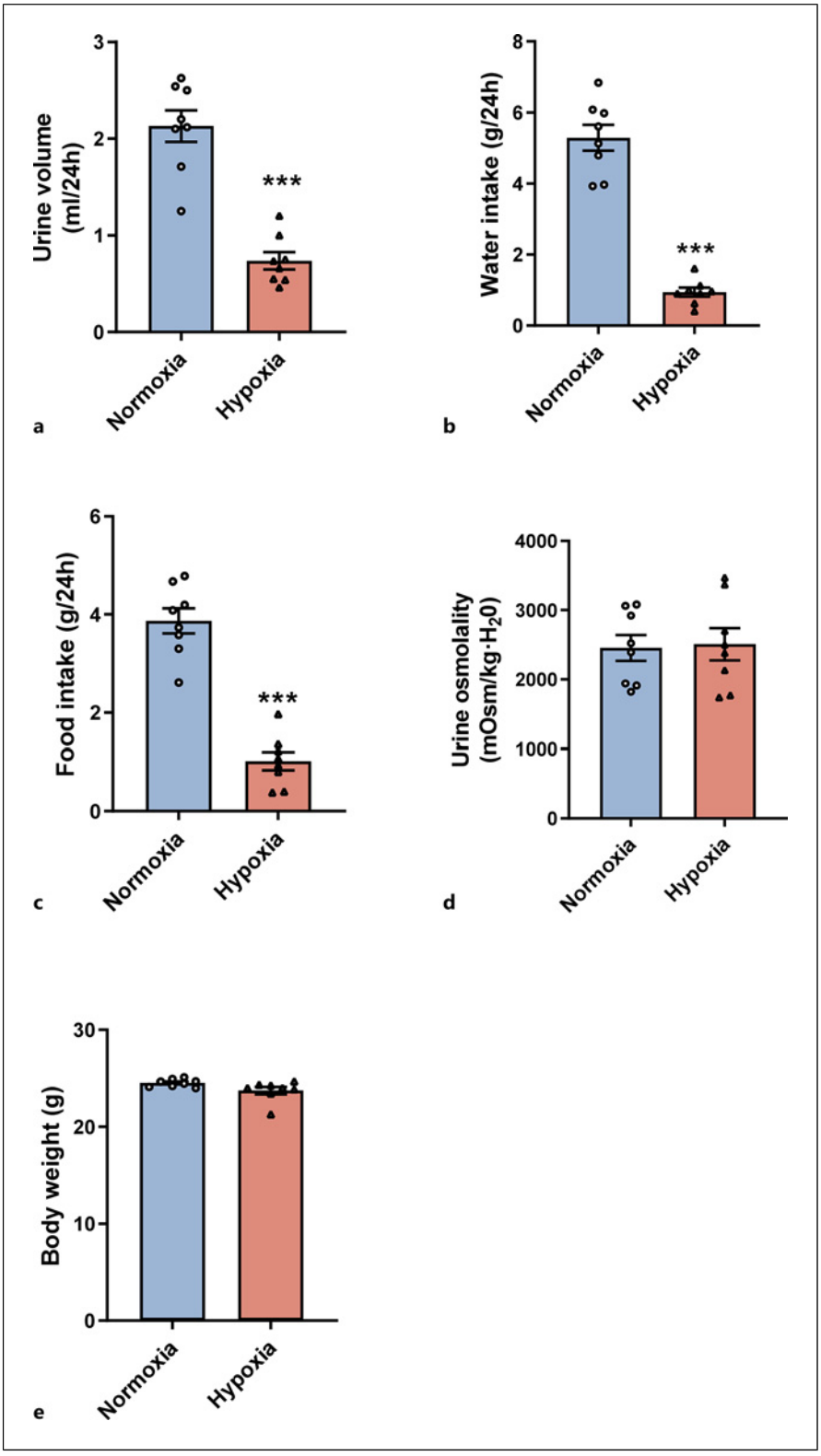


Fig. 1. Effects of hypoxia on mouse urine volume and osmolality. Male C57BL/6J mice aged 8 weeks were exposed to normoxic (21% O_2) or hypoxia (8% O_2) for 24 h. **a** Urine volume. **b** Water intake. **c** Food intake. **d** Urine osmolality. **e** Body weight. Results are presented as mean ± standard error, with statistical significance indicated as $^{***}p < 0.001$, $n = 8$.

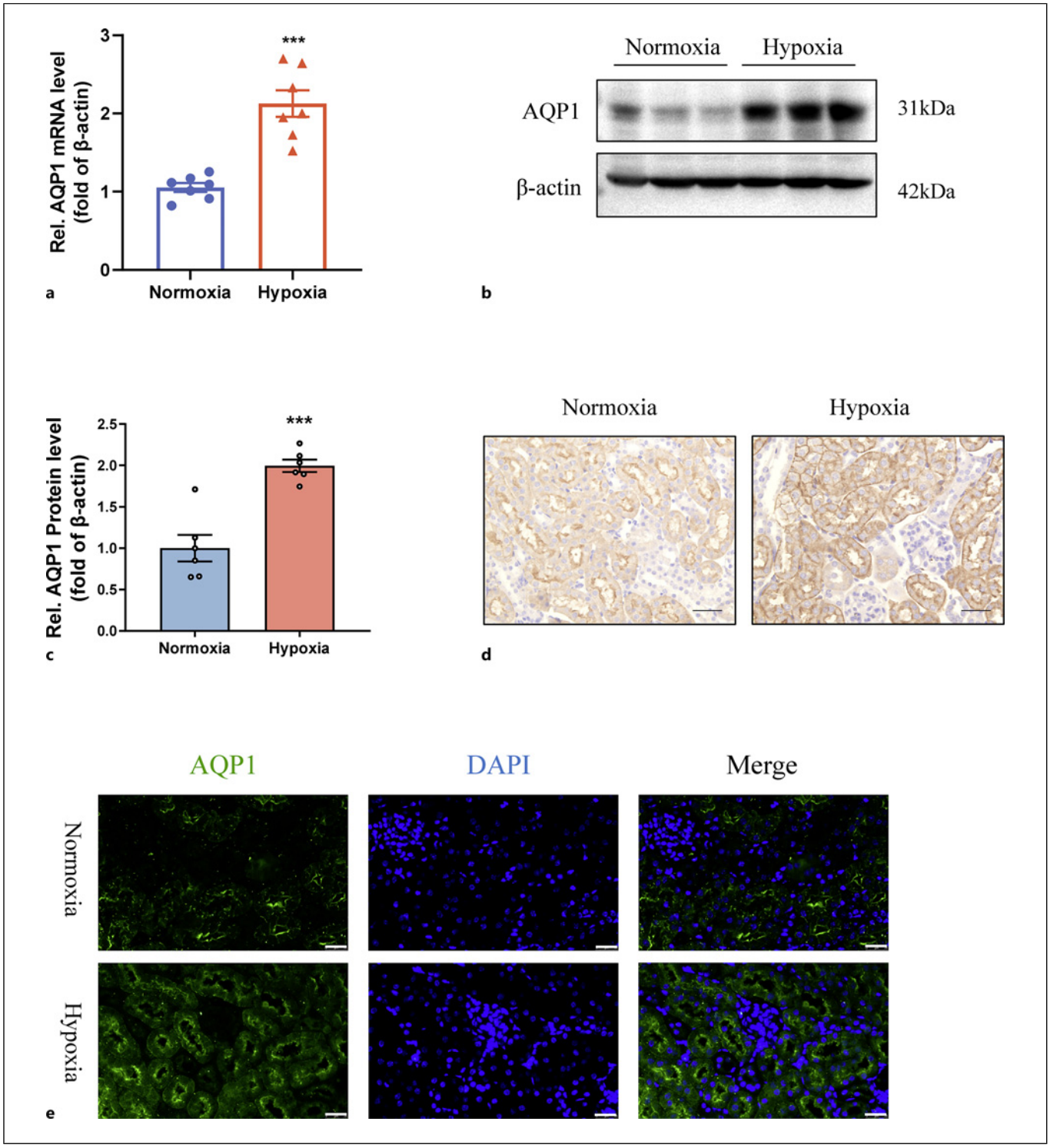


Fig. 2. Hypoxia increases AQP1 expression in mouse kidney. Mice were treated with hypoxia (8% O_2) for 24 h. **a** qPCR was used to detect the mRNA levels of AQP1 in mouse kidney tissues. **b** Western blot was conducted to assess the protein levels of AQP1 in mouse kidney tissues. **c** Quantitative analysis of AQP1 protein levels. **d** The representative images of immunohistochemistry staining showing the protein expression of AQP1 in mouse kidney tissues. **e** The representative images of immunofluorescence staining showing the protein expression of AQP1 in mouse kidney tissue. Scale bar = 25 μm. Results are presented as mean ± standard error, with statistical significance indicated as ***$p < 0.001$, $n = 6–7$.

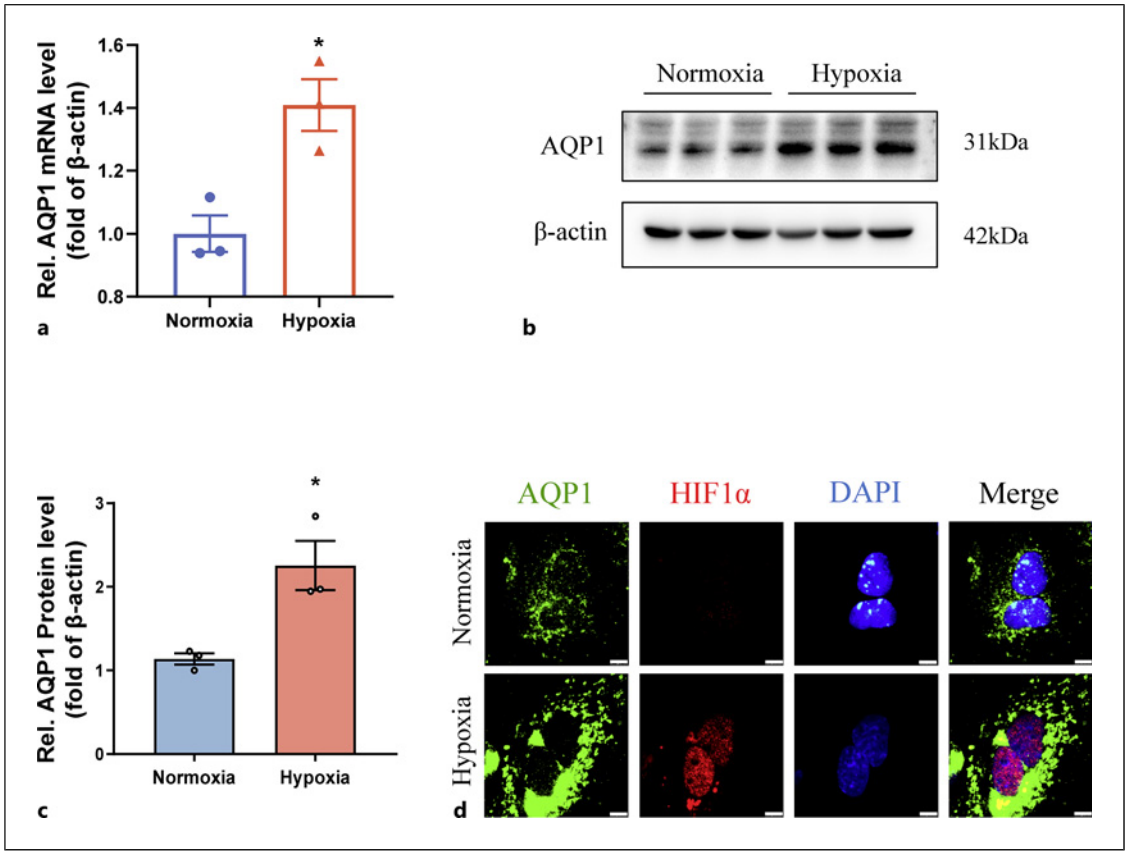


Fig. 3. Hypoxia increases AQP1 expression in mouse primary proximal tubular cells (mPTCs). The cells were treated with hypoxia (1% O_2) for 6 h. **a** qPCR was used to detect the mRNA levels of AQP1. **b** Western blot was conducted to assess the protein levels of AQP1. **c** Quantitative analysis of AQP1 protein levels in **b**. **d** The representative images of immunofluorescence staining showing AQP1 and HIF1α protein expression and subcellular localization. Scale bar = 7.5 μm. Results are presented as mean ± standard error, with statistical significance indicated as $^*p < 0.05$, $n = 3$.

Results

Hypoxia Exposure Reduces Urine Output in Mice

To assess the effect of hypoxia on urinary concentration, 8-week-old male C57BL/6J mice were allocated into normoxic (21% oxygen) and hypoxic (8% oxygen) groups and exposed to respective environments for 24 h. Mice were housed in the metabolic cages for 24 h with free access to water. Hypoxic treatment resulted in a significant decrease in urine volume compared to the normoxic group (Fig. 1a). Water intake and food intake were also significantly reduced in the hypoxic mice compared to that in the normoxic mice (Fig. 1b, c). No significant differences were observed in urine osmolality (Fig. 1d) and body weight (Fig. 1e) between two groups. Hypoxia had no significant impact on urinary Na^+ or K^+ concentration but reduced 24-h urinary Na^+ and K^+ excretion (online suppl. Fig. S1A–D; for all online suppl. material, see https://doi.org/10.1159/000542087). There was no significant difference in urinary pH levels (online suppl. Fig. S1E) between two groups. These results suggest that hypoxia exposure reduces urine output possibly by stimulating urinary concentrating ability.

Hypoxia Exposure Increases AQP1 Expression in Mouse Kidney

Given that AQP1 controls water reabsorption in the proximal tubules, the descending thin limbs of Henle, and the descending vasa recta, responsible for the reabsorption of about 80% of the water in primary urine, the effect of hypoxia on the expression of AQP1 in the kidney was determined. We treated mice with hypoxia (8% O_2) for 24 h. The effectiveness of hypoxia was verified by hematological analysis, which showed that the number of polychromatic erythrocytes and hematocrit levels were significantly increased in hypoxic mice (online suppl. Fig. S2A, B). Moreover, HE staining of mouse kidney tissues

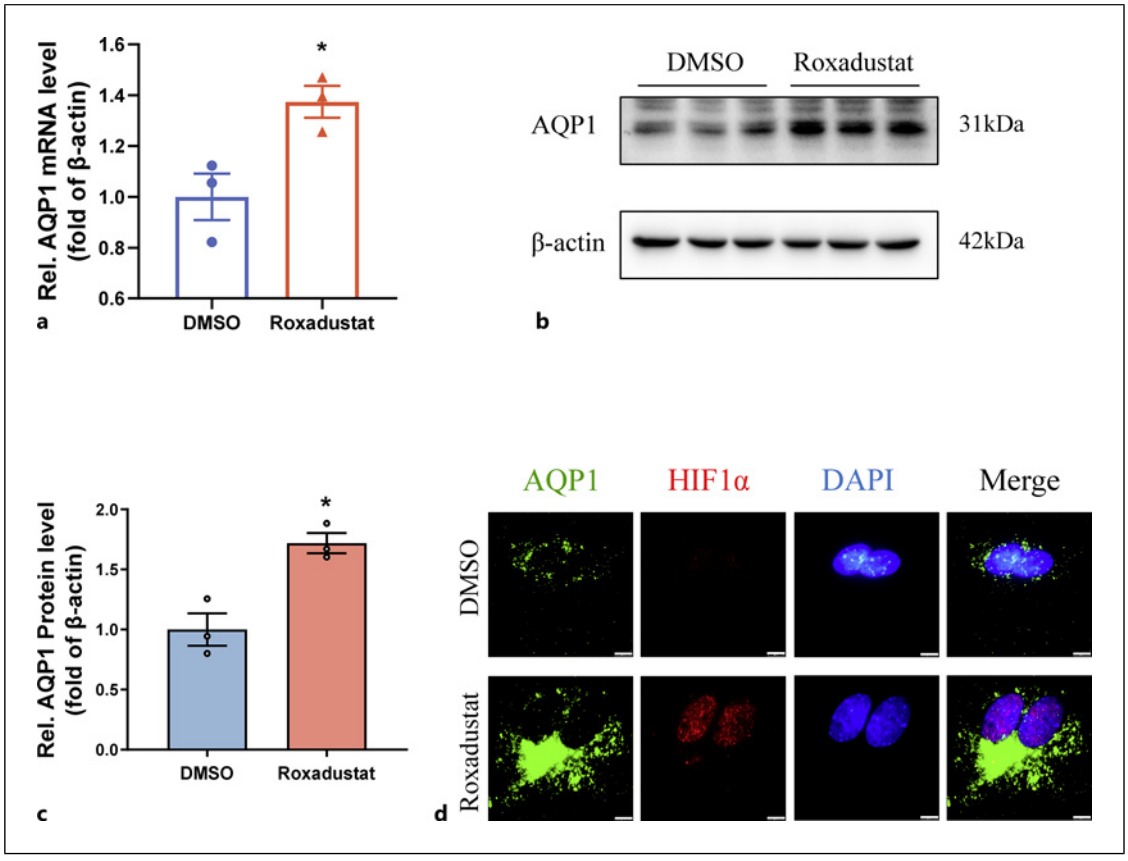


Fig. 4. Roxadustat increases AQP1 expression in mPTCs. The cells were treated with 20 μM roxadustat for 6 h. **a** qPCR was used to detect the mRNA level of AQP1. **b** Western blot was conducted to assess the protein levels of AQP1. **c** Quantitative analysis of AQP1 proteins in **b**. **d** The representative images of immunofluorescence staining showing AQP1 and HIF1α protein expression and subcellular localization. Scale bar = 7.5 μm. Results are presented as mean ± standard error, with statistical significance indicated as $*p < 0.05$, $n = 3$.

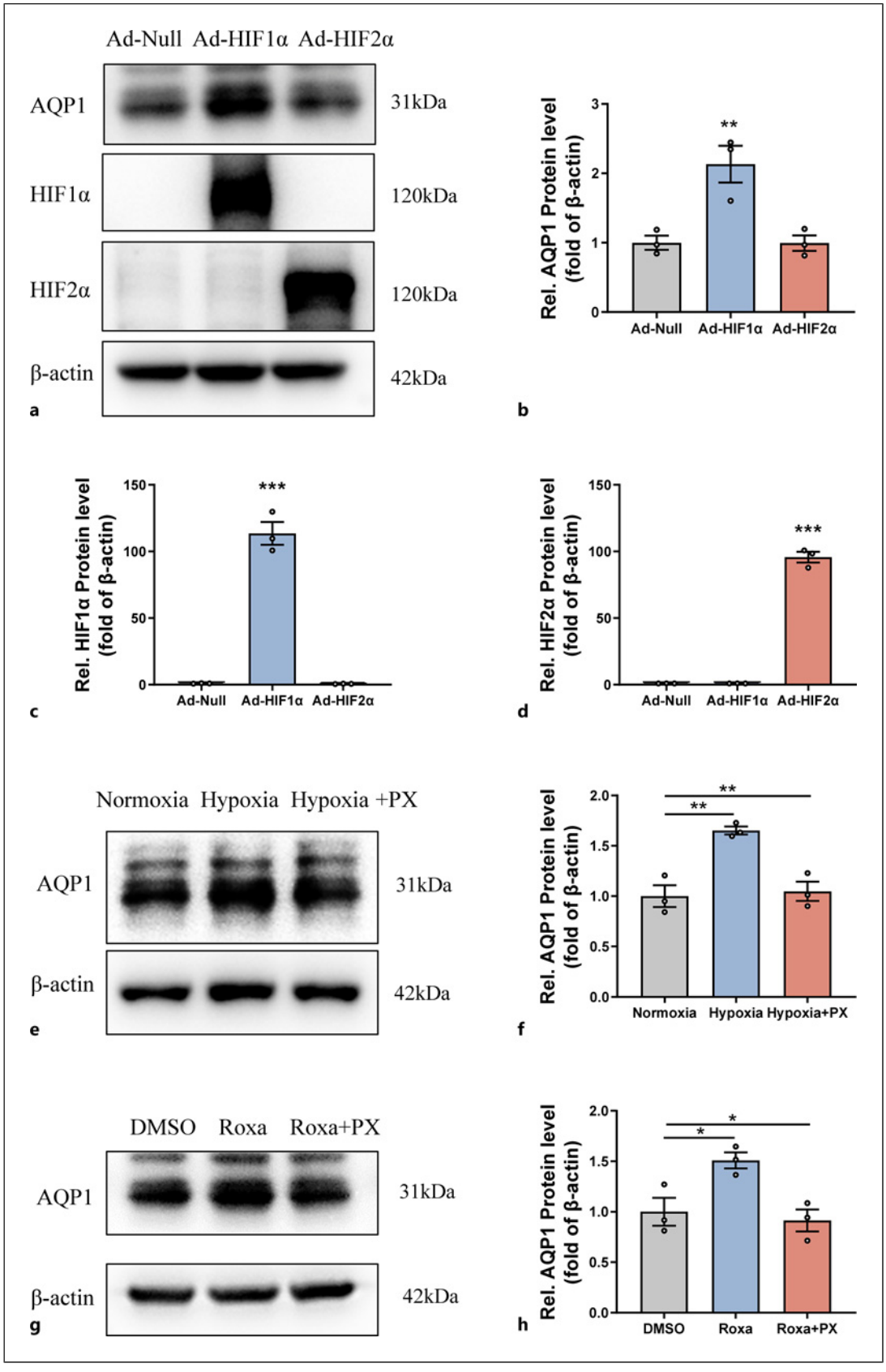
revealed that hypoxia did not cause morphological abnormalities (online suppl. Fig. S3A, B). By qPCR and Western blot assays, we found that hypoxia exposure for 24 h significantly increased AQP1 mRNA and protein expression (Fig. 2a–c). The results of immunofluorescence and immunohistochemistry also showed that hypoxia promoted the expression of AQP1 protein in mouse kidneys (Fig. 2d, e).

Hypoxia Increases AQP1 Expression in Primary Cultured Mouse Proximal Tubule Cells

Primary cultured mouse proximal tubular cells (mPTCs) were used to further validate the upregulation of AQP1 by hypoxia in vitro. We treated mPTCs with hypoxia (1% O_2) for 6 h. qPCR and Western blot analysis revealed an increase in both mRNA and protein levels of AQP1 with hypoxia exposure (Fig. 3a–c). Immunofluorescence results further showed that hypoxia increased AQP1 expression and induced nuclear localization of HIF1α (Fig. 3d), implying a potential regulatory role of HIF1α in AQP1 expression under hypoxic conditions. In addition, we used rat primary proximal tubule epithelial cells (rPTCs) for experiments and found similar results showing that hypoxia can elevate both mRNA and protein levels of AQP1 (online suppl. Fig. S4). Together, these findings demonstrate that hypoxia can induce AQP1 expression in cultured proximal tubule cells possibly via increasing HIF1α activity.

Roxadustast Increases AQP1 Expression in the Proximal Tubular Cells

Roxadustat is clinically used to treat renal anemia by stabilizing the HIF complex. We treated mPTCs with roxadustat for 6 h. Similar to hypoxia exposure, roxadustat up-regulated AQP1 expression at both mRNA and protein levels (Fig. 4a–c). Immunofluorescence



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results indicated an elevated AQP1 expression and HIF1α nuclear localization in mPTCs (Fig. 4d). Similarly, the experimental results in rPTCs also showed that roxadustat had the ability to upregulate AQP1 mRNA and protein levels (online suppl. Fig. S5). These results demonstrate that roxadustat can enhance AQP1 expression in cultured proximal tubule cells likely through stabilizing HIF1α protein levels.

The Upregulation of AQP1 Expression by Hypoxia Is Dependent on HIF1α

The above results demonstrate that hypoxia exposure and roxadustat treatment can increase AQP1 expression in renal proximal tubule cells. To determine which isoform mediates hypoxia-induced AQP1 upregulation, we infected mPTCs with HIF1α- and HIF2α-expressing adenoviruses respectively and found that only HIF1α adenovirus treatment increased AQP1 protein expression (Fig. 5a, b). The efficiency of adenovirus infection was shown in Figure 5c, d. Furthermore, we pretreated mPTCs with the HIF1α inhibitor PX-478 for 24 h before hypoxia exposure to determine the role of HIF1α in hypoxia-induced AQP1 expression. Western blot results revealed that PX-478 markedly blocked the upregulation of AQP1 by hypoxia (Fig. 5e, f). Similarly, PX-478 also blocked the upregulation of AQP1 by roxadustat (Fig. 5g, h). Together, these findings demonstrate that HIF1α mediates the hypoxia-induced AQP1 expression in the proximal tubule cells.

HIF1α Directly Enhances AQP1 Transcription

Sequence analysis of the promoter region of mouse AQP1 gene using the JASPAR CORE database showed a putative hypoxia response element (HRE) binding site between −1,047 bp and −1,043 bp upstream of the transcription start site (Fig. 6a), suggesting that *AQP1* gene might be directly regulated by HIF1α. Subsequently, the AQP1 promoter sequence containing the putative HRE site was inserted into a luciferase reporter vector (AQP1-Luc) and transfected into 293T cells along with an HIF1α expression vector. The result showed that HIF1α overexpression significantly increased transcription activity of mouse AQP1 gene promoter (Fig. 6b). To verify whether HIF1α could directly bind to the HRE site, ChIP assay was performed. In the basal condition, HIF1α indeed bound to the HRE site, which was further enhanced by hypoxia and roxadustat (Fig. 6c, d). Finally, to validate the specificity of the predicted binding site in the AQP1 gene promoter, EMSAs were performed using the nuclear extracts from HIF1α-overexpressing 293T cells. EMSA analysis showed a specific DNA-protein complex binding band (Fig. 6e, lane 2). Moreover, this binding was competitively inhibited upon the addition of a 200-fold molar excess of unlabeled AQP1 probe (Fig. 6e, lane 3), confirming the specificity of HIF1α binding to the predicted HRE in the AQP1 gene promoter. Collectively, these findings demonstrate that *AQP1* gene is a direct target gene of HIF1α and HIF1α directly enhances AQP1 gene transcription by binding to the HRE site in the AQP1 gene promoter.

Discussion

In the present study, we aimed to explore the effect and mechanism of hypoxia in the regulation of renal water reabsorption. Numerous studies have demonstrated that hypoxia alters body water homeostasis. Several studies reported that rapid ascent to high altitudes is associated with the occurrence of peripheral edema, decreased urine volume, and weight gain [7, 25–27]. However, some other studies suggested that acute hypoxia triggers a hypoxic diuretic response, possibly due to hypoxemia, hypocapnia, and a hypoxic ventilatory response [8, 9]. Since the maintenance of body fluid homeostasis involves multiple systems such as cardiovascular system, urinary system and respiratory system, and most of these studies were performed in human, making it impossible for monitoring many parameters at the same time. Therefore, to define the role of hypoxia on renal water reabsorption, we carried out animal and cell culture experiments. Here, we show

Fig. 5. HIF1α increases AQP1 expression in mPTCs. **a** mPTCs were infected with HIF1α, HIF2α, and null adenoviruses for 24 h, respectively. Western blot was conducted to assess the protein levels of AQP1, HIF1α, and HIF2α. **b** Quantitative analysis of AQP1 protein levels in **a**. **c** Quantitative analysis of HIF1α protein levels in **a**. **d** Quantitative analysis of HIF2α protein levels in **a**. **e** mPTCs were pretreated with the HIF1α inhibitor PX-478 for 24 h and then exposed to hypoxia for 6 h. Western blot was conducted to assess the protein levels of AQP1. **f** Quantitative analysis of AQP1 protein in **e**. **g** mPTCs were pretreated with HIF1α inhibitor PX-478 for 24 h and then treated by roxadustat for 6 h. Western blot was conducted to assess the protein levels of AQP1. **h** Quantitative analysis of AQP1 protein levels in **g**. Results are presented as mean ± standard error, with statistical significance indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$.

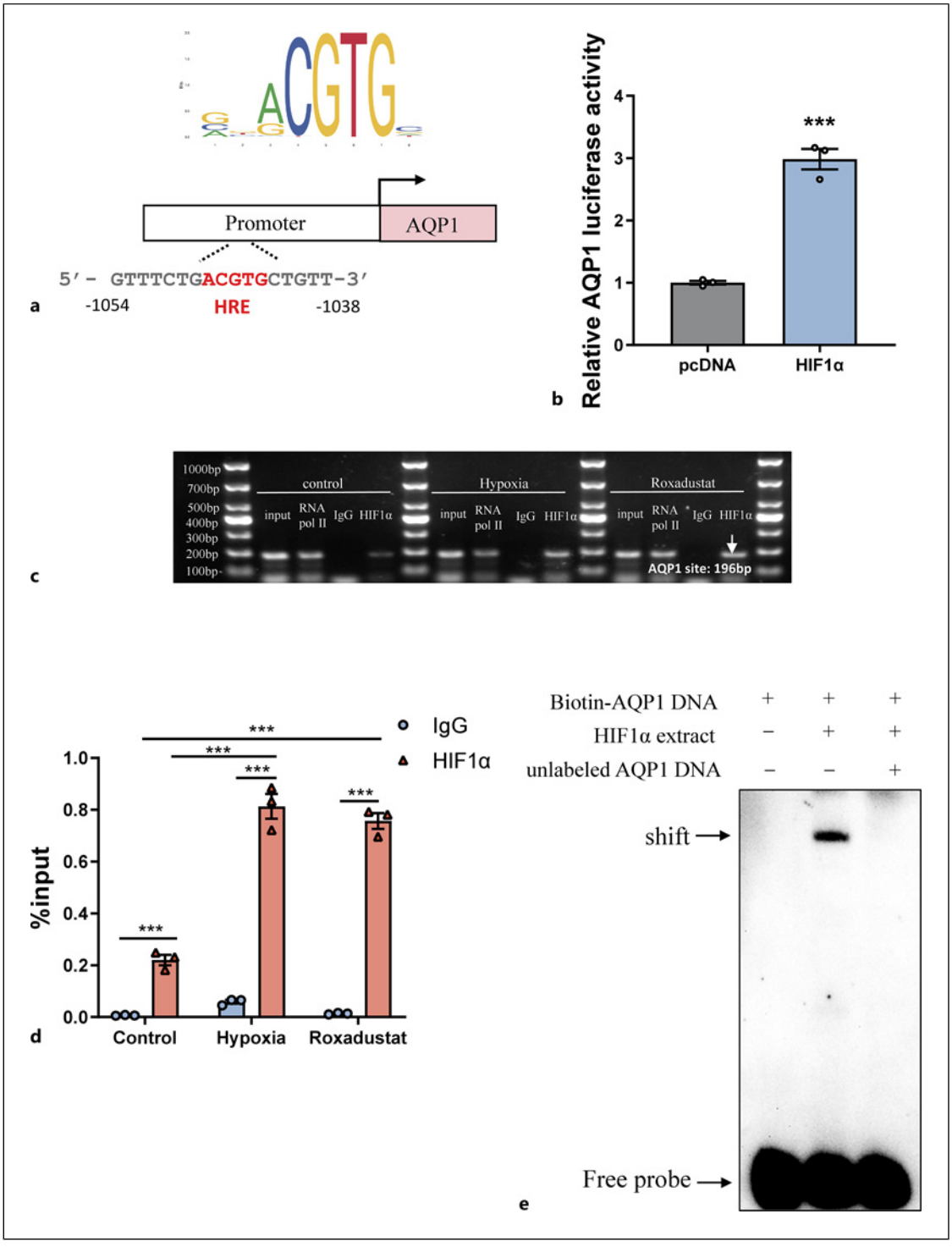

Fig. 6. HIF1α directly enhances AQP1 transcription. **a** Schematic representation of the predicted HIF1α binding site in the promoter region of mouse AQP1 gene. **b** Effect of HIF1α overexpression on AQP1 gene promoter-driven luciferase reporter gene activity. **c** ChIP agarose electrophoresis showing the binding of HIF1α to the AQP1 promoter region. **d** ChIP-qPCR analysis comparing HIF1α and IgG binding in mPTCs under normoxia, hypoxia, and roxadustat treatment. **e** EMSA assay demonstrating direct binding of HIF1α to the predicted HRE site in the AQP1 gene promoter region. Results are presented as mean ± standard error, with statistical significance indicated as ***$p < 0.001$, $n = 3$.

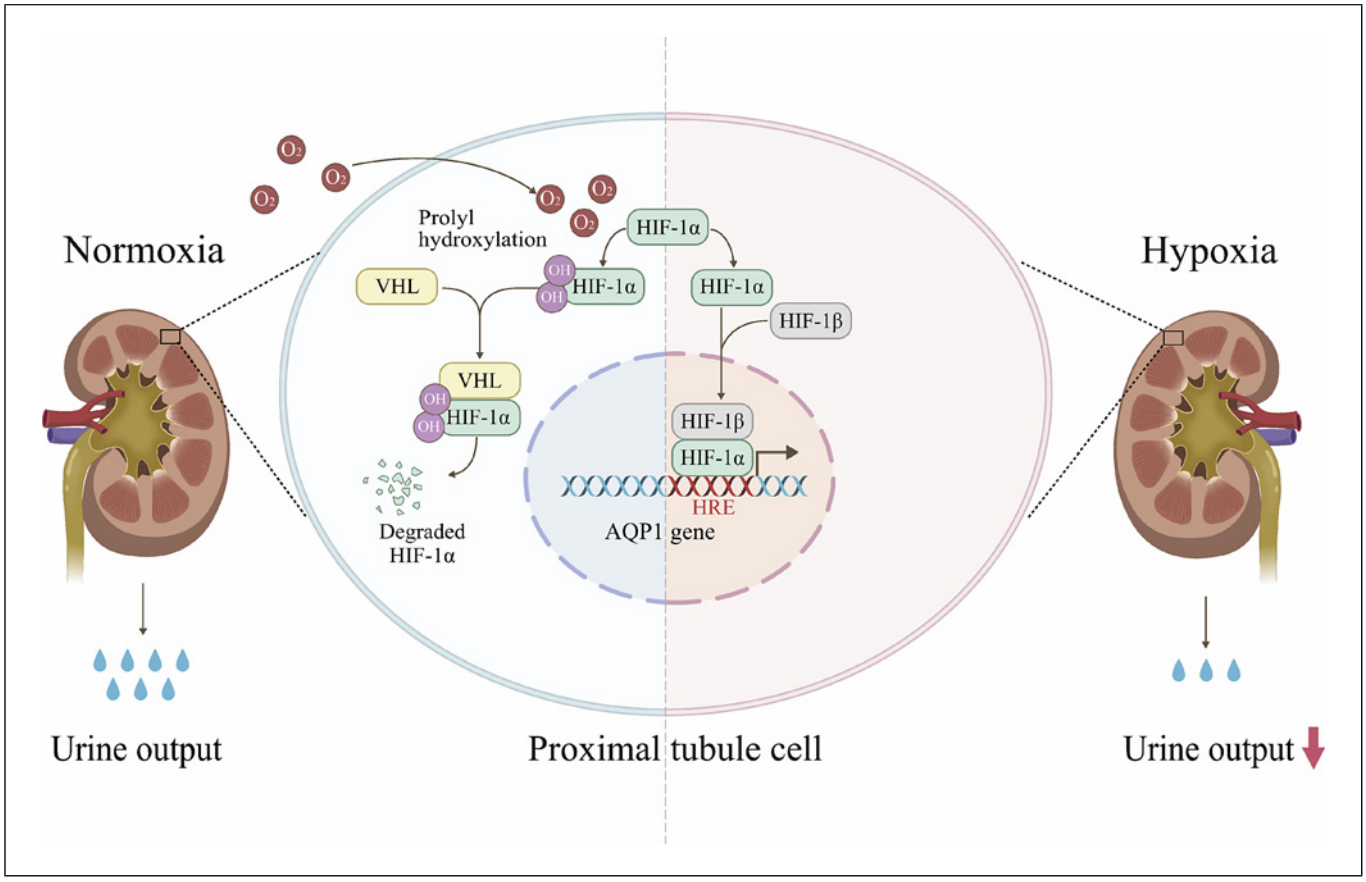

Fig. 7. Proposed mechanisms by which hypoxia reduces urine output through the HIF1α/AQP1 pathway. Upon hypoxic exposure, the degradation of HIFα protein ceases, and HIF1α and HIF1β form heterodimers which translocate into the nucleus to initiate the transcription of AQP1.

that hypoxia (8% O_2) exposure for 24 h significantly decreased 24-h urine volume in mice, with a marked increase in AQP1 expression in the proximal tubule cells. We further demonstrate that hypoxia exposure markedly enhances the AQP1 expression in an HIF1α-dependent manner.

The kidney is a key organ in maintaining water and solute homeostasis. Each day, an adult human produces ~1.5 L of urine despite of 180 L of fluid filtered through glomerular basement membrane. Approximately, 90% of glomerular filtrate is constitutively reabsorbed in the proximal tubules and descending limb of the loop of Henle through AQP1 [22, 28]. The proximal tubules are the most metabolically active part of the kidney, with AQP1 being the major channel for water reabsorption. AQP1 is expressed on both apical and basolateral membrane of the proximal tubule cells, as well as the thin descending limb of the loop of Henle and vasa recta. It has been reported that mice lacking AQP1 exhibited significantly impaired urine concentration function, evidenced by decreased basal urine osmotic pressure [29]. Similarly, urinary concentration deficits exist in humans with AQP1 gene mutations [30]. Therefore, the AQP1 is the major water channel responsible for the reabsorption of about 80% of water in the primary urine along the proximal tubules. In the present study, we showed that hypoxia can increase the expression of AQP1 mRNA and protein both in vivo and in vitro, suggesting that under hypoxia conditions, increased expression of AQP1 facilitates water reabsorption in the proximal tubules, leading to the reduction of urine volume in mice.

It is well known that in the kidney, there are four major AQPs responsible for water reabsorption, namely AQP1-4. As previously mentioned, AQP1 is expressed on both apical and basolateral membrane of the proximal tubule cells, as well as the thin descending limb of the loop of Henle and vasa recta. AQP2-4 is expressed predominantly in the principal cells of the

collecting ducts, responsible for about 20% of the reabsorption of filtered water. AQP2 is the predominant water channel on the apical plasma membrane, and AQP3 and AQP4 are located on the basolateral plasma membrane. Although the present study addressed the role of hypoxia in renal AQP1 expression, we could not rule out the possibility that hypoxia may also affect the expression of AQP2-4, contributing to hypoxia-associated dysregulation of water homeostasis. This issue warrants further investigation.

The α subunits of HIFs include HIF1α, HIF2α, and HIF3α. Among them, HIF1α and HIF2α are major regulators in modulating many gene expression in response to hypoxia. HIFs are heterodimeric proteins, which are composed of one of three O_2-dependent α subunits (HIF1α, HIF2α, and HIF3α) and a constitutively expressed O_2-insensitive subunit (HIF1β) [31, 32]. In the kidney, HIF1α is broadly distributed across various segments of renal tubules, whereas HIF2α is predominantly found in endothelial cells and myofibroblasts [33]. Several previous studies reported that HIF1α can regulate AQPs expression in other tissues or cells. Hoogewijs et al. [34] discovered a conserved HIF binding sites within close proximity to the translational start site of the AQP3 gene promoter, and identified that AQP3 is a HIF1α target gene in L929 fibrosarcoma cells. In the cerebellum of hypoxic mice, increased expression of HIF-1α correlates with upregulation of AQP4 mRNA and protein levels [35, 36]. In the present study, we explored whether the increase in AQP1 expression induced by hypoxia relies on HIF1α. We found that blocking HIF1α with a specific inhibitor markedly inhibited the upregulation of AQP1 induced by hypoxia and roxadustat, an HIF-stabilizing agent used to treat anemia, in mouse proximal tubule cells. In addition, infection of cells with the adenoviruses encoding HIF1α or HIF2α led to a selective increase in AQP1 protein expression by HIF1α, but not by HIF2α. Moreover, the luciferase, ChIP, and EMSA experiments all affirmed the direct involvement of HIF1α in the transcriptional regulation of AQP1, revealing that AQP1 is a novel target gene of HIF1α in the kidney.

The prevalence of AMS ranges from 40 to 90 percent, with high-altitude cerebral edema as the fatal end stage of severe AMS. It has been reported that timely hypoxic pre-acclimatization has ameliorative and preventive effects on AMS [37–41]. It would be important to test whether roxadustat treatment can simulate hypoxic pre-acclimatization in preventing the development of AMS. In addition, roxadustat, as a drug utilized clinically to treat renal anemia in CKD patients, frequently leads to peripheral edema [21]. Our findings that rosaduxtat mimics the effect of hypoxia on renal AQP1 expression indicate that increased expression of renal AQP1 is at least in part responsible for roxadustat-related water retention. Targeting renal AQP1 may help alleviate the adverse effect of roxadustat on body water homeostasis.

In conclusion, our study showed that hypoxia markedly reduces urine output with an increased AQP1 expression in the proximal tubule cells. Under hypoxic conditions, HIF1α, which is constitutively expressed in renal proximal tubule cells, promotes AQP1 gene transcription, thereby increasing water reabsorption, and ultimately leading to decreased urine volume in mice (Fig. 7). Our findings support a critical role of HIF1α in regulating AQP1 expression in renal proximal tubules and suggest HIF1α as a potential contributor to hypoxia-induced water retention.

Statement of Ethics

All experiments were reviewed and approved by the Animal Care and Use Review of Dalian Medical University (AEE22128).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.Q. and X.C.: designed the study and performed the experiments and wrote the manuscript. Y.H. and H.W.: participated in animal experiments and made the figures H.X.: analyzed the data. C.Z., C.D., J.C., W.M., Y.L., and Y.Q.: participated in vitro experiments. Y.G. and X.Z. designed and supervised the work and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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