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Original Paper

Ethyl Pyruvate Attenuates $CaCl_2$-Induced Tubular Epithelial Cell Injury by Inhibiting Autophagy and Inflammatory Responses

Jiawen Zhao[a] Jiwen Cheng[a] Chengyang Li[a] Mingbin Xu[a] Chenjun Ma[a] Lei Qin[a] Kai Yi[a] Naikai Liao[a]

[a]Department of Urology, the First Affiliated Hospital of Guangxi Medical University, Nanning, China

Key Words
Autophagy • Inflammation • Ethyl pyruvate • Tubular epithelial cell • Nephrolithiasis • Hypercalciuria

Abstract
Background/Aims: Nephrolithiasis is one of the most prevalent diseases of the urinary system. Approximately 80% of human kidney stones are composed of calcium oxalate (CaOx), and hypercalciuria is one of the most common metabolic disorders. Emerging evidence indicates that autophagy and inflammatory responses are related to the formation of CaOx nephrolithiasis. However, the roles of autophagy and inflammation in patients with hypercalciuria remain unclear. Ethyl pyruvate (EP) displays protective effects in experimental models of many illnesses. In this study, we investigated the protective effects of EP *in vitro* through its inhibition of autophagy and inflammatory responses after $CaCl_2$-induced tubular epithelial cell injury. ***Methods:*** First, we cultured human tubular epithelial (HK-2) cells in the presence of various concentrations of $CaCl_2$ (0, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 mg/ml) for 12 h and EP (0, 1.0, 2.5, 5.0, and 10.0 mM) for 2 h to select the optimum concentration using the Cell Counting Kit-8 assay and lactate dehydrogenase (LDH) assay. Cells in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). After the exposure, we detected the expression of inflammation-related proteins using an enzyme-linked immunosorbent assay and Western blot analysis. Finally, the levels of autophagy-related proteins were determined through Western blot analysis, and the number of GFP-LC3 dots and autophagic vacuoles was detected under confocal microscopy. ***Results:*** With the use of the Cell Counting Kit-8 assay and the LDH assay, we identified the optimum concentration for $CaCl_2$ (1.0 mg/ml) treatment and EP pretreatment (2.5 mM). Our research indicated that $CaCl_2$ can induce autophagy and inflammatory responses in HK-2 cells. Furthermore, treatment with EP prior to $CaCl_2$ stimulation attenuated HK-2 cell injury by inhibiting autophagy and inflammation. ***Conclusion:*** Our results provide evidence that EP attenuates $CaCl_2$-induced injury of HK-2 cells by downregulating the expression of inflammation and autophagy proteins

Chengyang Li, MD, PhD

Department of Urology, The First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning, 530021, Guangxi Zhuang Autonomous Region (China)
Tel. +86-0771-5356516, Fax +86-0771-5356516, E-Mail LCY_Urology@163.com

that may be associated with the inhibition of the high-mobility group box-1 (HMGB1)/toll-like receptor 4 (TLR4)/NF-κB pathway and the competitive interaction with Beclin-1 of HMGB1.

Introduction

Nephrolithiasis is a common clinical disease occurring in the urinary system. A majority of kidney stones (85%) are composed primarily of calcium oxalate (CaOx) mixed with calcium phosphate. However, the pathogenesis of nephrolithiasis remains unclear. Hypercalciuria is the most common predisposing factor in patients with CaOx kidney stones [1]. More than 50% of patients with calcium kidney stones present with an increased occurrence of urinary calcium excretion in clinical research studies [2]. Tubule calcium reabsorption in patients with hypercalciuria is reduced compared with that of patients with normal calcium excretion [3]. Emerging evidence indicates that autophagy and inflammatory responses are related to the formation of CaOx nephrolithiasis [4, 5]. Our previous study demonstrated that hypercalciuria may result in increased urinary excretion of various inflammatory cytokines, such as high-mobility group box-1 (HMGB1) and monocyte chemotactic protein-1[6]. Nevertheless, the exact pathogenesis of stone formation has not yet been completely elucidated.

Supersaturation and crystallization are strongly related. Kidney stone formation primarily involves urinary supersaturation, crystal nucleation, growth, aggregation, and migration to the renal epithelial cell surfaces [5]. Urinary supersaturation is the driving force for crystallization, and the main abnormalities are hypercalciuria, hyperoxaluria, hyperuricosuria, and hypocitraturia, either alone or in combination. CaOx crystal deposition in the kidney results in the increased expression of major markers of oxidative stress and inflammation [7]. Similarly, *in vivo* and *in vitro* studies demonstrate that renal tubular epithelial cells (HK-2) that are exposed to high amounts of calcium generate excessive reactive oxygen species (ROS), thereby causing oxidative stress and injury and the development of inflammation [6, 8]. Autophagy is a highly conserved and tightly regulated cellular process in which intracellular damaged organelles and proteins are eliminated via the lysosomal pathway; consequently, the necessary materials for the synthesis of new molecules are obtained to maintain intracellular homeostasis and cellular metabolism [9, 10]. Generally, the basal autophagy level is low in cells. Nonetheless, autophagy dysregulation can be triggered by stresses, such as nutrient starvation, inflammation, and infection [11]. Growing evidence suggests that autophagy is linked to renal disease, but its role in the development and progression of hypercalciuria remains poorly understood [12].

Ethyl pyruvate (EP), a stable and simple lipophilic ester derived from the endogenous metabolite pyruvic acid, is a protective agent in multiple inflammatory injuries, including sepsis and hemorrhagic shock [13, 14]. EP has also been safely administered to humans in a randomized controlled clinical trial [15]. In addition, EP exerts pharmacological effects on ROS scavenging, inhibition of apoptosis, and cellular ATP synthesis [16]. Several studies have shown that EP can effectively inhibit the secretion of HMGB1, which is a critical proinflammatory cytokine in the activation of inflammation [17, 18]. Shen et al. demonstrated that EP ameliorates hepatic ischemia-reperfusion injury by inhibiting apoptosis and autophagy, an effect that may occur through the downregulation of the HMGB1/TLR4/NF-κB axis and the interaction of HMGB1 with Beclin-1[19]. Moreover, delayed treatment with EP confers a prolonged survival time in a mouse model of endotoxic shock and decreases the Lipopolysaccharides(LPS)-induced tumor necrosis factor (TNF) release in RAW264.7 macrophages [17]. Nonetheless, no report is available on whether EP can regulate hypercalciuria-induced HK-2 cell inflammation and autophagy. The internal relationship has also not been illuminated. Thus, to simulate the hypercalciuria environment *in vivo*, we performed this experiment to establish a $CaCl_2$ concentration model *in vitro* by utilizing renal HK-2 cells. Afterward, we determined whether the effects of EP on inflammatory and autophagy markers were agent-specific.

Materials and Methods

Reagents and antibodies

Calcium chloride dihydrate (C7902), ethyl pyruvate (E47808), and rabbit anti-LC3B (L7543) for the Western blot (WB) (1:2000) analysis were purchased from Sigma-Aldrich. Rabbit anti-HMGB1 (18256), rabbit antireceptors for advanced glycation end-products (RAGE) (3611), and rabbit anti-TLR4 (13556) were purchased from Abcam for WB (1:1000). Mouse anti-Beclin-1 (3495), rabbit anti-NF-κB (8242), rabbit anti-IL-1β (12703), and rabbit anti-Bcl-2 (4223) were purchased from Cell Signaling Technology and diluted to a 1:1000 ratio. Mouse monoclonal anti-GAPDH (Proteintech, 60004-1-Ig) was used for WB (1:5000).

Cell culture

HK-2 cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. HK-2 cells were grown in a humidified incubator with 5% CO_2 and 95% air in a DMEM/F12 medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin).

Cell viability and lactate dehydrogenase (LDH) assay

We used the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's guidelines to determine the cell viability. Approximately 1×10^4 HK-2 cells were planted in each well of the 96-well plates and grown for 24 h. Subsequently, 10 μl of CCK-8 solution was added to each well. Subsequently, the 96-well plates were placed in an incubator for 2 h at 37 °C with 5% CO_2 and 95% air. Absorbance was measured at 450 nm using a microplate spectrophotometer. The LDH levels in the culture supernatant were examined using an LDH assay kit according to the manufacturer's guidelines (Jiancheng Bioengineering Institute, Nanjing, China).

The concentrations of $CaCl_2$ (0.1, 0.25, 0.5, 1.0 and 2.0 mg/ml) and EP (1, 2.5, 5 and 10 mM) were based on previous studies [6, 20, 21]. HK-2 cells were exposed to 0, 0.1, 0.25, 0.5, 1.0, and 2.0 mg/ml $CaCl_2$ for 12 h to select the optimum $CaCl_2$ concentration for further studies. HK-2 cells were also exposed to 0, 1.0, 2.5, 5.0, and 10.0 mM of EP for 2 h to determine the presence of cytotoxicity prior to analysis. Comparison among groups identified 1.0 mg/ml as the optimal $CaCl_2$ concentration.

We also identified the candidate concentration of pretreatment with EP, which was administered prior to $CaCl_2$ stimulation, by detecting cell viability and LDH release. After incubation of HK-2 cells with EP (0, 1, 2.5, 5, and 10 mM) for 2 h in a serum-free medium, the cells were stimulated with 1.0 mg/ml $CaCl_2$ for 12 h. The HK-2 cell injury levels that were induced by $CaCl_2$ were minimized by pretreatment with 2.5 mM EP, which was the candidate optimum concentration. All experiments were repeated at least three times.

Hoechst 33258 staining assay

Cells in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). We conducted the staining assay using the Hoechst 33258 Fluorescence Staining Kit (Solarbio Bioscience & Technology, Beijing, China). HK-2 cells in 6-well plates were washed three times with PBS and fixed with 4% paraformaldehyde for 10 minutes. Then, 1 ml Hoechst 33258 staining solution was added into each well of the 6-well plates. After incubating for 20 minutes, the HK-2 cells were washed twice with PBS and then observed under a fluorescence microscope at an excitation wavelength of 350 nm. Cells were marked as apoptotic if the nuclei presented chromatin condensation, marginalization, or nuclear beading.

Enzyme-linked immunosorbent assay (ELISA)

Cells in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). The IL-6 and TNF-α levels in the culture supernatant were examined by using the ELISA kit (Cusabio Biotech, Wuhan, China) according to the manufacturer's guidelines.

Western blot

Cells that had been plated on six-well dishes in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). Cells were washed three times with ice-cold PBS and the proteins were obtained with RIPA lysis buffer, which was supplemented with 1 mM PMSF. The protein concentrations were determined by a bicinchoninic acid (Solarbio Bioscience & Technology, Beijing,

China) protein assay. Each protein sample was resolved with 12% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% nonfat dry milk in TBST for 1 h at room temperature, the membranes were probed with primary antibodies overnight on ice. Membranes were incubated in secondary antibodies at room temperature for 1 h after washing. Membranes were subsequently reacted with the ECL Substrate kit (ThermoFisher, Rockford, IL), and chemiluminescent signals were captured by a CCD camera in a chemiDoc XRS (Bio-Rad) instrument with Image Lab software. We used Image Lab software to conduct a quantification analysis of the target protein by measuring the target protein/GADPH ratio. The final results in the form of bar charts were presented as the means ± SEM of three different quantification analyses.

Autophagy assay

The Ad-mRFP-GFP-LC3 adenovirus was purchased from Hanbio (Shanghai, China), transfected into HK-2 cells for 24 h according to the manufacturer's guidelines, and subjected to further research. The transfected cells were plated on six-well dishes in culture and stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). Then, the cells were washed with PBS, fixed with 4% paraformaldehyde and examined by confocal fluorescence microscopy. The numbers of GFP and mRFP dots were determined quantitatively by manual counting of the number of green or red puncta in each cell. In the merged images, the number of yellow puncta or free red puncta per cell were counted. The result of one single experiment contained the average number of dots in at least 30 cells. The final results are shown in bar charts and represent the means from five independent experiments. Briefly, the yellow spots (merge of red and green) that were observed under confocal microscopy indicated autophagosomes, and the red spots were suggestive of autolysosomes. The numbers of autophagosomes and autolysosomes were counted to determine the autophagy levels.

Statistical analysis

Data were analyzed statistically using the SPSS 22.0 software. Data are shown as the means ± SEM. Significant differences between groups were analyzed by one-way ANOVA. $P < 0.05$ was considered the threshold of significance.

Results

Effects of $CaCl_2$ and EP on cell vitality and LDH release

The cell proliferation assay displayed a negative correlation with various $CaCl_2$ concentrations compared with the controls (Fig. 1A), and different EP concentrations were not cytotoxic to the cultured HK-2 cells (Fig. 1B). A comparison among groups identified 1.0 mg/ml as the optimal $CaCl_2$ concentration. Afterward, we identified the candidate concentrations of EP pretreatment prior to $CaCl_2$ stimulation by detecting cell viability and LDH release. Treatment with EP prior to $CaCl_2$ stimulation increased the cell viability and significantly diminished the LDH release compared with those of the $CaCl_2$-only group (Fig. 1C and 1D). The injury levels of HK-2 cells due to $CaCl_2$ were minimized by pretreatment with 2.5 mM EP, which was the candidate optimum concentration.

Apoptosis detection

The Hoechst 33258 staining assay was used to detect apoptosis in the HK-2 cells, where the blue nuclei indicated living cells, and the white nuclei indicated apoptotic cells. $CaCl_2$ induced cellular apoptosis (Fig. 1E-c), and EP inhibited $CaCl_2$-induced apoptosis (Fig. 1E-b).

EP inhibits the release of inflammatory mediators

$CaCl_2$ significantly increased the release of IL-6 and TNF-α in the culture supernatant. Treatment with EP prior to $CaCl_2$ stimulation significantly decreased the release of IL-6 and TNF-α in the culture supernatant (Fig. 2A). Similarly, HK-2 cells that were exposed to $CaCl_2$ showed significant increases in RAGE, HMGB1, TLR4, IL-1β, NF-κB, and extracellular HMGB1 expression in the HK-2 cells. Treatment with EP prior to $CaCl_2$ stimulation attenuated the

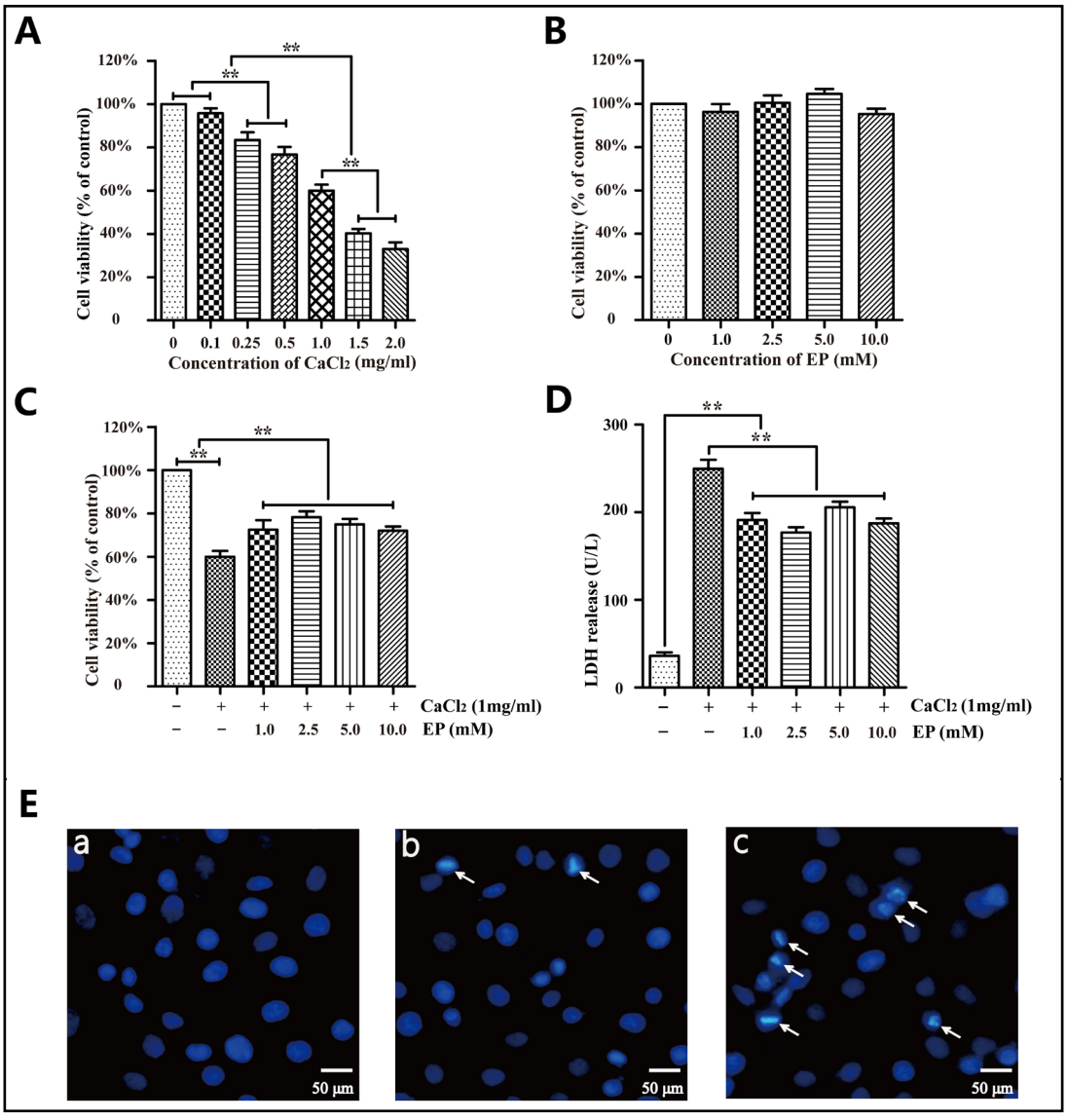

Fig. 1. (A) HK-2 cells were stimulated with different $CaCl_2$ concentrations (0, 0.1, 0.25, 0.5, 1.0, 1.5, and 2.0 mg/ml) for 12 h. Cell viability was examined by the CCK-8 assay. (B) HK-2 cells were incubated with different EP concentrations (0, 1.0, 2.5, 5.0, and 10.0 mM) for 2 h. Cell viability was examined by the CCK-8 assay. (C and D) After pretreatment with different EP concentrations (0, 1.0, 2.5, 5.0, and 10.0 mM) for 2 h, cells were stimulated with CaCl2 (1.0 mg/ml) for 12 h. Cell viability was examined by the CCK-8 assay. The lactate dehydrogenase (LDH) levels in the culture supernatant were examined using the LDH assay. (E) Apoptosis was assessed by Hoechst 33258 staining. (a) Cells in culture were stimulated with $CaCl_2$ (0 mg/ml, 12 h). (b) Cells were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) after EP pretreatment (2.5 mM, 2 h). (c) Cells were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h). White arrows indicate apoptotic cells (magnification: 100×). Data are presented as the means ± SEM from three experiments. **, P<0.01 (A, C, and D).

expression of these inflammatory mediators compared with that in the $CaCl_2$-only group (Fig. 2B).

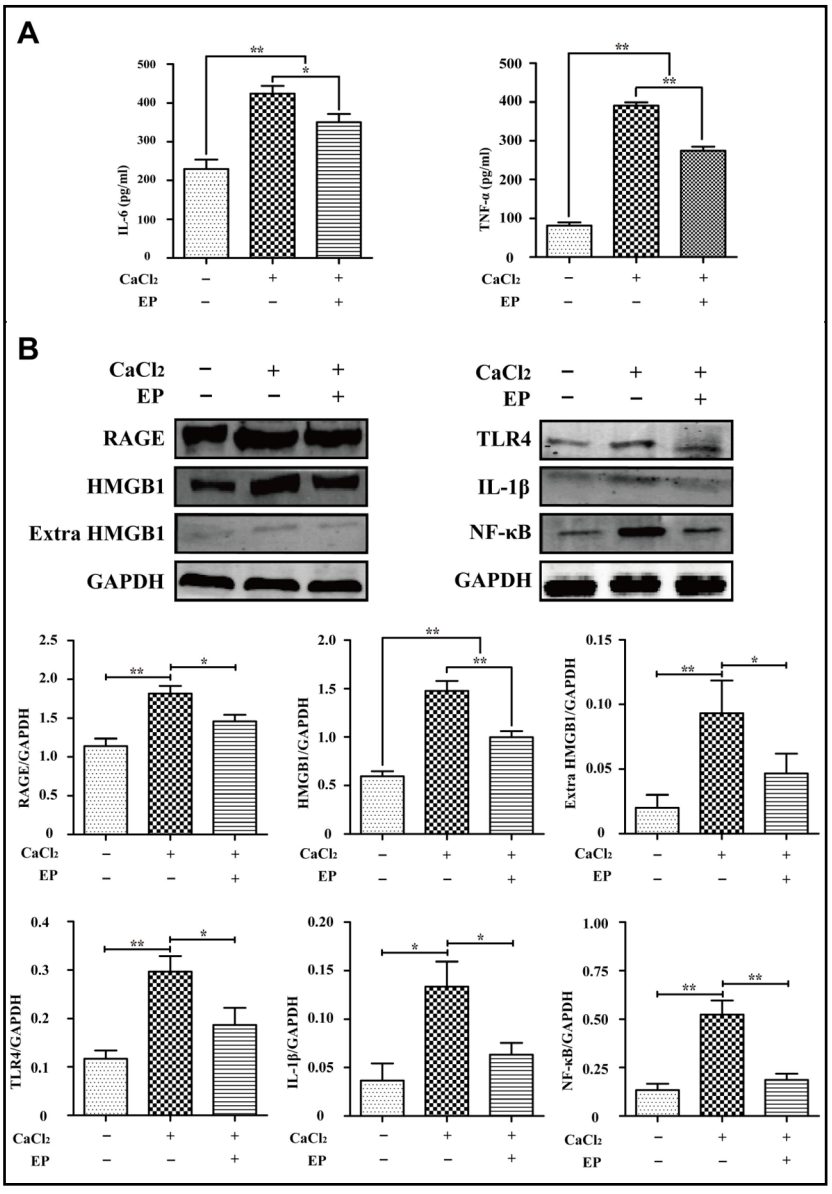

Fig. 2. (A) Cells in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). The levels of IL-6 and TNF-α in the culture supernatant were measured by using an enzyme-linked immunosorbent assay kit. Data are presented as the means ± SEM from three experiments. (B) Cells in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). Western blot and quantification analyses on RAGE, HMGB1, TLR4, IL-1β, and NF-κB in HK-2 cells and extracellular HMGB1. Data are presented as the means ± SEM of three experiments. *, P<0.05. **, P<0.01.

Autophagy detection

WB analysis demonstrated that the expression of LC3-II and Beclin-1 was significantly higher in HK-2 cells that were exposed to $CaCl_2$ than that of controls; the expression of Bcl-2 was significantly decreased. Treatment with EP prior to $CaCl_2$ stimulation decreased the expression of LC3 and Beclin-1 and increased the expression of Bcl-2 compared with that in the $CaCl_2$-only group (Fig. 3). The Ad-mRFP-GFP-LC3 adenovirus was transfected into HK-2 cells, stimulated with $CaCl_2$, and examined by confocal microscopy. Autophagosomes and autolysosomes were marked with yellow and red dots, respectively, in the merged images. The number of red and yellow spots markedly increased after treatment with $CaCl_2$ compared with that in the control group (Fig. 4). Treatment with EP prior to $CaCl_2$ stimulation evidently reduced the number of red and yellow spots compared with that of the $CaCl_2$-only group (Fig. 4).

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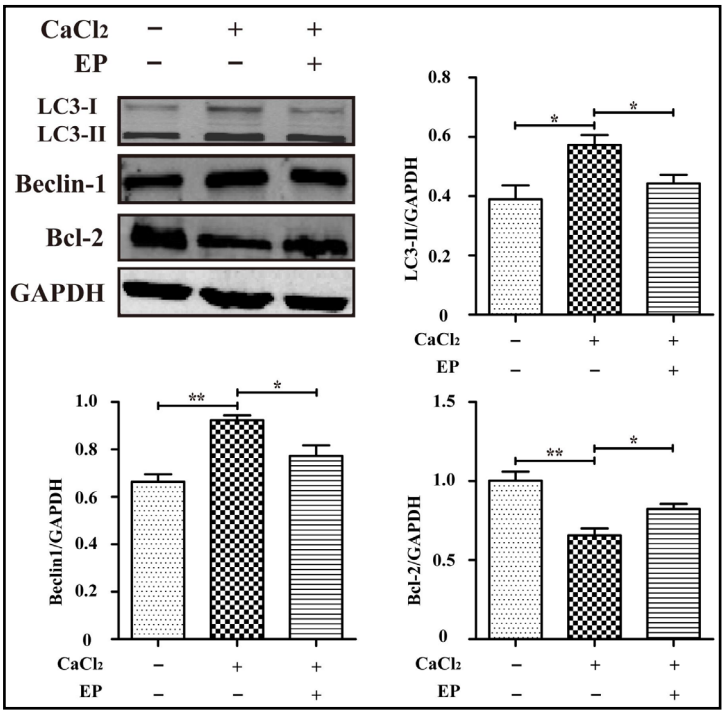

Fig. 3. Cells in culture were stimulated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). Western blot and quantification analyses of LC3II, Beclin-1, and Bcl-2 proteins in HK-2 cells. Data are presented as the means ± SEM of three experiments. *, P<0.05. **, P<0.01.

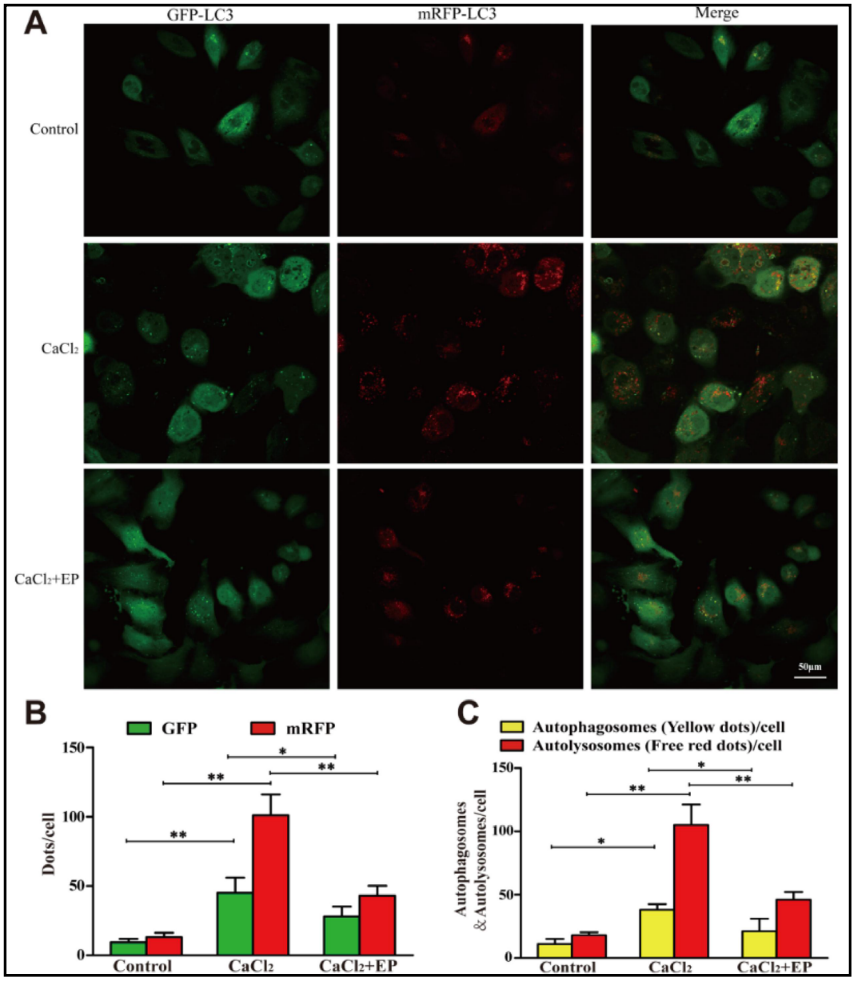

Fig. 4. Induction of autophagy in HK-2 cells that were transduced with the adenovirus Ad-mRFP-GFP-LC3 and incubated with $CaCl_2$ (1.0 mg/ml, 12 h) with or without EP pretreatment (2.5 mM, 2 h). (A) In the merged image, the yellow dots indicate autophagosomes, and the red dots indicate autolysosomes. (B) Means ± SEM of the numbers of green and red dots per cell. (C) Means ± SEM of the numbers of autophagosomes and autolysosomes per cell. Data shown are from at least five independent experiments. *,P<0.05. **,P<0.01.

Discussion

We established a $CaCl_2$ concentration model *in vitro* to simulate the hypercalciuria environment *in vivo.* We provided direct evidence that the exposure of HK-2 cells to $CaCl_2$ leads to simultaneous injury processes, including the induction of autophagy and inflammation. We also demonstrated that treatment with EP prior to $CaCl_2$ administration can attenuate HK-2 cell injury by inhibiting autophagy and inflammation. Similar to our previous investigations *in vivo* and *in vitro*, increased expression of urinary HMGB1 in patients with calcium nephrolithiasis and hypercalciuria may be a key influencing factor that is triggered by ROS[6]. Liu et al. also demonstrated that CaOx crystal retention can induce autophagy *in vivo* and *in vitro* and aggravate HK-2 cell injury. Chloroquine diphosphate inhibits autophagy, and renal injury and the formation of nephrolithiasis markedly decreases [22].

Our results indicated that exposure to $CaCl_2$ activated the inflammation pathway through the HMGB1-RAGE/TLR4-NF-κB axis and induced the secretion of proinflammatory cytokines (including IL-6, IL-1β, and TNF-α). EP treatment markedly attenuated the inflammatory response by downregulating the expression of the HMGB1 axis and of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) (Fig. 2). These findings suggested that the protective effect of EP may be closely associated with its reduction of the HMGB1-RAGE/TLR4-NF-κB pathway. The autophagy level was assessed by detecting the expression levels of the autophagy-related proteins LC3 and Beclin-1 in HK-2 cells that were exposed to $CaCl_2$. Detection with confocal microscopy demonstrated that the numbers of GFP-LC3 dots and autophagic vacuoles significantly increased under the $CaCl_2$ condition. In contrast, the EP treatment markedly decreased the expression of autophagy-related proteins and the numbers of GFP-LC3 dots and autophagic vacuoles.

HMGB1 is a highly abundant and well-conserved DNA-binding protein that plays a role in maintaining the chromatin structure and regulating transcription in cells [23]. In addition to its intranuclear distribution, HMGB1 can be passively released from injured or necrotic cells and actively secreted by activated inflammatory cells such as monocytes and macrophages [24]. Extracellular HMGB1 serves as a crucial cytokine that mediates the inflammatory response by binding with high-affinity TLRs and RAGE, thereby activating their downstream signaling pathways, mainly via NF-κB activation and the production of cytokines such as IL-6, IL-1β, and TNF-α[25]. Secreted TNF-α, IL-6, and HMGB1 can increase the excessive HMGB1 release, which amplifies the inflammatory response in a positive feedback loop [26]. Finally, an amplified inflammatory response occurs. Several studies have demonstrated that HMGB1 may play an amplifying role as a junction molecule between the initial injury and the subsequent inflammatory response in many inflammatory diseases, such as sepsis and rheumatoid arthritis, which ultimately cause organ or tissue damage [27, 28]. HMGB1 inhibition can effectively exert a protective effect against lethal sepsis in rat models and in *in vitro* models of vascular endothelial cell inflammatory injury [17, 29].

EP, a simple ester formed from pyruvic acid and ethanol, is safe and stable as an anti-inflammatory and antioxidant drug [30]. EP is also beneficial in the treatment of acute renal failure and hemorrhagic shock [13, 14]. However, the effects and mechanisms underlying the actions of EP against nephrolithiasis remain unexplored. The activation of NF-κB by HMGB1 is correlated with an increase in proinflammatory cytokines, including IL-6, IL-1β, and TNF-α[18]. The molecular action of EP involves the anti-inflammatory effects that primarily occur by specific interference with HMGB1 secretion from the nuclei to the cytoplasm, which downregulates the HMGB1-RAGE/TLR4-NF-κB axis and decreases the expression of proinflammatory cytokines, including TNF-α, IL-1β, and IL-6[25]. EP treatment reduces the ALT, AST, IL-6, and HMGB1 values and decreases the expression levels of TLR4 and NF-κB in a rat model of hepatic fibrosis [31]. EP decreases the lipopolysaccharide-stimulated NF-κB-p65 activity in RAW 264.7 cells and inhibits the binding of NF-κB to DNA[32]. Considering these data, we believe that the HMGB1-RAGE/TLR4-NF-κB axis can be associated with HK-2 cell

injury after $CaCl_2$ exposure and that EP treatment confers a protective effect via suppressing the HMGB1-RAGE/TLR4-NF-κB axis.

Autophagy is a process that is associated with a degradation in the quantity and quality of organelles and macromolecules through the lysosomal pathway to maintain cellular health [9, 10]. Autophagy is also associated with a variety of pathological conditions, such as neurodegeneration, cardiomyopathy, and cancer [33]. Liu et al. clearly demonstrated that autophagy activated via the reactive oxygen species pathway is deleterious in CaOx crystal-induced HK-2 cell injury [22]. The autophagy level that was observed in the current study was directly observed under confocal microscopy but also indirectly by the expression of LC3, Beclin-1, and Bcl-2 (Fig. 3 and 4). WB analysis showed that EP may attenuate the HK-2 cell injury by downregulating the expression of LC3 and Beclin-1. Further research with confocal microscopy verified our hypothesis that remarkably reduced levels of autophagosomes may be observed in the EP treatment group. Apoptosis and autophagy can be regulated by Bcl-2 family proteins. The Bcl-2 family proteins can interact with the BH3 domain of Beclin-1 to regulate autophagy. Pattingre and colleagues demonstrated that Beclin-1-Bcl-2 conjunction inhibits autophagy activity. Conversely, the dissociation of Bcl-2 from Beclin-1 may be a crucial mechanism for activating autophagy that may be triggered by stresses, primarily including nutrient starvation, inflammation, and infection [34]. Virtually all stimuli induce not only inflammation but also autophagy. HMGB1 can mediate autophagy when it is secreted to the extracellular milieu. Tang et al. showed that the inhibition of HMGB1 release decreases autophagy activity through HMGB1 short hairpin RNA (shRNA) or EP treatment in tumor cells, and this suggested a critical role for HMGB1 in the regulation of autophagy. One possible explanation is that excessive HMGB1 disrupts the Beclin-1/Bcl-2 interaction [35]. Similarly, Shen et al. revealed that EP inhibits the release of HMGB1 from the nuclei to the extracellular milieu, and this process suppresses the competitive combination of HMGB1 and Beclin-1 with Bcl-2 under hepatic ischemia-reperfusion injury and ultimately decreases autophagy activity [19]. We confirmed that EP attenuated the autophagy level under the $CaCl_2$ condition, possibly by interfering with the HMGB1 that was released outside of the HK-2 cells and inhibiting the competitive binding between HMGB1 and Beclin-1 with Bcl-2.

Conclusion

Our current data suggest that $CaCl_2$ induces tubular epithelial cell injury at least in part by activating the inflammation and autophagy responses. Furthermore, EP administration can significantly attenuate the $CaCl_2$-induced tubular epithelial cell injury by inhibiting the autophagy and inflammation responses. Nevertheless, this research is primarily limited by the fact that HMGB1 inhibition may be achieved by not only EP but also genetic manipulation such as by small interfering RNA. Further verification is required to verify this hypothesis.

Abbreviations

CaOx (Calcium oxalate); EP (Ethyl pyruvate); HK-2 cells (Human tubular epithelial cells); CCK-8 assay (Cell Counting Kit-8 assay); LDH (Lactate dehydrogenase); HMGB1 (High-mobility group box-1); TLR4 (Toll-like receptor 4); LPS (Lipopolysaccharides); RAGE (Advanced glycation end-products); ROS (Reactive oxygen species); TNF (Tumor necrosis factor); ELISA (Enzyme-linked immunosorbent assay)

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Disclosure Statement

All the authors declare no competing interests.

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