

## Original Article

# Effects of omi/HtrA2 on hypoxia/reoxygenation-induced apoptosis of rat renal tubular epithelial cells

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**Abstract:** The purpose of this research was to reveal the effects of knockdown of Omi/HtrA2 on rat renal tubular epithelial cells (NRK-52E cells) under conditions of hypoxia/reoxygenation. NRK-52E cells were randomized to five groups: a conventional culture group and four hypoxia/reoxygenation groups: model group (untransfected cells), HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK), shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1), and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2). Cells were incubated in anaerobic liquid to induce hypoxia/reoxygenation in the experimental models. Apoptosis was assessed by DNA Ladder electrophoresis and caspase-3/9 activity assays. Compared with the model group, electrophoretic assessment indicated that DNA damage was decreased after hypoxia/reoxygenation in the shRNA1 and shRNA2 groups. Additionally, the activity of caspase-3/9 was significantly decreased in shRNA1 and shRNA2 groups compared to the model group. Furthermore, expression of caspase-3/9 and Omi/HtrA2 proteins was significantly decreased in shRNA1 and shRNA2 groups compared to the model group. In conclusion, Omi/HtrA2 expression and apoptosis are significantly induced in NRK-52E cells by hypoxia/reoxygenation. Depletion of Omi/HtrA2 significantly attenuated apoptosis of NRK-52E cells induced by hypoxia/reoxygenation, indicating that therapies targeting Omi/HtrA2 could be an effective treatment for acute kidney failure.

**Keywords:** Omi/HtrA2, cell apoptosis, RNA interference, acute renal failure

## Introduction

Acute renal failure (ARF) is a common and severe medical condition with a high fatality rate of 30%-50% [1]. About three-quarters of ARF cases result from renal ischemia [2]. Renal ischemia-reperfusion injury (RIRI) is a major cause of ARF, and renal tubular epithelial cell apoptosis plays an important role in RIRI [3, 4]. Finding an effective method to reduce RIRI-induced renal tubular epithelial cell apoptosis is of great importance in developing better treatments for ARF.

Apoptosis, or programmed cell death, is a proactive and highly orderly cellular death process that involves a series of enzymes and is controlled by genes under specific physiological or pathological conditions. Characteristics of apoptosis include nuclear condensation, DNA ladder fragmentation, shrunken cytoplasm and

formation of apoptotic bodies [5]. The main pathways of apoptosis are the caspase-dependent and non-caspase-dependent apoptotic pathways [6]. In RIRI, apoptotic pathways are activated due to GTP depletion, lack of growth factors, and increases in reactive oxygen metabolites, which results in renal tubular epithelial cell apoptosis [7, 8]. The main apoptotic pathway activated in RIRI is caspase-dependent apoptosis that includes the intrinsic (mitochondrial) apoptotic pathway, the extrinsic (death receptor) apoptotic pathway, and the endoplasmic reticulum pathway [9].

Omi/HtrA2, a widely expressed member of the serine protease family, was recently reported to be an apoptosis-inducing factor [10, 11]. When the mitochondria is stimulated by apoptotic signals, permeability pores on the mitochondrial membrane open, and apoptosis-inducing factors such as Omi/HtrA2 and cytochrome C are

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released from the mitochondria into the cytoplasm, where they associate with inhibitor of apoptosis proteins (IAPs) resulting in the activation of caspase-9 and caspase-3, leading to cell apoptosis [12]. In addition, the proteolytic activities of Omi/HtrA2 also plays a role in the non-caspase-dependent apoptotic pathway [13].

RNA Interference (RNAi) is an effective gene-silencing approach that introduces double-stranded RNA complementary to a “target gene”, resulting in repression of target gene expression [14]. RNAi is widely used to study high-flux gene, gene knockout, gene therapy, and gene expression. After cells are transfected with shRNA plasmids, siRNAs can be highly expressed and achieve a stable and reproducible knockdown effect [15].

It has been reported that Omi/HtrA2 plays a significant role in the occurrence and development of prostate cancer and liver cancer [16, 17]. However, is known regarding the effect of Omi/HtrA2 on RIRI-induced renal tubular epithelial cell apoptosis. In this study, we constructed a hypoxia/reoxygenation model of rat renal tubular epithelial cells (NRK-52E) to determine the impact of hypoxia/reoxygenation on NRK-52E cell apoptosis and caspase-3/9 expression. Using this model, we transfected NRK52E cells with plasmids containing shRNAs targeting Omi/HtrA2 to explore the role and possible mechanisms of Omi/HtrA2 in renal tubular epithelial cell apoptosis.

### Material and methods

#### *Cell culture*

NRK-52E cells, a rat renal proximal tubular epithelial cell line (a gift from Professor Xue-qing Yu, Department of Nephrology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China), were cultured in routine conditions. NRK-52E cells in the logarithmic growth phase were seeded in 12-well plate at a concentration of  $5 \times 10^3$  cells/well for transfection with the HK (Pgenesil-1/HK), shRNA1, and shRNA2 constructs. In each well, 0.5  $\mu\text{g}$  of recombinant plasmid, 1.5  $\mu\text{l}$  of liposomes, and 75  $\mu\text{l}$  of DMEM/F12 culture medium (Invitrogen, Carlsbad, CA, USA) were combined, followed by the addition of 1 ml of DMEM/F12 culture medium. Eight hours later, the culture medium was replaced by media containing 10% fetal

bovine serum (Hyclone, Logan, UT, USA). After 24 h, culture medium was replaced by growth media containing 300  $\mu\text{g}/\text{ml}$  G418. Two weeks later, a stable monoclonal cell population was obtained and cultured in 50  $\mu\text{g}/\text{ml}$  G418.

#### *Grouping and preparation of hypoxia/reoxygenation models*

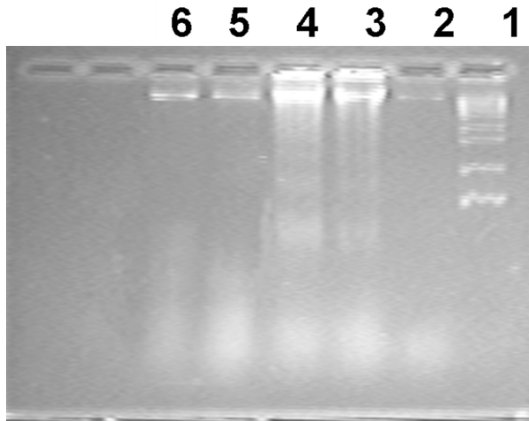
Experiments were divided into the five groups: five groups: a conventional culture group and four hypoxia/reoxygenation groups: model group (untransfected cells), HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK), shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1), and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2). Cells in the hypoxia/reoxygenation groups were incubated in hypoxic conditions in anaerobic liquid in an atmosphere of 95%  $\text{N}_2$  and 5%  $\text{CO}_2$  at 37°C for 45 min; reoxygenation was performed by incubation in normal culture medium under standard culture conditions for 90 min. After hypoxia/reoxygenation, cells from each group were collected in six 75 ml culture.

#### *Cell apoptosis observed by DNA Ladder*

To confirm the induction of apoptosis, DNA fragmentation was analyzed by a annexin V-FITC apoptosis detection test kit (Keygen Biotechnology, Nanjing, China). Briefly, cells were collected by centrifugation and washed twice with PBS. The cell pellet was resuspended in 0.1 ml lysis buffer and incubated with shaking at 37°C for 4 h. The lysate was treated with 20  $\mu\text{l}$  Solution A and 20  $\mu\text{l}$  Enzyme A for 1 h at 55°C, followed by reaction with 20  $\mu\text{l}$  Enzyme B for 1 h at 37°C. The upper phase was collected and precipitated with cold ethanol overnight at -20°C. DNA was collected by centrifugation (14,000 $\times$ g; 4°C; 20 min) and washed with 75% ethanol. The DNA pellet was resuspended in 100  $\mu\text{l}$  TE buffer and incubated at 65°C for 1 h to facilitate solubilisation. Finally, the DNA was subjected to electrophoresis on a 2% agarose gel (Invitrogen Life Technologies, Carlsbad, CA, USA) at 60 V for 2.5 h and observed and photographed under UV light.

#### *Colorimetric method for caspase-3/9 activity detection*

The activity of caspase-3/9 was measured by a colorimetric method using a Caspase Activity



**Figure 1.** Apoptosis observed by DNA fragmentation by gel. Lane identifications: 1) marker; 2) normal group; 3) model group; 4) HK group; 5) shRNA1 group; 6) shRNA2 group. normal: conventional culture group; hypoxia/reoxygenation groups: model group (untransfected cells); HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK); shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1); and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2).

Kit according to standard procedures (Caspase Activity Assay Kit, Beyotime Biotechnology, Shanghai, China).

#### Western blot analysis

Cytoplasmic proteins were extracted using a mitochondria/cytoplasmic separation and extraction kit (BioVision Inc, Milpitas, California, USA), and protein concentration was determined by a Bradford protein concentration detection kit (Sigma, St. Louis, MO, USA). Equal amounts of protein per sample (80  $\mu$ g) were separated by SDS-PAGE, transferred to nitrocellulose membrane under constant current. Membranes were blocked with 5% nonfat milk over night at 37°C, and then incubated with primary antibodies against Omi/HtrA2, caspase-3 or caspase-9 (each 1:1000 dilution, Santa Cruz, CA, USA), at room temperature for 4 hours. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (each 1:500 dilution, Maixin Biotechnology, Fuzhou, China), and then washed. Membrane were visualized after washing. Grey values were used for densitometric analysis of bands.  $\beta$ -actin band was used as a loading control reference for detection of grey values of each membrane.

#### Statistical analysis

One-way analysis of variance (ANOVA) was used for comparison of differences among groups. Tukey's honestly significant difference (HSD) test was utilized for comparisons between two groups. All *P* values were two-tailed and a *P* value < 0.05 was considered to indicate a statistically significant difference. All data were analyzed using the SPSS statistical package, version 13.0 (SPSS Inc., Chicago, IL, USA) for Windows. Results are expressed as mean  $\pm$  SE.

#### Results

##### Apoptosis observed by DNA Ladder

In the normal group, DNA electrophoresis identified a DNA fragment of high molecular weight that lacked gradient bands indicative of apoptosis. In the model and HK groups, DNA fragmentation in the nucleosome was observed as typical electrophoretic gradient bands, suggesting that apoptosis occurred after hypoxia/reoxygenation. There was no marked difference in DNA fragmentation between the model and HK groups. However, compared with model group, DNA fragmentation was significantly attenuated after hypoxia/reoxygenation in the shRNA1 and shRNA2 groups, suggesting that knockdown of Omi/HtrA2 decreased apoptosis (**Figure 1**).

##### Activity of caspase-3/9 determined with colorimetric method

Compared with the normal group, the activity of caspase-3/9 was significantly increased in the model, HK, shRNA1, and shRNA2 groups (*P* < 0.05). Compared with the model group, the activity of caspase-3/9 was significantly decreased in the shRNA1 and shRNA2 groups. (*P* < 0.05). There were no statistical differences in the activity of caspase-3/9 between the model and HK groups (**Table 1**).

##### Protein expressions of caspase-3/-9 and Omi/HtrA2 in the cytoplasm determined by western blotting

Compared with the normal group, the protein expressions of caspase-3/9 and Omi/HtrA2 were significantly increased in the model and HK groups (*P* < 0.05). Compared with model group, the protein expressions of caspase-3/9

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**Table 1.** Caspase-3/9 activity detected by colorimetric method

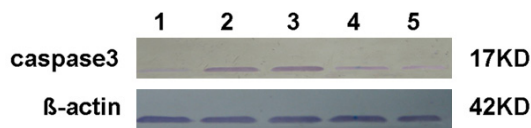
	Normal	Model	HK	shRNA1	shRNA2
caspase-3	0.32 ± 0.10	2.98 ± 0.15*	2.84 ± 0.18*	1.74 ± 0.08**	1.68 ± 0.09**
caspase-9	0.21 ± 0.08	1.76 ± 0.14*	1.63 ± 0.13*	1.23 ± 0.12**	1.31 ± 0.11**

\* $P < 0.05$  vs. normal group; \*\* $P < 0.05$  vs. model group. Values are mean ± SE, n = 6. Normal: conventional culture group; hypoxia/reoxygenation groups: model group (untransfected cells); HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK); shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1); and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2).

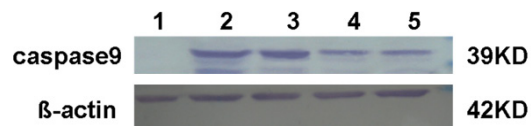
**Table 2.** Values of caspase-3/9 and Omi/HtrA2 determined by western blot

	Normal	Model	HK	shRNA1	shRNA2
caspase-3/β-actin	0.16 ± 0.04	0.86 ± 0.11*	0.84 ± 0.11*	0.69 ± 0.06**	0.64 ± 0.05**
caspase-9/β-actin	0.10 ± 0.03	0.63 ± 0.10*	0.66 ± 0.06*	0.32 ± 0.05**	0.35 ± 0.08**
Omi/HtrA2/β-actin	0.17 ± 0.06	0.77 ± 0.10*	0.74 ± 0.06*	0.34 ± 0.06**	0.36 ± 0.08**

\* $P < 0.05$  vs. normal group; \*\* $P < 0.05$  vs. model group. Values are mean ± SE, n = 6. Normal: conventional culture group; hypoxia/reoxygenation groups: model group (untransfected cells); HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK); shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1); and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2).

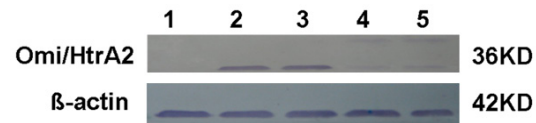


**Figure 2.** Protein expressions of caspase-3 determined by western blot. Lane identification: 1) normal group; 2) model group; 3) HK group; 4) shRNA1 group; 5) shRNA2 group. normal: conventional culture group; hypoxia/reoxygenation groups: model group (untransfected cells); HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK); shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1); and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2).



**Figure 3.** Protein expressions of caspase-9 determined by western blot. Lane identification: 1) normal group; 2) model group; 3) HK group; 4) shRNA1 group; 5) shRNA2 group. normal: conventional culture group; hypoxia/reoxygenation groups: model group (untransfected cells); HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK); shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1); and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2).

and Omi/HtrA2 were significantly decreased in the shRNA1 and shRNA2 groups ( $P < 0.05$ ). There were no statistical differences in the protein expression of caspase-3/9 and Omi/



**Figure 4.** Protein expressions of Omi/HtrA2 determined by western blot. Lane identification: 1) normal group; 2) model group; 3) HK group; 4) shRNA1 group; 5) shRNA2 group. normal: conventional culture group; hypoxia/reoxygenation groups: model group (untransfected cells); HK group (cells transfected with the non-silencing recombinant plasmid Pgenesil-1/HK); shRNA1 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA1); and shRNA2 group (transfected with Pgenesil-1/Omi/HtrA2 shRNA2).

HtrA2 between the model and HK groups, and between the shRNA1 and shRNA2 groups (Table 2 and Figures 2-4).

### Discussion

Apoptosis is a tightly regulated physiological process of cellular self-destruction, which plays an important role in removing abnormal or unwanted cells in multicellular organisms, facilitating the maintenance of internal homeostasis and preserving phylogenetic development [5, 18]. As a method of proactive cellular death, apoptosis has complex molecular biological mechanisms, including caspase-dependent and non-caspase-dependent apoptosis. Caspase-dependent apoptosis refers to apoptosis involving the successive activation of members of the caspase family of proteins by

stimulating factors, ultimately resulting in apoptosis. Based on the differences in activators and the types of caspases, caspase-dependent apoptotic pathways are divided into the death receptor pathway, the mitochondrial pathway, and the endoplasmic reticulum pathway. In the death receptor pathway, extracellular ligands activate caspase-8/10 through combination with their receptors; in the mitochondrial pathway, apoptosis inducing factors such as Omi/HtrA2, cytochrome C, and nuclear endonuclease G are released to activate caspase-9 following mitochondrial stimulation; in the endoplasmic reticulum pathway, after the endoplasmic reticulum is stimulated, calpain is released and activates caspase-12. In all the three pathways, activated caspase-3 is the ultimate pathway effector, resulting in DNA breakage and apoptosis. In non-caspase-dependent apoptosis, caspase activation is not involved. In general, there are both caspase-dependent and non-caspase-dependent pathways involved in most cellular apoptosis [19, 20]. In RIRI, the main pathway of apoptosis is the caspase-dependent pathways [21].

In this study, NRK-52E cells were transfected with shRNA1, shRNA2, and HK, and monoclonal cell cultures were obtained by G418 selection. Renal tubular epithelial cell apoptosis was induced in hypoxia/reoxygenation models. The results of this study indicated that expression of the Omi/HtrA2 and procaspase-3/9 proteins and cellular apoptosis were significantly increased in the model group. However, knockdown of Omi/HtrA2 resulted in decreased caspase-3/9 protein expression and relief of NRK-52E cell apoptosis after hypoxia/reoxygenation. These data suggest that Omi/HtrA2 induces renal tubular epithelial cell apoptosis through a caspase-dependent pathway.

Omi/HtrA2 is involved in apoptosis via a number of mechanisms. Hegde *et al.* [22] reported that Omi/HtrA2 is involved in mammalian apoptosis, and it is known that Omi/HtrA2 promotes apoptosis in a variety of tumor cells. In prostate cancer, hepatocellular cancer, and breast cancer expression of Omi/HtrA2 is elevated and is positively correlated with apoptosis [16, 17, 23]. Liu *et al.* [24] reported that during rat myocardial I/R administration of Omi/HtrA2 inhibitors induces a significant relief of apoptosis, decrease in XIAP protein expression, and inhibition of caspase-3/9 activity. It was reported

that the Omi/HtrA2 tetrapeptide domain consisting of the first four amino acid residues AVPS (or AVPA) may interact with the third BIR motif in the XIAP protein, resulting in an inhibition of the combination of XIAP and caspase-9, thus relieving the inhibitory effects of XIAP on caspase-9 and increasing apoptosis. Activated caspase-9 then activates caspase-3, and activated caspase-3 may also promote caspase-9 activation followed by endonuclease activation to promote apoptosis [25]. In addition, Omi/HtrA2 also can directly promote apoptosis through its protease activity.

Our study demonstrates that hypoxia/reoxygenation results in significantly increased expression of Omi/HtrA2 in NRK-52E cells. Furthermore, we show that blockade of Omi/HtrA2 expression using RNAi decreases the activity of caspase-3/9 protein and inhibits apoptosis in NRK-52E cells. shRNA targeting Omi/HtrA2 may be developed into an effective treatment for ARF resulting from RIRI.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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