JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC Article

Lysosomal Reacidification Ameliorates Vinyl Carbamate-Induced Toxicity and Disruption on Lysosomal pH

Yuting Li, Dongwen Hu, Jifeng Qi, Sunliang Cui, and Wei Chen*

Cite This: https://dx.doi.org/10.1021/acs.jafc.0c00534 Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Ethyl carbamate (EC) is a carcinogen toxicant, commonly found in fermented foods and beverages. The carcinogenic and toxic possibility of EC is thought to be related to its metabolite vinyl carbamate (VC). However, we found interesting mechanisms underlying VC-induced toxicity in this study, which were greatly different from EC. We first conducted a simple synthesis procedure for VC and found that VC possessed higher toxicity but failed to regulate levels of reactive oxygen species, glutathione, and autophagy. Notably, VC treatment resulted in upregulation of lysosomal pH, which was responsible for its cytotoxicity. Cyclic adenosine monophosphate (cAMP) pretreatment could enhance restoration of lysosomal acidity and ameliorate VC-induced damage. Inhibition of protein kinase A and cystic fibrosis transmembrane conductance regulator can block cAMP-induced cytoprotection. Together, our results provided the evidence for novel mechanisms of toxicity and possible protection method under VC exposure, which might give new perspectives on the study of EC-induced toxicity.

KEYWORDS: *vinyl carbamate, lysosomal reacidification, cAMP, cytotoxicity*

1. INTRODUCTION

Ethyl carbamate (EC) is present naturally during processing of fermented foods and beverages.[1,2] The concentration of EC in cigarette smoke is similar to that in foods.[3] EC, which can lead to development of lung, liver, pulmonary, gland, and skin tumors,[4−7] has been evaluated and classified as a probable carcinogen to human (Group 2A) by International Agency for Research on Cancer. Vinyl carbamate (VC) is a primary metabolite of EC via catalysis by a cytochrome P450 enzyme (CYP2E1) and found to possess higher carcinogenicity than EC.[8] VC exposure can cause higher levels of DNA adducts and greater numbers of tumors in lungs compared with EC.[7−9] EC and VC are both used as the inducer of lung tumors in rodents to investigate effective treatment on lung cancer and potential mechanisms of lung carcinogenesis.[10,11,12] In addition, previous studies indicate the potential toxicity and carcinogenicity caused by VC exposure in liver and small intestine.[13,14] Therefore, the toxic and carcinogenic possibility of EC might be related to its metabolite VC.[8]

Reactive oxygen species (ROS) have been shown to operate as intracellular signaling molecules driving various essential biochemical reactions.[15] Excessive ROS generated by exogenous toxicants, however, can damage normal cellular functions via toxicity of oxygen.[15,16] Our previous study found that redox disturbance led to a significant loss of cell viability in human normal hepatocyte L02 cells under EC exposure.[17] High levels of ROS can trigger the process of autophagy which serves as a cellular defense pathway.[18,19] Autophagy is an intracellular waste disposal pathway maintaining normal functions of cells.[20] The process of autophagy starts with the formation of autophagosomes containing cytoplasmic compounds and organelles, which are subsequently delivered to lysosome.[21] Lysosome, as the terminal organelle in the process of autophagy and other endocytic pathways, is involved in recycling cellular waste by digesting macromolecules and other materials.[22,23] Hydrolytic enzymes within the lysosome contribute to its multiple degradative functions and remain active with highly acidic pH.[24] Elevation in lysosomal pH, induced by exogenous toxicants or endogenous malfunction, can seriously damage activities of hydrolytic enzymes and restrict the degradation in lysosomes, which ultimately leads to cellular dysfunction and damage.[25,26] The process of lysosomal reacidification by treatment with exogenous reagents can restore the acidity of lysosomes and ameliorate cellular dysfunctions.[25]

Our study, therefore, is aimed at investigating the possible mechanism of toxicity from VC exposure and potential protective method for reducing VC-induced cellular damage. We found that VC did not regulate the process of ROS production and autophagy in L02 cells, which were greatly different from its parent compound EC.[17] Therefore, we tried to explore other possible mechanisms and found that lysosome alkalization might be the major reason for VC-induced cytotoxicity. Lysosome reacidification by cyclic adenosine monophosphate (cAMP) treatment could successfully rescue the disruption of lysosomal pH and cytotoxicity under VC exposure. We believe that our study may provide guidance for further studies on hepatotoxicity induced by EC and VC treatment.

Received: January 22, 2020
Revised: July 25, 2020
Accepted: July 29, 2020
Published: July 29, 2020

2. MATERIALS AND METHODS

2.1. Chemicals. Paraformaldehyde, dimethyl sulfoxide (DMSO), chloroquine (CQ), 3-Methyladenine (3-MA), rapamycin (Rap), trichloro acetic acid, Tris, sulforhodamine B (SRB), cAMP, H-89, cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor 172 (CFTRi) and 3-(4,5-dimthyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) were purchased from the Sigma-Aldrich (St. Louis, MO, USA). LysoTracker Red and LysoSensor Green were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dichlorodihydrofluorescein diacetate (DCFH-DA) and naphthalene-2,3-dicarboxal-dehyde (NDA) were obtained from Molecular Probes, Inc. (Eugene, OR, USA). All other reagents used were of analytical grade.

2.2. Synthesis of VC. Step 1. Iodobenzene (1 equiv weight, 1 equiv) and selenium powder (Se, 3 equiv) were added to a stirring suspension of cuprous iodide (CuI, 0.1 equiv) in DMSO. The reaction was stirred for 4 h. The reaction mixture was quenched with distilled water and then filtrated. The filtrate was extracted with EtOAc and brine in sequence. The combined organic layers were dried over Na_2SO_4, filtered, evaporated, and purified by silica gel column chromatography to give diphenyl diselenide (70% yield).

Step 2. Diphenyl diselenide (1 equiv) was dissolved in ethanol and then NaBH (3 equiv) was added portion wise. The reaction was stirred at room temperature for 15 min. After that, bromoethanol was slowly added and stirred overnight. The reaction solution was evaporated, re-dissolved in EtOAc, and then filtrated. The filtrate was washed with brine for three times, and the combined organic layers were dried over Na_2SO_4, filtered, evaporated, and purified by silica gel column chromatography to give compound 1 (95% yield).

Step 3. Compound 1 (1 equiv) and potassium cyanate (2.5 equiv) were dissolved in dichloromethane, and then trifluoroacetic acid (2 equiv) was slowly added to reaction solution and stirred overnight. After that, the reaction mixture was quenched with distilled water and then filtrated. The filtrate was extracted with dichloromethane and brine in sequence. The combined organic layers were dried over Na_2SO_4, filtered, evaporated, and purified by silica gel column chromatography to give compound 2 (85% yield). Compound 2 (1 equiv) and *m*-CPBA (1 equiv) were dissolved in dichloromethane and then stirred at room temperature overnight. After that, the reaction solution was filtrated, and the filtrate was evaporated to give compound 3 (80% yield). Compound 3 (1 equiv) and sodium carbonate (5 equiv) were dissolved in THF and stirred for 3 h. The reaction solution was filtrated. The filtrate was evaporated and purified by silica gel column chromatography to give VC (78% yield).

The structure of synthesized VC was characterized by NMR. The sample was dissolved in $CDCl_3$. ^1H and ^{13}C NMR analyses were performed on a Bruker AVANCE III spectrometer (14.1 T), operating at Larmor frequencies of 600 MHz for ^1H and 150 MHz for ^{13}C.

2.3. Cell Culture. Normal human hepatocyte L02 cell line was purchased from the Type Culture Collection of Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and incubated with an atmosphere of 5% CO_2 at 37 °C. The medium also contained 100 units/mL penicillin and 100 units/mL streptomycin.

2.4. Cell Viability Detection. The cell viability under VC exposure were determined using the MTT assay and SRB assay.[27,28] L02 cells were incubated with different concentrations (0, 0.625, 1.25, 2.5, 3.5, 5, and 10 mM) of VC or (2.5, 5, 20, 40, 60, and 80 mM) of EC for 24 h. Further study on cell viability under VC treatment was tested at the concentration of 2.5 mM, which was close to the IC_{50} value. To analyze the role of autophagy in VC-treated L02 cells, cells were incubated with Rap (1, 5, and 10 μM), 3-MA (0.5 mM) or CQ (5, 10, and 20 μM) for 1 h, and then with 2.5 mM VC for 24 h. To evaluate the effect of cAMP-induced lysosomal reacidification on VC-induced cytotoxicity, cells were first treated with 4 mM cAMP with or without 3 μM H-89 or 3 μM CFTRinh-172 (CFTRi) for 1 h and then incubated with 2.5 mM VC for 24 h. To determine the effect of CFTR on cell viability under VC exposure, L02 cells were first incubated with VX770, a CFTR potentiator, for 1 h and then treated with 2.5 mM VC for 24 h. For the MTT assay, formazan precipitate was dissolved in DMSO and detected at 490 nm after incubation with MTT for 4 h. For the SRB assay, cells were subjected to fixation with ice-cold trichloro acetic acid for 1 h and incubated with SRB solution for 30 min. Then, cells were washed with 1% acetic acid for three times. Remaining dye was dissolved in Tris solution and detected at 540 nm.

2.5. Determination of Intracellular ROS. Measurement of intracellular ROS levels was conducted according to the previous method with slight modifications.[29,30] After treatment with different concentrations (0, 0.625, 1.25, 2.5, 3.5, and 5 mM) of VC or (2.5, 5, 20, 40, 60, and 80 mM) of EC for 24 h, L02 cells were incubated with 10 μM DCFH-DA, a specific ROS fluorescence probe, for 30 min and photographed under a fluorescence microscope. H_2O_2 (600 μM) treatment were set as a positive control. The results were expressed as mean DCF fluorescence intensity.

2.6. Cellular Glutathione Measurement. The glutathione (GSH) concentration was detected using the GSH-specific probe NDA, as described previously.[31,32] After incubation with different concentrations of VC or EC for 24 h, L02 cells were stained with 50 μM NDA for 30 min and imaged under a fluorescence microscope. The results were expressed as mean NDA fluorescence intensity. GSH/GSSG ratio was determined by via the GSH and GSSG assay kit (Beyotime, China) based on the manufacturer's instruction.

2.7. LysoTracker Red Staining. The effect of VC on lysosome was determined using LysoTracker Red, as described previously with some changes.[33] L02 cells were treated with different concentrations of VC for 24 h. Based on results of the MTT assay, time-dependent changes of fluorescence intensity were tested at the concentration of 2.5 mM. To study the effect of CQ pretreatment on lysosome under VC exposure, cells were treated with 2.5 mM VC for 24 h after incubation with 20 μM CQ for 1 h. Then, L02 cells were incubated with 100 nM LysoTracker Red for 30 min and subjected to fluorescence microscope analysis. The results were expressed as mean fluorescence intensity.

2.8. Estimation of Lysosomal pH Using LysoSensor Green. The intralysosomal pH was detected using pH-sensitive probe LysoSensor Green, according to the manufacturer's instructions. L02 cells were treated with VC (0, 0.625, 1.25, and 2.5 mM) for 24 h. Effects of 2.5 mM VC treatment for different time (0, 3, 6, 9, 12, and 24 h) on LysoSensor Green fluorescence intensity were also tested. To evaluate the effect of CQ pretreatment on lysosomal pH under VC exposure, cells were treated with 20 μM CQ for 1 h and then with 2.5 mM VC for 24 h. To evaluate effect of cAMP-induced lysosomal reacidification, cells were treated with 2.5 mM VC for 24 h, after incubation with 4 mM cAMP with or without 3 μM H-89 or 3 μM CFTRi for 1 h. To determine the role of CFTR in lysosomal reacidification, L02 cells were first incubated with VX770 for 1 h, followed by treatment with 2.5 mM VC for 24 h. Then, cells were stained with 100 nM LysoSensor Green for 30 min and subjected to fluorescence microscope analysis. The results were expressed as mean fluorescence intensity.

2.9. GFP-RFP-LC3 Transfection Assay. L02 cells were transfected with GFP-RFP-LC3 plasmid and X-tremeGENE HP DNA transfection reagent (Roche, Switzerland) based on the manufacturer's instruction. Then, cells were treated with different concentrations of VC for 24 h. For detection of effects of CQ and cAMP on autophagy, cells were treated with 20 μM CQ or 4 mM cAMP for 1 h, followed by incubation with 2.5 mM VC for 24 h. After that, cells were observed under a fluorescence microscope.

2.10. Western Blot. Western bolt was conducted according to previous studies with slight modifications.[34,35] After treatment, protein samples were extracted using RIPA lysis buffer. The protein samples were subjected to gel electrophoresis. Then, the protein samples were transferred to polyvinylidene fluoride membranes. Blots were incubated in blocking buffer (10%, w/v, dried skimmed milk in PBST) for 1 h and then with primary antibodies diluted in 5% bovine serum albumin in PBST overnight at 4 °C. Anti-LC3 (Abcam, ab48394, 1:2000), anti-LAMP-1 (Santa Cruz, sc-20011, 1:1000), anti-

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

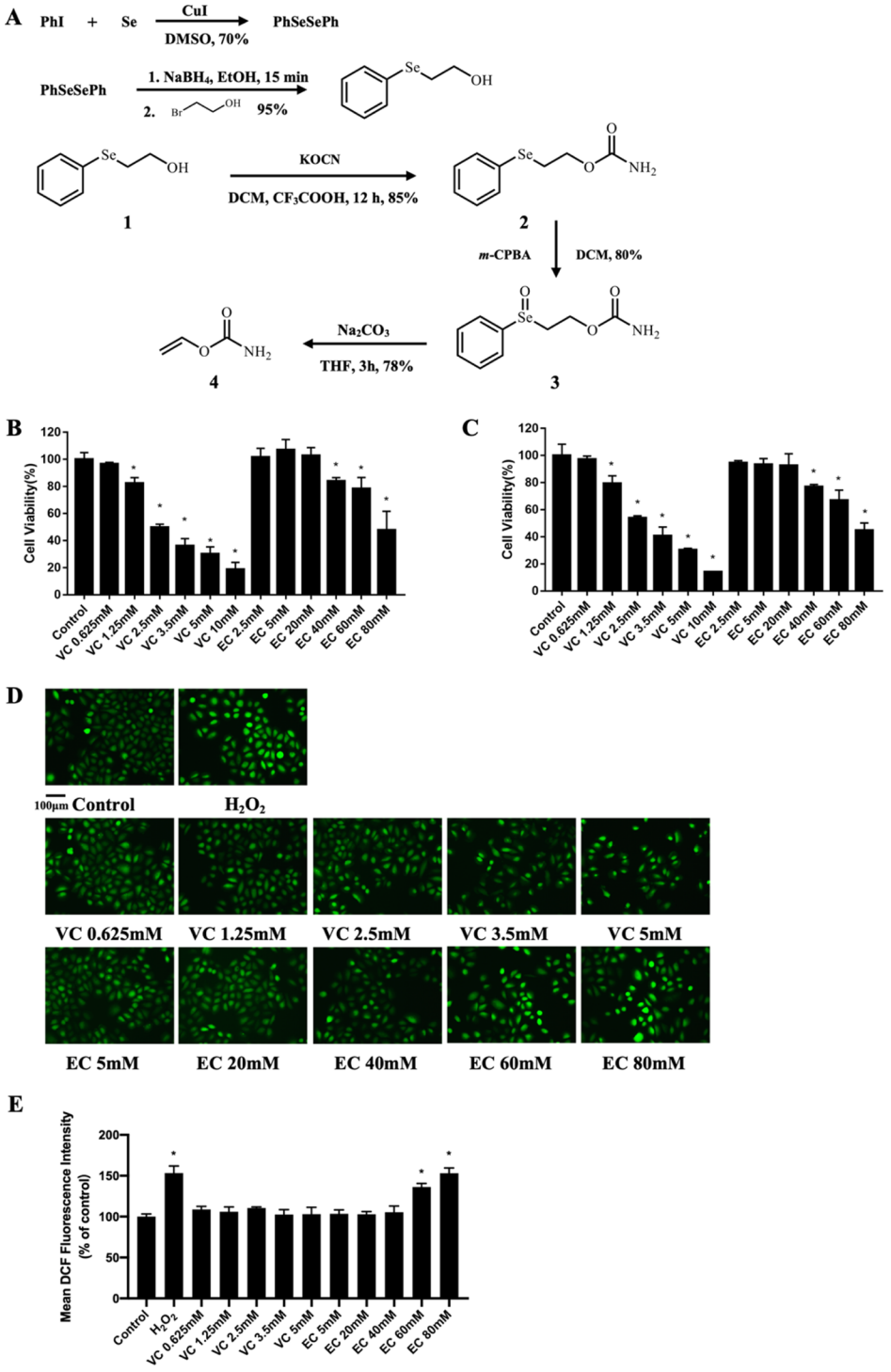

Figure 1. VC-induced cytotoxicity without affecting intracellular ROS. (A) Synthetic route of VC. (B,C) L02 cells were incubated with different concentrations (0, 0.625, 1.25, 2.5, 5, and 10 mM) of VC or different concentrations (2.5, 5, 20, 40, 60, and 80 mM) of EC for 24 h. H_2O_2 (600 μM) was set as a negative control. The quantitative data of cell viability determined by MTT and SRB assays. (D) Effect of VC treatment on intracellular ROS levels. (E) Quantitative data of panel (D) were calculated by ImageProPlus and expressed as mean DCF fluorescence intensity. VC, vinyl carbamate; ROS, reactive oxygen species; and DCFH-DA, 2′,7′-dichlorofluorescin diacetate. *$p < 0.05$.

cathepsin B (Cell Signaling Technology, 31718, 1:1000), anti-cathepsin D (Abcam, ab75852, 1:2000), and anti-GAPDH (Abcam, ab181602, 1:10,000) antibodies were used as primary antibodies in this study. After being washed with PBST, blots were further incubated with secondary antibodies for 1 h. The secondary antibodies were anti-rabbit and anti-mouse immunoglobulin B (Bio-Rad, 170-6515 and 170-6516, 1:1000). Blots were imaged by chemiluminescent HRP substrate (Millipore, USA).

2.11. Statistical Analysis. The value of IC_{50} was measured by software SPSS 22.0. For detection of fluorescence intensity, five randomized and independent microscopic fields were chosen for calculation using Image-Pro Plus. Each selected field contained at least 30 cells. Data were shown as means ± standard deviations (SD) of at least three independent experiments. One-way analysis of variance (Duncan test) conducted by software SPSS 22.0 was used for statistical analyses. $p < 0.05$ was considered as statistical significance.

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

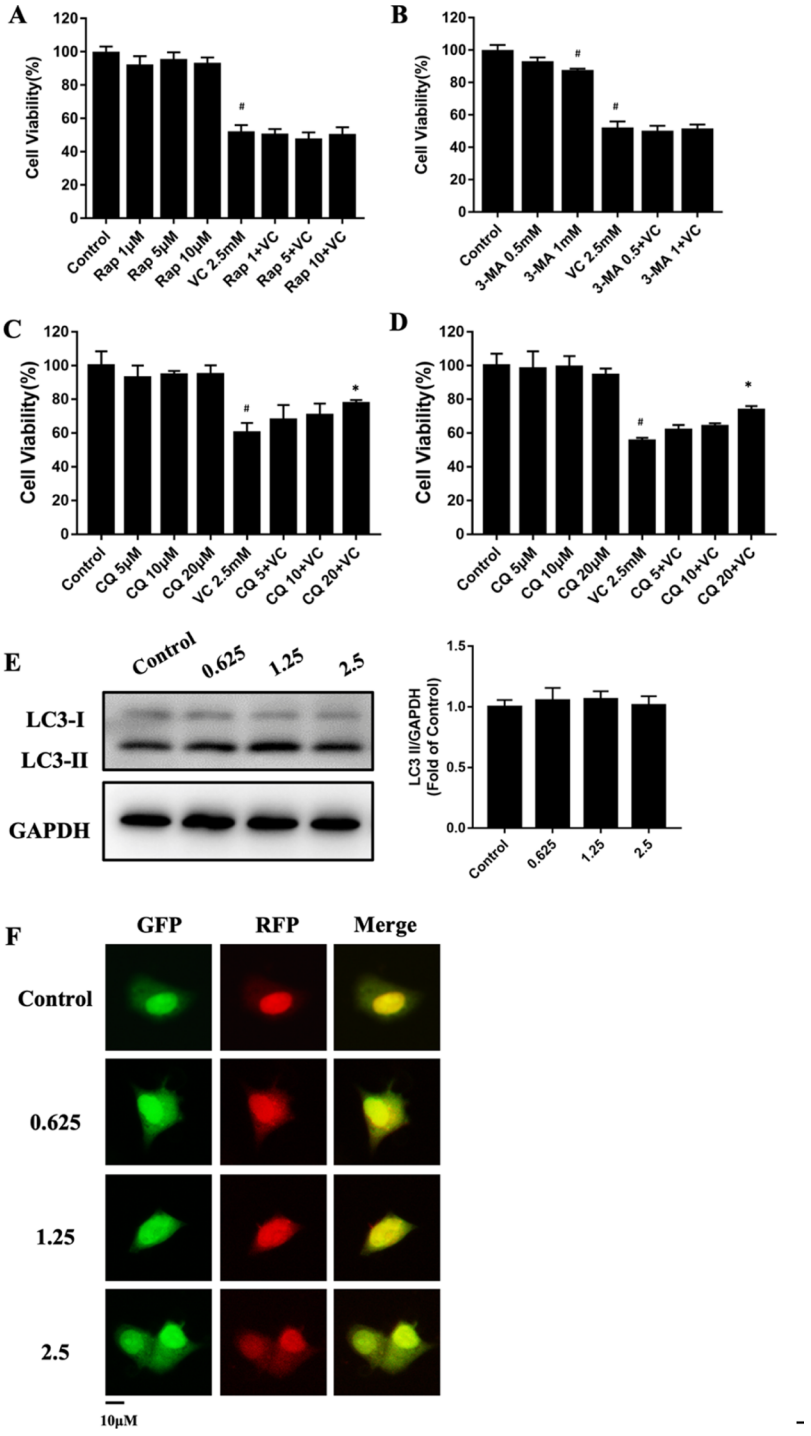

Figure 2. CQ protected against VC-induced cytotoxicity. (A–C) After incubation with Rap, 3-MA, or CQ for 1 h, L02 cells were treated with 2.5 mM VC for 24 h. Effect of Rap, 3-MA, or CQ on cell viability under VC exposure by the MTT assay. (D) Effect of CQ on VC-induced cytotoxicity tested by the SRB assay. (E) Immunoblot analysis of LC3 expression. (F) Morphology of L02 cells transfected with GFP-RFP-LC3. VC, vinyl carbamate; CQ, chloroquine; Rap, rapamycin; and 3-MA, 3-methyladenine. $^{\#}p < 0.05$ vs control group, $^{*}p < 0.05$ vs VC group.

3. RESULTS

3.1. Synthesis of VC and Structure Elucidation. A previous study has unveiled the possibility of hepatoxicity caused by VC treatment.[36] However, few studies have revealed further mechanisms of hepatoxicity under VC exposure. We first conducted a simple and effective synthesis of VC by chemical oxidation of 2-(phenylseleno) ethanol using *m*-CPBA. The detailed process of VC synthesis was shown in Figure 1. To confirm the structure of purified compound, LC–MS/MS and NMR experiments were performed (Figure S1). The results were listed as follows:

^{1}H NMR (600 M, $CDCl_3$): δ 7.148 (dd, J_1 = 13.8 Hz, J_2 = 6.0 Hz, 1H), 4.769 (dd, J_1 = 13.8 Hz, J_2 = 1.8 Hz, 1H), 4.468 (dd, J_1 = 6.6 Hz, J_2 = 1.9 Hz, 1H).

^{13}C NMR (150 M, $CDCl_3$): δ 153.8, 142.0, 96.1.

3.2. VC-induced Cytotoxicity in L02 Cells. The effect of VC treatment on hepatocyte L02 cells viability was

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX–XXX

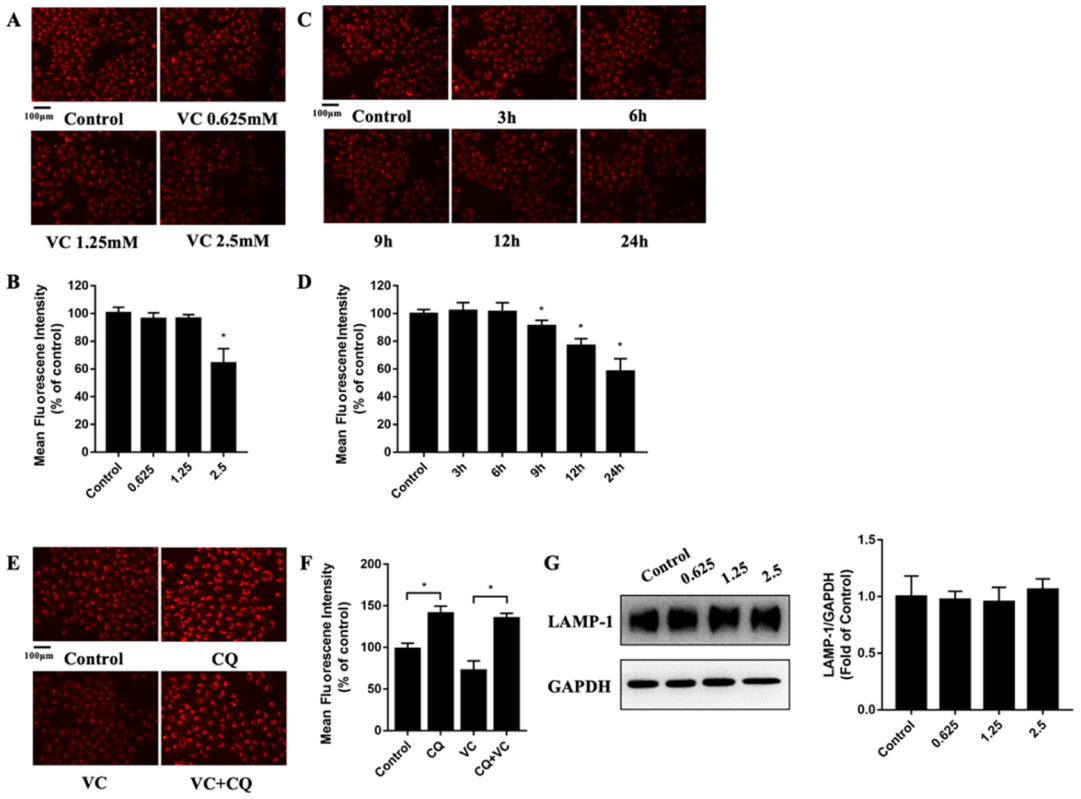

Figure 3. Effect of CQ and VC treatment on lysosome. (A) L02 cells staining with LysoTracker Red after VC treatment for 24 h. (B) Quantitative data of panel (A) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (C) L02 cells staining with LysoTracker Red after 2.5 mM VC treatment for 3, 6, 9, 12, and 24 h. (D) Quantitative data of panel (C) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (E) Cells were pretreated with 20 μM CQ for 1 h and then incubated with 2.5 mM VC for 24 h. After treatment, L02 cells were stained with LysoTracker Red. (F) Quantitative data of panel (E) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (G) Assessment of LAMP-1 expression by western blots. VC, vinyl carbamate. *$p < 0.05$.

subsequently evaluated by MTT and SRB assays. The MTT assay showed that cell viability after VC exposure for 24 h decreased dose-dependently (Figure 1B). A significant difference was not observed in 0.625 mM VC-treated group compared with the control group (Figure 1B). The cell viability decreased to 82, 50, 36, 30, and 19% under higher concentrations (1.25, 2.5, 3.5, 5, and 10 mM) of VC treatment, respectively. As a comparator, 40, 60, and 80 mM of EC treatment also significantly reduced cell viability to 84, 78, and 48%, which was similar to the previous study.[17] The IC_{50} value for VC was 2.9 mM based on the MTT assay. Similar results of the SRB assay were consistent with those of the MTT assay, as shown in Figure 1C. Given these results, 2.5 mM VC was chosen for following experiments. Hence, VC treatment could decrease viability of L02 cells, and its toxicity was found to be stronger than its parent compound EC.

3.3. VC Treatment did Not Regulate Levels of ROS and GSH in L02 Cells. ROS overproduction, usually activated under exposure of exogenous toxicants, can serve as an important initiator for cytotoxicity.[16] EC treatment was found to trigger redox disturbance in different cell lines.[17,37,38] As the primary metabolite of EC, VC was also speculated to induce intracellular ROS overproduction. Therefore, we measured ROS levels in VC-treated L02 cells using DCFH-DA, a ROS-specific fluorescence probe. Cells treated with 600 μM H_2O_2 were set as a positive control. H_2O_2 significantly increased ROS levels by 49% compared with the control (100%). No significant changes of fluorescence intensity were found after VC exposure for 24 h, while 60 and 80 mM EC enhanced ROS levels by 38 and 48%, respectively (Figure 1D,E). We also examined the intracellular GSH concentration after VC exposure. GSH serves as an essential antioxidant for maintaining redox homeostasis. We observed that GSH levels were markedly reduced to 77 and 58% when L02 cells were exposed to 60 and 80 mM of EC. On the contrary, no significant changes in GSH concentrations were found after VC treatment (Figure S2A,B). Different concentrations of VC did not affect the GSH/GSSG ratio, while 60 and 80 mM EC treatment significantly decreased the GSH/GSSG ratio compared with the control, which was consistent with results of NDA staining (Figure S2C). Taken together, 2.5 mM VC failed to affect intracellular ROS and GSH levels, which suggested that VC might possess different toxic mechanisms compared with EC.

3.4. VC Failed to Affect Autophagy in L02 Cells. Autophagy, stimulated under endogenous and exogenous cellular stress, serves as a cytoprotective system, which is an important source of essential compounds for normal cellular functions by lysosomal degradation of cytoplasmic materials and damaged organelles.[39,40] EC was found to promote the process of autophagy previously.[17] We, therefore, hypothesized that the severe cytotoxicity caused by VC might also enhance autophagy as a cell defense pathway. The MTT assay showed that Rap (an inducer of autophagy) or 3-MA (an inhibitor of autophagy) failed to affect cell viability compared with the VC-treated group (Figure 2A,B). However, the pretreatment of 20 μM CQ, an autophagy inhibitor, contributed to an increase of 17% in cell viability compared with the VC group (Figure 2C).

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

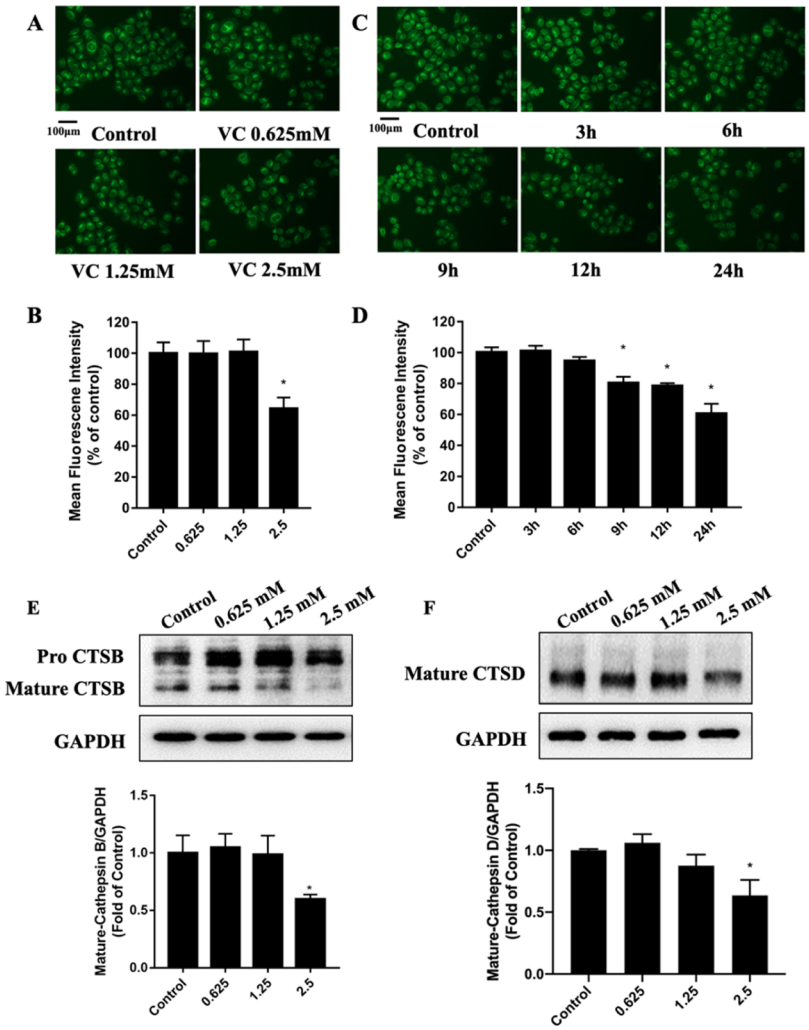

Figure 4. VC-induced lysosomal alkalization. (A) L02 cells were treated with different concentrations (0, 0.625, 1.25, and 2.5 mM) of VC for 24 h and then stained with LysoSensor Green. (B) Quantitative data of panel (A) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (C) L02 cells were treated with 2.5 mM VC for different time (0, 3, 6, 9, 12, and 24 h) and then stained with LysoSensor Green. (D) Quantitative data of panel (C) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (E,F) Immunoblot analysis of CTSB and CTSD expression. VC, vinyl carbamate; CQ, chloroquine; CTSB, cathepsin B; and CTSD, cathepsin D. *$p < 0.05$.

The results of SRB assays for CQ pretreatment were consistent with those of MTT assays (Figure 2D). To further determine whether VC treatment contributed to the regulation of autophagy, we conducted western blot to examine levels of LC3-II (a commonly used autophagosome marker). VC treatment failed to induce any significant changes of LC3-II levels (Figure 2E). We also conducted the GFP-RFP-LC3 transfection assay for further detection of autophagy flux. In total, 0.625, 1.25, and 2.5 mM VC failed to affect number of autolysosomes and autophagosomes (Figure 2F), which were consistent with LC3-II expression. Together, VC exposure did not affect the level of autophagy, which was also different from the results of EC.

3.5. VC Treatment Caused Lysosomal Alkalization in L02 Cells. The significant protection of CQ pretreatment against VC-induced cytotoxicity could ascribe to the regulation of lysosomal biogenesis and acidity.[41−43] Hence, we speculated that VC might affect lysosomes in L02 cells. The results of LysoTracker Red staining assay showed that VC induced a dose-dependent decrease in fluorescence intensity (Figure 3A,B). VC treatment with the concentration of 2.5 mM significantly reduced fluorescence intensity by 15% (Figure 3A,B). Besides, a time-dependent decrease in fluorescence intensity was also observed under the exposure of 2.5 mM VC (Figure 3C,D). We also observed effect of CQ pretreatment on fluorescence intensity of LysoTracker Red. As we expected, CQ pretreatment significantly increased fluorescence intensity compared with 2.5 mM VC-treated group, as shown in Figure 3E,F. Because CQ pretreatment could increase cell viability compared with VC treatment, we speculated that VC-induced toxicity might be related to the changes of lysosomes.

To further analyze the effect of VC exposure on the amount of lysosome, we conducted western blot on the expression of lysosomal-associated membrane protein 1 (LAMP-1). LAMP-1, the most abundant lysosomal membrane protein accounting for 50% of the total protein in the lysosomal membrane, is used as the marker for detection of lysosome numbers.[24] The results of western blot showed that VC treatment with different concentrations did not affect LAMP-1 expression (Figure 3G). Then, we evaluated the changes of lysosomal pH under VC treatment using a pH-sensitive fluorescence probe LysoSensor Green. The results were similar to those of the LysoTracker Red staining assay. Dose- and time-dependent decreases of fluorescence intensity were detected under VC exposure

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

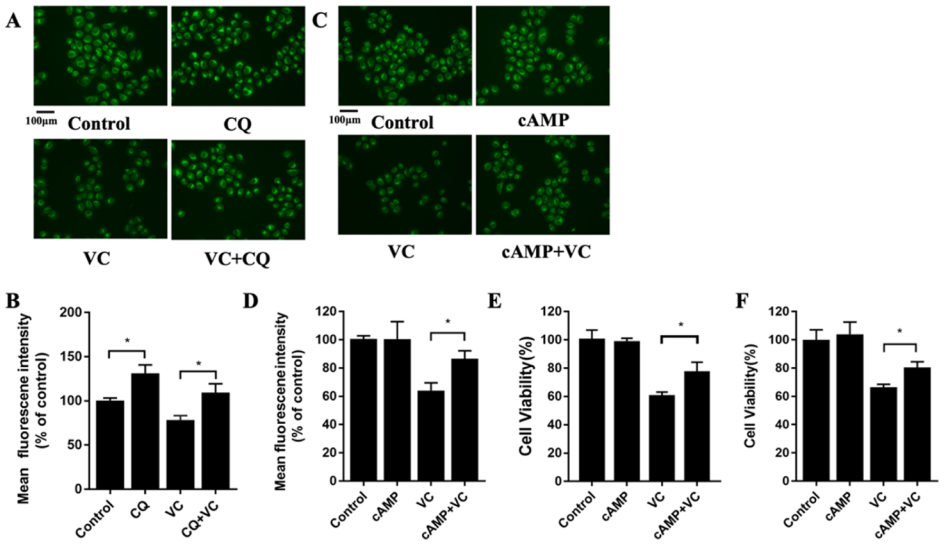

Figure 5. cAMP promoted lysosomal re-acidification and rescued cytotoxicity caused by VC. After incubation with 4 mM cAMP or 20 μM CQ for 1 h, L02 cells were treated with 2.5 mM VC for 24 h. (A,C) L02 cells staining with LysoSensor Green. (B) Quantitative data of panel (A) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (D) Quantitative data of panel (C) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (E,F) Effect of cAMP on cell viability under VC exposure tested by MTT and SRB assays. VC, vinyl carbamate; CQ, chloroquine; and cAMP, cyclic adenosine monophosphate. *$p < 0.05$.

(Figure 4A–D). Lysosomal acidity was crucial for lysosomal function. Inactive precursors of cathepsins are synthesized in cytoplasm and then cleaved to create the mature forms in lysosomes, which were responsible for lysosomal degradation function. 2.5 mM VC treatment also significantly inhibited the expression of mature cathepsin B and D (CTSB and CTSD), as shown in Figure 4E,F. Therefore, these results indicated that VC treatment could disrupt lysosomal pH and cause lysosomal dysfunction without affecting lysosome numbers in L02 cells.

3.6. CQ Activated Lysosomal Reacidification in VC-Treated L02 Cells. CQ can induce a lasting lysosomal reacidification in the previous study.[41] Therefore, we speculated that the protection of CQ pretreatment against VC-induced cytotoxicity was ascribed to the restoration of lysosomal acidity. CQ alone could cause higher fluorescence intensity of LysoTracker Red than that of the control group, which was consistent with the previous study.[41] Similar results are also shown in Figure 3E,F. Then, we confirmed our results using pH-sensitive LysoSensor Green to evaluate changes of lysosomal pH and observed similar results with LysoTracker Red (Figure 5A,B). Taken together, CQ pretreatment could activate lysosomal reacidification to protect against cytotoxicity caused by VC.

3.7. cAMP Promoted Lysosomal Reacidification and Reduced Cytotoxicity in L02 Cells. Despite the restoration of lysosomal acidity under CQ treatment,[41] CQ is also reported to raise the pH of cellular compartments, especially lysosomes.[44] Given these discrepancies on alteration of lysosomal pH by CQ treatment, we used cAMP, another effective reagent for lysosomal reacidification,[25,26] to restore lysosomal pH in VC-treated cells. We found that cAMP pretreatment could normalize lysosomal acidity of L02 cells under VC exposure (Figure 5C,D). Treatment with cAMP alone did not affect the basal acidity of lysosomes (Figure 5C,D), which was different from CQ exposure. Besides, we found that mature CTSB and CTSD levels of cAMP + VC groups were higher than the VC group, as shown in Figure S3A,B, which might ascribe to the elevation of lysosomal acidity. In addition, cAMP pretreatment significantly increased cell viability by 16% compared with VC-treated group according to the MTT assay (Figure 5E). SRB assays also showed similar results in Figure 6F. Thus, cAMP pretreatment not only promoted lysosomal reacidification but also suppressed VC-induced cellular damage, which further confirmed that lysosomal alkalization might be one of the main causes for cytotoxicity in VC-treated L02 cells.

We also investigated CQ and cAMP pretreatment on autophagy flux. Because CQ could inhibit autophagy flux and cause autophagosomes accumulation, LC3-II level of the CQ group was increased compared with the control (Figure S4A). No significant changes of LC3-II expression were shown in the CQ group and CQ + VC group, while cAMP pretreatment increased LC3-II expression compared with the VC group (Figure S4B). The GFP-RFP-LC3 transfection assay showed similar results (Figure S4C). The red puncta and yellow puncta represented autolysosomes and autophagosomes. The number of yellow puncta in the CQ group was similar to that in the VC + CQ group, which indicated that VC failed to affect autophagy flux. cAMP pretreatment significantly increased the numbers of red puncta, which suggested that cAMP pretreatment promoted autophagy flux. Based on these results, we further detected cAMP-triggered lysosomal reacidification in VC-treated cells.

3.8. PKA and CFTR Were Involved in Lysosomal Reacidification Caused by cAMP. Protein kinase A (PKA), a cAMP-dependent kinase, also has been found to promote lysosomal reacidification.[26] Therefore, we testified whether PKA was involved in the process of lysosomal reacidification caused by cAMP pretreatment in our study. The fluorescence intensity of LysoSensor Green in L02 cells treated with the PKA inhibitor H-89 alone was similar to that of the control and cAMP group (Figure 6A,B). However, H-89 could weaken the process of lysosomal reacidification caused by cAMP (Figure 6A,B). We further tested the effect of H-89 on increased cell viability under cAMP pretreatment. According to MTT and SRB assays, we found that H-89 significantly

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

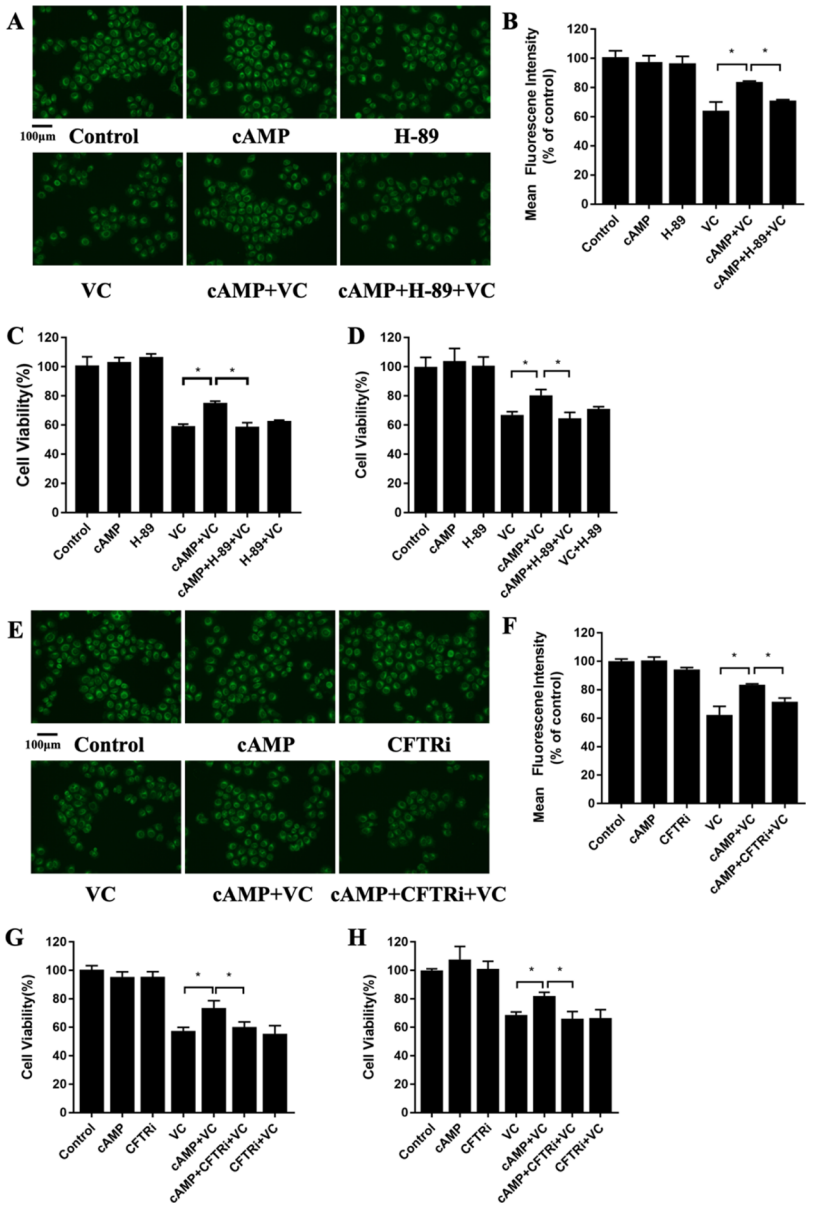

Figure 6. PKA and CFTR were critical for cAMP-induced lysosomal reacidification. L02 cells were incubated with 4 mM cAMP with or without 3 μM H-89 or CFTRi for 1 h and then treated with 2.5 mM VC for 24 h. (A,E) L02 cells staining with LysoSensor Green. (B) Quantitative data of panel (A) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (F) Quantitative data of panel (E) were calculated by ImageProPlus and expressed as mean fluorescence intensity. (C,D) Effect of H-89 and cAMP on VC-induced cytotoxicity tested by MTT and SRB assays. (G,H) Effect of CFTRi and cAMP on VC-induced cytotoxicity tested by MTT and SRB assays. VC, vinyl carbamate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; and CFTRi, CFTR inhibitor 172. *$p < 0.05$.

inhibited the protection from cAMP against VC-induced toxicity (Figure 6C,D). Therefore, PKA was involved in lysosomal reacidification and cytoprotection caused by cAMP.

The restoration of lysosomal pH activated by cAMP/PKA is usually related to CFTR, a PKA-mediated chloride channel that enhances lysosomal reacidification by regulating the exchanges of H^+/Cl^-.[45] The effect of CFTR on cAMP/PKA-induced pH restoration was further investigated in our study. L02 cells treated with CFTRi, a CFTR inhibitor, alone showed no significant changes in fluorescence intensity of LysoSensor Green compared with the control and cAMP group (Figure 6E,F). As expected, CFTRi inhibited restoration of lysosomal pH by cAMP pretreatment, which was similar to the results of H-89 (Figure 6E,F). MTT and SRB assays also showed significant reduction in cell viability caused by co-pretreatment of CFTRi and cAMP, compared with pretreatment of cAMP alone (Figure 6G,H). We further pretreated L02 cells with 5 μM VX770, a cAMP-independent CFTR potentiator,[46] for 1 h and followed by incubation with 2.5 mM VC for 24 h. VX770 pretreatment can significantly increase fluorescence intensity of LysoSensor Green and cell viability, compared with cells treated with VC alone (Figure S5A–D). These results, therefore, indicated that CFTR also served as an essential element for lysosomal reacidification.

4. DISCUSSION

In our study, we successfully synthesized VC, the primary metabolite of EC, and provided novel findings related to VC-induced cytotoxicity in human normal hepatocyte L02 cells.

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Despite its higher toxicity than EC, VC possesses greatly different toxic mechanisms compared with its parent compound. Interestingly, VC was found to induce lysosomal alkalization, which further triggered severe cellular damage. Lysosomal reacidification activated by cAMP pretreatment could reduce VC-induced cytotoxicity. To the best of our knowledge, this is the first study to elaborate the toxic mechanisms and potential protection against toxicity under VC exposure.

Our results first showed severe hepatoxicity of VC by observing significantly decreased cell viability under VC exposure in L02 cells. In our previous study, EC-induced cytotoxicity was related to excessive ROS generation accompanied with GSH depletion, and autophagy was enhanced as a defense pathway.[17] Conversely, VC treatment failed to regulate the levels of ROS and GSH, as well as the process of autophagy. The metabolism of EC is reported to generate ethanol, ammonia, carbon dioxide, and VC.[47] Ethanol and ammonia have direct and indirect regulation on ROS overproduction in the cytoplasm and mitochondria via the disturbance of antioxidant pathways.[48,49] Ethanol can also trigger high levels of autophagy as a defense mechanism in astroglia cells.[50] Therefore, excess ROS generation and enhancement of autophagy under EC exposure might ascribe to other metabolites or EC itself.

Despite the absence of autophagy in VC-induced cytotoxicity, we found CQ, a commonly used inhibitor for autophagy, could prevent the decrease in cell viability under VC exposure. CQ treatment has been found to induce a stable restoration of the lysosomal acidity.[41] Lysosome is the key degradative compartment for metabolic processes including the recycling of materials from organelles.[51] The degradative functions of lysosomes are potentiated by a profound luminal acidity.[23] Disruption of lysosomal pH caused by stimuli is treated as the main reason for cellular dysfunction and severe cytotoxicity.[26] We, thus, speculated that VC could upregulate lysosomal pH, and the protection from CQ against cytotoxicity might be related to the restoration of lysosomal acidity. In this study, we first found a significant decrease in fluorescence intensity of LysoTracker Red under high levels of VC. CQ pretreatment stimulated restoration of lysosomal pH by detecting increased fluorescence intensity. This fluorescence dye will lose its signals when the pH is over 6.5.[50] LysoSensor, a more sensitive fluorescence probe to pH, was used to further determine the alteration of lysosomal pH. The results of LysoSensor Green were in line with LysoTracker Red, which suggested that VC treatment could lead to lysosomal alkalization, and lysosomal reacidification induced by CQ could reduce its toxicity.

However, the effects of CQ treatment on lysosomal pH are controversial in current literature, and the mechanism of CQ-induced regulation on cellular metabolism and lysosomes was still vague.[41,42] Therefore, we used cAMP, another reagent possessing the possibility of lysosomal reacidification, to evaluate the role of lysosomal pH restoration in VC-induced toxicity. Pretreatment with cAMP promoted the process of lysosomal reacidification and remarkably rescued the cytotoxicity induced by high levels of VC, which indicated that lysosomal alkalization contributed to VC-induced cytotoxicity. We therefore focused on the role of lysosomal reacidification as a possible therapeutic method for cellular damage caused by VC.

The mechanism of lysosomal reacidification of cAMP might be related to PKA, a kinase activated by cAMP.[52] Treatment with H-89 alone, a commonly used inhibitor of PKA, did not alter basal pH in lysosomes, which is consistent with the previous study.[26] Co-pretreatment of H-89 with cAMP blocked the process of cAMP-induced lysosomal reacidification and protection against VC-induced cytotoxicity. The most attractive explanation for improvement of lysosomal acidity by the activation of cAMP/PKA would be through the enhancement of CFTR. CFTR, a cAMP/PKA-mediated chloride channel,[45] appears to be crucial for the process of lysosomal reacidification. The antagonist CFTRinh-172 (CFTRi) fail to affect basic levels of lysosomal acidity but significantly reduced the reacidification of compromised lysosomes under cAMP pretreatment. In addition, the inhibition of CFTR also suppressed cAMP-induced cytoprotection in VC-treated cells. Thus, CFTR and PKA were critical regulators in the pathway of cAMP-induced lysosomal reacidification and increased cell viability in L02 cells.

These findings provided new perspectives on potential mechanisms underlying hepatoxicity and possible protective methods under treatment of EC, the parent compound of VC. Besides VC, future studies should also focus on toxicity caused by other metabolites for further explanation of EC-induced damage.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c00534.

1H NMR and 13C NMR for VC; L02 cells treated with different concentrations of VC or EC; and L02 cells incubated with 4 mM cAMP, 20 μM CQ, and 5 μM VX770 and then treated with 2.5 mM VC (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Wei Chen − *Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing and Ningbo Research Institute, Zhejiang University, Hangzhou 310058, China;* orcid.org/0000-0002-2373-2437; Phone: +86 571 88982861; Email: zjuchenwei@zju.edu.cn; Fax: +86 571 88982191

Authors

Yuting Li − *Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou 310058, China*

Dongwen Hu − *Department of Food Science and Nutrition, Zhejiang Key Laboratory for Agro-Food Processing, Zhejiang University, Hangzhou 310058, China*

Jifeng Qi − *Institute of Drug Discovery and Design, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*

Sunliang Cui − *Institute of Drug Discovery and Design, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.0c00534

Notes

The authors declare no competing financial interest.

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

■ ACKNOWLEDGMENTS

This work was supported by grants from the Zhejiang Provincial Natural Science Foundation of China (LR18C200002) and the National Natural Science Foundation of China (21876152).

■ REFERENCES

(1) Riachi, L. G.; Santos, Â.; Moreira, R. F. A.; De Maria, C. A. B. A review of ethyl carbamate and polycyclic aromatic hydrocarbon contamination risk in cachaca and other Brazilian sugarcane spirits. *Food Chem.* **2014**, *149*, 159−169.

(2) Gowd, V.; Su, H.; Karlovsky, P.; Chen, W. Ethyl carbamate: An emerging food and environmental toxicant. *Food Chem.* **2018**, *248*, 312−321.

(3) Liu, H.; Cui, B.; Xu, Y.; Hu, C.; Liu, Y.; Qu, G.; Li, D.; Wu, Y.; Zhang, D.; Quan, S.; Shi, J. Ethyl carbamate induces cell death through its effects on multiple metabolic pathways. *Chem.-Biol. Interact.* **2017**, *277*, 21−32.

(4) Gurley, K. E.; Moser, R. D.; Kemp, C. J. Induction of Lung Tumors in Mice with Urethane. *Cold Spring Harb Protoc.* **2015**, *2015*, pdb.prot077446.

(5) Avanzo, J. L.; Mesnil, M.; Hernandez-Blazquez, F. J.; da Silva, T. C.; Fukumasu, H.; Mori, C. M. C.; Yamasaki, H.; Dagli, M. L. Z. Altered expression of connexins in urethane-induced mouse lung adenomas. *Life Sci.* **2006**, *79*, 2202−2208.

(6) Li, X.; Wu, J.; Zheng, J.; Li, Y.; Yang, T.; Hu, G.; Dai, J.; Yang, Q.; Dai, L.; Jiang, Y. Altered miRNA expression profiles and miR-1a associated with urethane-induced pulmonary carcinogenesis. *Toxicol. Sci.* **2013**, *135*, 63−71.

(7) Dahl, G. A.; Miller, E. C.; Miller, J. A. Comparative carcinogenicities and mutagenicities of vinyl carbamate, ethyl carbamate, and ethyl N-hydroxycarbamate. *Cancer Res.* **1980**, *40*, 1194−1203.

(8) Dahl, G. A.; Miller, J. A.; Miller, E. C. Vinyl carbamate as a promutagen and a more carcinogenic analog of ethyl carbamate. *Cancer Res.* **1978**, *38*, 3793−3804.

(9) Lee, R. P.; Forkert, P. G. Inactivation of cytochrome P-450 (CYP2E1) and carboxylesterase (hydrolase A) enzymes by vinyl carbamate in murine pulmonary microsomes. *Drug Metab. Dispos.* **1999**, *27*, 233−239.

(10) Liby, K.; Royce, D. B.; Williams, C. R.; Risingsong, R.; Yore, M. M.; Honda, T.; Gribble, G. W.; Dmitrovsky, E.; Sporn, T. A.; Sporn, M. B. The synthetic triterpenoids CDDO-methyl ester and CDDO-ethyl amide prevent lung cancer induced by vinyl carbamate in A/J mice. *Cancer Res.* **2007**, *67*, 2414−2419.

(11) Shen, T.; Jiang, T.; Long, M.; Chen, J.; Ren, D.-M.; Wong, P. K.; Chapman, E.; Zhou, B.; Zhang, D. D. A Curcumin Derivative That Inhibits Vinyl Carbamate-Induced Lung Carcinogenesis via Activation of the Nrf2 Protective Response. *Antioxid. Redox Signaling* **2015**, *23*, 651−664.

(12) Hernandez, L. G.; Forkert, P.-G. In vivo mutagenicity of vinyl carbamate and ethyl carbamate in lung and small intestine of F1 (Big Blue x A/J) transgenic mice. *Int. J. Cancer* **2007**, *120*, 1426−1433.

(13) Takahashi, K.; Dinse, G. E.; Foley, J. F.; Hardisty, J. F.; Maronpot, R. R. Comparative prevalence, multiplicity, and progression of spontaneous and vinyl carbamate-induced liver lesions in five strains of male mice. *Toxicol. Pathol.* **2002**, *30*, 599−605.

(14) Kim, S.; Surh, Y. J.; Sohn, Y.; Yoo, J. K.; Lee, J. W.; Liem, A.; Miller, J. A. Inhibition of vinyl carbamate-induced hepatotoxicity, mutagenicity, and tumorigenicity by isopropyl-2-(1,3-dithietane-2-ylidene)-2-[N-(4-methylthiazol-2-yl)carbamoyl]-acetate (YH439). *Carcinogenesis* **1998**, *19*, 687−690.

(15) D'Autréaux, B.; Toledano, M. B. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 813−824.

(16) Ott, M.; Gogvadze, V.; Orrenius, S.; Zhivotovsky, B. Mitochondria, oxidative stress and cell death. *Apoptosis* **2007**, *12*, 913−922.

(17) Li, Y.; Ye, X.; Zheng, X.; Chen, W. Transcription factor EB (TFEB)-mediated autophagy protects against ethyl carbamate-induced cytotoxicity. *J. Hazard. Mater.* **2019**, *364*, 281−292.

(18) Ding, R.; Zhang, C.; Zhu, X.; Cheng, H.; Zhu, F.; Xu, Y.; Liu, Y.; Wen, L.; Cao, J. ROS-AKT-mTOR axis mediates autophagy of human umbilical vein endothelial cells induced by cooking oil fumes-derived fine particulate matters in vitro. *Free Radic. Biol. Med.* **2017**, *113*, 452−460.

(19) He, Z.; Guo, L.; Shu, Y.; Fang, Q.; Zhou, H.; Liu, Y.; Liu, D.; Lu, L.; Zhang, X.; Ding, X.; Liu, D.; Tang, M.; Kong, W.; Sha, S.; Li, H.; Gao, X.; Chai, R. Autophagy protects auditory hair cells against neomycin-induced damage. *Autophagy* **2017**, *13*, 1884−1904.

(20) Rubinsztein, D. C.; Codogno, P.; Levine, B. Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat. Rev. Drug Discov.* **2012**, *11*, 709−730.

(21) Ueno, T.; Komatsu, M. Autophagy in the liver: functions in health and disease. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 170−184.

(22) DiCiccio, J. E.; Steinberg, B. E. Lysosomal pH and analysis of the counter ion pathways that support acidification. *J. Gen. Physiol.* **2011**, *137*, 385−390.

(23) Mindell, J. A. Lysosomal acidification mechanisms. *Annu. Rev. Physiol.* **2012**, *74*, 69−86.

(24) Settembre, C.; Fraldi, A.; Medina, D. L.; Ballabio, A. Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nat. Rev. Mol. Cell Biol.* **2013**, *14*, 283−296.

(25) Coffey, E. E.; Beckel, J. M.; Laties, A. M.; Mitchell, C. H. Lysosomal alkalization and dysfunction in human fibroblasts with the Alzheimer's disease-linked presenilin 1 A246E mutation can be reversed with cAMP. *Neuroscience* **2014**, *263*, 111−124.

(26) Folts, C. J.; Scott-Hewitt, N.; Pröschel, C.; Mayer-Pröschel, M.; Noble, M. Lysosomal Re-acidification Prevents Lysosphingolipid-Induced Lysosomal Impairment and Cellular Toxicity. *PLoS Biol.* **2016**, *14*, No. e1002583.

(27) Chen, W.; Xu, Y.; Zhang, L.; Su, H.; Zheng, X. Blackberry subjected to in vitro gastrointestinal digestion affords protection against Ethyl Carbamate-induced cytotoxicity. *Food Chem.* **2016**, *212*, 620−627.

(28) Zhang, L.; Xu, Y.; Li, Y.; Bao, T.; Gowd, V.; Chen, W. Protective property of mulberry digest against oxidative stress−A potential approach to ameliorate dietary acrylamide-induced cytotoxicity. *Food Chem.* **2017**, *230*, 306−315.

(29) Li, Y.; Bao, T.; Chen, W. Comparison of the protective effect of black and white mulberry against ethyl carbamate-induced cytotoxicity and oxidative damage. *Food Chem.* **2018**, *243*, 65−73.

(30) Chen, W.; Su, H.; Xu, Y.; Jin, C. In vitro gastrointestinal digestion promotes the protective effect of blackberry extract against acrylamide-induced oxidative stress. *Sci. Rep.* **2017**, *7*, 40514.

(31) Gowd, V.; Bao, T.; Wang, L.; Huang, Y.; Chen, S.; Zheng, X.; Cui, S.; Chen, W. Antioxidant and antidiabetic activity of blackberry after gastrointestinal digestion and human gut microbiota fermentation. *Food Chem.* **2018**, *269*, 618−627.

(32) Hu, D.; Xu, Y.; Xie, J.; Sun, C.; Zheng, X.; Chen, W. Systematic evaluation of phenolic compounds and protective capacity of a new mulberry cultivar J33 against palmitic acid-induced lipotoxicity using a simulated digestion method. *Food Chem.* **2018**, *258*, 43−50.

(33) Su, H.; Li, Y.; Hu, D.; Xie, L.; Ke, H.; Zheng, X.; Chen, W. Procyanidin B2 ameliorates free fatty acids-induced hepatic steatosis through regulating TFEB-mediated lysosomal pathway and redox state. *Free Radic. Biol. Med.* **2018**, *126*, 269−286.

(34) Chen, W.; Feng, L.; Nie, H.; Zheng, X. Andrographolide induces autophagic cell death in human liver cancer cells through cyclophilin D-mediated mitochondrial permeability transition pore. *Carcinogenesis* **2012**, *33*, 2190−2198.

(35) Chen, W.; Zhang, L.; Zhang, K.; Zhou, B.; Kuo, M.-L.; Hu, S.; Chen, L.; Tang, M.; Chen, Y.-R.; Yang, L.; Ann, D. K.; Yen, Y. Reciprocal regulation of autophagy and dNTP pools in human cancer cells. *Autophagy* **2014**, *10*, 1272−1284.

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(36) Kim, S.; Surh, Y. J.; Sohn, Y.; Yoo, J. K.; Lee, J. W.; Liem, A.; Miller, J. A. Inhibition of vinyl carbamate-induced hepatotoxicity, mutagenicity, and tumorigenicity by isopropyl-2-(1,3-dithietane-2-ylidene)-2-[N-(4-methylthiazol-2-yl)carbamoyl]-acetate (YH439). *Carcinogenesis* **1998**, *19*, 687−690.
(37) Chen, W.; Su, H.; Xu, Y.; Bao, T.; Zheng, X. Protective effect of wild raspberry (Rubus hirsutus Thunb.) extract against acrylamide-induced oxidative damage is potentiated after simulated gastro-intestinal digestion. *Food Chem.* **2016**, *196*, 943−952.
(38) Chun, S.-H.; Cha, Y.-N.; Kim, C. Urethane increases reactive oxygen species and activates extracellular signal-regulated kinase in RAW 264.7 macrophages and A549 lung epithelial cells. *Arch Pharm. Res.* **2013**, *36*, 775−782.
(39) Dikic, I.; Elazar, Z. Mechanism and medical implications of mammalian autophagy. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 349.
(40) Ueno, T.; Komatsu, M. Autophagy in the liver: functions in health and disease. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 170−184.
(41) Lu, S.; Sung, T.; Lin, N.; Abraham, R. T.; Jessen, B. A. Lysosomal adaptation: How cells respond to lysosomotropic compounds. *PLoS One* **2017**, *12*, No. e0173771.
(42) Martina, J. A.; Diab, H. I.; Lishu, L.; Jeong-A, L.; Patange, S.; Raben, N.; Puertollano, R. The nutrient-responsive transcription factor TFE3 promotes autophagy, lysosomal biogenesis, and clearance of cellular debris. *Sci. Signal.* **2014**, *7*, ra9.
(43) Settembre, C.; Zoncu, R.; Medina, D. L.; Vetrini, F.; Erdin, S.; Erdin, S.; Huynh, T.; Ferron, M.; Karsenty, G.; Vellard, M. C.; Facchinetti, V.; Sabatini, D. M.; Ballabio, A. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J.* **2012**, *31*, 1095−1108.
(44) Jacquin, E.; Leclerc-Mercier, S.; Judon, C.; Blanchard, E.; Fraitag, S.; Florey, O. Pharmacological modulators of autophagy activate a parallel noncanonical pathway driving unconventional LC3 lipidation. *Autophagy* **2017**, *13*, 854−867.
(45) Liu, J.; Lu, W.; Guha, S.; Baltazar, G. C.; Coffey, E. E.; Laties, A. M.; Rubenstein, R. C.; Reenstra, W. W.; Mitchell, C. H. Cystic fibrosis transmembrane conductance regulator contributes to reacidification of alkalinized lysosomes in RPE cells. *Am. J. Physiol. Cell Physiol.* **2012**, *303*, C160−C169.
(46) Van Goor, F.; Hadida, S.; Grootenhuis, P. D. J.; Burton, B.; Cao, D.; Neuberger, T.; Turnbull, A.; Singh, A.; Joubran, J.; Hazlewood, A.; Zhou, J.; McCartney, J.; Arumugam, V.; Decker, C.; Yang, J.; Young, C.; Olson, E. R.; Wine, J. J.; Frizzell, R. A.; Ashlock, M.; Negulescu, P. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 18825−18830.
(47) Jiao, Z.; Dong, Y.; Chen, Q. Ethyl Carbamate in Fermented Beverages: Presence, Analytical Chemistry, Formation Mechanism, and Mitigation Proposals. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 611−626.
(48) Hoyt, L. R.; Randall, M. J.; Ather, J. L.; DePuccio, D. P.; Landry, C. C.; Qian, X.; Janssen-Heininger, Y. M.; van der Vliet, A.; Dixon, A. E.; Amiel, E.; Poynter, M. E. Mitochondrial ROS induced by chronic ethanol exposure promote hyper-activation of the NLRP3 inflammasome. *Redox Biol.* **2017**, *12*, 883−896.
(49) Li, Y.; Zhang, J.; Xu, P.; Sun, B.; Zhong, Z.; Liu, C.; Ling, Z.; Chen, Y.; Shu, N.; Zhao, K.; Liu, L.; Liu, X. Acute liver failure impairs function and expression of breast cancer-resistant protein (BCRP) at rat blood-brain barrier partly via ammonia-ROS-ERK1/2 activation. *J. Neurochem.* **2016**, *138*, 282−294.
(50) Pla, A.; Pascual, M.; Guerri, C. Autophagy Constitutes a Protective Mechanism against Ethanol Toxicity in Mouse Astrocytes and Neurons. *PLoS One* **2016**, *11*, No. e0153097.
(51) Luzio, J. P.; Pryor, P. R.; Bright, N. A. Lysosomes: fusion and function. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 622−632.
(52) Alzamora, R.; Thali, R. F.; Gong, F.; Smolak, C.; Li, H.; Baty, C. J.; Bertrand, C. A.; Auchli, Y.; Brunisholz, R. A.; Neumann, D.; Hallows, K. R.; Pastor-Soler, N. M. PKA regulates vacuolar H+-ATPase localization and activity via direct phosphorylation of the a subunit in kidney cells. *J. Biol. Chem.* **2010**, *285*, 24676−24685.

https://dx.doi.org/10.1021/acs.jafc.0c00534
J. Agric. Food Chem. XXXX, XXX, XXX−XXX