

Copper Ion from Cu₂O Crystal Induces AMPK-Mediated Autophagy via Superoxide in Endothelial Cells

Youngsik Seo¹, Young-Sik Cho², Young-Duk Huh², and Heonyong Park^{1,*}

Copper is an essential element required for a variety of functions exerted by cuproproteins. An alteration of the copper level is associated with multiple pathological conditions including chronic ischemia, atherosclerosis and cancers. Therefore, copper homeostasis, maintained by a combination of two copper ions (Cu⁺ and Cu²⁺), is critical for health. However, less is known about which of the two copper ions is more toxic or functional in endothelial cells. Cubic-shaped Cu₂O and CuO crystals were prepared to test the role of the two different ions, Cu⁺ and Cu²⁺, respectively. The Cu₂O crystal was found to have an effect on cell death in endothelial cells whereas CuO had no effect. The Cu₂O crystals appeared to induce p62 degradation, LC3 processing and an elevation of LC3 puncta, important processes for autophagy, but had no effect on apoptosis and necrosis. Cu₂O crystals promote endothelial cell death via autophagy, elevate the level of reactive oxygen species such as superoxide and nitric oxide, and subsequently activate AMP-activated protein kinase (AMPK) through superoxide rather than nitric oxide. Consistently, the AMPK inhibitor Compound C was found to inhibit Cu₂O-induced AMPK activation, p62 degradation, and LC3 processing. This study provides insight on the pathophysiological function of Cu⁺ ions in the vascular system, where Cu⁺ induces autophagy while Cu²⁺ has no detected effect.

INTRODUCTION

Copper is an essential trace metal that plays an important role in energy production, catalytic activities of cuproenzymes and protecting from reactive oxygen species (ROS)-induced cell/tissue damages. Excess or depletion of copper ions has been known to induce a variety of pathological conditions including myocardial infarction, chronic inflammation, Alzheimer's

disease (Chevion et al., 1993; Hordyjewska et al., 2014; Rajendran et al., 2007; Singh et al., 2013). The level of copper in patients with ischemic cardiomyopathy appears higher than that in healthy people (Shokrzadeh et al., 2009), while copper depletion induces anemia (Gallagher et al., 1971; Hordyjewska et al., 2014). Such important physiological and/or pathological roles of the level of copper ions are largely associated with a variety of well-controlled and/or uncontrolled cellular reactions caused by the oxidation-reduction potential (ORP) of copper ions between cupric (Cu²⁺) and cuprous (Cu⁺) ions. In the vascular system, the reduction of cupric ion to cuprous ion is proposed to produce ROS, thereby causing ROS-associated cardiovascular diseases (Altekin et al., 2005; Bar-Or et al., 2001; Hordyjewska et al., 2014). However, due to technical difficulties originated from both instability and insolubility of the cuprous (Cu⁺) ion, the cellular roles of cuprous ion in the vascular system are difficult to identify and the pathophysiological role of the Cu⁺ ion remain to be elucidated.

Vascular endothelial cells remain largely viable for more than 20 years and do not easily turnover under normal physiological conditions (Clarke et al., 2007). However, ROS promotes endothelial cell death in atherosclerosis-prone regions (Abello et al., 1994; Dimmeler and Zeiher, 2000; Robaye et al., 1991). Based on these findings, pro-atherogenic factors have been suggested as pro-apoptotic stimulants, but remain controversial. Recently, a body of evidence has shown autophagy, another type of cell death, is responsible for various cardiovascular diseases (Glick et al., 2010).

Autophagy terminologically means 'self-eating', which originated from observations of the degradation of subcellular organelles including mitochondria within lysosomes. The formation of an isolation membrane (IM), called a phagophore, is an important signal for the progress of autophagy (Glick et al., 2010). The phagophore absorbs various protein aggregates and organelles, thereby enlarging into an autophagosome. This autophagosome fuses with the lysosome, then degrading the autophagosomal contents with lysosomal enzymes. These processes involving the subcellular organelles are controlled by molecular alterations in various signaling pathways. The signaling pathways reported to be involved in regulating autophagy are LC3 processing, interaction between Beclin 1 and VPS 34 in the endoplasmic reticulum, and inhibition of AMPK by mTOR, an autophagy blocker (Barth et al., 2010; Diaz-Troya et al., 2008; Glick et al., 2010; Kundu et al., 2008; Shaw, 2009). Additionally, BCL2 is known to inhibit the interaction between Beclin

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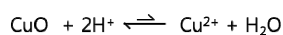
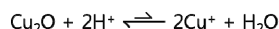
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1 and VPS 34, inhibiting autophagy (He et al., 2012). BCL2 mutation promotes autophagy through the degradation of p62. In cardiovascular diseases, hypoxia and oxidative stress promote autophagy, which is responsible for dilated cardiomyopathy (Shimomura et al., 2001), atherosclerosis and hypertension (Martinet et al., 2009; Ryter et al., 2010). However, their mechanisms remain poorly understood.

To gain a better understand of the relationship between oxidative stress and autophagy in endothelial cells, we constructed both cuprous oxide (Cu₂O) and copper oxide (CuO) crystals and analyzed their effects on endothelial cells. Cu₂O and CuO crystals slowly release Cu⁺ and Cu²⁺ ions, respectively, into aqueous systems as following reactions (Dortwegt et al., 2001; Palmer et al., 2004).



The effects of Cu²⁺ ions on endothelial cells can be examined using CuO crystals. However, Cu⁺ ions are unstable and easily oxidized to Cu²⁺ ions in aqueous systems by Fenton and/or Haber-Weiss reactions (Bar-Or et al., 2001; Rigo et al., 1977), therefore in blood, Cu⁺ ions are rapidly released and oxidized to Cu²⁺ ions.

In this study, copper ions generated by Cu₂O crystals promote cell death, whereas copper ions from CuO crystals at the similar level have no effect on endothelial cells. The copper induced cell death was caused by autophagy, not by apoptosis or necrosis, providing an insight into the toxicological features of the uncontrolled level of redox copper ions in the vascular system.

MATERIALS AND METHODS

Constructions of Cu₂O and CuO crystals

Cu(NO₃)₂·2.5H₂O (Sigma-Aldrich, USA), polyethylene glycol (PEG, Mw 20,000, Fluka, USA), NaOH (97%, Sigma-Aldrich), and ascorbic acid (Sigma-Aldrich) were used as received. For the preparation of cubic Cu₂O crystals, 2.4 g PEG was added to 50 ml of an aqueous solution of 0.04 M Cu(NO₃)₂ at room temperature and stirred for 30 min. After mixing, 50 ml of 0.12 M NaOH and 50 ml of 0.015 M ascorbic acid were added and stirred vigorously for an additional 20 min. The Cu₂O product was washed several times with water, and then dried at room temperature for 24 h. CuO crystals were prepared by the direct thermal oxidation of the Cu₂O precursor in air at 400°C for 5 h in a box-type furnace. The CuO was formed by the thermal oxidation of Cu₂O precursors reacted with the oxygen in air.

The structures of the Cu₂O and CuO crystals were characterized by powder X-ray diffraction (XRD, PANalytical, X'pert-PRO MPD, Netherland), and their morphologies were observed by scanning electron microscopy (SEM, Hitachi S-4300, Japan).

Cell culture

Bovine aortic endothelial cells (BAECs) obtained from descending thoracic aortas were cultured in DMEM (1 g/L glucose; Wel GENE Inc, Korea) containing 20% fetal bovine serum (FBS, Wel GENE Inc) with antibiotics (50 µg/ml of penicillin/streptomycin) at 37°C with 5% CO₂. Cells from passages 8 to 13 were used for the experiments.

Measurement of concentration of cupric ion

Various concentrations (0–500 µg/ml) of Cu₂O or CuO crystals were suspended in water or the growth culture media and incubated at 37°C for 12 h. Then, the amount of Cu²⁺ ions was

measured with QuantiChrom Copper Assay Kit (BioAssay System, USA) using manufacturer's protocol. Briefly, 100 µl of each Cu₂O or CuO suspension was aliquoted to an Eppendorf tube, then 35 µl of Reagent A (trichloroacetic acid) was added. Subsequently, 100 µl of each mixture was re-aliquoted into a 96-well plate (SPL Life Science, Korea) and then reacted with Reagents B and C for 5 min. Finally, optical density (O.D.) at 359 nm was measured for each sample. Cu²⁺ ions were measured against a standard solution plotted in a calibration curve to calculate the amount of Cu²⁺ ions in the sample.

Treatment of BAECs with various chemicals

Cu₂O or CuO crystals were dispersed in Dulbecco's phosphate buffered saline (PBS, Wel GENE Inc). BAECs grown to 90% confluency were then treated with the indicated amounts of the Cu₂O or CuO suspensions. For modulating the levels of superoxide and nitric oxide, BAECs were pretreated with a superoxide generator (Pyrogallol; Sigma), a nitric oxide generator (S-Nitroso-N-acetyl-DL-penicillamine (SNAP); Santacruz Biotechnology, USA) and an inhibitor of nitric oxide synthetase (N(G)-nitro-L-arginine methyl ester (L-NAME); Cell Signaling, USA) for 12 h.

Preparation of cell lysate

Cells grown on 6-well plates were washed in ice-cold PBS, scraped into 150 µl radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor cocktail (Roche Molecular Biochemicals, USA), and solubilized for 15 min at 4°C, as previously described (Lee et al., 2013). The soluble lysates were fractionated by centrifugation. Total protein amounts in the cell lysates were measured using a Micro BCA protein assay kit (Thermo Scientific, USA).

Cytotoxicity

For measuring cytotoxicity, the cells were serum-starved for 12 h and then treated with the crystals containing none, 250 µM of diethyldithiocarbamate (Sigma-Aldrich; a superoxide dismutase inhibitor), 3 mM of N-acetylcysteine (an ROS inhibitor; Sigma-Aldrich), 2 µM MHY 1485 (Sigma-Aldrich), 500 nM leupeptin (Sigma-Aldrich) or 20 µM of Compound C with/without 250 µM of AICAR (Sigma-Aldrich) for 12 h. Dead cells (round, shrunken cells) were examined under an optical microscope as previously reported (Kim et al., 2008).

Western Blots

Protein (20 µg) in the soluble lysates were resolved by 12% SDS-PAGE, then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was blocked in 5% non-fat milk in PBS containing 0.1% Tween-20 (PBST) for 1 h at room temperature, and probed with antibodies specific to LC3 (Novus, USA), caspase-3 (Cell signaling), p62 (Cell signaling), eNOS (Cell signaling), p-Ser¹¹⁷⁷-eNOS (Cell signaling), AMP-activated protein kinase (AMPKα1 and α2; Cell signaling) and p-AMPK (α1 and α2; Cell signaling). Subsequently, the membrane was thoroughly washed in PBST, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Finally, the membrane was developed using the enhanced chemiluminescence detection method (Amersham, USA).

Fluorescence-activated cell sorting analysis

BAECs grown to 80% confluency were treated with or without 100 µg/ml of Cu₂O. Then, BAECs were stained with propidium

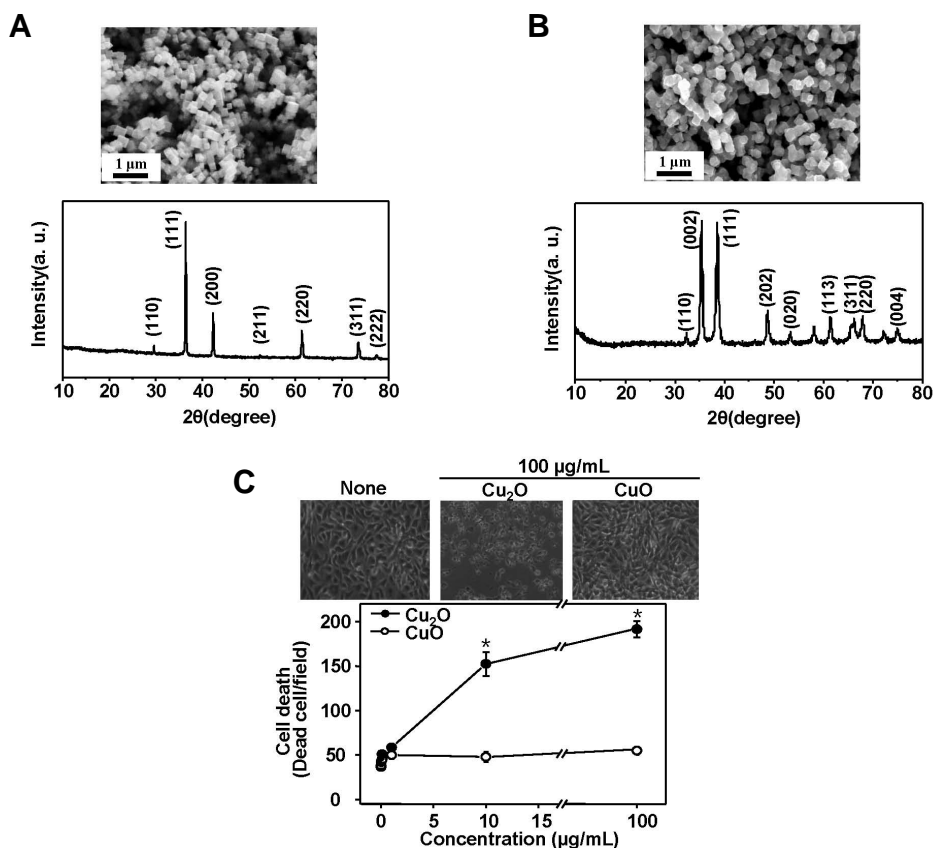


Fig. 1. SEM images, X-ray powder diffraction patterns and Miller indices of Cu₂O crystals (A) and CuO crystals (B). Cu₂O induces cell death, but does not activate caspase-3 (C). BAECs were grown to confluence in DMEM containing 20% serum and then serum-starved for 12 h before treating with the indicated concentrations of Cu₂O or CuO crystals for the indicated periods of time. Cells were then observed under a microscope (Top panels). Dead cells (round, shrunken cells) counted per visual field were plotted on bar graphs in the bottom panels (means ± S.E., n = 3). **P* < 0.05.

iodide (PI) and FITC-tagged Annexin V by using the ApoScan Annexin V FITC apoptosis detection kit (Biobud Co., LTD., Korea). Stained cells were detected by flow cytometry (Guava easyCyte, Millipore). To analyze necrosis, quadrants were drawn on a cytogram. A quadrant with annexin V+ (annexin V-stained cells) and PI+ (PI-stained cells) was set as a necrotic population, and a quadrant with annexin V+ (annexin V-stained cells) and PI- (PI-unstained or weakly PI-stained cells) was set as an apoptotic population.

Measurement of reactive oxygen species

BAECs were split into black 96-well plates (Thermo Scientific) with 10⁵ cells per well and incubated for 24 h. Then, cells were treated with 100 µg/ml of Cu₂O or CuO crystals for indicated periods of time. After removing the medium, the cells in the plates were washed with KRH buffer (129 mM NaCl, 5 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1 mM CaCl₂, 1.2 mM MgCl₂, 2.8 mM glucose, 10 mM Hepes pH 7.4) and then incubated in DMEM medium containing 100 µM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Life Technology, Korea) in 5% CO₂ at 37°C for 30 min. Finally, the cells were washed and incubated with KRH buffer. Then, fluorescence was measured with 485 nm excitation and 530 nm emission.

Immunostaining

Subcellular localization of LC3 was determined via immunostaining. BAECs were fixed in 4% paraformaldehyde for 10 min, permeabilized in PBS containing 0.25% Triton X-100 for 5 min, and blocked in PBS containing 2% BSA for 1 h. Fixed cells were incubated with anti-LC3 (Novus) primary antibody overnight at 4°C, followed by Alexa fluor 488-conjugated goat anti-rabbit antibody (Life Technology) for 1 h. Washed cells

were mounted with Slow-Fade mounting medium with DAPI, and observed under a fluorescence microscopy (Carl Zeiss, Axioplan 2, Germany).

Statistics

Statistical significance was evaluated by Student's *t*-test. The minimum level of significance was set at *p* < 0.05. Various sample groups were compared with one way ANOVA.

RESULTS

To characterize the role of copper ion on the vascular system, we first prepared CuO and Cu₂O crystals which generate Cu²⁺ and Cu⁺ ions, respectively to determining the roles of Cu²⁺ and Cu⁺ ions in endothelial cells.

Figure 1A represents the SEM images of the Cu₂O crystals prepared from the reduction of aqueous copper solution by ascorbic acid and CuO crystals prepared from thermal oxidation of the Cu₂O precursor. Perfect cubic-shaped Cu₂O crystals with a mean size of 200 nm were obtained, as shown in Fig. 1A. The CuO crystals prepared by direct thermal oxidation of the Cu₂O precursor in air at 400°C for 5 h can be seen in Fig. 1B. The shape of the CuO crystals resembled the Cu₂O precursor, however the surfaces of the cubic CuO crystals were slightly roughened. Figures 1A and 1B show the XRD patterns of the Cu₂O and CuO crystals. The crystals matched reported data of cubic Cu₂O (JCPDS 05-0667, *a* = 0.4269 nm) and monoclinic CuO (JCPDS 45-0937, *a* = 0.4685 nm, *b* = 0.3425 nm, *c* = 0.5130 nm, and β = 99.549°) (Zhao et al., 2012). Since no impurities were observed in the XRD patterns, pure Cu₂O and CuO crystals were formed.

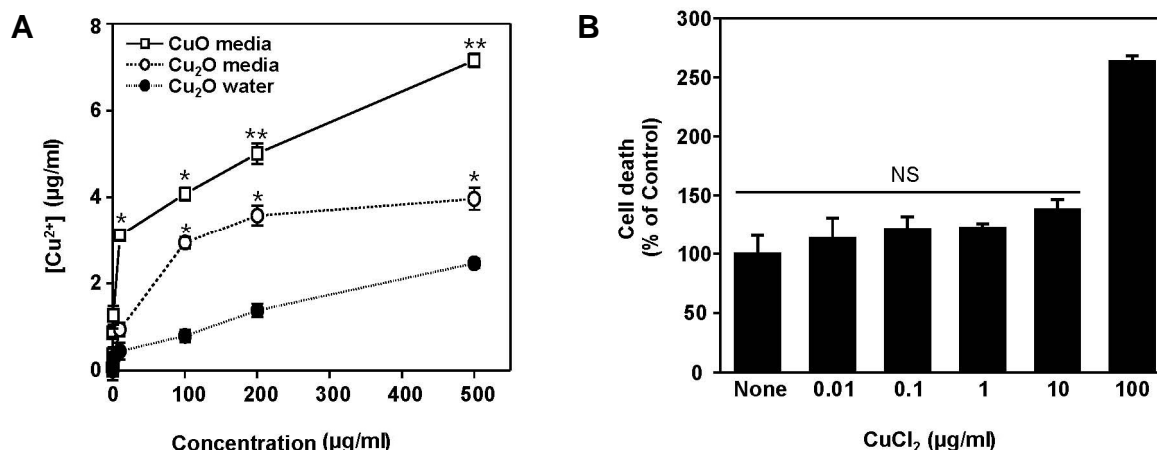


Fig. 2. Measurement of copper ions of Cu₂O or CuO crystals dispersed in water and growth media (A). In (A), data were plotted as line graphs (means ± S.E., n=3). **P* < 0.05, ***P* < 0.01. The amounts of Cu²⁺ ions were measured as described in “Materials and Methods”. Dead cells (BAEC) were counted per visual field after cells were treated with various concentrations of water-soluble CuCl₂ (B). NS, statistically non-significant difference.

To examine the effects of cuprous oxide (Cu₂O) or cupric oxide (CuO) crystals on endothelial cell death, BAECs were treated with cuprous oxide or cupric oxide crystals in dose curve experiments. As shown in Fig. 1C, only cuprous oxide appeared to induce cell death at concentrations higher than 10 µg/ml, whereas cupric oxide has no effect, suggesting Cu⁺ ions to be more toxic than Cu²⁺ ions. Cu⁺ or Cu²⁺ ions generated from Cu₂O and CuO crystals were measured. In the growth media, soluble oxygen oxidizes Cu⁺ ions to Cu²⁺ ions with the production of reactive oxygen species (ROS) (Rael et al., 2007). Therefore, we measured Cu²⁺ from both Cu₂O and CuO crystals to monitor the corrosion levels of the crystals in the growth media. As shown in Fig. 2A, ~3 µg/ml of Cu²⁺ was detected from the 100 µg/ml suspension of the Cu₂O crystal, whereas around 4 µg/ml of Cu²⁺ was detected in the CuO crystal. As concentrations of Cu₂O crystals increased, higher amounts of Cu²⁺ ions were detected. In addition, the concentration of Cu²⁺ ions for Cu₂O crystals in the growth media appeared higher than that in water (Fig. 2A). To know the toxicity of Cu²⁺ ions, we treated cells with a highly soluble form of CuCl₂ and monitored the cell death. As shown in Fig. 2B, Cu²⁺ ion at the concentration of ≤10 µg/ml of CuCl₂ was found to have no effect on the cell death. These data indicate that the toxicity of Cu₂O is not caused by Cu²⁺ ion but rather due to the oxidation mediated by Cu⁺ ion.

Different types of cell deaths are known, such as apoptosis, necrosis and autophagy. Results from cell death experiments show cuprous oxide induced neither apoptotic nor necrotic cell death, as the Cu₂O crystals had no effect on caspase-3 activation and PI staining, known critical signaling markers for apoptosis and necrosis (Figs. 3A and 3B). A series of experiments was executed to determine if cuprous oxide induces autophagy by measuring the LC3 conversion and the amount of p62. Interestingly, LC3 conversion and p62 degradation were observed in BAECs treated for longer than 6 h with 100 µg/ml of cuprous oxide (Figs. 3C and 3D), indicating autophagy (Ahn et al., 2014). In addition, LC3-punctated staining (Fig. 3E) and death-minimizing effects of autophagy inhibitors such as MHY1485 and leupeptin (Fig. 3F) confirmed that Cu₂O induces autophagy (Choi et al., 2012).

The autophagic induction by Cu₂O without a noticeable effect by CuO, gives rise to a hypothesis that the reduction-oxidation

power of Cu⁺ from Cu₂O generates ROS to elicit autophagy. ROS was measured using dichlorodihydrofluorescein diacetate (DCF-DA) which becomes fluorescent by a reaction with ROS. The level of ROS was elevated with a longer incubation times when cells were treated by Cu₂O, while the level of ROS was not altered by CuO (Fig. 4A). We further found that Cu₂O crystals appeared to activate eNOS (Fig. 4B).

Considering Fenton and Haber-Weiss reactions, the Cu₂O crystals seem likely to generate superoxide (O₂⁻) and NO. In such a case, peroxynitrite (ONOO⁻) can be produced from a reaction between superoxide and NO, and can examine autophagy triggering by ROS generators such as Pyrogallol (a superoxide generator), SNAP (S-nitroso-N-acetylpenicillamine, an NO generator) and SIN-1 (3-morpholininosynonimine, a superoxide/NO co-generator producing peroxynitrite). As shown in Fig. 4C, both Pyrogallol and SIN-1 at the concentration of 5 mM induce the conversion of LC3 and the degradation of p62 to lead to autophagy. However, SNAP had no effect, indicating that NO has little or no effect on endothelial cell autophagy. The eNOS activation by Pyrogallol (see Fig. 4D) supported a concept that eNOS can be largely activated by Cu₂O via superoxide. Furthermore, to understand NO contribution in Cu₂O-induced autophagy, we tested down-regulation of Cu₂O-induced autophagy by eNOS inhibitor L-NAME. From data showing that L-NAME had no effect on the Cu₂O-induced autophagy (see Fig. 4E), we determined that NO has a minimal effect on the Cu₂O-induced autophagy. Meanwhile, SIN-1 appeared to elevate autophagy, indicating that peroxynitrite promotes autophagy in endothelial cells. The SOD inhibitor, diethyldithiocarbamate, was also tested and shown to have an effect on the cell death induced by Cu₂O. As shown in Fig. 4F, the SOD inhibitor exacerbated Cu₂O-induced cell death, whereas an ROS scavenger reduced the Cu₂O-induced cell death. These results confirm the critical superoxide role in the Cu₂O-induced cell death.

Based on previous reports (Anna et al., 2008; Ishida et al., 2013) showing that copper deficiency activates AMPK, we tested if cuprous oxide activates AMPK. As shown in Fig. 5A, cuprous oxide appeared to phosphorylate AMPKα2 in a time-dependent manner. Pyrogallol also appeared to activate AMPKα2 (Fig. 5B), confirming that AMPK is an important factor for superoxide-associated autophagy. Unexpectedly,

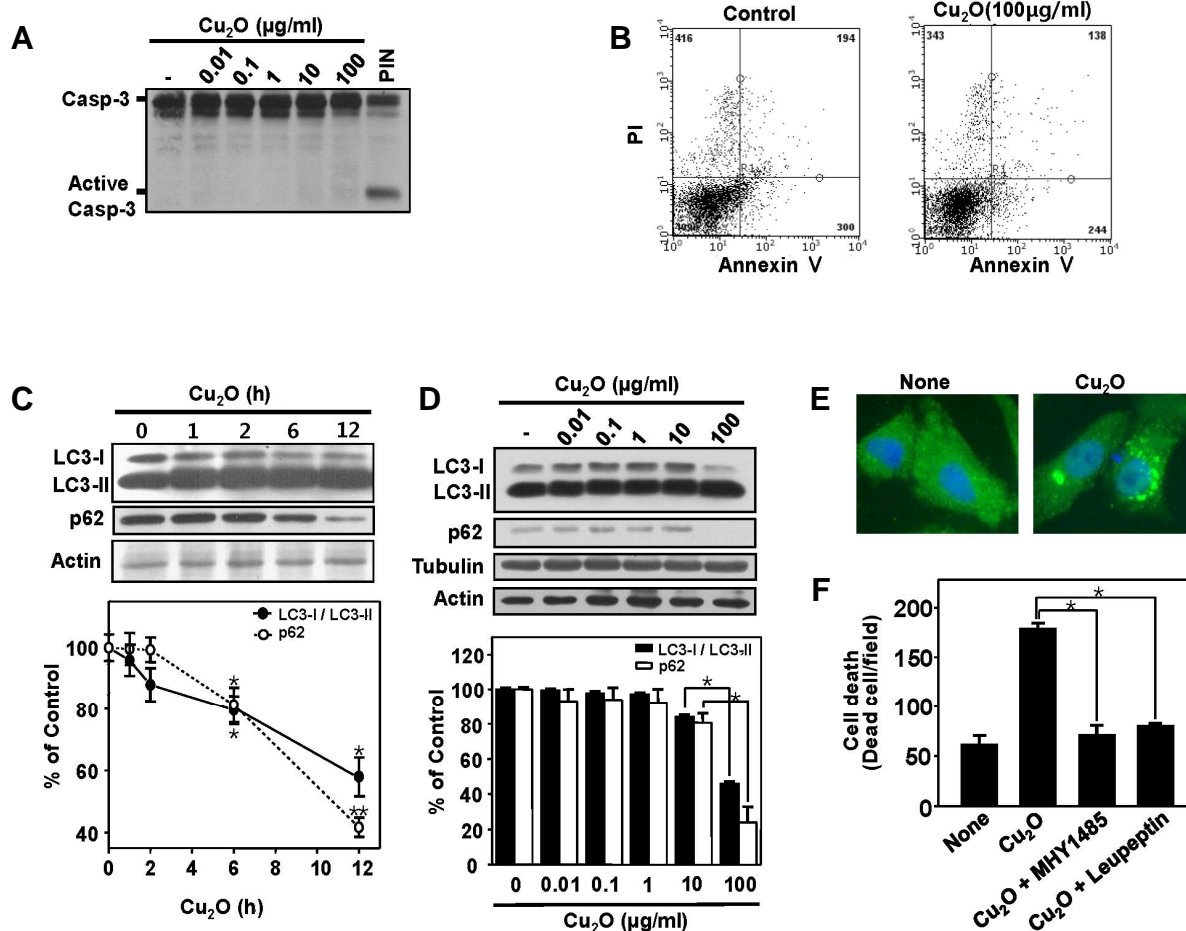


Fig. 3. Cu₂O induces cell death via autophagy, neither by apoptosis nor necrosis. (A) Cell lysates were obtained from BAECs treated with Cu₂O crystals, resolved by SDS-PAGE, and then immunoblotted with antibodies for caspase-3. Pinosylvin (PIN; 100 µM, 12 h) was used as a pro-apoptotic agent. (B) BAECs were treated with or without 100 µg/ml Cu₂O, then stained with FITC-tagged annexin V and propidium iodide (PI), as described in “Materials and Methods”. Stained cells were analyzed with flow cytometry. (C) BAECs were treated with 100 µg/ml of Cu₂O crystals for various periods of time. Line graphs represent means ± S.E. (n = 3). *P < 0.05, **P < 0.01 (Student *t*-tests vs. the untreated control). (D) BAECs were serum-starved for 12 h and then treated with the indicated concentrations of Cu₂O crystals for 6 h. The total proteins in the cell lysate were then immunoblotted with the proper antibodies. Quantification was performed using densitometry. In the bottom panels, data were plotted as bar graphs (means ± S.E., n = 3). *P < 0.05. (E) Serum-starved BAECs were treated with or without 100 µg/ml of Cu₂O crystals for 12 h and then immunostained with LC3 antibody. (F) Serum-starved BAECs were treated with 100 µg/ml of Cu₂O crystals in the presence of MHY 1485 (2 µM) or leupeptin (500 nM). Then cell death was analyzed as mentioned above. Data were plotted as bar graphs (means ± S.E., n = 3). *P < 0.05.

SIN-1 had no effect on the AMPK α phosphorylation, giving rise to an assumption that AMPK is not involved in the peroxynitrite-induced autophagy.

As shown in Fig. 5A, AMPK activation was markedly enhanced after 2 h of treatment with 100 µM of cuprous oxide. Therefore, we hypothesized that AMPK may be a mediator for the cuprous oxide-induced autophagy of BAECs. To test this hypothesis, the BAECs were pre-treated with the AMPK inhibitor, Compound C (CC), before induction of autophagy with cuprous oxide. As shown in Fig. 5C, treatment with CC almost completely reverses the cuprous oxide-induced LC3 conversion and p62 degradation. These findings support that cuprous oxide induces autophagy via AMPK activation.

Cupric oxide was found to have no effect on cell death in BAECs (Fig. 1C). Consistently, cupric oxide was also found to

have no effect on LC3 conversion and p62 degradation, whereas cuprous oxide promoted both LC3 conversion and p62 degradation (Fig. 5C). Furthermore, AMPK phosphorylation was not stimulated by cupric oxide, whereas AMPK α 2 was phosphorylated by cuprous oxide, as shown in Fig. 5D. These data support the notion that the Cu⁺ ion is a powerful inducer of autophagy, whereas Cu²⁺ was not responsible for autophagy in BAECs. Surely, CC appeared to inhibit the Cu₂O-induced cell death (Fig. 5E). This inhibitory effect of CC was partly diminished by AICAR, convincingly showing that AMPK is a key molecule for the Cu₂O-induced autophagy.

DISCUSSION

In this study, we prepared two identical shaped crystals containing

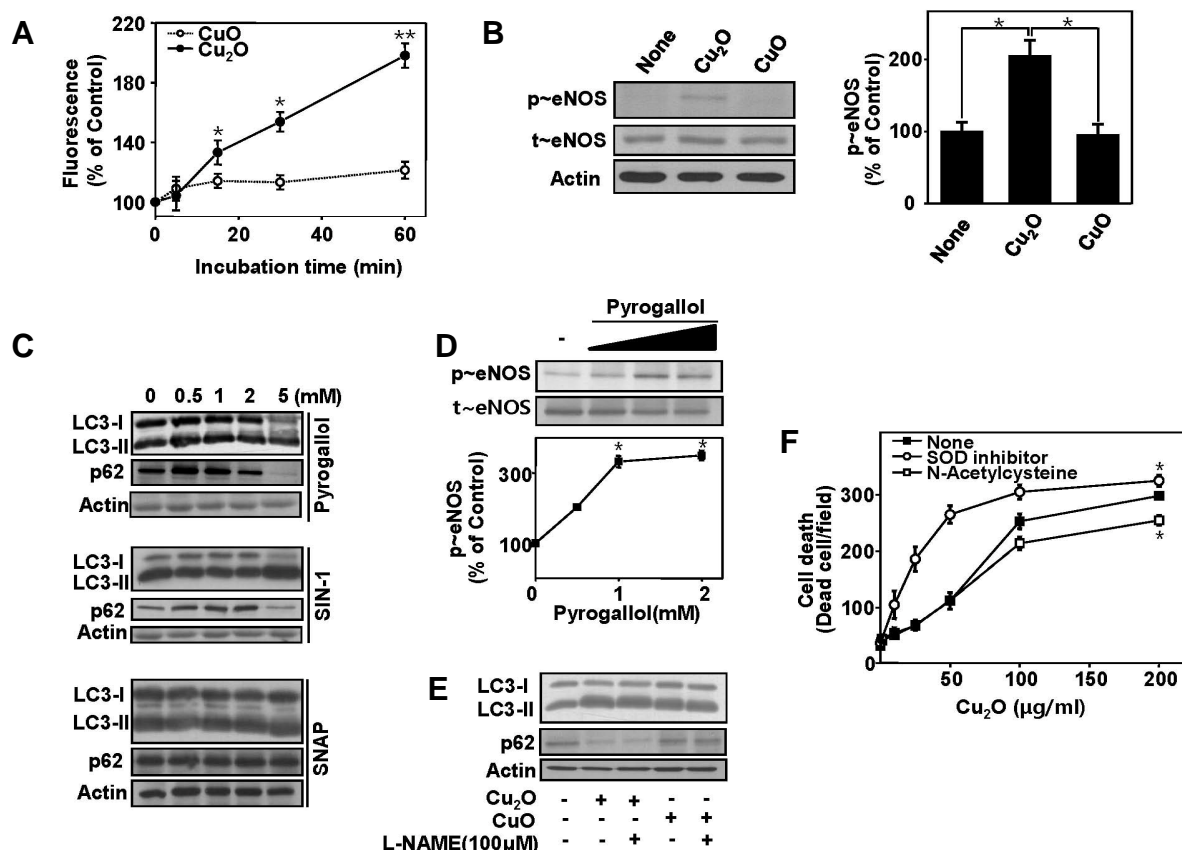
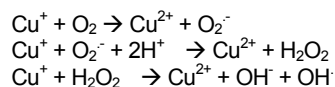


Fig. 4. Superoxide plays an important role in the Cu₂O-induced autophagy. (A) BAECs were treated with 100 μg/ml of Cu₂O or CuO crystals for indicated periods of time. Then, fluorescence intensities of DCF-DA (2',7'-dichlorodihydrofluorescein diacetate) in the cell lysates were measured as described in Methods. Line graphs represent means ± S.E. (n = 3). *P < 0.05, **P < 0.01 (Student t-tests vs. the untreated control). (B) BAECs were treated with 100 μg/ml of Cu₂O or CuO crystals for 12 h. Subsequently, immunoblots were performed using antibodies for p-S¹¹⁷⁷ eNOS (p-eNOS), total eNOS (t-eNOS) and actin. Quantification data were shown in bar graphs (means ± S.E., n = 3). *P < 0.05. (C) Serum-starved BAECs were treated with the indicated concentrations of Pyrogallol, SIN-1 and SNAP for 12 h. Immunoblots were conducted using antibodies for LC3, p62 and actin. (D) BAECs were treated with Pyrogallol as abovementioned. Immunoblots were performed using p-eNOS and t-eNOS. Quantification data were shown in the line graph (means ± S.E., n = 3). *P < 0.05. (E) BAECs were treated with various combinations of 100 μg/ml of Cu₂O or CuO crystals and 100 μM of L-NAME for 12 h. Then, immunoblots were conducted using antibodies for LC3, p62 and actin. (F) BAECs were treated with various concentrations of Cu₂O crystals and with or without 250 μM of diethyldithiocarbamate (an SOD inhibitor) or 3 mM N-acetylcysteine (an ROS inhibitor) for 12 h. Dead cells were counted as described in Fig. 1C. Data were plotted as line graphs (means ± S.E., n = 3). *P < 0.05 (Student t-tests vs. the untreated control at 200 μg/ml of Cu₂O).

Cu⁺ and Cu²⁺ to characterize effects in endothelial cells and better understand roles of the two types of crystals. First, to rule out shape-derived differences, the two forms of cuprous oxide and cupric oxide implemented in the experiments were crystallized with the same shape. Interestingly, our data showed that only cuprous oxide (Cu₂O) induced autophagy. Although poorly understood still, we conjecture that cuprous oxide (Cu₂O) and cupric oxide (CuO) generate Cu⁺ and Cu²⁺ ions, respectively, in the water-based blood by following reactions such as the Fenton reaction and the Haber-Weiss reaction (Bar-Or et al., 2001; Barbusiński, 2009; Rigo et al., 1977).



The Cu⁺ ion rapidly donates an electron and converts into

Cu²⁺, while Cu²⁺ does not donate electrons. This concept was supported by our data showing that the only copper ion dissolved from Cu₂O crystals induces cell death. Similar levels of Cu²⁺ ions were found in media from either Cu₂O crystals or CuO crystals (see Fig. 2A). Concentrations of dissolved Cu²⁺ ions in each suspension of 100 μg/ml (a level inducing death) of Cu₂O or CuO were shown as 3 and 4 μg/ml, respectively. Nevertheless, the cell death is unlikely to occur at a range of concentrations (less than 10 μg/ml) of Cu²⁺ dissolved from CuCl₂ solutions. Non-toxic effect of Cu²⁺ ion at 10 μg/ml of CuCl₂ (see Fig. 2B) confirms that cytotoxicity is likely to be caused by Cu⁺ ions and not by Cu²⁺ ions.

As a consequence, the oxidative process of Cu⁺ ion possibly provides oxidative stresses to the mitochondria of the BAECs. These oxidative stresses may cause autophagy of the endothelial cells. The Cu₂O crystal did not induce apoptosis or necrosis, indicating that the oxidative stress originated from Cu⁺ ions

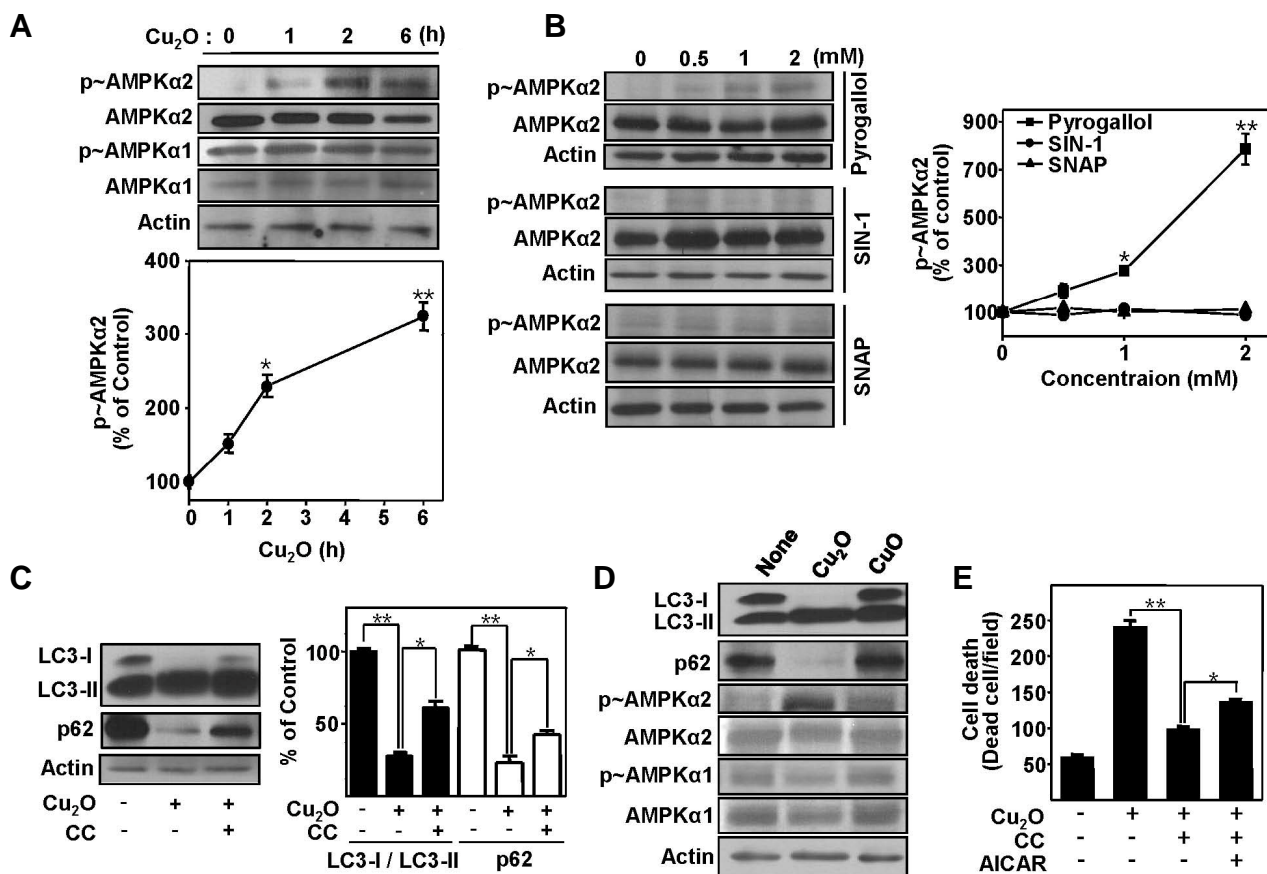


Fig. 5. Cu₂O induces autophagy via AMPK. (A) BAECs were treated with 100 μg/ml of Cu₂O crystals for various periods of time. The total proteins in the cell lysate were then immunoblotted with antibodies for AMPK, p-AMPK, and actin. Quantification was performed using densitometry. In the bottom panels, line graphs represent means ± S.E. (n = 3). **P* < 0.05, ***P* < 0.01 (Student *t*-tests vs. the untreated control). (B) Serum-starved BAECs were treated with the indicated concentrations of Pyrogallol, SIN-1 and SNAP for 12 h. Western blotting for each sample was performed with antibodies for AMPK, p-AMPK, and actin. Quantification was shown in line graphs (means ± S.E., n = 3). **P* < 0.05, ***P* < 0.01. (C) BAECs were pre-conditioned under serum starvation for 24 h to activate AMPK in the presence or absence of 20 μM Compound C (CC). Western blotting was then executed with antibodies for LC3, p62 and actin. Bar graphs represent means ± S.E. (n = 3). **P* < 0.05, ***P* < 0.01. (D) BAECs were untreated (Control) or treated with 100 μg/ml Cu₂O, or 100 μg/ml CuO. Immunoblots were then executed as mentioned above. (E) Cell death was measured in BAECs treated with Cu₂O (100 μg/ml) in the presence of Compound C (CC, 20 μM) or AICAR (250 μM) for 12 h. Bar graphs represent means ± S.E. (n = 3). **P* < 0.05

stimulates autophagy.

The present study strongly suggests that an elevation of the level of ROS by Cu₂O resulted from generations of superoxide (O₂⁻), nitric oxide (NO) and peroxynitrite (ONOO⁻) (see Fig. 4B). Peroxynitrite is known to be generated by an 1:1 stoichiometric reaction between superoxide and NO (Szabó et al., 2007). The agents producing superoxide and peroxynitrite appeared to induce autophagy, while an agent generating NO did not. Cu⁺ seems to trigger autophagy via superoxide and peroxynitrite, however this autophagy-inducing effect of Cu₂O is not consistent with our further mechanistic studies showing that only the superoxide generator stimulates AMPK activation but peroxynitrite- and NO-generators does not. Given the production of peroxynitrite by 1:1 reaction between superoxide and NO, peroxynitrite-mediated autophagy by Cu₂O would minimally contribute to endothelial cell death, due to little or no possibility of the productions of superoxide and NO with an 1:1 stoichiometric ratio by Cu⁺. Cu⁺-induced autophagy largely occurs by an AMPK-dependent pathway and minimally by a non-AMPK-

peroxynitrite-dependent pathway.

CuO nanoparticles are known to be cytotoxic in a variety of cells, including A549 cells, H1650 cells, CNE-2Z cells, and MCF cells (Laha et al., 2014; Sun et al., 2012). In contrast, our data showed that the CuO crystals did not induce autophagy in BAECs. The greater cytotoxicity of CuO nanoparticles may be due to (1) the nanoparticle or (2) cell-type specificity. First, the average size of our crystals was much larger than that of reported nanoparticles. The bulky size of the CuO crystals may not allow them to be easily ionized, thereby generating less oxidative stress compared to CuO nanoparticles, suggested by Yu et al. (2013). Zinc oxide (ZnO) nanoparticles generate reactive oxygen species (ROS), inducing autophagy. A nanoparticle has a higher amount of water-accessible surface area per mass, potentially generating more ROS. Accordingly, nanoparticles are possibly more cytotoxic than larger crystals. Second, BAECs are more apoptosis-resistant than cancer cell lines and CuO nanoparticles may be toxic in BAECs.

Autophagy in the cardiovascular system has been poorly

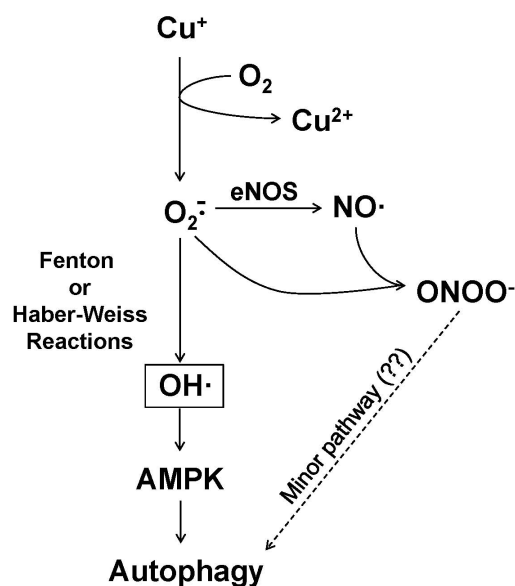


Fig. 6. Schematic diagram showing Cu⁺ ion induction of autophagy in BAECs.

characterized. Previously, autophagy was shown to occur in heart exposed to hypoxia (Shimomura et al., 2001; Tanaka et al., 2000). However, apoptosis was mostly observed along with the autophagy. In this study, cuprous oxide crystals were demonstrated to induce autophagy but not apoptosis or necrosis. Accordingly, our current *in vitro* model system is a prominent case for the induction of autophagy in endothelial cells, although the detailed mechanisms of Cu₂O-induced autophagy remain unknown. Nevertheless, the cytotoxicity of Cu⁺ ion shown in this study correlates with a wide number of previous reports proving that alterations of metabolism and mobilization of copper tightly link to various cardiovascular diseases including ischemia-reperfusion injury, inflammation and atherosclerosis (Hordyjewska et al., 2014; Linder et al., 1996; Rajendran et al., 2007). Such publications have suggested that severe cellular and tissue damages caused by copper ions are associated with ROS formations (Powell et al., 1999). Shokrzadeh et al. (2009) have recently proposed that copper may play an important role in developing ischemic cardiomyopathy, showing that interventions with copper chelators relieve symptoms of ischemic cardiomyopathy and decline its progression. Our findings showing the Cu⁺ induced autophagy in endothelial cells may provide an insight into underlying mechanisms of such clinical cases, because both copper and autophagy have been shown to contribute to myocardial infarction (Chevion et al., 1993; Glick et al., 2010).

Collectively, our findings are more likely to support the limited utilization of materials containing Cu⁺ ions in the vascular system. Considering the longer viability of the endothelium, the Cu⁺ induced endothelial autophagy suggests that excessive utilization of materials containing Cu⁺ as pharmaceuticals or nutraceuticals may cause deleterious tissue processes. Meanwhile, our data give also an insight into medical applications of the Cu₂O crystal, perhaps as a tool to slowly generate cuprous ions (Cu⁺) and recruit Cu⁺ ions to local lesions with bypassing various copper-removal pathways. If copper ions are utilized as pharmaceuticals or nutraceuticals, both copper-binding and

redox-control properties would be available. For instance, copper-binding molecules are known as albumin, ceruloplasmin, and transcuperin which prevent the toxicity of Cu⁺ ions (Linder et al., 1996). In addition, the redox imbalance caused by alterations of the level of copper ions can be negatively regulated by diverse cellular redox molecules, e.g., glutathione, cysteine and thioredoxin, to maintain the copper homeostasis (Szabó et al., 2007). Such protective molecules decline the serous level of free copper ions, thereby quenching a redox reactivity of the Cu⁺ ion. If Cu₂O crystals can be developed to reside for an extended period at local lesions, these crystals can be utilized for medical purposes requiring slow release of Cu⁺ ions.

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