Research Article

7,8-DHF Treatment Induces Cyr61 Expression to Suppress Hypoxia Induced ER Stress in HK-2 Cells

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Acute kidney injury (AKI) is a common syndrome which is strongly linked to high morbidity and mortality. Hypoxia is the leading cause of AKI and the proximal renal tubular cells are the most damaged part in the kidney during this period. It has been observed that 7,8-dihydroxyflavone (7,8-DHF) plays a protective role by acting on antiapoptosis and antioxidative stress. In this study we explored functions of 7,8-DHF in protecting human proximal tubular cell line HK-2 from hypoxia insults. We observed that treatment of 7,8-DHF could improve the viability of ischemic cell. Mechanistically, we found that 7,8-DHF could elevate the expression of cysteine-rich protein 61 (Cyr61), a protective immediate early gene in AKI. In addition, treatment of 7,8-DHF decreased CCAAT/enhancer-binding protein homologous protein (CHOP) expression, which is a marker protein during endoplasmic reticulum (ER) stress activation. Intriguingly, overexpression of Cyr61 significantly reduced CHOP expression. Taken together, our results provide novel insights into the possible protective role of 7,8-DHF by activating Cyr61 signaling and suppressing ER stress in hypoxic HK-2 cells which have potential clinical implications for the treatment of AKI.

1. Introduction

There has been a gradually increasing mortality and morbidity of acute kidney injury (AKI) worldwide [1]. A wide range of pathogenesis including ischemia, hypertension, and infections could result in AKI [2], among which ischemia is known as a main leading insult that causes dysfunction of kidney [3]. Some studies reported that AKI could increase the risk of chronic kidney disease (CKD) development and end-stage renal disease (ESRD) with time. So far, there is no effective therapeutic strategy though dialysis and renal transplantation could help to some degree. Therefore, exploring effective treatments of AKI with the ultimate goal of halting renal disease progression is of great interest.

The pathogenesis of AKI involves multiple stresses including inflammatory response, hypoxia, nutrient starvation, and other environmental insults, by which apoptosis, necrosis, and autophagy could happen, especially in the most sensitive part, proximal tubular cells [4]. In addition, there

is growing evidence suggesting that endoplasmic reticulum (ER) stress is also involved in AKI pathology [5–7]. Under normal physiological conditions, ER performs cellular activities, such as biosynthesis, folding, and trafficking modification of proteins [8]. When the balance breaks down within a variety of environmental insults including hypoxia, oxidative stress, and cell starvation, unfolded and malfolded proteins are unable to transport from ER lumen to other parts of cells or space out of cells and then loaded in the ER to trigger ER stress [9]. With the injury lasting long and progressing, ER stress known as prodeath pathway will result in apoptosis and other responses [10].

7,8-Dihydroxyflavone (7,8-DHF) is a kind of flavone derivative which was demonstrated to be a promising small molecule tyrosine kinase B receptor (TrkB) agonist. Numerous evidences have been reported that 7,8-DHF produces pivotal biological functions mainly through activating TrkB receptors. Notably, it plays an important role in promoting neuron regeneration in some neurodegenerative diseases,

such as Alzheimer's disease and Parkinson's [11–13]. It could also improve memory and ameliorate depressive status [14, 15]. Moreover, it displayed a therapeutic efficacy in metabolic diseases on the basis of TrkB signaling to inhibit obesity [16]. Choi et al. found that 7,8-DHF was able to inhibit adipogenesis of preadipocyte cells and induced apoptotic cell death [17]. Mechanistically, 7,8-DHF could protect cells from oxidative stress. For example, treatment with 7,8-DHF protects retinal ganglion cells from excitotoxic and oxidative stress-induced apoptosis and cell death [18]. And previous studies have found that 7,8-DHF could prevent C2C12 myoblasts and endothelial cells from H_2O_2-induced oxidative cytotoxicity [19, 20]. Furthermore, 7,8-DHF has been found to induce apoptosis in some malignant diseases, including oral squamous cancer and leukemia [21, 22]. However, functions of 7,8-DHF in kidney diseases are still seldom clarified. Since the role of 7,8-DHF in antioxidant stress has been proved, we speculated that it may have a protective effect in AKI.

In this study we investigated the protecting roles of 7,8-DHF in human proximal tubular cell line HK-2 which was exposed to hypoxia condition. We demonstrated that 7,8-DHF could effectively improve ischemic HK-2 cell viability. Mechanistically, we found that 7,8-DHF could attenuate the ER stress by suppressing expression of CCAAT/enhancer-binding protein homologous protein (CHOP), a key regulator of ER stress. In addition, the cysteine-rich protein 61 (Cyr61) expression was elevated upon 7,8-DHF treatment. Interestingly, forced expression of Cyr61 could downregulate CHOP expression. Thus, our study indicated the hypoxia induced HK-2 protective property of 7,8-DHF by controlling Cyr61 and ER stress signaling, which may provide a novel therapeutic strategy of AKI.

2. Materials and Methods

2.1. Cell Culture and Induction of Hypoxia.
HK-2 cells were cultured in DMEM/F12 medium (Thermo Scientific, USA), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Gibco, USA) at 37°C in 5% CO_2 atmosphere. The experimental model of hypoxia injury of HK-2 cells was established in a hypoxia incubator chamber (Billups-Rothenberg, USA). In brief, hypoxia induced HK-2 cells were incubated in D-MEM without glucose with 95% N_2 and 5% CO_2 for 12 h.

2.2. CCK-8 Cell Proliferation and Viability Assay.
Cells were seeded into 96-well plates (1×10^4 cells per well) and treated with dimethyl sulfoxide (DMSO) (Sigma, USA) or 7,8-DHF (TCI Laboratories, Japan) under indicated condition for 12 h at 37°C. Then the solution was removed and 10 μl CCK-8 (Dojindo, Japan) diluted in 100 μl DMEM was added to each well. After 1 to 4 h incubation, the absorbance was measured using a 96-well plate reader at 450 nm.

2.3. Plasmid and Western Blotting.
Cyr61 was subcloned into PiggyBac (PB) vector. The plasmids were transfected with Lipofectamine 3000 (Invitrogen, USA) into cells following the instructions from the manufacturer. To obtain cell protein, the cells were washed by cold PBS twice and extracted with SDS lysis buffer. Cell lysates were boiled for 15 minutes at 98°C and vortexed 3 times during this period. After quantification with BCA Protein Assay Kit, 20 μg total proteins was loaded. After transferring to the PVDF, membrane was blocked with 5% nonfat milk for 1 h at room temperature. Then indicated antibodies including cleaved Caspase-3 (1 : 1000), Cyr61 (1 : 1000, Cell Signaling Technology, USA), CHOP (1 : 1000, Elabscience Biotechnology, China), p-AKT/AKT (1 : 3000, CST, USA), TrkB (1 : 500, Boster, China), and β-actin and β-tubulin (1 : 3000, Beyotime Institute of Biotechnology, China) were incubated at 4°C overnight. This study used β-actin and β-tubulin as loading control. The membranes were detected by chemiluminescence (ECL) reagents in autoradiography machine Vilber Lourmat fusion Fx7.

2.4. Flow Cytometry for Apoptotic Determination.
Quantification of apoptosis cells was detected by annexin V FITC/PI staining (Jamay Biotech, China). The cells in suspension were incubated by 5 μl annexin V FITC and 5 μl propidium iodide (PI) at room temperature without light for 15 min according to the manufacturer's instructions. Then they were detected by a flow cytometry. The analysis showed entire population of viability cells (FITC−/PI−), early apoptotic cells (FITC+/PI−), late apoptotic cells (FITC+/PI+), and necrotic cells (FITC−/PI+).

2.5. Quantitative Real-Time PCR Studies.
Total RNA was extracted with TRIzol reagent (Invitrogen, USA). Reverse transcriptase and oligo'dT primer were used to obtain cDNA by 500 ng RNA referring to the manufacturer's instructions (Takara, Japan). And the expression level of target mRNA was detected by real-time PCR using SYBR Green Master Mix. The relative expression of target mRNA was evaluated by $2^{-\Delta\Delta ct}$. All PCR reactions were performed in duplicate. Primer sequence could be found in the supplementary data.

2.6. Statistical Analysis.
All data were expressed as means ± SEM with using SPSS. One-way analysis of variance (ANOVA) was used among diverse groups and independent samples were analyzed by Student's t-test when appropriate. A value of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. 7,8-DHF Improved the Cell Viability of Hypoxia Treated HK-2 Cells.
To observe the protective effect of 7,8-DHF, we first observed the appropriate concentration of 7,8-DHF in HK-2 cell in normal condition and the cell viability was detected by CCK-8 assay. Our result showed that 7,8-DHF at 50, 100, 150, and 200 μM significantly increased the proliferation of HK-2 cells but the cells were damaged with the concentration of 250 μM (Figure 1(a)).

We then used the most effective concentration of 7,8-DHF at 100 μM and 150 μM to evaluate 7,8-DHF efficacy in suppressing HK-2 cells from hypoxia induced insults. After pretreatment of 7,8-DHF for 1 h the cells were incubated for 12 h under hypoxia condition. Then we tested cell viability by CCK-8 assay. Compared to control cells at normal condition,
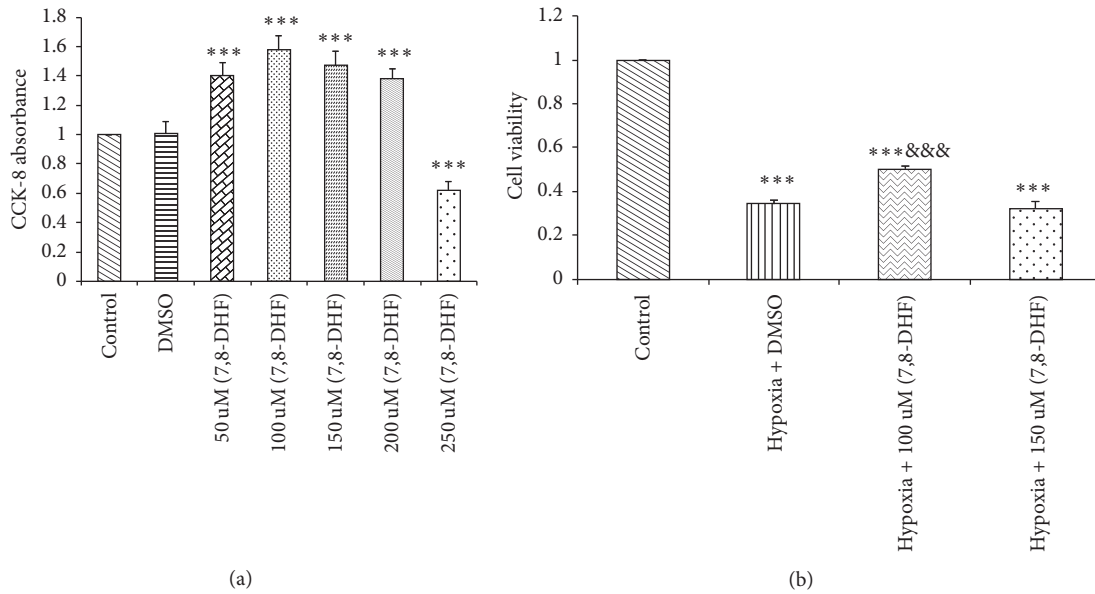
(a)

(b)

FIGURE 1: 7,8-DHF improved cell viability of hypoxia treated HK-2 cells. (a) HK-2 cells were treated with 7,8-DHF (50–250 μM) for 12 h. (b) Cells were pretreated with 7,8-DHF (100 and 150 μM) for 1 h and then exposed to hypoxia for 12 h. Data were expressed as the mean ± SD ($n = 5$ per group). ***$P < 0.001$ versus control group. &&&$P < 0.001$ versus hypoxia alone (DMSO) group.
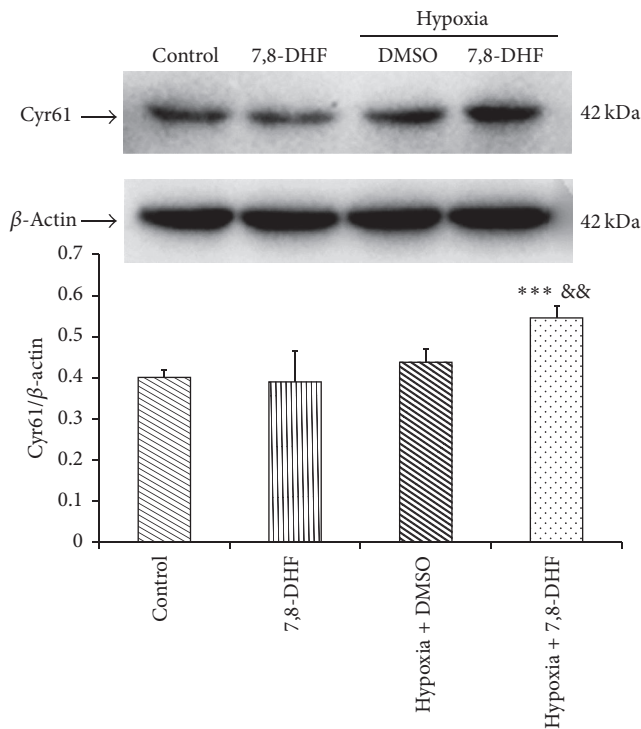

FIGURE 2: 7,8-DHF upregulated protein level of Cyr61 in HK-2 cells damaged by hypoxia. Data were presented as the mean ± SD ($n = 5$ per group). ***$P < 0.001$ versus control group. &&$P < 0.01$ versus hypoxia alone (DMSO) group.

ischemic cell viability was dramatically decreased. However, 7,8-DHF protected cells from hypoxia induced cell death. The results demonstrated that at a concentration of 100 μM

7,8-DHF has the optional effect which was able to improve cell viability (Figure 1(b)). Based on this study, the concentration of 100 μM 7,8-DHF was used in the subsequent studies.

3.2. 7,8-DHF Upregulated the Protein Expression Levels of Cyr61 in HK-2 Cells. Cyr61 has been shown to play a protective role in AKI. Next we tested whether 7,8-DHF controls expression of Cyr61. The protein level of Cyr61 was significantly elevated by 7,8-DHF with the concentration of 100 μM in HK-2 cells under hypoxia condition, respectively, compared with control group (Figure 2). This investigation suggested that 7,8-DHF induced the activity of Cyr61 in HK-2 cells treated with hypoxia.

3.3. Cyr61 Protected HK-2 Cells against Hypoxia Induced Apoptosis. It is well known that hypoxia could reduce cell viability by apoptosis [23]. To determine the protective role of Cyr61 which was increased by 7,8-DHF in hypoxia induced HK-2 cells, we overexpressed Cyr61 and exerted FACS analysis by annexin V FITC/PI staining. The expression of Cyr61 protein was significantly increased in O/E group compared to the control cells (Figure 3(a)).

After we confirmed that Cyr61 was overexpressed in HK-2 cells, flow cytometry was measured. The results showed that the percentage of annexin V(+)/PI(−) cells in hypoxia group dramatically increased ($P < 0.05$), compared with the control group. In hypoxia + O/E group, overexpression of Cyr61 significantly attenuated the portion of annexin V(+)/PI(−) cells ($P < 0.05$), which suggested the antiapoptotic role of Cyr61 in hypoxia induced HK-2 cells (Figure 3(b)).

It was known that during apoptosis Caspase-3 is cleaved and activated [24]. Therefore, next we examined the changes of Caspase-3, a molecular marker of apoptosis, in HK-2 cells under hypoxia condition, and whether Cyr61 could inhibit
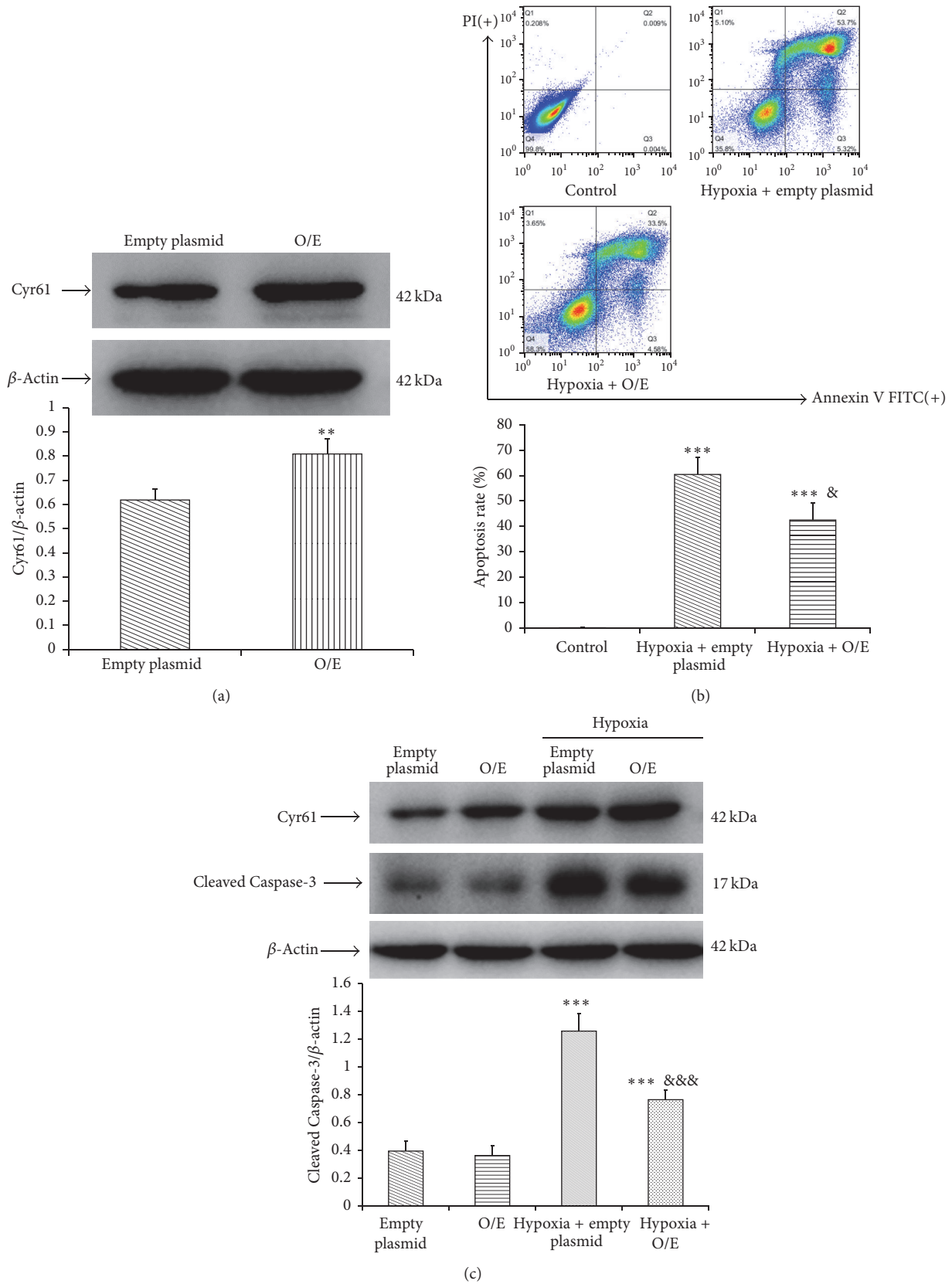
(a)

(b)

(c)

FIGURE 3: Protective effect of Cyr61 in hypoxia induced HK-2 apoptosis. (a) The changes of Cyr61 protein level after transfection. (b) Quantitative assessment of apoptotic cells by annexin V FITC/PI staining. (c) The changes of cleaved Caspase-3 protein level. Data were presented as the mean ± SD ($n = 5$ per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control group. $^{\&}P < 0.05$ and $^{\&\&\&}P < 0.001$ versus hypoxia alone (empty plasmid) group.
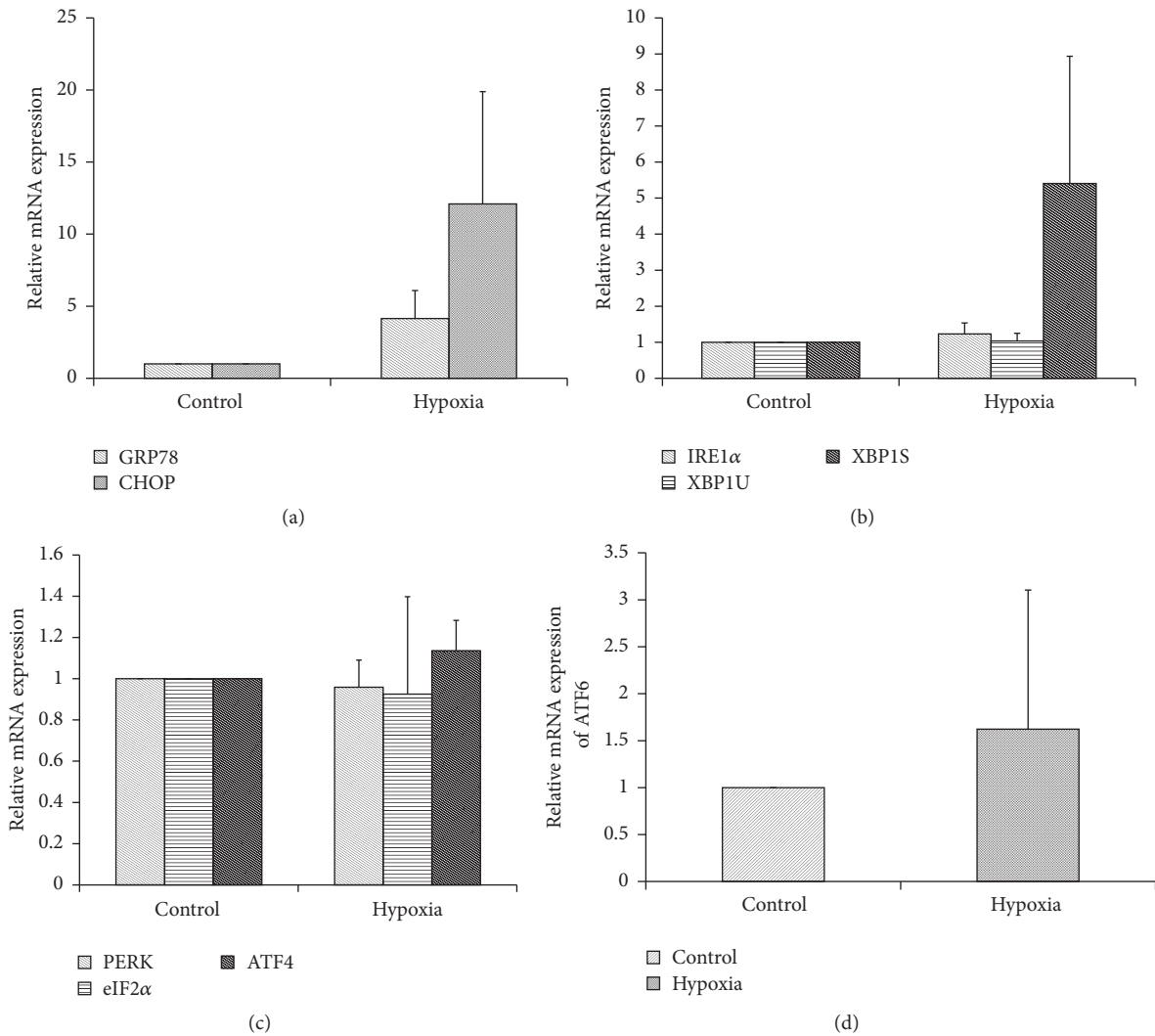
(a)

(b)

(c)

(d)

Figure 4: Hypoxia induced ER stress in HK-2 cells. (a) Effect of hypoxia on the mRNA expression level of GRP78 and CHOP. (b) Effect of hypoxia on mRNA expression level of PERK signaling pathway, including PERK, eIF2α, and ATF4. (c) Effect of hypoxia on the mRNA expression level of IRE1α signaling pathway, including IRE1α, XBP1u, and XBP1s. (d) Effect of hypoxia on the mRNA expression level of ATF6 signaling pathway. Data were presented as the mean ± SD (n = 4 per group).

the activation of Caspase-3 by Western blotting analysis. Compared with the control group, cleaved Caspase-3 was increased in the hypoxia induced HK-2 cells. And overexpression of Cyr61 downregulated the level of cleaved Caspase-3 protein (Figure 3(c)). Hence, our observation suggested that Cyr61 could inhibit apoptosis in HK-2 cells with hypoxia insult.

3.4. *Hypoxia Activated ER Stress in Renal Proximal Tubular Epithelial Cells.* In order to demonstrate whether ER stress participates in ischemic insult, we detected the expression of a variety of molecules in ER stress pathways in hypoxia treated HK-2 cells. We found that hypoxia induced the unfolded protein response (UPR) as revealed by elevated mRNA expression of the glucose-regulated protein 78 (GRP78). In addition, the mRNA level of CHOP, a key regulator of ER stress, was increased compared to the control group (Figure 4(a)).

In inositol-requiring 1α (IRE1α) pathway, splicing X-box-binding protein-1 (XBP1s) was dramatically upregulated upon hypoxia (Figure 4(c)). However, expression of protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) had no obvious changes (Figures 4(b) and 4(d)). Taken together, these results confirmed that ER stress was activated in hypoxia induced HK-2 cells.

3.5. *7,8-DHF Decreased Protein Expression of CHOP.* Growing studies have demonstrated that ER stress is involved in the cell viability. Thus, we were wondering whether 7,8-DHF could affect ER stress during hypoxia. Our results showed that under hypoxia condition CHOP mRNA expression was decreased upon 7,8-DHF treatment (Figure 5(a)) whereas there was no obvious change for other ER stress molecules (Figures 5(b), 5(c), and 5(d)). Consistent decrease of CHOP protein expression has been confirmed by Western blot
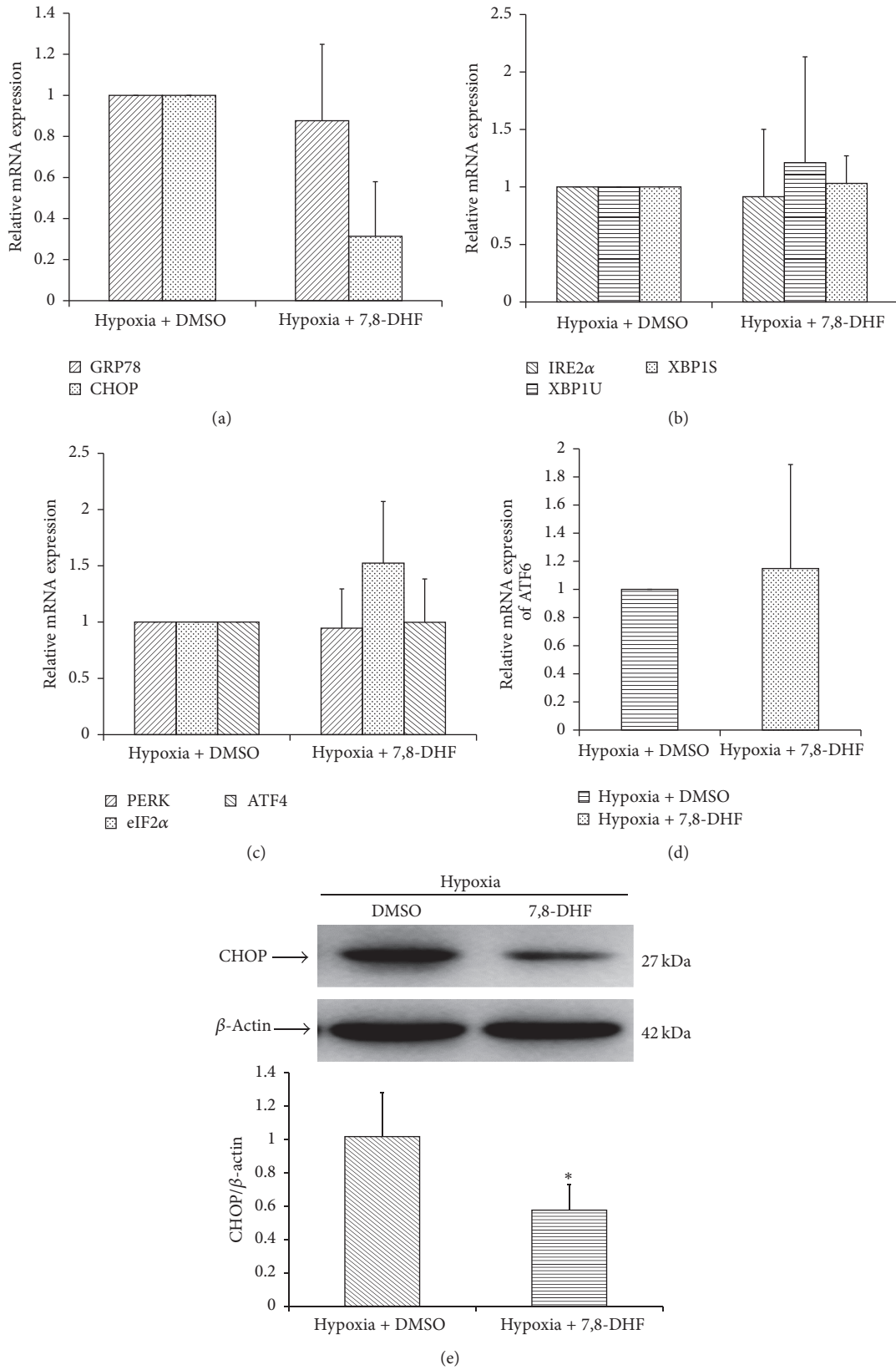
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FIGURE 5: 7,8-DHF suppressed expression of CHOP. (a), (b), (c), and (d) mRNA expression of ER stress biomarkers after 7,8-DHF treatment in hypoxia induced HK-2 cells. (e) The changes of Cyr61 protein level after 7,8-DHF treatment. Data were presented as the mean ± SD ($n = 4$ per group). $^{*}P < 0.05$ versus hypoxia alone (DMSO) group.
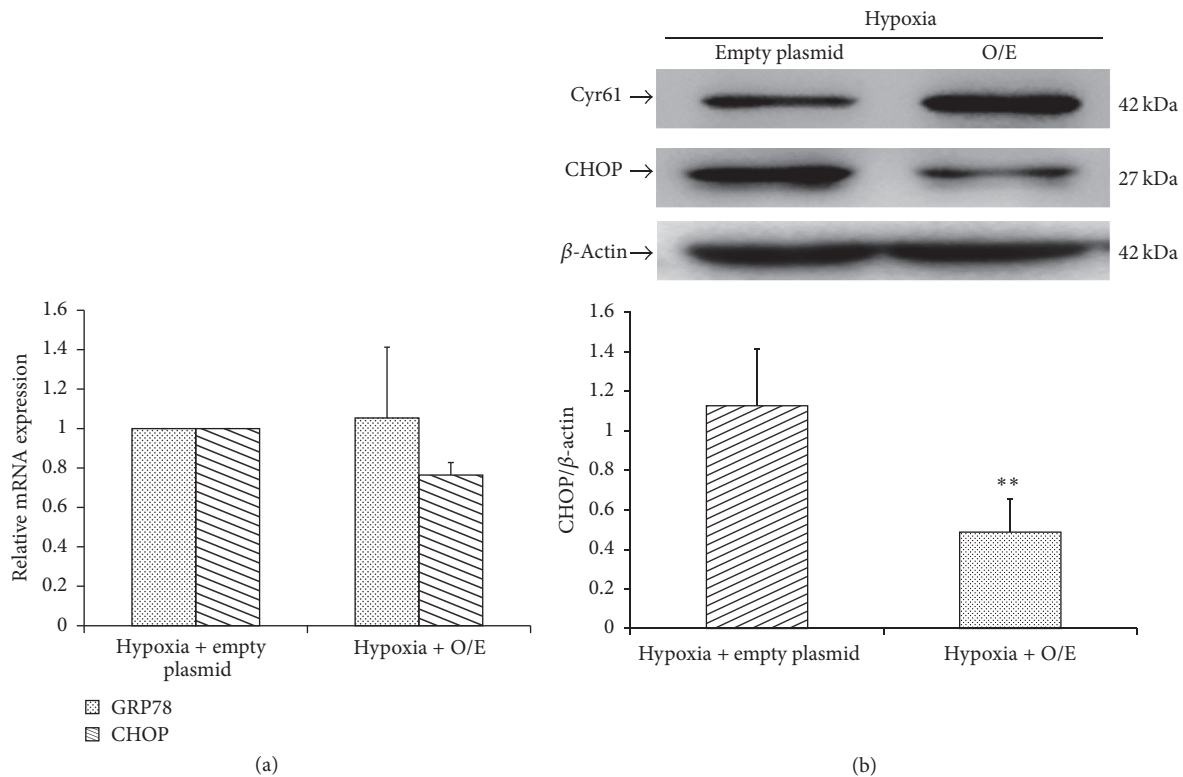
FIGURE 6: Overexpression of Cyr61 downregulated CHOP protein level. (a) mRNA expression level of GRP78 and CHOP upon Cyr61 overexpression in hypoxia cultured HK-2 cells. (b) The changes of CHOP protein level with overexpression of Cyr61. Data were presented as the mean ± SD ($n = 4$ per group). $^{**}P < 0.01$ versus hypoxia alone (empty plasmid) group.

analysis (Figure 5(e)). Thus, our studies suggest that 7,8-DHF may inhibit ER stress to protect cells from hypoxia injury.

3.6. Cyr61 Reduced ER Stress in HK-2 Cells Damaged from Hypoxia. To elucidate whether Cyr61 prevents HK-2 cells from ischemia damage through its anti-ER stress action, we detected mRNA level of GRP78 and CHOP. CHOP mRNA was decreased while GRP78 expression has no change of Cyr61 overexpression (Figure 6(a)). Western blot assay confirmed the dramatic reduction of CHOP protein level upon overexpression of Cyr61 (Figure 6(b)). These results indicated that Cyr61 could have protective effects on hypoxia induced HK-2 cells through suppressing ER stress.

4. Discussion

7,8-DHF is a member of the flavonoid family and has been shown as a selective small molecule agonist for TrkB receptor. Previous researches mainly focused on its protective effects on nervous system [25–27]. 7,8-DHF has been demonstrated to protect neurons from ischemic stroke, which may be at least attributable to its antiapoptotic, antioxidative, and anti-inflammatory actions [27–29]. Since hypoxia is the leading cause of AKI, which involves apoptosis, oxidative stress, and inflammation, we thought that 7,8-DHF may have evident effects on AKI. Therefore, we investigated its function in the proximal tubular epithelial cells damaged from hypoxia. We observed that 7,8-DHF was able to improve the viability of

HK-2 cells from injury caused by hypoxia exposure, which may provide a new approach for AKI treatment.

Recently 7,8-DHF was found to interact with VEGFR2 and block the activity of VEGFR2 in the 661W photoreceptor cells and rat retina [30]. VEGFR2 as a tyrosine kinase receptor plays an important role in the regulation of vasculogenesis and angiogenesis. In normal kidney, VEGFR2 is mainly expressed in endothelial cells in the peritubular capillaries and glomerular capillary loops [31], whereas VEGF expression is most prominent in glomerular podocytes and tubular epithelial cells [32, 33]. Although it has been documented that VEGF is a protective factor in CKD, excessive VEGF expression in the tubular cells also displays a detrimental effect. Increased VEGF expression leads to significant fibrosis and cyst formation in the VEGF transgenic mice [34]. Interestingly, VEGFR2 blockade in endothelial cells markedly attenuated fibrosis and capillary rarefaction in UUO mice, which was attributed to preventing pericyte differentiation and proliferation [35]. Thus, the role of 7,8-DHF targeting VEGFR2 or other VEGF receptors in kidney diseases is complicated and needs further investigation.

It has been reported that the cytoprotective effects of TrkB agonist 7,8-DHF are mediated by activation of PI3K/AKT signaling [19, 25, 26, 36, 37]. First we found that TrkB is expressed in the HK-2 cells (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/5029797). Our study also found that 7,8-DHF could increase the phosphorylation of AKT (Figure S2), indicating that

7,8-DHF may act as TrkB agonist in HK-2 as other cell lines. Thus, some key downstream regulators of PI3K/AKT signaling could be potential candidates. Our and many other studies have reported that Cyr61 could control cell cycle, remodel the cell matrix, and promote angiogenesis via interacting with cells or extracellular matrix [38]. Cyr61 has been found to be rapidly upregulated in the renal outer medulla and urine after ischemia kidney injury [39]. Moreover, Cyr61 and its interacting protein Caprin-1 were modulated by PI3K/AKT signaling activation in prostate carcinoma PC-3 cells [40], osteosarcoma tumor, and lung cancer cells [41]. Considering the emerging data, we speculated that 7,8-DHF may protect HK-2 cells from hypoxia damage via inducing Cyr61. Intriguingly the results showed that under hypoxia condition elevated expression level of Cyr61 was detected in HK-2 cells treated with 7,8-DHF compared to the control. It has been previously reported that inhibition of PI3K/AKT signaling could decrease expression of Cyr61 in HUVEC cells under hypoxia, which is consistent with our results that LY294002 blocked the elevation of Cyr61 expression upon 7,8-DHF and hypoxia treatment (Figure S2) [42]. In addition, Cyr61 increased PI3K/AKT signaling activity in different types of cells, such as HUVEC, gastric cancer cells, breast cancer cells, glioma cells, and renal cell carcinoma [42–46]. It seems that Cyr61 and PI3K/AKT signaling promote each other positively. The treatment of 7,8-DHF could trigger the beneficial cycle.

Many studies have shown that ER stress is a major contributor to cellular apoptosis and damage after hypoxia. Our study revealed that incubation of HK-2 cells in hypoxia condition increased the mRNA expression of GRP78 and CHOP. Moreover, increased level of XBP1s and ATF6 indicated several signaling pathways of ER stress were activated by hypoxia. In addition, our results showed that 7,8-DHF treatment significantly decreased the expression of CHOP. Western blot assay was exerted to further confirm the role of Cyr61 in control of ER stress. We detected the level of CHOP was significantly downregulated upon overexpression of Cyr61 in HK-2 cells. Therefore, our results suggested that the protective effects of 7,8-DHF via Cyr61 against renal hypoxia injury were mediated, at least in part, by the inhibition of renal ER stress although the underlying mechanisms still need to be explored.

5. Conclusion

In summary, our study investigated the role of 7,8-DHF in protecting ischemic HK-2 cells. To our knowledge it is the first time that we provide an in vitro study of potential therapeutic value of 7,8-DHF in HK-2 cells damaged by hypoxia. Mechanistically, 7,8-DHF could suppress ER stress pathway by upregulating Cyr61. Thus, 7,8-DHF may be a promising compound that could protect renal tubular cells from AKI damage.

Glossary

AKI: Acute kidney injury
7,8-DHF: 7,8-Dihydroxyflavone

ATF4:   Activating transcription factor 4
ATF6:   Activating transcription factor 6
CHOP: CCAAT/enhancer-binding protein
              homologous protein
CKD: Chronic kidney disease
Cyr61:   Cysteine-rich protein 61
DMSO: Dimethyl sulfoxide
eIF2α: Eukaryotic initiation factor 2α
ER: Endoplasmic reticulum
ESRD: End-stage renal disease
GRP78: Glucose-regulated protein 78
HK-2:   Human proximal tubular cell line
IRE1α: Inositol-requiring enzyme 1α
O/E: Overexpression
PERK: Protein kinase-like ER kinase
TrkB: Tyrosine kinase B receptor
UPR: Unfolded protein response
XBP1:   X-box-binding protein-1
XBP1s: Splicing X-box-binding protein-1
XBP1u: Unsplicing X-box-binding protein-1.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Rui Ma and Jisheng Zhang are both the first authors in this article, and they contributed equally to this work.

Acknowledgments

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