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THE ROLE OF OMI/HTRA2 PROTEASE IN NEONATAL POSTASPHYXIAL SERUM-INDUCED APOPTOSIS IN HUMAN KIDNEY PROXIMAL TUBULE CELLS

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Abstract - Omi/HtrA2, a proapoptotic mitochondrial serine protease, is involved in both caspase-dependent and caspase-independent apoptosis. A growing body of evidence indicates that Omi/HtrA2 plays an important role in the pathogenesis of a variety of ischemia-reperfusion (I/R) injuries. However, the role of Omi/HtrA2 in renal injuries that occur in neonates with asphyxia remains unknown. The present study was designed to investigate whether Omi/HtrA2 plays an important role in the types of renal injuries that are induced by neonatal postasphyxial serum. Human renal proximal tubular cell line (HK-2) cells were used as targets. A 20% serum taken from neonates one day after asphyxia was applied to target cells as an attacking factor. We initially included control and postasphyxial serum-attacked groups and later included a ucf-101 group in the study. In the postasphyxial serum-treated group, cytosolic Omi/HtrA2 and caspase-3 expression in HK-2 cells was significantly higher than in the control group. Moreover, the concentration of cytosolic caspase-3 was found to be markedly decreased in HK-2 cells in the ucf-101 group. Our results suggest both that postasphyxial serum has a potent apoptosis-inducing effect on HK-2 cells and that this effect can be partially blocked by ucf-101. Taken together, our results demonstrate for the first time that postasphyxial serum from neonates results in Omi/HtrA2 translocation from the mitochondria to the cytosol, where it promotes HK-2 cell apoptosis via a protease activity-dependent, caspase-mediated pathway.

Key words: Asphyxia, serum, apoptosis, Omi/HtrA2, renal injury

INTRODUCTION

Asphyxia is one of the major factors that can cause death in neonates during the perinatal period. The essence of asphyxia is hypoxia and ischemia, which can lead to damage in almost every organ in the body. Several studies have indicated that injury to the organs of postasphyxial neonates was associated with ischemia/reperfusion. Neonatal sequelae, in many cases, may be accounted for by perinatal asphyxia damage to organs. As the kidneys are very sensitive to oxygen deprivation (renal injury may occur within 24 h of a hypoxic ischemic episode) renal damage has a higher incident rate in asphyxiated neonates than any other type of hypoxic ischemia-related injury, occurring in more than 50% of neonatal asphyxia cases (Gupta et al., 2005). However, the precise mechanism of renal injury in postasphyxial neonates is still unknown. Understanding this mechanism could lead to the development of more efficient drugs to prevent cases of asphyxia from developing renal injury in newborns. Omi/HtrA2 is the mammalian homolog of the prokaryotic HtrA protein. Omi/HtrA2 is a nuclear-encoded mitochondrial serine protease and is expressed generally in almost every tissue and organ of the

body (Rami et al., 2010). Omi/HtrA2 is formed as a precursor in the cytoplasm, where it is then translocated to the mitochondria. In the mitochondria it is processed to its mature form via the removal of an amino-terminal domain (amino acids 1 to 133), which exposes the AVPS motif. Upon induction of apoptosis by cellular stresses, mature Omi/HtrA2 is released from the mitochondria into the cytoplasm, where it binds to the baculovirus IAP repeat domain of IAPs via its AVPS sequence motif. Following a specific cleavage and degradation of the IAPs, Omi/HtrA2 initiates caspase-dependent apoptosis. Omi/HtrA2 is also able to induce apoptosis in human cells in a caspase-independent protease pathway (Verhagen et al., 2002). Previous studies have demonstrated that apoptosis is involved in ischemia/reperfusion renal injuries (Zhao et al., 2009). Recent studies from several different researchers have independently demonstrated that Omi/HtrA2 plays an important role in ischemia/reperfusion injuries. Althaus et al. (2007) reported that focal cerebral ischemia/reperfusion results in Omi/HtrA2 translocation from the mitochondria to the cytosol, where it participates in neuronal cell death. Prior to ischemia treatment with ucf-101, the specific inhibitor of Omi/HtrA2 reduced both the infarct size and the number of apoptotic cells in the focal cerebral ischemia/reperfusion in animal models. Hui-Rong Liu and colleagues demonstrated that myocardial ischemia/reperfusion significantly increased cytosolic Omi/HtrA2 content and markedly increased apoptosis (Liu et al., 2005). Omi promotes cardiomyocyte apoptosis via a protease activity-dependent, caspase-mediated pathway. Treatment with ucf-101 exerts significant cardioprotective effects. The aim of the present study was to investigate the effect of Omi/HtrA2 on the renal injury that occurs in postasphyxial neonates and to explore further the mechanisms involved.

MATERIALS AND METHODS

Cell culture

Human kidney proximal tubular cell line (American Type Culture Collection) HK-2 cells were grown in DMEM (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO), and 15 mM HEPES, at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly by trypsinization (0.25% trypsin, 0.02% EDTA) after formation of a confluent monolayer. The cultured cells were placed in serum-free media 24 h before stimulation and were divided into a control group, a postasphyxial serum-attacking group, and a ucf-101 group. The cells of the control group were grown in a normal nutritive medium. The cells of the postasphyxial serum-attacking group were grown in a medium with 20% postasphyxial serum. The cells of the ucf-101 group were treated with ucf-101 (10 μmol/L) and grown in 20% postasphyxial serum. During the preliminary study, we only examined the control group and the postasphyxial serum-attacking group to investigate whether Omi/HtrA2 was involved in postasphyxial serum-induced injury to the human kidney proximal tubular cell line HK-2 cells. In the next stage of our study, we included the ucf-101 group to determine the intracellular signal transduction pathway of Omi/HtrA2 in such injuries. Cells from all groups were harvested and assayed after 24 h of treatment.

Preparation of postasphyxial serum in neonates

Neonates (mean gestational age, 37-40 weeks) with neonatal asphyxia (Apgar score lower than 7 points at 1 minute) admitted to our neonatal intensive care unit (NICU) between March 2007and December 2007 were enrolled in this study. The parents of the neonates gave informed consent for their participation. None of the patients was administered immune depressants or exposed to infectious disease. Blood was aspirated from the femoral vein within 24 h of birth (5 ml from each, anticoagulated with Liquemine, about 100 ml in total), then the serum was gathered by 3000 rpm centrifugation for 20 min. The serum was inactivated in a thermostatic water bath at 56°C. The serum was filtered and adjusted to 200 ml/L by DMEM medium (Zhao et al., 2009).

Determination of cell viability

Cell viability was determined by MTT assay. Exponentially growing HK-2 cells from each group were seeded in 96-well culture plates in serum-free medium at optimal density. After 24 h of incubation, the cells were treated as described above. After 24 h, 20 μl (5 g/L) MTT (Sigma) solution was added to each well. After 4 h of incubation, the supernatant was removed and 150 μl DMSO was added to each well and swung for 10 min. The optical density at 590 nm was determined with an enzyme linked immunosorbent assay (ELISA) reader. The results were calculated as mean values of eight wells per treatment group.

Detection of the expression of Omi/HtrA2 and caspase-3

The expression of Omi/HtrA2 and caspase-3 in cytoplast was detected by SP immunocytochemical staining. Immunostaining was carried out according to the standard-procedure (SP) method and the manufacturer's instructions. Cells were incubated overnight with the primary antibody at 4°C. The same process with PBS instead of primary antibody was used as a control. After incubation with the secondary antibody at 37°C for 10 min and then DAB staining, cells were mounted and observed under a microscope. Five high-power fields were randomly selected. The positive cells appeared brownish-yellow in the cytoplast area. The expression of Omi/HtrA2 was determined by counting the number of positive cells among 200 cells. The expression of caspase-3 was analyzed with the Image-Pro Plus6.0 Image Analyzing System.

Confocal microscopy for Omi/HtrA2 translocation

Cells cultured on glass cover slips in orifice were incubated with MitoTracker Red 580 (1:50000 dilution) for 20 min at 37°C in the dark. The cells were then fixed on cover slips with 4% paraformaldehyde (15 min, room temperature) and washed with PBS followed by a permeabilization step with 0.1% Triton X-100 in PBS for 15 min at room temperature. After several washes with PBS, the cover slips were incubated sequentially with blocking buffer for 20 min at room temperature, an anti-human Omi/HtrA2 rabbit polyclonal antibody for 1 h at 37°C (5μg/mL), and secondary antibody (goat anti-Rb IgG/FITC) diluted in blocking buffer (1:150) for 30 min at 37°C in the dark. The cover slips were washed with PBS several times and mounted onto glass slides (VWR) using glycerin. Omi/HtrA2 translocation was observed by confocal microscopy. The images were acquired in confocal microscope at excitation and emission wavelengths of 579 nm and 495 nm, respectively.

Flow cytometric analysis of cell apoptosis

Cell apoptosis was determined by flow cytometry after staining with propidium iodide (PI). HK-2 cells (at least 1×10^6 per sample) from each group were harvested by trypsinization (0.25% trypsin), washed by PBS, and fixed in 70% pre-cooled ethanol. The tubes containing the cells were stored at 4°C for 24 h. The cells were washed by centrifugation at 2000 rpm for 5 min in PBS and stained with PI solution (0.05g/L, 5g/L RNase A, 10g/L Triton X-100) in the dark for 40 min at 37°C. Cells were then resuspended in PBS and centrifuged at 2000 rpm for 5 min. before being resuspended in 500 μl PBS for flow cytometric analysis.

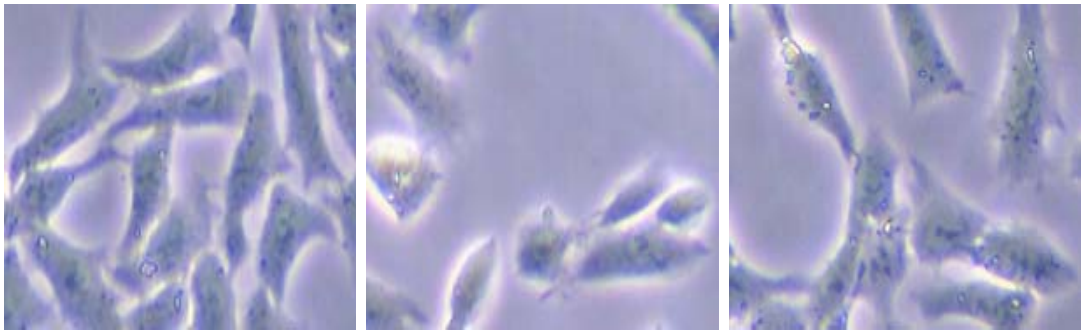
Statistical analyses

All parameters were presented as mean±SEM ($\bar{x}\pm S$). The translocation and expression of Omi/HtrA2 between groups were analyzed via T test. The other data among groups were analyzed using a one-way ANOVA followed by a mean comparison using a *post hoc* LSD test. Probabilities of 0.05 or less were considered statistically significant. The statistical analyses were performed using SPSS 10.0 software.

RESULTS

Changes in morphology and viability of HK-2 cells

Under inverted microscopy, HK-2 cells from the control group showed normal morphology with tight connections to neighboring cells (Fig. 1A). Com-



A B C

Fig. 1. The effect of ucf-101 on the morphology of HK-2 cells treated with postasphyxial serum (inverted microscopy×400). A - control group; B - postasphyxial serum-attacking group; C - ucf-101-treated group.

pared to those of the control group, the cells from the postasphyxial serum-attacking group assumed an off-normal rounded or ellipsoid appearance. The refraction rate was lower and contour was enhanced. Vacuoles, lipid droplets, and granulation appeared in the cytoplasm. There was a great deal of cell debris in the accrescent intercellular space (Fig. 1B). Relative to those of the postasphyxial serum-attacking group, the changes in morphology of the HK-2 cells of the ucf-101-treated group were clearly improved (Fig. 1C).

Relative to the control group (0.47±0.02), the cell viability (optical density, OD) was significantly decreased in the postasphyxial serum-attacking group (0.22±0.02). Relative to the postasphyxial serum-attacking group, cell viability was clearly increased in the ucf-101-treated group (0.36±0.02, $P<0.05$) but not to the same level as the control group (Fig. 2).

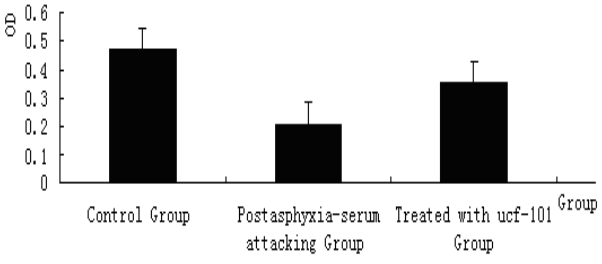


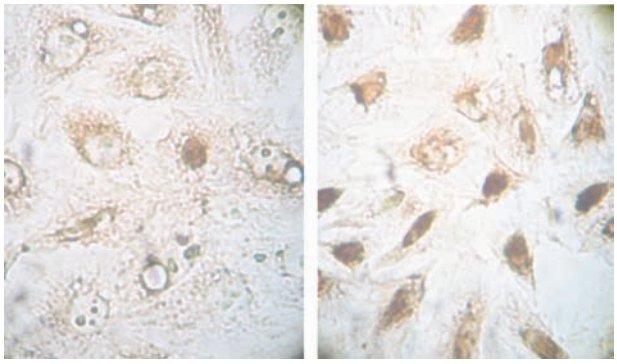
Fig. 2. The effect of postasphyxial serum on the cell viability of HK-2 cells. Cell viability was determined by MTT assay. The control group shows the highest cell viability of all three groups. After postasphyxial serum challenge, the cell viability of the control group decreased remarkably but was partly reversed by treatment with ucf-101.

Effects of postasphyxial serum on Omi/HtrA2 expression in HK-2 cells

Several studies have reported that Omi/HtrA2 plays an important role in post-ischemic organ injury (Bhuiyan and Fukunaga, 2007; Saito et al., 2004). Following ischemia/reperfusion in the kidney, the proteolytic activity of Omi/HtrA2 is markedly upregulated (Faccio et al., 2000). To investigate whether Omi/HtrA2 was involved in postasphyxial serum-induced injury to HK-2 cells, we examined the level of expression of Omi/HtrA2 in HK-2cells by SP immunocytochemical staining. As shown in Fig. 2, Omi/HtrA2 was stained inaurate or yellow-brown and appeared localized in the cytoplasm. The percentage of control group cells expressing Omi/Htra2 was 9.00±2.50% (Fig. 3A). After postasphyxial serum challenge, the percentage of the postasphyxial serum-attacking-group Omi/Htra2-positive cells increased remarkably (25.15±3.50%, $P < 0.05$) (Fig. 3B).

Effects of postasphyxial-serum on Omi/HtrA2 translocation from mitochondria to cytoplasm

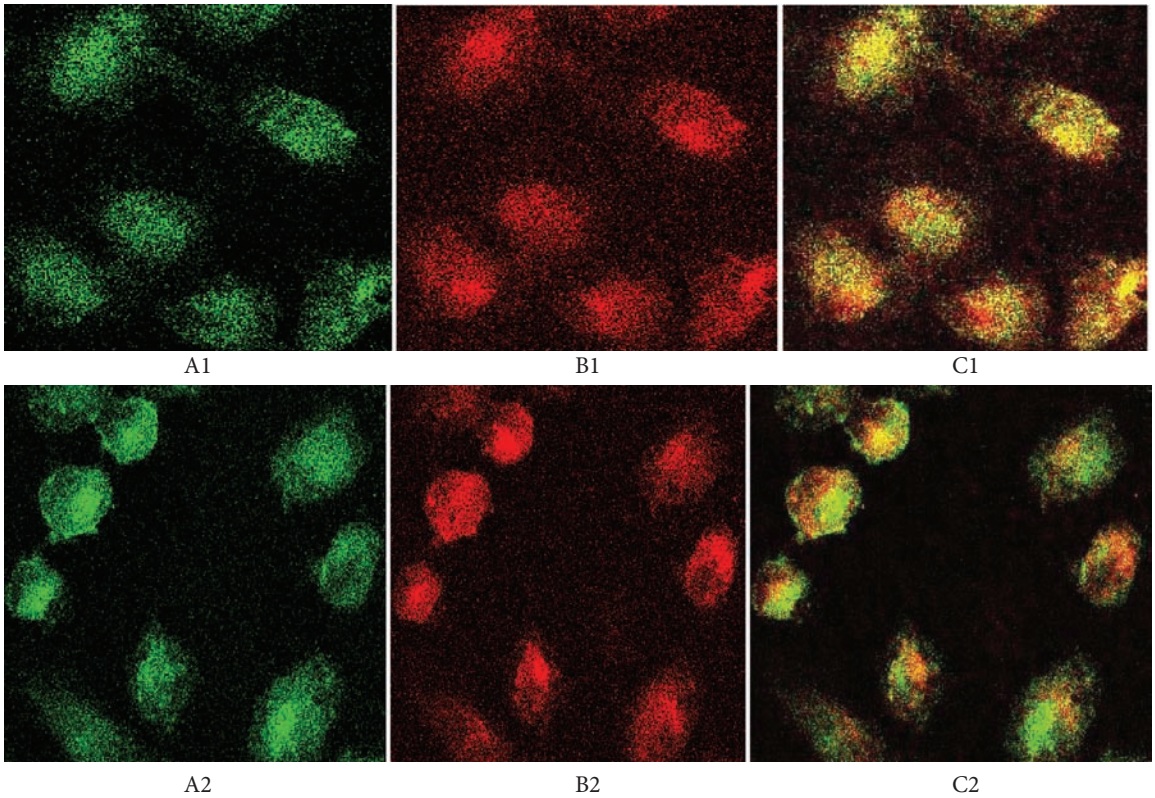
The mature form of Omi/HtrA2 is localized in the mitochondria and may have a distinct function involved in the maintenance of mitochondrial homeostasis (Jones et al., 2003). In the cytoplasm, mature Omi/HtrA2 can induce apoptosis in human cells,



A B

Fig. 3. Postasphyxial serum-induced changes in expression of Omi/HtrA2 in HK-2 cells. The level of expression of Omi/HtrA2 was detected by SP immunocytochemical staining. The percentage of cells expressing Omi/HtrA2 is small in the control group (Fig. 3A). After postasphyxial serum challenge, the percentage of postasphyxial serum-attacking-group cells expressing Omi/HtrA2 increased remarkably (Fig. 3B).

either in a caspase-independent manner through its protease activity or in a caspase-dependent manner. The key step in this apoptotic pathway is the release of Omi/Htra2 from the mitochondrial intermembrane space. To confirm that the Omi/HtrA2 protein is translocated from mitochondria to cytoplasm in HK-2 cells treated with postasphyxial serum, we compared the fluorescence-staining pattern of cells stained with Omi/HtrA2 antibodies and MitoTracker Red 580. The results of the two staining patterns were almost identical in the control group, indicating that Omi/HtrA2 is localized predominantly in the mitochondria in normal HK-2 cells (Fig. 4C1). The two staining patterns were distinctive in the postasphyxial serum-attacking group, indicating that Omi/HtrA2 is translocated from the mitochondrial intermembrane space to the cytoplasm (Fig. 4C2).



A1 B1 C1

A2 B2 C2

Fig.4. The effects of postasphyxial serum on the translocation of Omi/HtrA2 in human renal tubular cell (HK-2). Confocal microscope images of HK-2 cells (1. Control group, 2. postasphyxial serum-attacking group) stained with anti-Omi/HtrA2 (green) and MitoTracker Red 580 (red). Merged images from A1 and B1 are shown in C1. A2 and B2 are shown in C2. In the control group, Omi/HtrA2 protein staining colocalized with MitoTracker staining (C1). After exposure to postasphyxial serum, the two staining patterns were distinctive (C2).

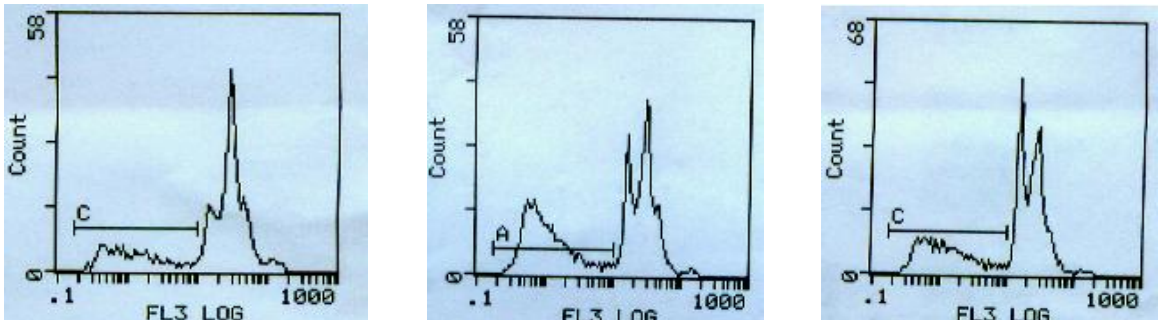


Fig. 5. The effect of postasphyxial serum on apoptosis in HK-2 cells. HK-2 cell apoptosis was detected by flow cytometry. The proportion of cells undergoing apoptosis is small in the control group (Fig. 5A).The proportion of cells undergoing apoptosis increased significantly in the postasphyxial serum-attacking group (Fig. 5B). Relative to the postasphyxial serum-attacking group, the proportion of cells undergoing apoptosis decreased significantly in the group treated with ucf-101 (Fig. 5C).

Effects of postasphyxial serum on apoptosis in HK-2 cells

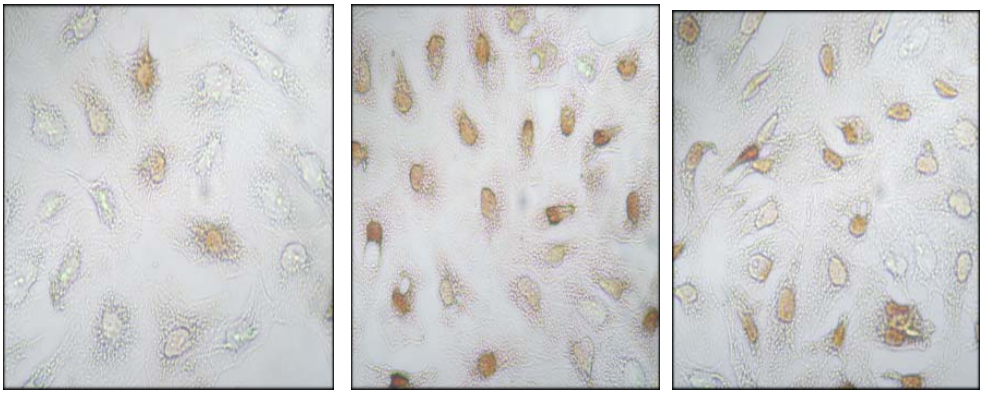
Apoptosis plays an important role in the pathogenesis of a variety of ischemia/reperfusion (I/R) injuries. We postulate that apoptosis is associated with postasphyxial serum-induced injury to HK-2 cells. Apoptosis of HK-2cells was evaluated by quantitative determination of apoptosis using flow cytometric DNA analysis following propidium iodide (PI) staining. The data were presented as the percentage of apoptotic cells. As shown in Fig. 4, the proportion of cells undergoing apoptosis increased from fewer than 15% in the control group (Fig. 5A) to 36% after being attacked by postasphyxial serum (Fig. 5B, $P<0.05$). The proportion of cells undergoing apoptosis decreased from 36% in the postasphyxial serum-attacking group to 26% after ucf-101 treatment (Fig. 5C, $P<0.05$).

The effect of postasphyxial serum on caspase-3 expression in HK-2 cells

The caspase family is closely connected with many apoptotic processes. In the caspase family, caspase-3 is a common executer of apoptosis (Thornberry and Lazebnik, 1998). Multiple lines of evidence have demonstrated that caspase-3 plays a vital role in I/R injury. To confirm that caspase-3 participates in postasphyxial serum-induced injury to HK-2, we examined the level of expression of caspase-3 in HK-2cells. As shown in Fig. 5, caspase-3 was inaurate or yellow-brown and appeared localized in the cytoplasm of HK-2 cells. After being attacked by postasphyxial serum, the level of expression of caspase-3 was significantly increased, as indicated by the stronger staining in the postasphyxial serum-attacking group (Fig. 6B) relative to the control group (Fig. 6A, $P < 0.01$). After treatment with ucf-101 (10 μmol/L), the expression of caspase-3 was significantly decreased (Fig. 6C) relative to the postasphyxial serum-attacking group ($P<0.05$).

DISCUSSION

It has been reported that ischemia/reperfusion induces apoptosis in many cells through different mechanisms. As a mitochondrial serine protein, mature Omi/HtrA2 is localized in mitochondria. Once released into the cytosol following apoptosis stimuli, it promotes cell death by antagonizing IAPs (in a caspase-dependent fashion) and via its proteolytic activity (in a caspase-independent fashion) (Saelens et al., 2004). Several studies have demonstrated that overexpression of Omi/HtrA2 markedly increases apoptosis (Kim et al., 2010; Tun et al., 2007). In this study, we demonstrated that postasphyxial serum incubation can significantly induce apoptosis in human HK-2 cells and that this effect is mediated by overexpression and



A B C

Fig. 6. The effect of postasphyxial serum on the expression of Omi/HtrA2 in HK-2 cells. The expression of caspase-3 was detected by SP immunocytochemical staining. The proportion of the cells expressing caspase-3 was small in the control group (Fig. 6A). After postasphyxial serum challenge, the percentage of cells expressing caspase-3 increased remarkably, as shown in this image of the postasphyxial serum-attacking group (Fig. 6B). After ucf-101 treatment, the percentage of cells expressing caspase-3 decreased remarkably (Fig. 6C) but not to the level of the control group.

mitochondrial release of the Omi/HtrA2 protein into cytoplasm.

Omi/HtrA2 is a member of the HtrA family of serine proteases, which shows extensive homology to the *Escherichia coli* HtrA genes that are essential for bacterial survival at high temperatures. The serine protease Omi/HtrA2 is synthesized as a precursor (premature Omi/HtrA2) and translocated into the mitochondria. Upon apoptotic stimuli, the N-terminal amino acids preceding alanine 134 are cleaved and the resulting mature Omi/HtrA2 is released from the mitochondria into the cytoplasm, where it induces apoptosis (Suzuki et al., 2004). Transcription of Omi/HtrA2 has been shown to be upregulated in ischemic human kidneys. The enzymatic activity of Omi/HtrA2 is substantially enhanced ischemic/reperfusing mouse kidneys (Faccio et al., 2000). Here, we demonstrated that Omi/HtrA2 was overexpressed in HK-2 cells after these cells are attacked by postasphyxial serum. Arnold Levine and colleagues have identified Omi/HtrA2 as a p53-targeted gene (Jin et al., 2003). Kelly et al. reported that p53 protein levels increase significantly in the kidney over 24 h post-ischemia (Kelly et al., 2003). In postasphyxial serum-treated HK-2 cells, activation of the p53 protein increases the transcription of the Omi/HtrA2 gene.

The key step in the role that Omi/HtrA2 plays in postasphyxial serum-induced HK-2 cell apoptosis is its release from the mitochondrial intermembrane space. After I/R injury, Ca2+ influx through the L-type calcium channel triggers Ca2+ release from the InsP3R, which activates a cascade of Ca2+ release from the ER storage area (Wu et al., 2008). However, in ischemia/reperfusion injuries, severe depletion of ATP leads to failure of the pump-leak balance mechanism, leading to an influx of Na+ that results in overload of Na+ in the cytosol. This increased Na+ level activates Na+-K+-ATPase and consumes ATP, which further activates nonselective Ca2+ channels, resulting in massive cytosolic Ca2+ accumulation. Increased cytoplasmic Ca2+ may activate calpain, and calpain can cleave Bid (Chen et al., 2001b). After cleavage, the truncated C-terminal portion of Bid (tBid) translocates to the mitochondria and is inserted into the outer membrane via its tail. It then binds to Bax, facilitating its insertion into the outer mitochondrial membrane and creates pores (Eskes et al., 2000). In addition, monomeric Bax and tBID together can induce lipid remodeling following the permeabilization of the outer mitochondrial membrane (Kuwana et al., 2002). Furthermore, full-length Bid may directly translocate to the mitochondria, where it may cause effects similar to those of Bax (Pei et al., 2007; Verhagen et al., 2002). As the permeability of

the outer mitochondrial membrane increases, Omi/HtrA2 is released from the mitochondria to the cytoplasm.

TNF-α may also play an important role in the translocation of Omi/HtrA2 from the mitochondria to the cytoplasm. In renal ischemia/reperfusion, TNF-α gene transcription is primarily regulated by NF-κB activation (Donnahoo et al., 2000). Usually, NF-κB is localized in the cytoplasm in an inactive state due to its association with a class of inhibitory proteins termed inhibitory κB (IκB). During cellular ischemia/reperfusion injury, phosphorylation and subsequent ubiquitination of IκB leads to the release and nuclear translocation of NF-kB, where it promotes the transcription of genes such as TNF-α (Chen et al., 2001a). Recent studies have demonstrated that TNF-α involves a sequential signaling complex termed "complex I," which contains TNFR-associated factor 2 (TRAF2) and promotes the activation of the cytoprotective transcription factor NF-κB (Micheau and Tschopp, 2003). When NF-κB is activated by complex I, a positive feedback for TNF-α gene transcription is formed. On one hand, TNF-α-induced oxidative stress alters redox homeostasis by impairing the MPTP protein adenine nucleotide translocator and voltage-dependent anion channel, thereby resulting in the opening of pores and inducing the release of Omi/HtrA2 (Mariappan et al., 2007). On the other hand, the increased TNF-α level in the cytosol activates caspase-8, a proximal effector protein from the tumor necrosis factor receptor family. Even small amounts of activated caspase-8 are able to cleave Bid efficiently, the truncated form of which translocates to mitochondria and induces the release of Omi/HtrA2 through the mechanism described above.

After being released from the mitochondria, Omi/HtrA2 may play an important role in apoptosis by means of binding and cleaving IAP proteins and relieving their inhibitory effect on caspases (Verhagen et al., 2007; Yang et al., 2003). The protease activity of Omi/HtrA2 is central to its function. Ucf-101, a specific Omi/HtrA2 inhibitor, is able to inhibit the protease activity of Omi/HtrA2 (Kim et al., 2010). Here we report that HK-2 cells treated with ucf-101 are resistant to postasphyxial serum-induced cell apoptosis. Our data demonstrate that Omi/HtrA2 plays a significant role in postasphyxial serum-induced HK-2 cell apoptosis and that its serine protease activity is necessary and essential for its proapoptotic function in this system. The role of Omi/HtrA2 in the cytoplasm is associated with caspase-3. Many studies have demonstrated that caspase-3 is upregulated in organs affected by ischemia/reperfusion injury (Li et al., 2008; Teruya et al., 2008). Our results demonstrate that the level of expression of caspase-3 is upregulated in HK-2 cells attacked by postasphyxial serum. Caspase-3 can also cleave and activate Bid after the onset of apoptosis as part of a positive feedback loop (Slee et al., 2000). HK-2 cells treated with ucf-101 are resistant to postasphyxial serum-induced caspase-3 expression. Our findings suggest that Omi/HtrA2 is associated with the expression of caspase-3 in HK-2 cells treated with postasphyxial serum.

Our studies suggest that Omi/HtrA2 plays an important role in neonatal postasphyxial serum-induced injury in renal tubular cells. After HK-2 cells are attacked by neonatal postasphyxial serum, Omi/HtrA2 was released from the mitochondria to the cytoplasm where it induced caspase-dependent apoptosis in HK-2 cells through its proteolytic activity. Our findings point to a novel way of relieving postasphyxial serum injuries to human kidney cells through inhibiting the proteolytic viability of Omi/HtrA2.

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