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Ming Wu#, Min Hu#, Huansheng Tong, Junying Liu, Hui Jiang, Ming Zhang, Lei Su, Mingli Li*, Yongwen Feng*, Biao Cheng*

Regulatory mechanism of ulinastatin on autophagy of macrophages and renal tubular epithelial cells

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Abstract: Kidney ischemia and hypoxia can cause renal cell apoptosis and activation of inflammatory cells, which lead to the release of inflammatory factors and ultimately result in the damage of kidney tissue and the whole body. Renal tubular cell and macrophage autophagy can reduce the production of reactive oxygen species (ROS), thereby reducing the activation of inflammatory cytoplasm and its key effector protein, caspase-1, which reduces the expression of IL-1β and IL-18 and other inflammatory factors. Ulinastatin (UTI), as a glycoprotein drug, inhibits the activity of multiple proteases and reduces myocardial damage caused by ischemia-reperfusion by upregulating autophagy. However, it can be raised by macrophage autophagy, reduce the production of ROS, and ultimately reduce the expression of inflammatory mediators, thereby reducing renal cell injury, promote renal function recovery is not clear. In this study, a series of cell experiments have shown that ulinastatin is reduced by regulating the autophagy of renal tubular epithelial cells and macrophages to reduce the production of reactive oxygen species and inflammatory factors (TNF-α, IL-1β and IL-1), and then, increase the activity of the cells under the sugar oxygen deprivation model. The simultaneous use of cellular autophagy agonists Rapamycin (RAPA) and ulinastatin has a synergistic effect on the production of reactive oxygen species and the expression of inflammatory factors.

Keywords: Renal ischemia and hypoxia; Ulinastatin; Autophagy; Inflammatory response.

1 Introduction

Kidney ischemia and hypoxia-induced acute renal injury (AKI) can induce systemic inflammatory response, which can promote or accelerate the death of patients [1,2]. Activation of inflammatory cells in the kidney, the release of inflammatory mediators and changes of inflammatory cell microenvironment is caused by hemorrhagic shock kidney injury and is one of the important reasons. Inflammatory cells (neutrophils, lymphocytes, monocytes/macrophages, dendritic cells (DCs))and other immune cells in the kidney mediate uncontrolled release of cytokines (TNF-α, IL-1β etc) by TLRs / NF-κB signaling pathway, which leads to acute renal injury (AKI) [2,3]. Macrophages play a key role in acute kidney injury, which both regulates kidney damage and promotes repair of damaged renal tissue [4]. When the exogenous adverse stimuli (including ischemia-reperfusion injury, stress, microbial) attack the body, monocyte-macrophages recognize the pathogen-associated molecular patterns (PAMP) through the toll-like receptors (TLRs). Then, it will activate nuclear transcription factor-kappaB (NF-κB) through a series of

***Corresponding authors: Mingli Li**, Department of interventional therapy, Shen Zhen No.2 People's Hospital, Shenzhen 518035, P.R. China, Email: boshiyy@163.com; **Yongwen Feng,** Department of Critical Care Medicine,ShenZhen No.2 People's Hospital, Shenzhen 518035, China, Email: fengyongwen2008@126.com
Biao Cheng, Department of Plastic Surgery, Guangzhou School of Clinical Medicine, Southern Medical University (Guangzhou General Hospital of Guangzhou Military Region) Guangzhou 510010, China, Email: chengbiaocheng@163.com
Ming Wu, Ming Zhang: Southern Medical University, Guangzhou 510515, China
Ming Wu, Hui Jiang: Department of Critical Care Medicine, Shen Zhen No.2 People's Hospital, Shenzhen 518035, China
Min Hu: Emergency Department, Changhai Hospital, Shanghai 200433, China
Huansheng Tong, Lei Su: Department of Intensive Care Unit, Guangzhou School of Clinical Medicine, Southern Medical University (Guangzhou General Hospital of Guangzhou Military Region) Guangzhou 510010, China
Junying Liu: Department of Endocrinology, Shen Zhen No.2 People's Hospital,Shenzhen 518035, China
Ming Zhang: Department of Critical Care Medicine ,The People's Hospital of Qin Yuan, Qin Yuan, 511500, China
# these authors contributed equally to this study

signal transduction, leading to cascade amplification of inflammation and the formation of systemic inflammatory response syndrome (SIRS). However, if the macrophage autophagy can be upregulated, it will promote the protection after the renal ischemia-reperfusion injury.

Autophagy is a process in which cells use a large amount of lysosomes to degrade long-lived proteins, macromolecules, ribosomes and damaged organelles [5,6]. It has shown that ischemia-reperfusion can induce autophagy, whereas autophagy protects the kidneys by inhibiting the release of inflammatory factors [6,7]. In the mouse renal ischemia-reperfusion injury, autophagy in renal tissue was significantly enhanced during reperfusion, indicating that autophagy was involved in the AKI process [6,8,9]. Hypoxia can induce autophagy of proximal renal tubular cells, and both the application of autophagy inhibitor 3-methylpurine and the knock-out of Beclin-1 and ATG5 genes of renal tubule cells by small RNA increased the sensitivity to hypoxia, which then induces the cell apoptosis [10]. After the perfusion of the proximal tubule-specific Atg5-deficient mice, the expression of LC3 in the proximal tubule cells was inhibited, and the accumulation of autophagic substrate and serum creatinine and urea nitrogen were obviously increased compared to the control group [11]. Moreover, autophagy protects proximal tubular cells by inhibiting mitochondrial stress induced by cyclosporin A [11]. Saitoh *et al* found that after LPS stimulation, macrophages of autophagy gene Atg16L1-deficient mouse released more inflammatory cytokines IL-1β and interleukin-18 (IL-18) than normal cells [12]. In RAW264.7 macrophages, inhibition of autophagy with autophagy inhibitor 3-methyladenine (3-MA) significantly increased the production of inflammatory cytokines IL-1β [13,14]. However, the autophagy-upregulated human monocytes induced by starvation pretreatment significantly reduced the muramyl dipeptide-induced IL-1β secretion [15,16]. It was also found that mechanism of reduction of IL-1β, IL-18 and other inflammatory factors is mainly by removing damaged mitochondria, reducing reactive oxygen species (ROS) production and releasing Mitochondrial DNA (mitochondrial DNA, mtDNA) into the cytoplasm, thereby the activations of inflammasomes and the key effector protein caspase-l will be decreased, which reduces the release of inflammatory factors [16]. These reactive O_2 metabolites, including superoxide anion, hydrogen peroxide and hydroxyl radical are usually collectively referred to as ROS. ROS generated within cells or in a tissue environment, which were originally characterized in terms of their harmful effects on cells and invading microorganisms, are increasingly implicated in various cell fate decisions and signal transduction pathways [17,18]. Previous studies have indicated that Ulinastatin (UTI) can protect kidney function by inhibiting oxygen free radicals and tumor necrosis factors, stabilizing lysosomal membrane, increasing the stability of mitochondria and endoplasmic reticulum [19]. Upregulation of autophagy in monocyte-dendritic cells would inhibit the expression of ROS and mtDNA and down-regulate inflammation [20].

UTI is a glycoprotein that is isolated and purified from fresh urine of healthy adult men and is also a protease inhibitor [21]. Its clinical application is mainly for acute pancreatitis and severe sepsis treatment. UTI can mitigate the damaged liver after ischemia-reperfusion injury by suppressing neutrophil aggregation [22], which promotes the regeneration of the liver after the majority of liver resection [23]. UTI also decreases the content of pulmonary water in hemorrhagic shock rats and reduces lung histopathological changes [24]. Both the ileal mucosal cell apoptosis in hemorrhagic shock rat and the damage caused by acute renal ischemia were decreased [21]. Therefore, it is particularly important to explore the molecular mechanism of UTI for its clinical application. Recent studies have shown that UTI can upregulate autophagy through the mTOR pathway to protect myocardial cells from ischemia-reperfusion injury [25]. So, it is still unclear whether UTI can increase the expression of ROS by up-regulating the autophagy of macrophages and ultimately decrease the expression of TNF-α, IL-1β, IL-18 and other inflammatory mediators to change the renal macrophage microenvironment and promote renal function recovery.

Therefore, this study aims to illuminate the effect of UTI on the phenotype of primary renal macrophages *in vitro* and on the biological activity of renal tubular cells co-cultured with primary renal macrophages by using the oxygen glucose deprivation model (OGD). This will reveal the effect of UTI on macrophage LC3 and the molecular mechanism for renal injury protection, which will provide a theoretical basis for the clinical treatment of renal injury by hemorrhagic shock

2 Materials and methods

2.1 Isolation and culture of rat macrophages and rat renal tubular epithelial cells

The animal procedures were performed by following the Institutes of Health Guide for the Care and Use of Laboratory Animals [26], and the experiments were approved by the

Animal Use Committee of Shengzhen Second Hospital (ID# Number: 20160528). Rat macrophages were isolated from the peritoneal cavity of wild-type (WT) rats. WT rats were intraperitoneally injected with 400 mL phorbol myristate acetate (PMA) overnight to stimulate the accumulation of macrophages. After anesthesia with CO_2, the rats were killed and after disinfection with 70% ethanol a small incision was made along the midline of each rat with sterile scissors. Ice-cold PBS (3 mL) was injected into the peritoneal cavity of each rat and the cell suspension was extracted from the peritoneal cavity with 5-mL syringes. The cell suspension was centrifuged at 1200 rpm room temperature for 10 min and the supernatant was discarded. The cell pellet was re-suspended into 10 mL basic DMEM/F-12 medium (Gibco, NY, USA). After cell counting, 3×106 cells were seeded into 6-well plates. The floating cells were removed by gently washing with PBS 3 times. After a 2 hour incubation, the adherent cells were cultured in DMEM/F-12 medium supplemented with 10% FBS (Gibco, NY, USA). By this stage, over 90% cells were determined as macrophages [25]. Rat renal tubular epithelial cells were purchased from Biopike (Peking, China) and they were cultured in DMEM medium supplemented with 10% FBS (Gibco, NY, USA).

2.2 Cell Viability Analysis

To assess the effects of UTI in cell viability, cells were seeded into a 96-well plate at 5×10^3 cells/well. After incubation for 24 h, the cells were exposed to OGD and/or UTI for 24 h. Cells were then treated with 0.5 mg/mL MTT. After cells were cultured for an additional 4 h, the cell culture medium was replaced by 150 μL DMSO and the absorbance at 570 nm was measured with a spectrometer (Model 680, Bio-Rad, Hercules, CA, USA).

2.3 Western Blot Analysis

After treatment with different drugs, the cells were washed on ice with cold PBS and lysed on ice for 30 min in radioimmunoprecipitation (RIPA) buffer. The cell lysis was centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was then transferred to another tube and the total protein concentration was determined by BCA assay kit. Western blotting was performed by following the standard protocol. The primary antibodies were LC3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was the corresponding HRP-conjugated secondary antibody (1:5000; Zhongshan Golden Bridge Biotechnology, Nanjing, China). Finally, the blotting bands were visualized by the Gel Image System (Tannon, Biotech, and Shanghai, China) and the acquired bands were quantified with the Quantity One Software (Bio-Rad).

2.4 Real-time polymerase chain reaction (q-PCR)

Total RNA was extracted using TRIzol reagent (Takara, Dalian, China) and cDNA synthesis was carried out using PrimeScriptTM RT reagent kit (Takara). qRT-PCR was performed using 2× SYBR® Premix Ex TaqTM II and primers for rat LC3 (5-TGAATATGAGCGAACTCATCAAG-3' forward; 5'-CATGCTGTGCCCATTCAC-3' reverse) and Beta-actin (5-CCCATCTATGAGGGTTACGC-3' forward; 5'-TTTAATGTCACGCACGATTTC-3' reverse). Experiments were performed in triplicate for each sample on Bio-Rad iQ5 system and the acquired data were analyzed using the Bio-Rad Optical System Software (Bio-Rad). Amplification of the rat Beta-actin gene was used to normalize the experimental data.

2.5 ELISA assay

ELISA experiment was performed by following the manufacturer's instructions (Senxiong Technology Industrial Co., Ltd., Shanghai, China). The absorbance at 450 nm was detected by a microplate reader, and all the results were standardized using a standard curve.

2.6 Statistical analysis

Experimental data were analyzed by SPSS 16.0 statistical software and the results were presented as the mean ± SD (standard deviation). The multiple groups were statistically analyzed by one-way ANOVA, and pairing groups were analyzed by Student's t-test. When the P value was less than 0.05 ($P < 0.05$), data were considered significantly different.

3 Results

3.1 Effects of UTI on the activity of Rat renal tubular epithelial cells under the OGD condition

In order to detect the effect of UTI on the viability of rat renal tubular epithelial cells, 3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide assay (MTT) was applied to test the cell biological activities of these cells In vitro. The result indicates that in OGD model the cell viability of rat renal tubular epithelial cell was significantly decreased comparing with the control group, but UTI can significantly improve the cell viability caused by the oxygen glucose deprivation (Figure 1).

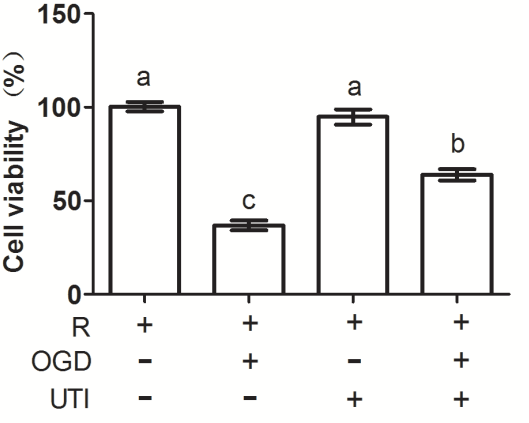

Figure 1: UTI increased the viability of renal tubular epithelial cells in OGD condition. R: Renal tubular epithelial cells; OGD: Oxygen-glucose deprivation; UTI: Ulinastatin. Bars with different letters are significantly different ($p < 0.05$).

3.2 Autophagy is involved in the regulation of cell viability by UTI

The rat renal tubular epithelial cells were co-cultured with the isolated primary macrophages and treated differently: oxygen glucose deprivation (OGD group), UTI treatment (UTI group), oxygen glucose deprivation and then UTI treatment (OGD + UTI group), oxygen glucose deprivation and then treated with UTI and cell autophagy inhibitor 3MA (OGD + UTI + 3MA group) or cell autophagy agonist RAPA (OGD + UTI + RAPA group). The results showed that UTI can significantly save the decrease of renal tubular epithelial cell viability caused by OGD (Figure 2). Moreover, the activity of rat renal tubular epithelial cells in OGD + UTI + 3MA group was lower than that in OGD + UTI group, and the activity of OGD + UTI + RAPA group was significantly higher than that of OGD + UTI group. These results suggest that autophagy is involved in regulation of cellular viability by UTI, and the increase of autophagy can work with UTI to increase cell viability.

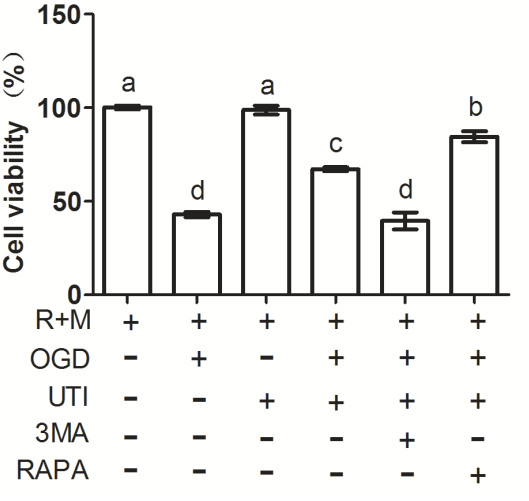

Figure 2: Cell autophagy influenced the regulation of UTI on the activity of renal tubular epithelial cells. R: Renal tubular epithelial cells; M: Macrophage; OGD: Oxygen glucose deprivation; UTI: Ulinastatin.; 3MA: 3-Methyladenine; RAPA: Rapamycin. Bars with different letters are significantly different ($p < 0.05$).

3.3 UTI affects the expression of inflammatory factors in macrophages.

ELISA was applied to detect whether UTI affects the secretion of macrophage inflammatory factors. It can be seen that OGD can increase the secretion of TNF-α, IL-18 and IL-1β in macrophages (Figure 3). Compared with OGD group, OGD + UTI group has lower concentration of inflammatory factors. The concentration of TNF-α, IL-18 and IL-1β in OGD + 3MA group was significantly higher than the control group, while the concentration of TNF-α, IL-18 and IL-1β in OGD +RAPA group is lower than the OGD + 3MA group. These results suggest that UTI may decrease the expression of TNF-α, IL-18 and IL-1β by up-regulating macrophage autophagy levels.

3.4 UTI regulates macrophage autophagy

qRT-PCR and Western blotting were used to detect the effect of UTI on macrophage autophagy. It can be seen that the OGD treatment significantly decreased mRNA level and protein expression of LC3 in the macrophage

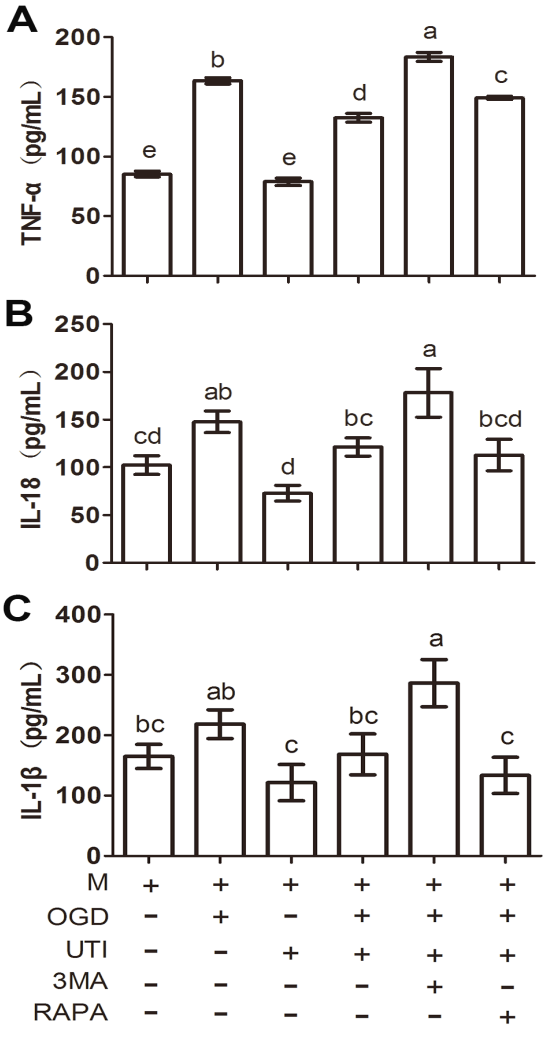

Figure 3: UTI regulated the expression of inflammatory cytokines in macrophages. A: ELISA analysis of the concentration of THF-α in macrophage culture supernatant; B: The concentration of IL-18 in macrophage culture supernatant; C: The concentration of IL-1β in macrophage culture supernatant by ELISA analysis. M: Macrophage; OGD: Oxygen glucose deprivation; UTI: Ulinastatin; 3MA: 3-Methyladenine; RAPA: Rapamycin. Bars with different letters are significantly different ($p < 0.05$).

(Figure 4), but UTI can promote the expression of LC3 and improve macrophage autophagy. The autophagy inhibitor 3MA can significantly inhibit the mRNA level and protein expression of LC3, while the autophagy agonist RAPA can significantly increase the expression of LC3 and improve the autophagy. The results also indicate that UTI could synergistically promote the expression of LC3 with RAPA to enhance macrophage autophagy.

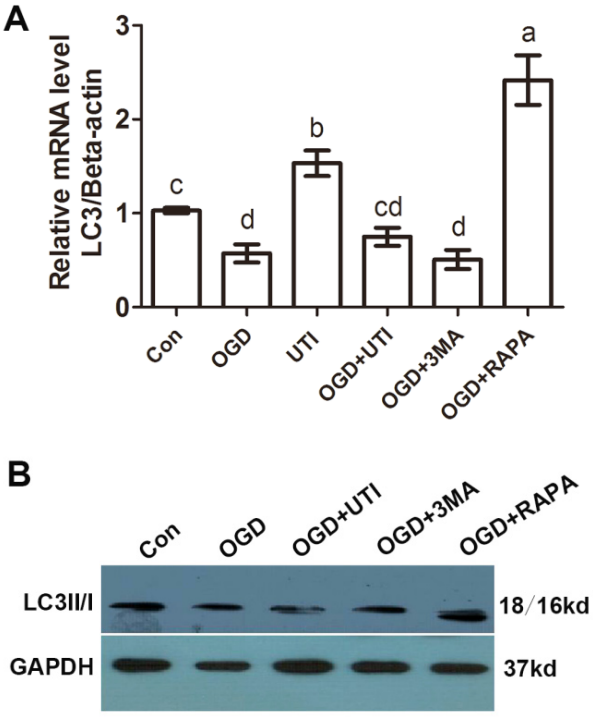

Figure 4: UTI regulated macrophage autophagy. A: Relative mRNA expression of LC3 in macrophages. B: Western blotting analysis of LC3 II/I protein expression levels in macrophages.OGD: Oxygen glucose deprivation; UTI: Ulinastatin; 3MA: 3-Methyladenine; RAPA: Rapamycin. Bars with different letters are significantly different ($p < 0.05$).

3.5 UTI affects the expression of inflammatory factors by autophagy

ELISA was used to detect the expression of inflammatory factors in cultured rat renal tubular epithelial cells or the cells co-cultured with macrophages in vitro. The results showed that the expression of TNF-α and IL-18 was significantly decreased in UTI-treated rat renal tubular epithelial cells in vitro (Figure 5A, 4B); and UTI could reduce the secretion of inflammatory factor TNF-α under the OGD condition (Figure 5A). The secretion of TNF-α, IL-18 and IL-1β in the OGD group was significantly higher than that in the control group ($P < 0.05$), while the secretion of the inflammatory factors of UTI group is similar to the control group in the co-cultured rat renal tubular epithelial cells and macrophages (Figure 5). The secretions of TNF-α, IL-18 and IL-1β in the OGD+UTI+RAPA group were significantly higher than the OGD + UTI group and the control group. The expressions of these three inflammatory factors in OGD + UTI + RAPA were significantly lower than those in OGD + UTI group. Thus, the inhibition of autophagy increases

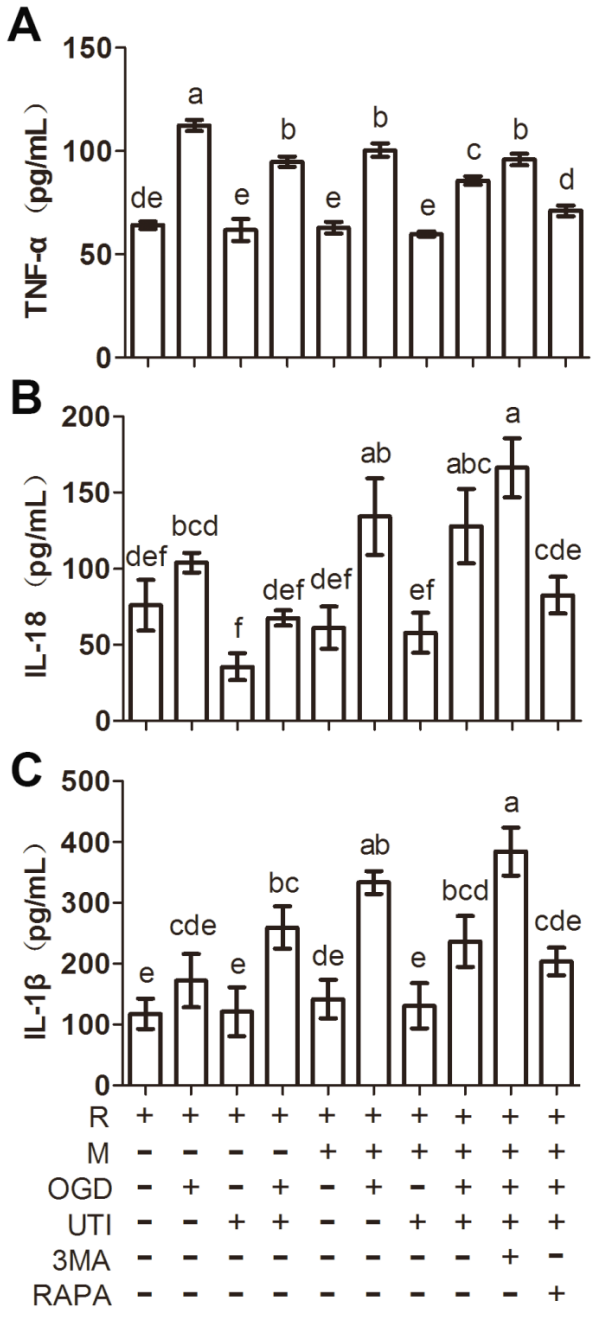

Figure 5: UTI affected the expression of inflammatory factors through autophagy. A: The concentration of TNF-α in the supernatant of renal tubular epithelial cells and macrophage and renal tubular epithelial cells co-culture system by ELISA analysis. B: ELISA analysis of the concentration of IL-18 in the supernatant of renal tubular epithelial cells and macrophage and macrophages co-culture system; C: The concentration of IL-1β in the supernatant of renal tubular epithelial cells and macrophages and renal tubular epithelial cells co-culture system by ELISA analysis. R: Renal tubular epithelial cells; M: Macrophage; OGD: Oxygen glucose deprivation; UTI: Ulinastatin.; 3MA: 3-Methyladenine; RAPA: Rapamycin. Bars with different letters are significantly different ($p < 0.05$).s

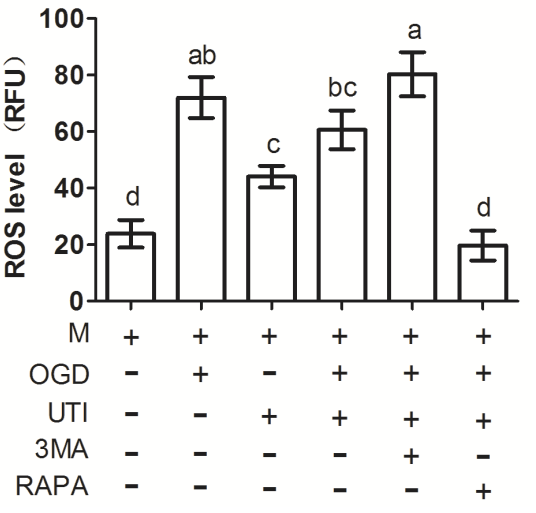

Figure 6: UTI affected the ROS level in macrophages through autophagy. M Macrophage; OGD: Oxygen glucose deprivation; UTI: Ulinastatin.; 3MA: 3-Methyladenine; RAPA: Rapamycin. Bars with different letters are significantly different ($p < 0.05$).

the secretion of inflammatory factors, but the autophagy agonist decreases the secretion of inflammatory factors co-cultured cells. The results showed that UTI could decrease the expression and secretion of inflammatory factors TNF-α, IL-18 and IL-1β. After 3MA inhibition of autophagy, UTI could not inhibit the expression of inflammatory cytokines. But, after RAPA activation of autophagy, inhibition of secretion of Inflammatory factors (TNF-α and IL-18) by UTI was enhanced. Therefore, it is hypothesized that UTI inhibits the expression of TNF-α, IL-18 and IL-1β by activation of autophagy.

3.6 UTI affects the ROS level in macrophage by autophagy

In order to detect the effect of UTI on the production of reactive oxygen species (ROS) after autophagy, a ROS assay kit was used to measure the content of ROS in macrophage. The amount of active oxygen production of macrophages was significantly increased after OGD treatment in comparison with the control group. However, UTI could obviously decrease the production of ROS. Compared with OGD + UTI group, the amount of ROS in UGD + UTI + 3MA group was significantly higher than the 3MA-treated group, indicating that autophagy inhibitor 3MA could block UTI-induced autophagy, which resulted in the increase of ROS level. In contrast, the ROS level was decreased after administration of autophagy activator RAPA (OGD + UTI + RAPA group). Thus, UTI can reduce

the expression of inflammatory factors and the content of ROS by increasing the autophagy.

4 Discussion

The results show that OGD can induce cell apoptosis and decrease the viability of renal tubular epithelial cells and macrophages, but UTI can significantly improve the activity of renal tubular epithelial cells and macrophages pretreated with OGD. In the past study, it had been found that the mechanism of apoptosis in OGD-induced nephritis model is mainly through the release of cytokines (TNF-α, IL-1β, etc.). This study found that OGD can increase the expression of TNF-α in renal tubular epithelial cells, but the induction of IL-1β and IL-18 is not obvious. However, OGD can not only improve the expression of TNF-α in renal tubular epithelial cells, but also induce the expression of IL-1β and IL-18. This reflects that inflammatory factors (IL-1β and IL-18) are predominantly expressed in macrophages and are relatively low expressed in epithelial cells.

The use of UTI reduces the expression of three inflammatory factors (TNF-α, IL-1β and IL-18) in renal tubular epithelial cells and macrophages under the model of glucose deprivation, and its effect is affected by autophagy Inhibitor (3MA) and cell autophagy agonist (RAPA). UTI also reduced the expression of IL-1β and IL-18 in macrophages under normal culture conditions, which indicated that UTI had a certain regulation effect on the transformation of macrophage function. When UTI and cell autophagy inhibitor 3MA are used simultaneously, the expression of inflammatory factors cannot be inhibited, indicating that UTI induces cell autophagy to reduce the expression of inflammatory factors and improve cell activity. UTI and cell autophagy agonist RAPA can synergistically inhibit the expression of inflammatory factors, and the effect is better than the use of UTI alone. The expression of LC3 was in contrast to the expression of inflammatory cytokines. Under the OGD condition, the regulation of expression of inflammatory factors by UTI was inhibited by cell autophagy inhibitor 3MA, but was promoted by cell autophagy agonist RAPA. These suggest that cell autophagy agonist (RAPA) is a synergistic drug with UTI, which then enhances autophagy and inhibits inflammation.

The previous studies have shown that lowering intracellular reactive oxygen species can downregulate the activation of inflammatory agents and their key effector protein, caspase-1, thereby reducing the release of inflammatory factors. In this study, we found that UTI can reduce the production of reactive oxygen species. The cell autophagy inhibitor 3MA increased the production of reactive oxygen species and cell autophagy agonist RAPA can significantly inhibit the production of reactive oxygen species. This shows that cell autophagy is the upstream regulation of reactive oxygen species, and regulates the expression and release of inflammatory factors.

Although UTI can promote cell autophagy to inhibit inflammation and enhance its activity, its mechanism of interactions with the receptors on the cells and the mechanism of transformation of macrophage phenotype are still not clear. More experimental studies are needed to further elucidate the molecular mechanisms of UTI interaction with renal tubular epithelial cells and macrophages, as well as the mechanism by which macrophages are transformed from inflammatory cells into autophagic cells that inhibit inflammation. The effect of UTI and cell autophagy agonist RAPA on the inhibition of inflammation in vivo will also need to be demonstrated by further animal experiments.

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