Abstract. Imbalance in mitochondrial fusion/fission is one of the mechanisms leading to sepsis-induced mitochondrial dysfunction and cell apoptosis. The present study examined the effects of human trypsin inhibitor (UTI), a well-known antioxidant and anti-inflammatory substance, on mitochondrial dynamics and cell apoptosis in lipopolysaccharide (LPS)-induced human kidney-2 (HK-2) cells. The HK-2 cells were incubated for 24 h either with LPS (800 ng/ml) or LPS (800 ng/ml) mixed with UTI (250 U/ml). Cell viability was assessed using a3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay. Oxidative activities (estimated by maleic dialdehyde and superoxide dismutase), levels of inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α, and levels of ATP were measured using an enzyme-linked immunosorbent assay. The expression levels of the mitochondrial fission protein, death-associated protein kinase 2 (DAPK-2), mitofusin (Mfn)1 and Mfn2 mitochondrial fusion proteins, and apoptotic and anti-apoptotic biomarkers, including cytochrome c, caspase-3, caspase-9, B-cell lymphoma (Bcl)-2, Bcl-extra large and poly ADP-ribose polymerase (PARP), were assessed using western blot analyses. The changes in mitochondrial membrane potential were analyzed following JC-1 staining. Annexin V/propidium iodide assays were used to evaluate cell apoptosis. The results showed that the balance of mitochondrial dynamics was towards mitochondrial fusion in the UTI group, as a reduced expression of DAPK2, and increased expression levels of Mfn1 and Mfn2 were detected (P<0.05, vs. LPS group). In addition, a decline in the levels of the inflammatory cytokines, TNF-α and IL-6, and the oxidative activities, reflected by an increase in SOD and a decrease in MDA (P<0.05, vs. LPS group) were observed. Cell apoptosis was inhibited following co-treatment with UTI (P<0.05, vs. LPS group). It was concluded that UTI may protect mitochondrial functions by promoting mitochondrial fusion and limiting mitochondrial fission, thus reducing the apoptosis of LPS-induced HK-2 cells.

Introduction

Acute kidney injury (AKI) is a common complication of sepsis, which increases mortality rates to as high as 70% (1). However, the pathophysiology of sepsis-induced AKI remains to be fully elucidated (2). Excessive fission and/or insufficient fusion of mitochondria, which contributes to the progression of sepsis, may be evoked by oxidative stress, and can lead to the loss of mitochondrial function and the apoptosis of tubular cells under stress (3,4).

Human trypsin inhibitor (urinary trypsin inhibitor; UTI) is a Kunitz-type protease inhibitor, which inhibits the activity or release of lysosomal enzymes (5,6). The effects of UTI include the protection of endothelial cells, suppression of excessive superoxide anion radicals, and reductions in systemic inflammation and oxidative stress (7). It has been reported that UTI can protect mitochondria from ischemia functionally and morphologically, thus benefiting renal function (8,9). However, the mechanism remains to be elucidated.
In the present study, it was hypothesized that UTI may have beneficial effects on regulating mitochondrial dynamics. To confirm this hypothesis, the levels of the mitochondrial fission protein, death-associated protein kinase 2 (DAPK-2), and two types of mitochondrial fusion proteins, mitofusin-1 (Mfn1) and mitofusin-2 (Mfn2), were examined in lipopolysaccharide (LPS)-induced human kidney-2 (HK-2) cells incubated with or without UTI. The oxidative activities of inflammatory cytokines, indicated by maleic dialdehyde (MDA) and superoxide dismutase (SOD), and cell apoptosis were also measured.

Materials and methods

Drugs, reagents and kits. LPS from Escherichia coli 055:B5 and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). UTI was purchased from Techpool Bio-pharma Co., Ltd. (Guangzhou, China). MitoProbe J-aggregate (JC-1; 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carboxyanineiodide), propidium iodide (PI), the Annexin V-FITC Apoptosis Detection kit, SOD assay kit (cat. no. KGT0050-1) and MDA assay kit (cat. no. KGT004) were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). The ATPlite 1 step Luminescence ATP Detection Assay system was obtained from PerkinElmer, Inc. (Waltham, MA, USA). The following primary antibodies were used: Caspase-3 (cat. no. SC-7272; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), caspase-9 (cat. no. 9502S; 1:1,000; CST Biological Reagents Company Ltd., Shanghai, China), PARP (cat. no. ab96476; 1:1,000; Abcam, Cambridge, UK), B-cell lymphoma (Bcl)-2 (cat. no. 2870S; 1:1,000; CST Biological Reagents Company Ltd.), Bcl-extra large (Bcl-xL; cat. no. 2764; 1:1,000; CST Biological Reagents Company Ltd.), β-actin (cat. no. AP0060; 1:3,000; Bioworld Technology, Inc., St Louis Park, MN, USA), DAPK-2 (cat. no. 3432-1; 1:1,000; Epitomics; Abcam), Mfn1 (cat. no. 5870-1; 1:1,000; Epitomics; Abcam) and Mfn2 (cat. no. 3272-1; 1:1,000; Epitomics; Abcam). Secondary antibody (horseradish peroxidase-labeled goat anti-mouse immunoglobulin G) was obtained from Abgent Biotechnology Co., Ltd. (cat. no. LP1002a; 1:10,000; Suzhou, China).

Cells culture. The HK-2 cells, an immortalized proximal tubular epithelial cell line from the normal adult human kidney (10), were obtained from American Type Culture Collection (Manassas, VA, USA). The HK-2 cells were cultured in Dulbecco's modified Eagle's medium F-12 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA; cat. no. SH30023.01) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA; cat. no. 10099-141; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere of 5% CO₂ at 37°C.

LPS administration. This procedure was performed to determine the appropriate concentration of LPS to use in the experiments. The cell culture medium was replaced with complete medium supplemented with 0.5% fetal bovine serum and various concentrations of LPS (0, 100, 200, 400, 800, 1,600 and 3,200 ng/ml). Each sample was incubated for different durations (3, 6, 12 and 24 h) at a density of 2,500 cells/well at 37°C, which was performed three times.

Cell viability assay. The cell viability was assessed using a commercial MTT-based assay. This assay detects viable cells based on the production of the purple compound, formazan, in viable cells. Following incubation with LPS or LPS+UTI, 10 μl of MTT (5 mg/ml) was added to each well, and the plates were incubated at 37°C for 4 h. The content of the wells was eluted and the precipitate was dissolved with 150 μl of MTT solubilization reagent (dimethyl sulfoxide). The optical density value (OD value) was read at a wavelength of 490 nm using the Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was determined as the ratio of surviving cells in each group divided by the number in the control ethanol-treated group. The LPS concentration shown to reduce cell viability the most, but affect the surrounding environment of the cells the least, was selected.

UTI administration. This procedure was performed to determine the appropriate concentrations of UTI to use in the experiments. The cell culture media were replaced with complete media supplemented with 10% fetal bovine serum containing LPS at a final concentration of 800 ng/ml (based on the results of the LPS intervention experiment) and various concentrations of UTI (100, 150, 200, 250, 300, 350 and 400 U/ml). In the control group, neither LPS nor UTI was administered. The cell viability assay was then repeated for selection of the UTI concentrations causing a significant increase in cell viability.

Groups. Based on the results of the UTI intervention experiment, the HK-2 cells were randomly divided into a control group, LPS group and UTI group. The cells were treated with LPS at a final concentration of 800 ng/ml in the LPS group, and 800 ng/ml LPS together with 250 U/ml UTI in the UTI group. All cells were cultured at a density of 2,500 cells/well and maintained at 37°C for 24 h.

Measurement of inflammatory response and oxidative activity. Enzyme-linked immunosorbent assay (ELISA) kits were used in this procedure. Following treatment of cells with LPS and UTI, as described above, the cell supernatants were collected (centrifuged at 12,000 x g for 5 min at 37°C) and stored at -80°C. The levels of inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) were measured to evaluate the inflammatory response. SOD and MDA were measured to estimate the oxidative activities. In all cases, a standard curve was constructed from standards provided by the manufacturer.

Analysis of mitochondrial fission/fusion and apoptotic proteins. Western blot analysis was performed to detect the levels of total proteins. Following treatment with LPS and UTI, as described above, the cells were lysed using radioimmunoprecipitation assay buffer [0.6% SDS, 4% glycerine, 12.5% Tris-HCl (1 M; pH 6.8), H₂O], and the concentrations of total proteins in each sample were measured using a BCA protein assay kit. The proteins (50 μg/lane) were separated using SDS-PAGE on a 10% gel and transferred onto PVDF membranes; the membranes were then blocked in 5% fat-free milk at room temperature for 2 h. Following incubation with primary antibodies against caspase-3, caspase-9, PARP, Bcl-2,
Bcl-xL and β-actin, and primary antibodies against DAPK-2, Mfn 1 and Mfn 2 at a dilution of 1:1,000 at 4˚C overnight, the membranes were probed with goat-anti-mouse secondary antibodies at a dilution of 1:10,000 at 37˚C for 1 h. The signals were detected and then analyzed using SensiAnsys software (version JS-680D; Shanghai Peiqing Science & Technology Co., Ltd., Shanghai, China).

Mitochondrial membrane potential (MMP) analysis. The fluorescent probe JC-1 was used to detect the MMP. Following treatment of the cells with LPS and UTI, as described above, the culture medium was removed and 1-2×10⁶ cells were harvested by trypsinization with 0.25% Trypsin-EDTA. The cells were washed twice with PBS and centrifuged at 170 x g for 5 min at room temperature. For each sample, the cells were suspended in 500 µl of JC-1 stock solution (final concentration, 2 mg/ml; diluted by 1X incubation buffer) and then incubated for 15-20 min at 37˚C. The staining procedure was performed according to the manufacturer's protocol. The stained cells were then centrifuged at 700 x g for 5 min at room temperature, washed twice in 1X incubation buffer and re-suspended in 500 µl 1X incubation buffer. JC-1 aggregates (red fluorescence) favor high MMP intact cells and, in response to the loss of MMP, JC-1 monomers are formed showing green fluorescence. The percentage of cells showing a decrease in red fluorescence was evaluated to determine the extent of depolarization. The fluorescence intensity of the red/green ratio was determined semi-quantitatively using flow cytometry (Beckman Coulter, Inc., Brea, CA, USA). Cells with collapsed MMPs exhibited a decrease in the red/green fluorescence intensity ratio.

Measurement of intracellular ATP. Intracellular levels of ATP were measured using a luminescence ATP detection assay according to the manufacturer's protocol. Following treatment of the cells with LPS and UTI, as described above, and equilibrating the microplate at room temperature for 30 min, 100 µl of the reconstituted lyophilized substrate solution was added to each well. Each lyophilized substrate solution vial was reconstituted with 10 ml substrate buffer and agitated gently until homogenous. The 96-well microplate was then shaken for 5 min, and the relative light unit (RLU) was measured using the JANUS® Automated Workstation (PerkinElmer, Inc.) within 10 min in the dark.

Cell apoptosis assay. The Annexin V-FITC kit was used to detect the externalization of phosphatidylserine of the cell membrane, one of the typical markers of early apoptosis. Following treatment of cells with LPS and UTI as described above, the culture medium was removed and 1-5×10⁵ cells were harvested by trypsinization without Trypsin-EDTA, washed twice with PBS and centrifuged at 700 x g for 10 min at room temperature. Then the cells were re-suspended in 500 µl of binding buffer. Subsequently, 5 µl Annexin V-FITC and 5 µl of propidium iodide (PI) were added into each 500 µl of solution according to the manufacturer's protocol. The cells were then gently vortexed and incubated for 5-15 min at room temperature in the dark. Flow cytometry (Beckman Coulter, Inc.) was used to analyze the samples within 1 h. The excitation wavelength was 488 nm and the emission wavelength was 530 nm. The percentage of Annexin V(+)PI(-) and Annexin V(+)/PI(+) cells represent the ratios of early and late stage of apoptosis respectively.

Statistical analysis. All experiments were performed three times. All numerical data are expressed as the mean ± standard deviation. Multiple comparison between the groups was performed using one-way analysis of variance and the least significant difference method. SPSS 22.0 software (IBM SPSS, Armonk, NY, USA) was used. P<0.05 was considered to indicate a statistically significant difference. All statistical graphs were produced using OriginLab Corporation, Northampton, MA, USA).

Results

Cell viability. As shown in Fig. 1A, cell viability was reduced as LPS concentration increased. LPS at concentrations of 1,600 and 3,200 ng/ml caused a rapid reduction in cell viability in all groups, which may have resulted from the high osmotic pressure induced by the high drug concentration on the surrounding environment of the cells. In the 3 and 6 h groups, no significant changes in cell viability were observed with LPS concentrations of 50-800 ng/ml. In the 12 and 24 h groups, cell viability was significantly decreased with LPS concentrations of 50-800 ng/ml (P<0.05, vs. control) the lowest level was observed at 800 ng/ml in the 24 h group. Based on these results, the HK-2 cells were treated with LPS at 800 ng/ml for 24 h in the subsequent experiments.

As shown in Fig. 1B, following co-treatment with different concentrations of UTI for 24 h, cell viability was positively correlated with drug concentrations at 100-200 U/ml, but decreased rapidly at concentrations >250 U/ml, which suggested that concentrations of UTI >250 U/ml led to an excessive increase in osmotic pressure. No significant differences in cell viability were observed between concentrations of UTI at 250 and 200 U/ml. Therefore, 250 U/ml of UTI was used to evaluate its protective function in subsequent experiments.

Release of inflammatory cytokines and oxidative factors induced by LPS is reduced by UTI. As shown in Fig. 2A and B, following treatment with LPS for 24 h, significant increases in the release of IL-6 and TNF-α were detected in the HK-2 cells (P<0.05, vs. control). In the UTI group, the increase in these two inflammatory cytokines were significantly lower (P<0.05 vs. LPS group). The oxidation product, MDA, was significantly increased, whereas the antioxidant enzyme, SOD, was decreased following treatment with LPS (P<0.05 vs. control). These effects were less marked in the UTI group (P<0.05 vs. LPS group; Fig. 2C and D).

LPS-induced increase in mitochondrial fission and reduction in mitochondrial fusion are reversed by UTI. As shown in Fig. 3, the expression of DAPK2 was increased, and the levels of Mfn1 and Mfn2 were decreased in the LPS group (P<0.05 vs. control). These changes suggested that LPS induced increased fission and weakened fusion of mitochondria. In the cells co-treated with UTI, decreased expression of DAPK2, and enhanced expression of Mfn1 and Mfn2 were
found, compared with the levels in the UTI group (P<0.05, vs. LPS group). These changes indicated that UTI increased mitochondrial fusion and limited mitochondrial fission.

**LPS-induced decreases in MMP and intracellular ATP are reversed by UTI.** As shown in Fig. 4A and B, LPS stimulation led to fewer high red JC-1-positive cells (69.4±0.75%) compared with the control (98.1±0.3; P<0.05), which indicated a decrease in MMP. The decrease in MMP was less marked in the UTI group, compared with that in the LPS group (97.23±0.25 vs. 69.4±0.75%; P<0.05), which indicated amore stabilized mitochondrial membrane. As presented in Fig. 4C, RLU was significantly decreased following treatment with LPS for 24 h (0.12±0.05 vs. 0.33±0.03; P<0.05), compared with that in the negative control, and this reduction was
reversed in the UTI group, compared with that in the LPS group (0.22±0.04 vs. 0.12±0.05; P<0.05). These results indicated that co-treatment with UTI prevented the mitochondrial dysfunction caused by LPS.

LPS-induced cell apoptosis is inhibited by UTI. As shown in Fig. 1B, cell viability was preserved in the UTI groups, compared with that in the LPS group (P<0.05), although UTI concentrations ≥250 U/ml led to a descending trend of cell viability. This suggested that co-treatment with UTI prevented the cell apoptosis induced by LPS.

The hypothesis of the present study was confirmed using Annexin V and PI staining. As shown in Fig. 5, the increase in the percentage of Annexin V-positive cells was marked in the LPS group (34.42±0.64%), compared with that in the negative control (6.47±0.17; P<0.05), whereas cell apoptosis was inhibited following co-treatment with UTI (10.52±0.24), compared with the LPS group (34.42±0.64%; P<0.05).
As shown in Fig. 6, significantly higher expression levels of cytochrome c, caspase-3 and caspase-9 were observed in the HK-2 cells following incubation with LPS for 24 h (P<0.05, vs. control), whereas lower expression levels of cytochrome c, caspase-3 and caspase-9 were observed in the LPS+UTI group. In addition, as shown in Fig. 7A-C, the protein levels of Bcl-2 and Bcl-xL were lower in HK-2 cells treated with LPS (P<0.05 vs. control), whereas their expression levels were increased in the LPS+UTI group (P<0.05). As shown in Fig. 7D, cleavage of PARP was reduced in the LPS group (Fig. 7D; P<0.05, vs. control), but levels of PARP were increased in the UTI group (P<0.05, vs. LPS group).

Discussion

In previous studies, it was found that neither hemodynamic failure nor ischemia were the primary pathogenic factors in septic renal damage. Lerolle et al analyzed postmortem histopathological findings of 19 patients with septic AKI and reported that tubular cell apoptosis was the major histological abnormality. Even in patients with severe septic shock, tubular necrosis was minimal (11). It was reported that inflammation and renal cellular apoptosis were the essential factors (12,13).

Mitochondria are dynamic organelles, which constantly undergo fission and fusion, during which they may exhibit a tubular or fragmented morphology, or they may be assembled into networks in response to cellular energy demands and environmental challenges (14,15). In mammalian cells, DAPK2 (also known as Drp1) is one of the primary mitochondrial fission proteins, and the large mitochondrial GTPases, Mfn1 and Mfn2 are important in mitochondrial fusion (16).

The suppression of Drp-1 has been reported to maintain mitochondrial function and reduce the apoptosis of tubular...
cells induced by ischemic injury, rhabdomyolysis and cisplatin nephrotoxin (17,18). MFN2 deficiency has been shown to promote Bcl-2-associated X protein-mediated mitochondrial outer membrane injury and increase the apoptosis of tubule epithelial cells under stress (19). These findings indicate that the regulation of mitochondrial dynamics may be one approach in protecting mitochondrial function and reducing tubule epithelial cell apoptosis. However, there have been no studies focusing on the mitochondria dynamics of tubule epithelial cells in septic AKI.

In the present ex vivo study, it was confirmed that excessive mitochondrial fission and insufficient mitochondrial fusion were present in LPS-induced HK-2 cells. Following stimulation with LPS, the HK-2 cells showed significantly increased expression of DAPK2, and decreases in the expression levels of Mfn1 and Mfn2, indicating an increase of mitochondrial fission and decrease of mitochondrial fusion. Decreased mitochondrial dysfunction and cell apoptosis were also detected, in addition to increased levels of cytochrome c, caspase-3 and caspase-9, and decreased levels of Bcl-2, Bcl-xL and PARP.

It has been shown previously that UTI can suppress the excessive generation of superoxide anion radicals, systemic inflammation, oxidative stress and endothelial injury caused by endotoxins (7), particularly against LPS-induced kidney injury (20). In a previous rat model of ischemia, UTI was shown to have protective effects on mitochondria in the kidney (8,9).

In the present study, when co-treated with UTI, the HK-2 cells showed higher expression levels of Mfn1 and Mfn2, and lower expression levels of Drp1, suggesting a positive role of UTI in preventing excessive mitochondrial fission. Mitochondrial dysfunction and cell apoptosis were also decreased as a subsequent effect. As previous studies have already shown that oxidative stress and inflammation can induce disordered mitochondrial fission (21,22), the present study hypothesized that the regulatory effect of UTI on mitochondrial dynamics may function by suppressing the oxidative and inflammatory damage caused by LPS. The decreased levels of IL-6, TNF-α and MDA, and the increased expression of SOD support this hypothesis.

Taken together, the results of the present study suggested that UTI protected human tubular epithelial cells from apoptosis by preventing the excessive mitochondrial fission induced by LPS. The effect may result from its antioxidant and anti-inflammatory effects. Future investigations are required to evaluate the effect of UTI on mitochondrial dynamics in an animal model of sepsis.

In conclusion, the present study showed that UTI prevented mitochondrial dysfunction caused by LPS by promoting mitochondrial fusion and limiting mitochondrial fission, thus reducing the apoptosis of LPS-induced HK-2 cells. This protective effect may be accomplished through decreasing the release of inflammatory cytokines and limiting oxidative activities.

Acknowledgements

This study was part of the Program of The National Natural Science Foundation of China (grant no. 81201452).
References