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Original Paper

Mitophagy Plays a Protective Role in Iodinated Contrast-Induced Acute Renal Tubular Epithelial Cells Injury

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Key Words
Acute kidney injury • Contrast media • Mitophagy • ROS • Autophagy

Abstract
Background/Aims: Contrast induced-acute kidney injury (CI-AKI) is one of the most common causes of acute kidney injury (AKI) in hospitalized patients. Mitophagy, the selective elimination of mitochondria via autophagy, is an important mechanism of mitochondrial quality control in physiological and pathological conditions. In this study, we aimed to determine effects of iohexol and iodixanol on mitochondrial reactive oxygen species (ROS), mitophagy and the potential role of mitophagy in CI-AKI cell models. ***Methods:*** Cell viability was measured by cell counting kit-8. Cell apoptosis, mitochondrial ROS and mitochondrial membrane potential were detected by western blot, MitoSOX fluorescence and TMRE staining respectively. Mitophagy was detected by the colocalization of LC3-FITC with MitoTracker Red, western blot and electronic microscope. ***Results:*** The results showed that mitophagy was induced in human renal tubular cells (HK-2 cells) under different concentrations of iodinated contrast media. Mitochondrial ROS displayed increased expression after the treatment. Rapamycin (Rap) enhanced mitophagy and alleviated contrast media induced HK-2 cells injury. In contrast, autophagy inhibitor 3-methyladenine (3-MA) down-regulated mitophagy and aggravated cells injury. ***Conclusions:*** Together, our finding indicates that iohexol and iodixanol contribute to the generation of mitochondrial ROS and mitophagy. The enhancement of mitophagy can effectively protect the kidney from iodinated contrast (iohexol)-induced renal tubular epithelial cells injury.

Introduction

Acute kidney injury (AKI) is a clinical syndrome with abnormal renal function or structure that occurs within 48 h and the leading clinical cause of chronic kidney disease,

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with high morbidity and mortality [1-3]. With the widely utilization of contrast media in the angiography and percutaneous coronary intervention, contrast-induced acute kidney injury (CI-AKI) has generally been considered as the third most common cause of hospital-acquired acute kidney injury (AKI) [4]. CI-AKI is also related with increased mortality, prolonged hospitalization, high incidence of complications and expensive treatment costs. In addition, up to 7%-36% of CI-AKI patients require incident hemodialysis and have an increased risk of developing into end stage kidney disease [5, 6].

The exact underlying pathogenesis and pathophysiology of CI-AKI have not yet been fully elucidated. Recently, studies have shown that renal ischemia, direct nephrotoxicity, and oxidative stress are responsible for the pathogenesis of CI-AKI [7, 8]. Our previous study showed that free radical scavengers, such as pentoxifylline, could effectively reduce reactive oxygen species (ROS), protecting the kidney from iodinated contrast-induced injury [9]. Additionally, scavenging reactive oxygen species (ROS) by recombinant manganese superoxide dismutase prevented reduction of eGFR and renal histologic damage that follow the administration of contrast media [10]. All these evidences suggest that ROS plays an important role in the pathogenic process of CI-AKI. Mitochondria are the main source of intracellular ROS genesis and main organelle target of ROS [11]. Plenty of evidences showed that mitochondria injury plays a critical role in the pathogenesis of AKI, manifesting as significant changes in mitochondrial morphology, viability and function of renal tubular epithelial cells [12-14]. However, it is unclear whether iso-osmolar (IOCM) and low-osmolar contrast media (LOCM) have effects on mitochondrial ROS and mitochondrial damage in acute renal epithelial cells injury.

Mitophagy is the main way of mitochondrial degeneration. Mitochondrial dysfunction had been implicated in a series of diseases [15, 16]. A previous study has proved that mitophagy plays a protective role in cisplatin induced renal tubular epithelial cells injury [15]. However, it remains unclear whether mitophagy is associated with acute renal epithelial cells injury induced by contrast media.

Iodinated contrast media are classified into high-, iso- or low-osmolar and iodixanol (IOCM) as well as iohexol (LOCM) is commonly used in clinical practice [17]. HK-2 cell-line is derived from human renal proximal tubular cells. Proximal tubular cells are particularly vulnerable to a variety of injuries including hypoxia and toxins due to their high energy demands and relatively poor antioxidant ability [18]. In the present study, we investigated the change of mitophagy and mitochondrial ROS in HK-2 cells treated with iodinated contrast media of different osmotic pressures and concentrations. Furthermore, we further studied the potential role and mechanism of mitophagy in iodinated contrast-induced renal tubular epithelial cell injury.

Materials and Methods

Cell culture

The human proximal tubular cell line HK-2 (ATCC, Rockville, USA) was used for *in vitro* studies. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO2. The culture medium (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) contained 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Iohexol and iodixanol were purchased from Shanghai General Pharmaceutical Co., Ltd (Shanghai, China), and 3-methyladenine (3-MA) and rapamycin (Rap) were obtained from Selleck Chemicals (Houston, USA).

Cell treatment

HK-2 cells were incubated in the FBS-free DMEM/F12 medium for 12 h. The cytotoxicity of HK-2 cells were evaluated after exposure to different concentrations of iohexol or iodixanol at the time points of 0 h, 2 h, 4 h, 6 h, 8 h. An injured cell mitophagy model was chosen based on our previous study [19]. Rap (100 nM) or 3-MA (10 mM) were used to enhance or inhibit the formation of autophagosomes and mitophagy

respectively. HK-2 cells were divided into 4 groups. Control: control group (DMEM/F12 with 1% FBS); Iohexol: 200 mg iodine/ml iohexol treated for 6 h. Control + Rap or 3-MA: HK-2 cells were treated with Rap or 3-MA for 30 min without iohexol. Iohexol + Rap or 3-MA: HK-2 cells were treated with Rap or 3-MA for 30 min, and then exposed to iohexol (200 mg iodine/ml) for 6 h.

Cell viability analysis

The cell viability of HK-2 cells was assayed by cell counting kit-8 (CCK-8, Beyotime Institutes of Biotechnology, Shanghai, China) according to manufactory's instructions. Cells were seeded in 96-well plates at 37 °C in humidified 5% CO_2 atmosphere. 10 μl of CCK-8 solution was added to each well and then incubated for 4 h. The absorbance of 450 nm was spectrophotometrically measured by a microplate ELISA reader (Molecular devices corporation, California, US). HK-2 cells cultured in DMEM/F12 medium and culture media without cells were treated as negative and blank controls respectively. The proliferation rate was expressed as a percentage of the absorbance of the treated group to that of control group cell absorbance.

Western blot assay

Cells were lysed with cell lysis buffer (RIPA: PMSF = 100: 1, Beyotime Institutes of Biotechnology, Shanghai, China) on ice for 30 minutes and then centrifuged at 8500 × g for 5 minutes at 4 °C. The protein concentration was determined by BCA Protein Assay Reagent Kit (Beyotime Institutes of Biotechnology, Shanghai, China). Equal amount of protein was loaded in each lane of SDS-polyacrylamide electrophoresis gel. Then resolved protein was transferred to polyvinylidene difluoride membrane, which was then blocked with 5% bovine serum albumin for 1 h at room temperature and further incubated with a specific primary antibody. The blot membrane was rinsed with TBST for 4 times and subsequently incubated with horseradish peroxidase-conjugated second antibodies. The results were analyzed with Tanon 5200 Multi image analysis software (Tanon, Shanghai, China).

Measurement of mitochondrial ROS

HK-2 cells growing in cell culture plates were treated with iohexol, iodixanol or only DMEM/F12 medium as a control for 6 h. Then cells were rinsed once with phosphate buffered solution (PBS). After incubation with 4% paraformaldehyde for 25 minutes, these cells were rinsed with PBS 3 times again and incubated in MitoSOX (Invitrogen Corporation, USA) for 8 minutes at 37 °C in the dark. MitoSOX is a specific red mitochondrial superoxide indicator in live-cells. Finally, DAPI (Invitrogen Corporation, USA) were used for nuclear staining for 5 minutes, and the distribution of ROS could be observed by a confocal laser scanning microscope LSM780 (Carl Zeiss Jena, German).

Mitochondrial membrane potential assay

TMRE Mitochondrial Membrane Potential Assay Kit (Invitrogen Corporation, USA) was used to measure mitochondrial membrane potential (MMP) of HK-2 cells according to the manufacturer's instructions. HK-2 cells growing in glass bottom cell culture dish were loaded with 200 μl TMRE for 30 minutes at 37 °C in the dark. Cells were washed 3 times with 0.1 M PBS, and then measured by a confocal laser scanning microscope LSM780 (Carl Zeiss Jena, German) with settings suitable for TMRE.

Immunofluorescence colocalization analysis

Cells were cultured on polyline-coated glass slides (CITOTEST, Jiangsu, China) in 6 well plates with the density of $1 \sim 5 \times 10^5$/ml, incubated in MitoTraker Red for 8 min, then fixed in 4% paraformaldehyde for 15 min, rinsed in PBS and permeabilized with 0.1% Triton-X-100 (Tianjin Kemiou Chenmical Co., Ltd, China) for 5 min, followed by incubation with the LC3 (Cell Signaling Technology, Boston, USA) primary antibody for 1 h at room temperature and incubation with a fluorescent secondary antibody (fluorescence FITC, 1:100; Abcam, Cambridge, England). DAPI (Invitrogen Corporation, USA) was added to the cells in dark for 5 min. A confocal laser scanning microscope LSM780 (Carl Zeiss Jena, German) was used to capture images.

Transmission electron microscope analysis

HK-2 cells were cultured in 6 well plates with the density of $1 \sim 5 \times 10^5$/ml and were treated as described. The cells were digested and collected routinely. The cells were suspended and fixed with 2.5 %

glutaraldehyde in 0.1 mM PBS (pH 7.4) at 4 °C for 2 h. After washing twice with PBS, cells were treated with conventional dehydration, osmosis, embedding, sectioning and staining [20]. The ultrastructure of the cells was observed under a Hitachi H7700 electron microscope.

Statistical analysis

The Graph-pad prism and SPSS 19.0 software were used for the statistical analysis. Data were presented as mean values ± standard deviation (Mean ± SD) for independent experiments. For comparison between two groups, a paired or unpaired t-test was performed. Comparisons among more than two groups were assessed by one-way analysis of variance (ANOVA) followed by LSD test for post-hoc comparisons. $P<0.05$ was considered statistically significant.

Results

Iohexol and iodixanol induce cytotoxicity in HK-2 cells

To examine the toxic effects of iohexol and iodixanol, HK-2 cells were treated under various concentrations of iohexol and iodixanol at different time points. The viability of the manipulated cells was assessed by CCK-8 kit. Iohexol and iodixanol caused a concentration- and time-dependent decrease in cell viability (Fig. 1). Interestingly, iohexol induced a more dramatic decrease in cell viability than iodixanol ($p<0.05$). Collectively, these results demonstrate that iohexol and iodixanol treatments lead to a partial loss of viability of HK-2 cells.

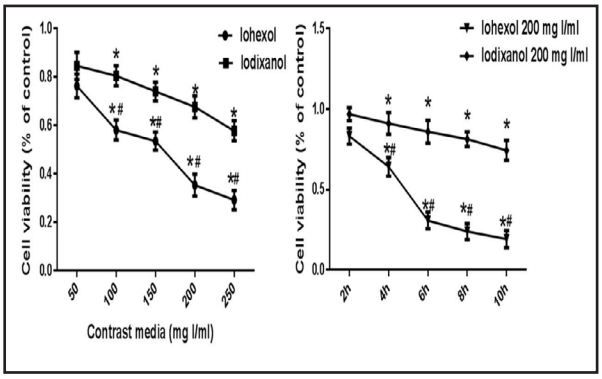

Fig. 1. Iohexol and iodixanol treatments cause cytotoxicity in HK-2 cells. Cell viability was measured by CCK-8 assay. Iohexol and iodixanol (mg iodine/ml, mg I/ml) caused decreased cell viability in a concentration- and time-dependent manner. Data were presented as mean ± SD (n=6). *$p<0.05$ vs. preceding time point or concentration group, #$p<0.05$ vs. cells treated with the same concentration of iohexol.

Mitochondrial damage and ROS generation are caused by iohexol and iodixanol treatments

Based on previous study [19], we select the concentration of 200 mg iodine/ml of iohexol or iodixanol and treatment duration of 6 h for further

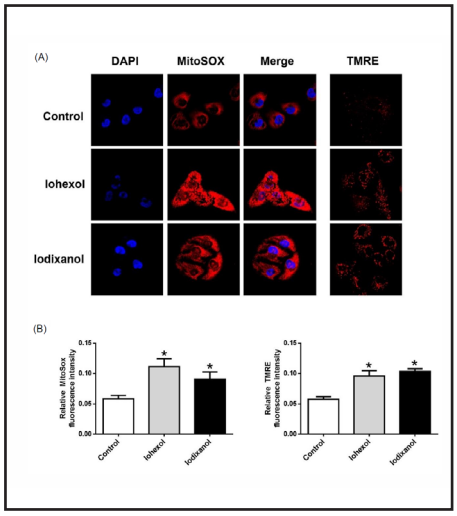

Fig. 2. Effect of iohexol and iodixanol exposure on mitochondrial ROS generation and membrane potential. (A) Representative images of mitochondrial ROS generation and mitochondrial membrane potential. HK-2 cells were treated with iohexol or iodixanol (200 mg iodine/ml) for 6 h respectively. DAPI staining was performed to label nucleus (blue). Mitochondrial ROS was labeled by MitoSOX (red). Mitochondrial membrane potential was stained with TMRE (red). Fluorescence images were taken by LSM780 confocal microscope(magnification, × 630) (B) Quantification of intracellular ROS generation and mitochondrial membrane potential, the density of fluorescent signals was measured normalized to control quantity. Values were presented as mean ± SD (n=6), *$p<0.05$ vs. control.

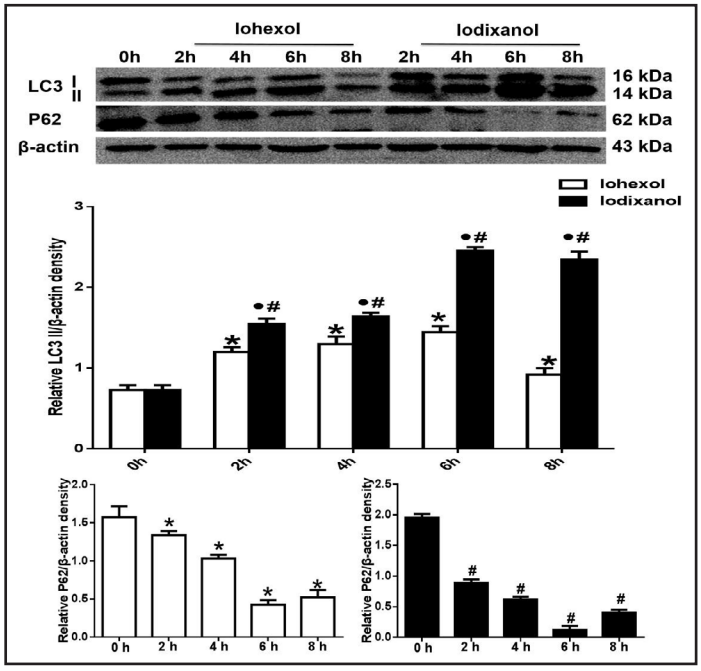

Fig. 3. Iohexol and iodixanol treatments induce autophagy in HK-2 cells. Western blot analysis of the expression of LC3Ⅱ/Ⅰ and P62. Cells were treated with iohexol and iodixanol (200 mg iodine/ml) respectively for different time periods, and then the whole cells lysates were collected for western blot analysis. Data were presented as mean ± SD (n=3). *p<0.05, #p<0.05 vs. control respectively. •P< 0.05 vs. cells treated with iohexol at the same time periods.

study on mitochondrial damage and ROS generation. The exposure of iohexol or iodixanol led to a significant increase in the signals of fluorescent MitoSOX, suggesting a reinforcement in ROS production (Fig. 2). In addition, we evaluated the MMP in HK-2 cells exposed to iohexol or iodixanol via TMRE staining (Fig. 2). Quantitative analysis showed that iohexol and iodixanol exposure dramatically increased mitochondrial ROS generation and TMRE staining red fluorescent (P<0.05); and there was no obvious difference between iohexol group and iodixanol group (P>0.05). These findings indicate that iohexol and iodixanol exposure induced mitochondrial injury, whereas there is no significant difference observed in the severity of damage to mitochondria caused by the two types of iodinated contrast media.

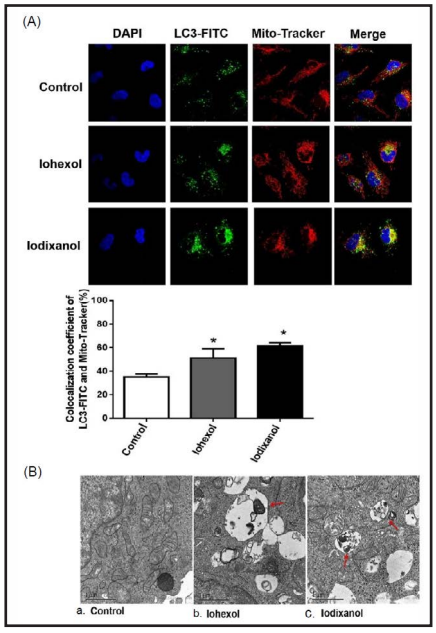

Fig. 4. Mitophagy is induced in HK-2 cells. (A). Representative images. HK-2 cells were exposed to iohexol or iodixanol (200 mg iodine/ml) for 6 h, and then treated with LC3-FITC (green) and MitoTracker Red to label autophagosome and mitochondria respectively. The distribution of LC3-FITC and MitoTracker Red was analyzed by confocal microscope. Colocalization of LC3-FITC and MitoTracker Red was defined as overlapped red and green peaks. Data were presented as mean ± SD (n=3), *p<0.05 vs. control. (B). a). Electron micrographs (× 20 K) showed normal cytoplasm and no typical autophagosomes in the control group. b). Increased number of mitophagic vesicles were observed in HK-2 cells after treatment of iohexol (200 mg iodine/ml) for 6 h. c). Increased mitophagic vesicles were observed in HK-2 cells after treatment of iodixanol (200 mg iodine/ml) for 6 h. Red arrows indicated typical mitophagy, which was visualized as mitochondria-containing autophagosome.

Mitophagy is induced by iohexol and iodixanol treatments in HK-2 cells

To determine whether iohexol and iodixanol induce autophagy, we examined the expression of LC3Ⅱ and P62 by western blot analysis. During autophagy, a subpopulation of LC3Ⅰ is converted to LC3Ⅱ that localizes to autophagosomal membranes

[21]. To identify upregulation of LC3 Ⅱ level was due to increased autophagy initiation, we tested the expression of P62 [22]. As shown in Fig. 3, iohexol and iodixanol induced an increased expression of LC3 Ⅱ and decreased expression of P62. Interestingly, we also found iodixanol contributed to a higher expression of LC3 Ⅱ than iohexol (P<0.05).

Then we identified whether mitophagy was activated by iohexol and iodixanol exposure in HK-2 cells. We initially examined the colocalization of autophagosome with mitochondria by fluorescent microscope. MitoTracker Red and LC3-FITC were used to label mitochondria and mitophagosomes respectively in HK-2 cells. The fluorescent confocal of LC3-FITC and MitoTracker Red were more obviously upregulated in iohexol and iodixanol group, compared with control group (P<0.05) (Fig. 4A).

Next, we employed transmission electron microscope to detect the effects of iohexol and iodixanol on mitochondrial damage and mitophagy. Normal mitochondria morphology and no typical autophagosomes were observed in control group (Fig. 4B a). However, in iohexol and iodixanol treated group, there appeared a marked accumulation of autophagosomes containing mitochondria, of which cytoplasmic material and/or membrane vesicles were encapsulated in vacuoles. Partial autophagic vacuoles contained mitochondrial specific bilayer membrane, cristae and other structures (red arrows). Mitochondria became swollen. Mitochondrial cristae fully disintegrated (Fig. 4B b) or partly disintegrated (Fig. 4B c). There was a decrease in number of mitochondria (Fig. 4B b).

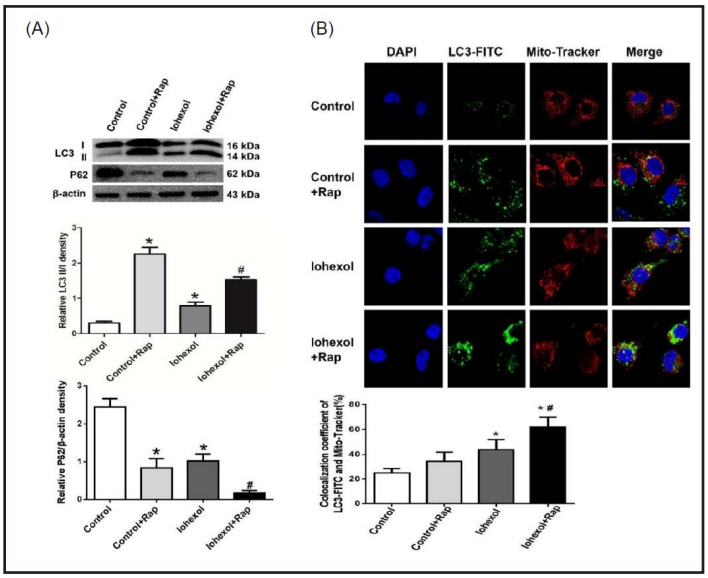

Fig. 5. Rap enhances mitophagy in iohexol treated HK-2 cells. HK-2 cells were treated with Rap (100 nM) for 30 min, and then exposed to iohexol (200 mg iodine/ml) for 6 h. (A). Western blot analysis of LC3 and P62 expression in the whole cells. (B) Representative images. HK-2 cells were treated with LC3-FITC (green) and MitoTracker Red to label autophagosomes and mitochondria respectively. The distribution of LC3-FITC and MitoTracker Red was analyzed by confocal microscope. Colocalization of LC3-FITC and MitoTracker Red was defined as overlapped red and green peaks. Data were presented as mean ± SD (n=3). *p<0.05 vs. control, #p<0.05 vs. iohexol group.

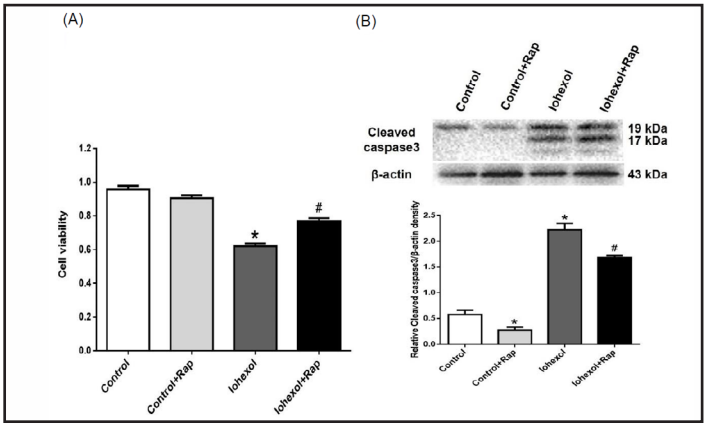

Fig. 6. Enhancement of mitophagy by Rap attenuates cell injury induced by iohexol in HK-2 cells. HK-2 cells were treated with Rap (100 nM) for 30 min, and then exposed to iohexol (200 mg iodine/ml) for 6 h. (A). Iohexol-induced decreased cell viability was alleviated by Rap (n=6). (B). Western blot was used to detect the expression of cleaved caspase 3 in HK-2 cells (n=3). Data were presented as mean ± SD. *p<0.05 vs. control, #p<0.05 vs. iohexol group.

Taken together, based on previous study [19] and our experimental results, both iohexol and iodixanol caused mitochondrial damage, autophagy, and mitophagy. However, iohexol induced more markedly decrease in cell viability than iodixanol. After exposure to 200 mg iodine/ml iohexol for 6 h, cell viability decreased more than 50% compared with control group. In the continuation of this study, HK-2 cells treated with 200 mg iodine/ml iohexol for 6 h was used to establish the CI-AKI cell model.

Enhancement of mitophagy by Rap protects against iohexol induced cytotoxicity in HK-2 cells

Rap inhibiting the activity of mTOR, thus activates the signaling pathway of autophagy [23]. Pro-apoptotic events were assessed by cleaved caspase 3 activity assay. HK-2 cells were pretreated with Rap prior to iohexol treatment. The overlapping signals of LC3- FITC and MitoTracker Red were more obvious in iohexol and Rap treated group (Fig. 5B), and the expression of LC3 Ⅱ was significantly increased, compared with the group treated with iohexol only (Fig. 5A). Cells pretreated with Rap alleviated the decreased cell viability induced by iohexol (Fig. 6A). Expression of cleaved caspase 3 and P62 were also significantly decreased by enhancement of mitophagy (Fig. 5A, 6B). These findings demonstrate that Rap can enhance mitophagy and prevent HK-2 cells from apoptosis induced by iohexol.

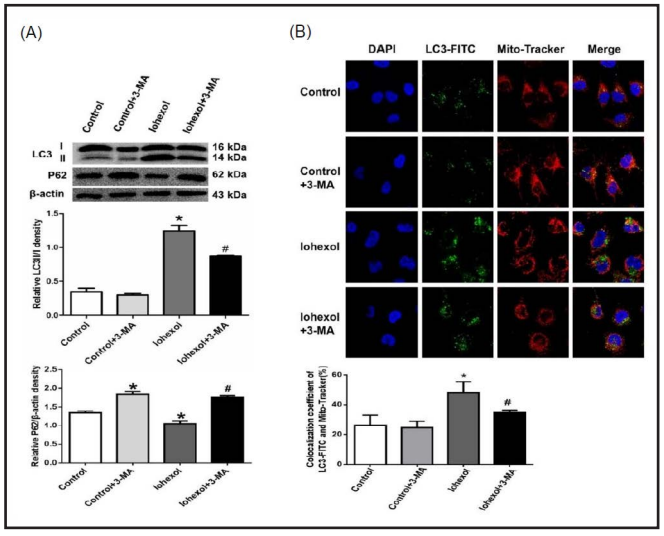

Fig. 7. 3-MA inhibits mitophagy in iohexol treated HK-2 cells. HK-2 cells were treated with 3-MA (10 mM) for 30 min, and then exposed to iohexol (200 mg iodine/ml) for 6 h. (A). Western blot analysis of LC3 and P62 expression in the whole cells. (B) Representative images. HK-2 cells were exposed to iohexol (200 mg iodine/ml) for 6 h, then treated with LC3-FITC (green) and MitoTracker Red to label autophagosomes and mitochondria respectively. The distribution of LC3-FITC and MitoTracker Red was analyzed by confocal microscope. Colocalization of LC3-FITC and MitoTracker Red was defined as overlapped red and green peaks. Data were presented as mean ± SD (n=3). *p<0.05 vs. control, #p<0.05 vs. iohexol group.

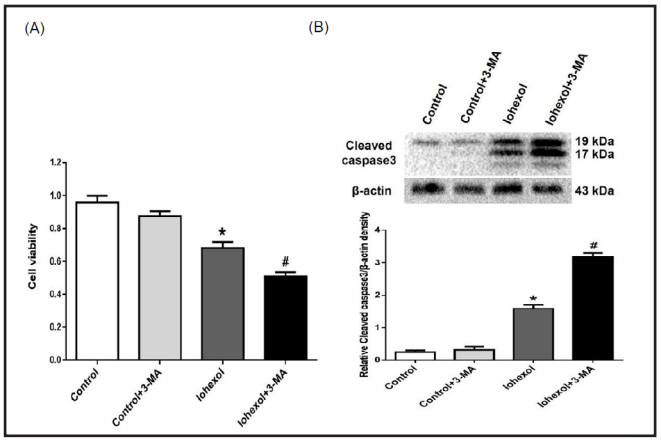

Fig. 8. Inhibition of mitophagy by 3-MA aggravates iohexol-induced cell injury of HK-2 cells. HK-2 cells were treated with 3-MA (10 mM) for 30 min, and then exposed to iohexol (200 mg iodine/ml) for 6 h (A). Iohexol-induced decreased cell viability was aggravated by 3-MA (n=6). (B). Western blot was used to detect the expression of cleaved caspase 3 in HK-2 cells (n=3). Data were presented as mean ± SD. *p<0.05 vs. control, #p<0.05 vs. iohexol group.

Inhibition of mitophagy by 3-MA exacerbates iohexol induced cell injury in HK-2 cells

Potent autophagy inhibitor 3-MA was used to determine if suppression of mitophagy accelerated iohexol induced cells injury. 3-MA inhibits autophagy

by blocking autophagosome formation via inhibition of type Ⅲ phosphatidylinositol 3 kinases (PI3 kinase) [24]. When cells were pretreated with 3-MA before iohexol, it not only decreased cell viability (Fig. 8A) and expression of LC3Ⅱ in HK-2 cells (Fig. 7A), but also increased the expression of cleaved caspase-3 and P62 (Fig. 7A, 8B). The overlapping signals of LC3-FITC and MitoTracker Red were significantly decreased in iohexol and 3-MA group, compared with the group treated with iohexol only (Fig. 7B). All these evidences indicate that 3-MA effectively inhibits mitophagy and the inhibition of mitophagy aggravates HK-2 cells injury induced by iohexol.

Discussion

In this study, we firstly demonstrate that iohexol and iodixanol exposure can induce mitophagy, mitochondrial ROS generation and mitochondrial damage in HK-2 cells. In addition, enhancement of mitophagy with Rap protects HK-2 cells from contrast media induced apoptosis, and inhibition of autophagy and mitophagy by 3-MA aggravates HK-2 cell injury. Therefore, mitophagy was cytoprotective against HK-2 cells apoptosis induced by contrast media.

Iodinated contrast-induced AKI is a clinically important complication of intravascularly applied contrast media. A predominant toxic effect of iodinated contrast media on renal tubules has been shown in previous clinical trials and animal experiments. Animal experiments have shown that IOCM are less nephrotoxic than low-osmolar contrast media LOCM *in vivo* [25]. Also, clinical trials have proven a significantly lower nephrotoxicity of IOCM compared with LOCM [26]. A recent comprehensive meta-analysis by John Eng, which included 29 randomized control trials, showed superiority of IOCM (iodixanol) over LOCM [27]. However, in both European Society of Urogenital Radiology and the Kidney Disease: Improving Global Outcomes guidelines, either IOCM or LOCM was recommended in patients with increased risk of CI-AKI [28, 29]. Mounting evidence demonstrated that ROS generation and mitochondrial membrane depolarization may lead to DNA damage and apoptosis [30, 31]. Recent studies indicated that an excess generation of mitochondrial ROS played an important role in the pathogenesis of AKI [32, 33]. The damaged mitochondria are first marked by a change of mitochondrial membrane potential. Mitochondrial damage and dysfunction are recognized as a leading etiological factor to many acute renal diseases [34]. Other studies have demonstrated that ROS plays a crucial role in regulating mitophagy. Cadmium induced ROS-mediated mitophagy through PINK1/Parkin pathway in kidneys of mice [35, 36]. However, the effects of IOCM and LOCM on mitochondrial ROS generation and mitochondrial damage still remain unclear. Based on previous studies [37, 38], we selected cell viability, mitochondrial ROS and MMP to evaluate cytotoxicity of IOCM and LOCM at cellular and mitochondrial levels. Our study showed that iohexol and iodixanol caused a concentration- and time-dependent decrease in cell viability. At different time points, iohexol contributed to more significantly decreased cell viability, compared with iodixanol, which suggests that IOCM may have lower toxicity than LOCM. Besides, the results obtained from this study are the first to demonstrate that no significant difference of ROS generation and mitochondrial membrane depolarization level between iohexol and iodixanol treated groups. However, iohexol and iodixanol had different effects on cell viability, but similar effects on mitochondrial damage and ROS generation. There are several possible explanations. Firstly, the results of cytotoxicity induced by IOCM and LOCM are related to the selected injury indexes. Secondly, the pathogenesis of CI-AKI is multifactorial, such as renal ischemia, direct nephrotoxicity, oxidative stress, autophagy, apoptosis, etc. Thirdly, as will be narrated later, the more activated autophagy pathway by iodixanol treatment may partially lessen its cytotoxic effect on HK-2 cells.

Autophagy is the major intracellular degradation system by which cytoplasmic materials are delivered to and degraded in the lysosome [39]. Mitophagy, one type of autophagy, is

known as selective removal of damaged and depolarized mitochondria. Although initially identified in familial Parkinson's disease [40], recent studies showed that autophagy in kidney was of great significance for the homeostasis of renal tubular [41, 42]. Autophagy and mitophagy have been well studied in renal ischemia-reperfusion, sepsis and cisplatin induced acute kidney injury [15, 43-45]. However, there are few researches on the effect and possible disparities of LOCM and IOCM on mitophagy and its role in renal injury induced by iodinated contrast media. We found out that autophagy and cell viability induced by 200 mg iodine/ml of LOCM was significantly lower than those of IOCM in different time points, suggesting that lower cytotoxicity of IOCM may be associated with higher levels of autophagy. We further identified that mitophagy was induced by iohexol and iodixanol in HK-2 cells. But there were no significant differences between these two groups. In order to further support our studies, cells were treated with Rap or 3-MA respectively. Pretreatment with Rap could effectively enhance mitophagy, alleviate cells apoptosis. However, pretreatment of cells with 3-MA could remarkably inhibit mitophagy and caused more severe cell toxicity. Our study shows that mitophagy plays a cytoprotective role in cytotoxicity induced by iodinated contrast media.

The current study has some limitations. Firstly, there lacks *in vivo* experiments to support the results obtained from our *in vitro* experiments. Secondly, gene silencing or knockout technologies are required to elucidate the possible mechanism of mitophagy in CI-AKI. Therefore, in iodinated contrast media induced AKI, the observed effects of mitophagy need to be further validated. Knockdown studies *in vivo*/*vitro* or some data from human in this work will make the conclusion more convincing in further studies.

In conclusion, iohexol and iodixanol induce mitochondrial ROS generation and mitophagy in HK-2 cells. Mitophagy plays an important role in mitochondrial quality control and tubular cell survival during CI-AKI cell models. The enhancement of mitophagy probably offers a novel therapeutic target for CI-AKI.

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Disclosure Statement

No conflict of interests exists.

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