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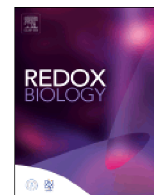

Research Paper

Autophagy inhibition attenuates hyperoxaluria-induced renal tubular oxidative injury and calcium oxalate crystal depositions in the rat kidney

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ABSTRACT

Hyperoxaluria-induced oxidative injury of renal tubular epithelial cell is a casual and essential factor in kidney calcium oxalate (CaOx) stone formation. Autophagy has been shown to be critical for the regulation of oxidative stress-induced renal tubular injury; however, little is known about its role in kidney CaOx stone formation. In the present study, we found that the autophagy antagonist chloroquine could significantly attenuate oxalate-induced autophagy activation, oxidative injury and mitochondrial damage of renal tubular cells in vitro and in vivo, as well as hyperoxaluria-induced CaOx crystals depositions in rat kidney, whereas the autophagy agonist rapamycin exerted contrasting effects. In addition, oxalate-induced p38 phosphorylation was significantly attenuated by chloroquine pretreatment but was markedly enhanced by rapamycin pretreatment, whereas the protective effect of chloroquine on rat renal tubular cell oxidative injury was partly reversed by a p38 protein kinase activator anisomycin. Furthermore, the knockdown of Beclin1 represented similar effects to chloroquine on oxalate-induced cell oxidative injury and p38 phosphorylation in vitro. Taken together, our results revealed that autophagy inhibition could attenuate oxalate-induced oxidative injury of renal tubular cell and CaOx crystal depositions in the rat kidney via, at least in part, inhibiting the activation of p38 signaling pathway, thus representing a novel role of autophagy in the regulation of oxalate-induced renal oxidative injury and CaOx crystal depositions for the first time.

1. Introduction

Kidney stone is one of the most common diseases in urology and recur in up to 50% of patients within 5–10 years after the initial stone episode. Calcium-containing stones constitute approximately 80% of cases of kidney stones [1,2]. Despite the performance of an extensive number of studies, the exact mechanism responsible for kidney stone formation remains poorly understood; thus, developing effective means of preventing stone formation and recurrence remains an important challenge [3,4]. Accumulating numbers of studies have demonstrated that high oxalate- and/or CaOx-induced oxidative injury of renal tubular epithelial cell is a pivotal factor in kidney stone formation, as this type of injury not only promotes the crystallization of CaOx crystals by providing substances for their heterogeneous nucleation but also enhances the adhesion of CaOx crystals to renal epithelial cells [5–8]. Therefore, inhibiting the renal oxidative injury may have beneficial effects on renal function and decrease the rate of kidney stone recurrence [9].

Autophagy is a cellular process that contributes to the degradation of endogenous cellular protein aggregates and damaged organelles via the lysosomal pathway and plays important roles in the pathogenesis of a variety of diseases, including kidney injury and kidney diseases [10–13]. Emerging studies recently indicated that autophagy is involved in the development of many kidney diseases, such as diabetic nephropathy, glomerular diseases, ischemia-reperfusion (I/R)-induced kidney injury, renal cancer and renal fibrosis; however, little is known about the role of autophagy in the development of kidney stones, which are a form of chronic kidney injury and disease [10,14,15]. In addition, emerging investigations have confirmed that autophagy dysfunction results in impaired mitochondrial function, reactive oxygen species (ROS) accumulation and oxidative stress [16–19].

Thus, we hypothesized that autophagy is involved in the regulation of oxalate-induced oxidative injury of renal tubular cells and investigated its role in the regulation of hyperoxaluria-induced renal

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oxidative injury and CaOx crystal depositions in the present study.

2. Materials and methods

2.1. Animal and materials

Forty male Sprague-Dawley rats (6–8 weeks old, 180–220 g) were randomly divided into the following four groups: a control group, a model group, and groups treated with rapamycin or chloroquine, respectively. The control group rats had free access to tap water. The hyperoxaluria rat model was induced by allowing the rats in the EG group free access to drinking water containing 1% EG, as described in previous studies [20–22]. The rats in the treatment groups were daily intraperitoneally injected with chloroquine (30 mg/kg/d, Sigma-Aldrich, USA) or rapamycin (0.25 mg/kg/d, Sigma-Aldrich, USA) for 4 weeks, whereas the control rats received an equal volume of normal saline. Rapamycin and chloroquine were dissolved in DMSO and PBS, respectively, as a stock solution of 10 mM, and were resuspended in saline before injection. All rats had free access to regular rat chow and were maintained at 25 °C and under a light-dark cycle during the experimental period. All procedures were performed in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Animal Care Commission of the First Affiliated Hospital of Guangzhou Medical University. Oxalate was prepared as described previously [23]. Briefly, a stock solution of 50 mM sodium oxalate was prepared in sterile PBS and was diluted to a concentration of 0.75 mM in defined medium. Anisomycin and JC-10 were purchased form Selleck and Abcam, respectively.

2.2. Cell culture and transfection

NRK-52E cells were purchased from ATCC (USA) and cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 10 mM Hepes buffer as ATCC's suggestion in a humidified incubator containing 5% CO_2 at 37 °C. The pEGFP-LC3 and ptfLC3 plasmids used in the study were gifts from Tamotsu Yoshimori (Addgene plasmid #21073 and #21074). Small interfering RNA (siRNA) was synthesized by RiBoBio Co. Ltd. (Shanghai, China). The sequences of the siRNAs used in the study were as follows: 5′-CGAUCAAUAAUUUCAGACU-3′(siRNA-Beclin-1) and 5-UUCUC CGAACGUGUCACGU-3′ (siRNA-NC). The transfections were conducted with indicated plasmids or siRNAs using Lipofectamine LTX according to the manufacturer's instructions (Invitrogen).

2.3. Cell viability assay

Cell viability was assessed using a Cell Titer 96® AQueous One Solution Cell Proliferation Assay Kit (MTS, Promega). For this experiment, the cells were treated with 10 μL of MTS (5 mg/mL in PBS) and then incubated for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Relative cell viability was assessed with an ELISA reader with a 490-nm filter.

2.4. Western blot analysis

Western blot analysis was conducted as described previously. Briefly, the cells were lysed in RIPA buffer, and equal amounts of protein were separated on an SDS polyacrylamide gel, transferred to an NC membrane (Millipore) and then immunoblotted with antibodies. Primary antibodies against the following proteins were used in this study: Beclin1(#3495), SQSTM1/P62 (#5114), LC3A/B (#12741), phospho-p38 (T180/Y182, #4511) and p38 (#9212) were purchased from Cell Signaling Technology, 8-OHdG (ab10802) and SOD1 (ab51254) were purchased from Abcam, GAPDH (sc-365062) was purchased from Santa Cruz. The following secondary antibodies used in the study were purchased from Santa Cruz: HRP-conjugated anti-mouse and anti-rabbit IgG (sc-2005 and sc-2004). The band intensities were quantified and normalized to the band intensities of GAPDH using ImageJ software. The data were presented as bar graphs after their statistical validity was tested.

2.5. Determination of lactate dehydrogenase (LDH) release

NRK-52E cells were seeded into 96-well plates (5×10^3 cells/well). After the cells received the appropriate treatments, the medium from the control and experimental groups was centrifuged to remove crystals and cellular debris. LDH release was then quantified using a CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega, USA), according to the manufacturer's instructions. The absorbance was read at 490 nm using a multimode reader (SynergyH1, BioTek), and the results were expressed as the percent LDH release. The results for the treated samples were normalized to those for the control samples.

2.6. Determination of glutathione (GSH) content

GSH content was quantified by a GloMax® 96 Microplate Luminometer, according to the instructions for the GSH-Glo™ Glutathione Assay Kit (Promega, USA). The values for the treated samples were normalized to those for the control samples.

2.7. Measurement of intracellular ROS levels

Dihydroethidium (DHE) fluorescence dye was used to evaluate intracellular ROS generation. For this experiment, the cells were incubated with DHE (Sigma-Aldrich, USA) solution (5 μM) in DMEM in the dark for 30 min at 37 °C. Excess DHE was removed via two rinses with PBS solution, and the images were captured with a fluorescence microscope (IX-71, Olympus) immediately thereafter. ROS levels were semi-quantified by ImageJ software. All data pertaining to the ROS levels for the experimental group were normalized to those for the control group.

2.8. Measurement of mitochondrial membrane potential (Δψm)

JC-10 dye was used to monitor mitochondrial integrity. Briefly, NRK-52E cells were seeded in black 96-well plates (1×10^4 cells/well). After receiving the appropriate treatments, the cells were incubated with JC-10 (10 μg/mL) for 15 min at 37 °C and then washed twice with PBS. For signal quantification, we measured red (excitation 560 nm, emission 595 nm) and green fluorescence (excitation 485 nm, emission 535 nm) intensities using a multimode reader (Synergy H1, BioTek). Mitochondrial membrane potential (Δψm) was calculated as the JC-10 red/green fluorescence intensity ratio, and this value was normalized to the corresponding value for the control group.

2.9. Transmission electron microscope (TEM) assay

Kidney tissue specimens from the rats and NRK-52E cells were fixed in 2.5% glutaraldehyde (2.5% in 0.1 Mphosphate buffer, pH 7.4) and dehydrated in a graded series of ethanol. Ultrathin sections were obtained and stained as described previously [24], and then they were observed by a JEM-100CXII TEM (JEOL, Japan). The autophagosomes and autolysosomes were identified as described previously [25].

2.10. Preparation of ponceau-S-labeled COM crystals

Calcium oxalate monohydrate (COM) crystals were prepared according to protocols established previously [26]. Briefly, $CaCl_2$ (10 mM) and $Na_2C_2O_4$ (1 mM) were diluted to final concentrations of 5 mM and 0.5 mM, respectively, in Tris buffer containing 90 mM NaCl (pH 7.4) and ponceau-S (22.5 mg/mL). The mixture was incubated overnight at

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