Effect of deubiquitinase USP8 on hypoxia/reoxygenation-induced inflammation by deubiquitination of TAK1 in renal tubular epithelial cells

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Abstract. Intermittent hypoxia/reoxygenation (IHR), characterized by repeated episodes of hypoxia interspersed with periods of reoxygenation, has been found to induce pro-inflammatory cytokine production and is increasingly recognized as a major pathophysiological factor in various disease processes with distinct cell and molecular responses. The present study is the first, to the best of our knowledge, to investigate the effects of ubiquitin-specific peptidase 8 (USP8) on IHR-induced inflammation in renal tubular epithelial cells and examine the underlying mechanism. Following transfection of plasmids, HK-2 and TCMK-1 cells were incubated for eight cycles of IHR treatment including 3 h of hypoxic incubation followed by 3 h of normoxic culture. It was demonstrated that the expression of USP8 was decreased in IHR conditions but not in normoxic or continuous hypoxic conditions. In addition, IHR-induced inflammation was suppressed in USP8-overexpressing renal tubular epithelial cells and the silencing of USP8 markedly aggravated inflammation. Furthermore, it was found that the overexpression of USP8 inhibited the IHR-induced activation of nuclear factor-xB and it was demonstrated that USP8 interacted with transforming growth factor-β-activated kinase-1 (TAK1) and deubiquitinated the K63-linked ubiquitination of TAK1. Taken together, the results demonstrated the role of USP8 in IHR-induced inflammation and suggested USP8 as a potential and specific therapeutic target for IHR-related diseases.

Introduction

Intermittent hypoxia/reoxygenation (IHR) has been reported to contribute to the pathogenic process of obstructive sleep apnea (OSA); it also appears to be involved in promoting resistance to radiotherapy and chemotherapy in solid tumors, and is encountered in a wide range of respiratory and cardiac disorders, including chronic obstructive pulmonary disease and congestive cardiac failure (1-5). During these disease processes, IHR is always associated with increased reactive oxygen species (ROS) production, oxidative stress, and inflammation (6,7). Emerging evidence indicates that chronic kidney disease is a prevalent complication of untreated OSA with symptoms of polyuria and proteinuria (8,9). However, the precise mechanisms between these two diseases remain to be fully elucidated.

In contrast to the adaptation effect in response to continuous hypoxia (CH) through hypoxia-inducible factor 1 (HIF-1) signaling, the body undergoes tissue damage and cellular inflammation, mainly caused by nuclear factor-xB (NF-xB) signaling, following IHR challenge (10). NF-xB is considered the main pro-inflammatory family of transcription factors (11,12), NF-xB is released consequently and translocates to the nucleus where it activates the expression of NF-xB-related inflammatory genes (13). In this process, transforming growth factor-β-activated kinase-1 (TAK1) has been reported to be crucial through its kinase activity to phosphorylate and activate inhibitor of NF-xB (IxB) kinase (IKK) β, facilitating IKKβ-mediated IxB degradation and downstream activation of NF-xB (14). However, the role of TAK1 in the IHR-induced activation of NF-xB has not been reported.

Deubiquitination is catalyzed by deubiquitinating (DUB) enzymes, which are proteases that cleave ubiquitin or ubiquitin-like proteins from target proteins (15). Ubiquitin-specific peptidase 8 (USP8; also known as Ubp5) is a cysteine protease and member of the ubiquitin-specific protease family of DUB enzymes capable of catalyzing the complete breakdown of K48- and K63-linked polyubiquitin into its component monomers (16,17). USP8 has an important physiological function in cell growth, and the deletion of USP8 causes embryonic death in mice (18,19). USP8 has been reported to negatively regulate the ubiquitination of epidermal growth factor receptor, neuregulin receptor degradation protein-1, and β-site amyloid precursor protein-cleaving enzyme to regulate the process of cell proliferation, neuroinflammation and neurological disorder (20-23). However, the effect of USP8 on IHR-induced inflammation and the underlying targets in the IHR-induced

Key words: ubiquitin-specific peptidase 8, intermittent hypoxia/reoxygenation, inflammation, transforming growth factor-β-activated kinase-1

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activation of NF-κB remain to be fully elucidated. The present study demonstrated for the first time, to the best of our knowledge, that the expression of USP8 was decreased in IHR conditions but not in normoxic or CH conditions in renal tubular epithelial cells. Furthermore, the overexpression of USP8 significantly suppressed IHR-induced pro-inflammatory cytokine expression and inhibited the activation of NF-κB. It was also observed that USP8 interacted with TAK1 and deubiquitinated the K63-linked ubiquitination of TAK1, leading to the inactivation of TAK1 and NF-κB. In conclusion, the present study revealed an anti-inflammatory role of USP8 in IHR-induced inflammation and suggested USP8 as a potential target for the treatment of IHR-related diseases.

Materials and methods

Cell culture and hypoxia treatment. The HK-2 (human renal tubular epithelial cells) and TCMK-1 (murine renal tubular epithelial cells) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The hypoxia treatment was performed as described previously (24). Briefly, for the normoxic cultures, the cells were cultured in a humidified incubator (37°C, 21% O2, 74% N2 and 5% CO2) for 48 h. For the CH cultures, the cells were grown in a sealed hypoxic incubator for 48 h (37°C, 1% O2, 94% N2 and 5% CO2). For the IHR cultures, the cells were subjected to a specified number of hypoxia cycles (37°C, 1% O2, 94% N2 and 5% CO2) and reoxygenation cycles (37°C, 21% O2, 74% N2 and 5% CO2). Western blot analysis was performed as described previously (27). Equal amounts of protein (50 µg) per lane were loaded on 10% SDS-PAGE for separating them and then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk for 1 h, the membranes were probed with primary antibodies at 4°C overnight. The membranes were washed three times with TBS and 0.1% Tween 20 (TBST) for 1 h at room temperature. Following washing with TBST, the immunoreactive protein bands were visualized by using Western Blotting Luminol Reagent (cat no. sc-2048; Santa Cruz Biotechnology, Inc.) and detected with Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc.). The antibodies targeting USP8 (1:1,000; cat. no. 8728), p65 (1:1,000; cat. no. 8943), p-IKKβ (1:1,000; cat. no. 2697), IKKβ (1:1,000; cat. no. 8943), p-IKKβ (1:1,000; cat. no. 2697), Flag (1:1,000; cat. no. 14793), Myc (1:1,000; cat. no. 20276), ubiquitin (linkage-specific K63; 1:500; cat. no. 5621) and β-actin (1:2,000; cat. no. 3700) were all purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Statistical analysis. All data are presented as the mean ± standard deviation of three experiments. Statistical significance was determined with (two-tailed) Student's t-test, P<0.05 was
considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS software (SPSS program, version 13.0, SPSS, Inc., Chicago, IL, USA).

**Results**

**Expression of USP8 is decreased in IHR-treated renal tubular epithelial cells.** The present study first examined the expression of USP8 in normoxia, IHR or CH. As shown in Fig. 1A and B, the expression of USP8 was not affected by the CH conditions, however, it was significantly decreased in the IHR-treated HK-2 and TcMK-1 cells. The association between the expression of USP8 and IHR cycles was then examined. It was demonstrated that the mRNA and protein levels of USP8 were attenuated in a time-dependent manner (Fig. 1c and d), which indicated that, as the number of IHR cycles increased, the expression of USP8 was decreased.

**Overexpression of USP8 alleviates IHR-induced inflammation.** It has been reported that USP8 is essential in downregulating neuroinflammation (23); the present study investigate whether USP8 regulates the inflammation induced by IHR. First, USP8 was overexpressed in HK-2 and TCMK-1 cells, and the overexpression of USP8 was confirmed as shown in Fig. 2A and B. The mRNA and protein levels of pro-inflammatory cytokines were detected, and it was demonstrated that these cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and IL-1β, were all downregulated in the USP8-overexpressing HK-2 cells following IHR challenge (Fig. 2C and D). Consistently, similar results were observed in the TCMK-1 cells (Fig. 2E and F).

**Silencing of USP8 aggravates IHR-induced inflammation.** An shRNA plasmid of USP8 was purchased to knock down the expression of USP8, and the effect of shRNAs was detected (Fig. 3A and B). As presented in Fig. 3c and d, the silencing of USP8 enhanced the IHR-induced production of TNF-α, IL-6, IL-8 and IL-1β in the HK-2 cells. Consistent with these overexpression results, it was found that the expression of pro-inflammatory cytokines in TcMK-1 cells was also increased following USP8 shRNA treatment in IHR conditions.

**USP8 inhibits IHR-induced activation of NF-κB.** It has been reported that IHR-induced inflammation occurs mainly through NF-κB signalling (10). The present study investigated whether USP8 also regulates IHR-induced pro-inflammatory cytokine production through the NF-κB pathway. As presented in Fig. 4A, a Dual-Luciferase Reporter Assay was used to examine the activation of NF-κB signaling, and it was found that the overexpression of USP8 significantly suppressed the activation of NF-κB in the IHR-treated HK-2 cells. Consistently, it was demonstrated that silencing of USP8 enhanced the IHR-induced

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<tr>
<td>1</td>
<td>USP8</td>
<td>F AAA GAC AGG CAG GAG GAA GC</td>
</tr>
<tr>
<td>2</td>
<td>USP8</td>
<td>R CCA ATG TGC CAC CAT CCT CT</td>
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<tr>
<td>3</td>
<td>TNF-α</td>
<td>F CTA AGA GGG AGA GAA GCA</td>
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<td>5</td>
<td>IL-6</td>
<td>F GCC ACC ACg TCT TCT TGT CT</td>
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<td>6</td>
<td>IL-6</td>
<td>R CCA GTG CCT CTT TGC TGC TTT</td>
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<td>7</td>
<td>IL-1β</td>
<td>F AGC TAC GAA TCT CCG ACC</td>
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<td>10</td>
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<td>11</td>
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USP8, ubiquitin-specific peptidase 8; TNF-β, transforming growth factor-β; IL, interleukin; CXCL15, chemokine (C-X-C motif) ligand 15; F, forward; R, reverse.

**Table I. List of primers used in the present study.**
activation of NF-κB (Fig. 4B). Subsequently, the phosphorylation of p65 and IKKβ was examined, and it was demonstrated that the overexpression of USP8 inhibited the IHR-induced phosphorylation of p65 and IKKβ (Fig. 4C and D).

**USP8 interacts with TAK1 and deubiquitinates the K63-linked ubiquitination of TAK1.** TAK1 has been demonstrated to be essential in regulating the activation of NF-κB (14). The present study examined whether USP8 regulates TAK1 and thus affects the NF-κB pathway. As presented in Fig. 5A, it was demonstrated that, following the overexpression of USP8, the TAK1-induced activation of NF-κB was suppressed, whereas IKKβ-induced NF-κB activation was not affected. IP experiments were performed to examine the interaction between TAK1 and USP8, and it was found that USP8 interacted with TAK1 (Fig. 5B). On examining whether the expression of TAK1 was regulated by USP8, however, it was observed that the expression of TAK1 was not affected by the overexpression of USP8 (Fig. 5C). USP8 is reported to be a deubiquitinating enzyme, and it has been reported to deubiquitinate the K63-linked ubiquitination of diverse targets (16,17), therefore, the present study examined the K63-linked ubiquitination

**Figure 1.** Expression of USP8 is decreased in IHR-treated renal tubular epithelial cells. (A) mRNA and protein levels of USP8 in HK-2 cells in normoxia, IHR or CH conditions. (B) mRNA and protein levels of USP8 in TCMK-1 cells in normoxia, IHR or CH conditions. (C) mRNA and protein levels of USP8 in HK-2 cells in different cycles of IHR. (D) mRNA and protein levels of USP8 in TCMK-1 cells in different numbers of cycles of IHR. Data are representative of three independent experiments (mean ± standard deviation). "P<0.01; ""P<0.001. USP8, ubiquitin-specific peptidase 8; N, normoxia; CH, continuous hypoxia; IHR, intermittent hypoxia/reoxygenation.
Figure 2. Overexpression of USP8 alleviates IHR-induced inflammation. (A) mRNA and protein levels of USP8 in USP8-overexpressing HK-2 cells. (B) mRNA and protein levels of USP8 in USP8-overexpressing TcMK-1 cells. (C) mRNA and (D) protein levels of TNF-α, IL-6, IL-8 and IL-1β in USP8-overexpressing HK-2 cells in IHR conditions. (E) mRNA and (F) protein levels of TNF-α, IL-6, CXCL15 and IL-1β in USP8-overexpressing TcMK-1 cells in IHR conditions. Data are representative of three independent experiments (mean ± standard deviation). *P<0.05; **P<0.01; ***P<0.001. USP8, ubiquitin-specific peptidase 8; IHR, intermittent hypoxia/reoxygenation; EV, empty vector; TNF-α, tumor necrosis factor-α; IL, interleukin; CXCL15, chemokine (C-X-C motif) ligand 15.
Figure 3. Silencing of USP8 aggravates IHR-induced inflammation. (A) mRNA and protein levels of USP8 in USP8-silenced HK-2 cells. (B) mRNA and protein levels of USP8 in USP8-silenced TcMK-1 cells. (C) mRNA and (D) protein levels of TNF-α, IL-6, IL-8 and IL-1β in USP8-silenced HK-2 cells in IHR conditions. (E) mRNA and (F) protein levels of TNF-α, IL-6, CXCL15 and IL-1β in USP8-silenced TcMK-1 cells in IHR conditions. Data are representative of three independent experiments (mean ± standard deviation). *P<0.05; **P<0.01; ***P<0.001. USP8, ubiquitin-specific peptidase 8; IHR, intermittent hypoxia/reoxygenation; TNF-α, tumor necrosis factor-α; IL, interleukin; CXCL15, chemokine (C-X-C motif) ligand 15; shRNA, short hairpin RNA; NC, negative control.
Figure 4. USP8 inhibits IHR-induced NF-κB activation. (A) HK-2 cells were transfected with control or USP8 expression plasmid, followed by transfection with the NF-κB reporter plasmid together with the phRL-TK plasmid (internal control); 36 h later the cells were treated with eight cycles of IHR, the activation of NF-κB promoter was measured using a Dual-Luciferase reporter gene assay. (B) HK-2 cells were transfected with the shRNA-NC or shRNA-USP8 plasmid, followed by transfection with the NF-κB reporter plasmid together with the phRL-TK plasmid (internal control); 36 h later the cells were treated with eight cycles of IHR, the activation of NF-κB promoter was measured using a Dual-Luciferase reporter gene assay. (C) Phosphorylation of p65 and IKKβ were examined by a western blot assay in USP8-overexpressing HK-2 cells treated with eight cycles of IHR and (D) protein levels protein levels of p-p65 and p-IKKβ were quantified. Data are representative of three independent experiments (mean ± standard deviation). *P<0.05; **P<0.01; ***P<0.001. USP8, ubiquitin-specific peptidase 8; shRNA, short hairpin RNA; NC, negative control; N, normoxia; IHR, intermittent hypoxia/reoxygenation; NF-κB, nuclear factor-κB; IKKβ, inhibitor of NF-κB kinase β; p-, phosphorylated.
level of TAK1. As shown in Fig. 5D, following IHR treatment, the overexpression of USP8 downregulated the K63-linked ubiquitination of TAK1, indicating that USP8 suppressed the activation of TAK1 through regulating TAK1 K63-linked ubiquitination.

**Discussion**

To the best of our knowledge, the present study is the first to demonstrate the essential role of USP8 in IHR-induced inflammation in renal tubular epithelial cells. It is also the first report to reveal that TAK1 is one of the targets of USP8 and provide evidence to illustrate that USP8 negatively regulates the activity of TAK1 through deubiquitinating the K63-linked ubiquitination of TAK1.

During the progression of acute kidney injury, renal tubular epithelial cells undergo ischemia and hypoxia, which include a series of events that cause pathological damage (28,29). Patients with severe OSA also exhibit a high prevalence of chronic kidney disease and it has been found to accelerate the loss of kidney function (30). Therefore, it is of significance to examine the underlying pathogenesis of the disease and identify other appropriate drug targets. In the present study, it was found that, in cultured human and murine renal tubular epithelial cells, the expression of USP8 was significantly decreased in IHR conditions, however, the expression of USP8 was not
affected in CH conditions. It was hypothesized that this may relate to the oxidative stress and production of ROS induced by IHR, and future investigations aim to investigate this to confirm this hypothesis.

It has been reported that NF-κB-dependent inflammatory gene expression is induced by IHR (10). Consistent with previous studies, the present study found in renal tubular epithelial cells that the activation of NF-κB and pro-inflammatory cytokine production was increased in IHR conditions. It was also observed that USP8 markedly suppressed the IHR-induced activation of NF-κB and cytokine production, indicating that USP8 has an anti-inflammatory effect on IHR-induced inflammation. For the first time, to the best of our knowledge, the present study revealed that TAK1 is a target of USP8. TAK1 directly interacted with USP8, and the overexpression of USP8 significantly suppressed the TAK1-induced activation of NF-κB. These findings also indicated that TAK1 is the key regulator of IHR-induced pro-inflammatory cytokine production, which improves current understanding of TAK1-related diseases.

Ubiquitin has seven lysines (K6, K11, K27, K29, K33, K48 and K63), all of which can be conjugated to another ubiquitin to form a polyubiquitin chain through different lysine linkages to serve distinct functions in the cells (31). The K63-linked polyubiquitination of TAK1 has been reported to be crucial in the activation of TAK1 and NF-κB (32,33). In the present study, it was found that E3 ligase USP8 directly interacted with TAK1, but the overexpression of USP8 did not affect the expression of TAK1, indicating that the K48-linked polyubiquitination of TAK1 was not regulated by USP8. Finally, it was observed that USP8 cleaved K63-linked polyubiquitin chains from TAK1 to inhibit TAK1 kinase activity, leading to the attenuated activation of NF-κB and IHR-induced inflammation. This provides novel ideas for drug development for IHR-related diseases, including OSA.

In conclusion, the present study found that the expression of USP8 was decreased in renal tubular epithelial cells in IHR conditions, and USP8 suppressed the IHR-induced activation of NF-κB and pro-inflammatory cytokine production, mainly through deubiquitinating the K63-linked ubiquitination of TAK1. The results demonstrated the role of USP8 in IHR-induced inflammation and suggested USP8 as a potential and specific therapeutic target for IHR-related diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

YWZ and YQL performed the experiments and drafted the manuscript. YW and HL assisted with the cell culture and western blot. YY and QW conceived and designed the study, analyzed and interpreted the data, and critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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