Salubrinal suppresses cadmium-induced cell death by affecting endoplasmic reticulum stress/autophagy in SH-SY5Y human neuroblastoma cells

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Abstract

Background: Salubrinal, inhibits the dephosphorylation of eukaryotic translation initiation factor 2 subunit α (eIF2α), provides protection against cadmium toxicity. However, underlying mechanisms of salubrinal for ER stress/autophagy remain unknown in SH-SY5Y human neuroblastoma cells following exposure to cadmium.

Methods: Cells were exposed to 1.0 μM $CdCl_2$ and 10 μM salubrinal for 24 h. Cytotoxicities and viabilities were evaluated by using a WST-8 assay. The expression of ER stress– and autophagy–related genes was analyzed by immunoblotting. To evaluate lysosomal pH and autophagosomal formation, fluorescence signals of LysoTracker and Cyto-ID were determined by confocal laser scanning microscopy, respectively. To discriminate autophagic impairment or autophagic activation, flux assay was performed with bafilomycin A1.

Results: Salubrinal suppressed cadmium-induced cell death. Treatment with salubrinal led to increased levels of phosphorylated eIF2α and 78-kDa glucose-regulated protein and a decrease in mRNA level of CCAAT/enhancer-binding protein homologous protein (CHOP) in cells exposed to cadmium. p62 protein and microtubule-associated protein light chain 3B-II (LC3B-II) was increased in cells treated with both cadmium and salubrinal. Flux assays showed that the increase in LC3B-II expression was enhanced by treatment with salubrinal and bafilomycin A1.

Conclusions: Salubrinal suppresses cadmium-induced CHOP expression and activates autophagic flux, thereby promoting cell survival.

Background

Salubrinal was identified in a screening of 19,000 chemicals that protect PC12 rat pheochromocytoma cells from endoplasmic reticulum (ER) stress–induced apoptosis [1]. Salubrinal selectively inhibits the dephosphorylation of eukaryotic translation initiation factor 2 subunit α (eIF2α), resulting in the upregulation of eIF2α phosphorylation and the attenuation of environmental pollutant–induced cell damage [2]. Several recent reports suggest that salubrinal provides protection against cadmium toxicity in rat cerebral cortical neurons [3], ARPE-19 human retinal pigment epithelial cells [4], and HK-2 human renal proximal tubular cells [5]. However, the precise regulatory mechanisms underlying the effects of salubrinal remain unknown in SH-SY5Y human neuroblastoma cells.

Cadmium is a well-known occupational and environmental pollutant, and the toxicological mechanism has been studied. Cadmium exposure can lead to the accumulation of unfolded or misfolded proteins within the ER lumen, resulting in a condition referred to as ER stress [6–8]. ER stress initiates the activation of three ER membrane sensors; protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) [9–11]. The downstream targets of PERK, IRE1α, and ATF6 are involved in the perturbation of protein synthesis, trafficking, degradation, and apoptosis. In the PERK signaling pathway, activated PERK phosphorylates the downstream target,

namely eIF2α [12]. Phosphorylation of eIF2α in turn results in global repression of protein synthesis and induction of ATF4 translation [13]. Elevated ATF4 expression can lead to the induction of additional transcriptional regulators, such as 78-kDa glucose-regulated protein (GRP78) and CCAAT/enhancer-binding protein homologous protein (CHOP) [14, 15]. The expression of these proteins is related to the induction of apoptosis. Recent studies indicate that cadmium affects the PERK signaling pathway by elevating the level of ER stress [16–18]. Thus, the PERK signaling pathway is a target in cadmium-induced apoptosis.

Autophagy plays a role in the cellular homeostasis, stress, pathophysiology and cell death [19]. The process of autophagy is consist of three steps [20]. First, the target proteins and organelles are enclosed by a double-membraned vesicle. These vesicles are known as autophagosome. Second, autophagosome fuses with lysosome. These fusions are known as autolysosome. Finally, the internalized proteins and organelles are degraded by lysosomal hydrolases in autolysosome. As autophagy is a dynamic and complex process, it is essential to determine the autophagic flux. Autophagic flux is defined as the amount of autophagic degradation [21–23]. Several reports have suggested that exposure to heavy metals such as cadmium can impair autophagic flux [24, 25]. In addition, palladium was shown to impair autophagic flux in human prostatic cancer cell lines (e.g., PC-3 and LNCaP cells) [26]. However, the mechanism by which salubrinal regulates cadmium-mediated autophagic impairment remains unclear.

As the ER is closely associated with autophagy, it has been suggested that ER stress and autophagy are linked via a specific axis [27]. Autophagy facilitates the degradation of accumulated misfolded and unfolded proteins generated by ER stress [28, 29]. Moreover, the ER serves as the source of membrane for autophagosome formation [30, 31]. In the present study, we investigated the effect of salubrinal on the PERK signaling pathway during ER stress and autophagic flux in cadmium-exposed SH-SY5Y human neuroblastoma cells.

Methods

Cell culture and treatment

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown and maintained as mentioned below. D-MEM (dulbecco's modified eagle's medium)/ F-12 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin were purchased from GIBCO (Thermo Fisher Scientific Inc., Waltham, MA, USA). The condition of cell culture is under a 5% CO_2 atmosphere at 37°C. Cells were seeded on a six-well plastic plate at 4.0 x 10^5 cells/well, and were pre-incubated for 24 h. The cells were harvested 24 h after the subjected to analysis as mentioned below. Cells were incubated in medium without fetal bovine serum containing an appropriate concentration of $CdCl_2$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h. Salubrinal (Merck Millipore, Burlington, MA, USA) and bafilomycin A1 (Adipogen Corp., San Diego, CA, USA) were dissolved in dimethyl sulfoxide (DMSO). Cells were incubated in serum-free

medium containing DMSO (0.1%) and 10 μM salubrinal for 2 h and then treated with 0 to 2.0 μM $CdCl_2$ and 10 μM salubrinal for an additional 24 h.

Cytotoxicity

Cytotoxicity and viabilities were evaluated by using a WST-8 assay (Nacalai Tesque, Kyoto, Japan), which is a modification of the methods for a mitochondrial function of redox potential. A total of 10 μl of 5 mM WST-8 was added to each well of a 96-well plastic culture plate. In each well, the absorbance of redox chemical form of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was determined at 450 nm as measurement wavelength and at 655 nm as a reference wavelength according to the manufacturer's instructions.

Western blotting

After incubation with $CdCl_2$ and/or salubrinal in medium for 24 h, cells were washed with phosphate-buffered saline (PBS) and lysed by Laemmli sample buffer (BIO-RAD Laboratories, Hercules, CA, USA) supplemented with 5% 2-mercaptoethanol (Nacalai Tesque). The experiments of electrophoresis and electrotransfer were reported previously [32]. The transferred membranes were incubated overnight with primary antibodies against eIF2α (#9722), phospho-eIF2α (Ser51) (#3597), LAMP-1 (#9091), TFEB (#4240), SQSTM1/p62 (#8025), LC3B (#3868) (Cell Signaling Technology, Inc., Beverly, MA, USA), ATF4 (sc-390063), GRP78 (sc-13539), and actin (sc-1616) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) respectively. The membrane was then incubated with the secondary antibodies, namely, donkey anti-goat IgG-HRP (sc-2020) (Santa Cruz Biotechnology, Inc.) against for sc-1616, goat anti-rat IgG-HRP (sc-2006) (Santa Cruz Biotechnology, Inc.) against for sc-13539, anti-rabbit IgG-HRP-linked antibodies (#7074S) (Cell Signaling Technology, Inc.) against for #9722, #3597, #9091, #4240, #8025, #3868 and sc-390063, in TBST containing 5% skimmed milk powder (Nacalai Tesque), and washed three times with TBST. The detection of blots from membrane was detected by chemiluminescent reagents (20X LumiGLO® Reagent and 20X Peroxide, Cell Signaling Technology, Inc.). The intensities of individual bands on the developed films (Amersham Hyperfilm™ ECL, cytiva, Shinjuku, Tokyo, Japan) were quantified using image processing programmed software (ImageJ 1.42, National Institutes of Health, Bethesda, MD, USA), and normalized to the intensity of actin.

Quantitative real-time PCR

Total RNA was extracted and isolated by RNeasy® Plus Mini kit (Qiagen, Venlo, Netherlands) according to the protocol provided by instruction. Aliquots of total RNA (1.0 μg) were reverse-transcribed into cDNA by a PrimeScript™ 1st strand cDNA Synthesis kit (Takara Bio Inc., Kusatsu, Shiga, Japan) according to the protocol provided by instruction. Reverse transcription reaction of cDNA at 42°C for 60 min, denaturation with reverse transcriptase at 95°C for 5 min. Quantitative real-time PCR was performed with a PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific Inc.). Thermal cycler program for 40 cycle was following below. Denaturation of cDNA at 95°C for 3 s. Annealing and extension at 60°C for 30 s. The sequences of gene-specific primers as follows: CHOP, 5'-TGGAAGCCTGGTATGAGGAC-3' (forward) and 5'-

AGTCAGCCAAGCCAGAGAAG-3′ (reverse); GAPDH, 5′-AATCCCATCACCATCTTCCA-3′ (forward) and 5′-TGGACTCCACGACGTACTCA-3′ (reverse). The expression level of CHOP mRNA was normalized to that of GAPDH mRNA. Fluorescence intensity of the amplified PCR products was determined by StepOne™ Real-Time PCR System (Thermo Fisher Scientific Inc.).

Fluorescence imaging of lysosomal pH and autophagosomal formation

LysoTracker® Blue DND-22, a specific imaging fluorescence probe for lysosomal pH, was purchased by Life Technologies Japan Ltd. (Shibaura, Tokyo, Japan). Cyto-ID®/Hoechst® 33342, a specific imaging fluorescence probe for autophagosome with cell nucleus, was purchased by Enzo Life Sciences Inc. (Farmingdale, NY, USA). SH-SY5Y cells were seeded on the glass bottom dish (Matsunami Glass, Ind., Ltd., Wada, Osaka, Japan) for 24 h according to the same maintain protocol as that mentioned above. Cells were incubated under a 5% CO_2 atmosphere at 37°C with LysoTracker® Blue DND-22 and Cyto-ID®/Hoechst® 33342 for 30 min, respectively. After 30 min, adherent cells on the glass bottom dish were washed with PBS, and then fluorescence images in living cells were observed by a confocal laser scanning microscope (LSM-710, Carl Zeiss, Jena, Thuringia, Germany).

Statistics

Results are presented as mean ± standard error of the mean (SEM) of three independent samples. The statistical significance of differences between two groups was calculated by the Student's *t*-test. A *P* value of less than 0.05 was considered indicative of a statistically significant.

Results

Salubrinal suppresses $CdCl_2$-induced cellular damage and restores cell viability

We have initially determined the possible cellular damage induced by salubrinal treatment for 24 h (the concentrations from 5 μM to 40 μM) in SH-SY5Y cells. (data not shown). Exposure to $CdCl_2$ at a concentration of 1.0 μM for 24 h caused SH-SY5Y cells to float from adhesion on culture dish bottoms (Fig. 1A). This cellular damage was reduced by treating cells with both 1.0 μM $CdCl_2$ and salubrinal at a concentration of 10 μM for 24 h (Fig. 1A). The viability of cells decreased in exposed to $CdCl_2$ at a concentration of 1.0 μM and 2.0 μM (Fig. 1B). In contrast, treatment with salubrinal at a concentration of 10 μM for 24 h significantly suppressed the toxic effects of $CdCl_2$ at a concentration of 1.0 μM and 2.0 μM (Fig. 1B).

Expression of ER stress–related genes in cells treated with $CdCl_2$ and salubrinal

To elucidate the effects of salubrinal on $CdCl_2$-induced cell death, the expression of ER stress–related genes was analyzed by immunoblotting of lysates of SH-SY5Y cells exposed to 0–2.0 μM $CdCl_2$ for 24 h. The levels of phosphorylated eIF2α and GRP78 increased in a dose-dependent manner (Fig. 2A). The levels of phosphorylated eIF2α and GRP78 in cells treated with both 1.0 μM $CdCl_2$ and 10 μM salubrinal

were significantly higher compared with cells treated with $CdCl_2$ only (Fig. 2B). In contrast, there were no differences in the total amounts of eIF2α and ATF4. CHOP, also known as transcription factor growth arrest and DNA damage–inducible gene 153 (GADD153), plays a role in ER stress–mediated apoptosis [14]. To assess transcription of the CHOP gene, quantitative real-time PCR was used to measure the expression of CHOP mRNA in SH-SY5Y cells following exposure to $CdCl_2$ with and without salubrinal for 24 h. The level of CHOP mRNA increased in cells exposed to $CdCl_2$ (0.5–2.0 μM) (Fig. 3A). However, the level of CHOP mRNA in cells treated with both 1.0 μM $CdCl_2$ and 10 μM salubrinal was significantly lower than in cells treated with $CdCl_2$ only (Fig. 3B).

Expression of autophagy-related proteins in cells treated with $CdCl_2$ and salubrinal

Lysosomal-associated membrane protein 1 (LAMP-1) is known as major component of lysosomal membrane and it is necessary for autophagic regulations [33]. In the present study, levels of LAMP1 were not affected by treatment with $CdCl_2$ and salubrinal (Fig. 4A and B). Transcription factor EB (TFEB) is known to play an important role to the regulation of autophagy and lysosomal biogenesis [34, 35]. Levels of TFEB decreased in cells treated with 2.0 μM $CdCl_2$ for 24 h (Fig. 4A). The levels of TFEB in cells treated with both 1.0 μM $CdCl_2$ and 10 μM salubrinal were significantly lower compared with cells treated with $CdCl_2$ only (Fig. 4B). SQSTM1/p62 (p62) binds autophagosomal membrane protein light chain 3 (LC3), and then recruiting to the autophagosome [36]. The expression levels of phosphatidylethanolamine-conjugated form of LC3B (LC3B-II) are correlated with autophagosome [37]. Lysosomal infusion of autophagosomes leads to a decrease p62 and LC3B-II [38, 39]. Therefore, p62 and LC3B-II has been used as indicators of autophagy. In the present study, levels of p62 and LC3B-II increased in a dose-dependent manner in cells treated with $CdCl_2$ (0.5–2.0 μM) for 24 h (Fig. 4A). The levels of p62 and LC3B-II in cells treated with both 1.0 μM $CdCl_2$ and 10 μM salubrinal were significantly higher compared with cells treated with $CdCl_2$ only (Fig. 4B).

Effects of $CdCl_2$ and salubrinal on lysosomal pH and autophagosomal formation

To determine the effects of exposure to $CdCl_2$ and/or salubrinal on lysosomal pH and autophagosomal formation, respectively, LysoTracker was used for the pH changes of lysosome and Cyto-ID was used for the formation of autophagosome in living cells [40–42]. As shown in Fig. 5A, confocal laser scanning microscopy analyses revealed a decrease in LysoTracker fluorescence signals following exposure to 1.0 μM $CdCl_2$ for 24 h. In contrast, salubrinal had no effect for alkalization induced by 1.0 μM $CdCl_2$. Although treatment with $CdCl_2$ decreased LysoTracker florescence signals, no further alteration was observed following co-treatment with salubrinal (Fig. 5A). As shown in Fig. 5B, Cyto-ID signals were markedly increased in cells following treatment with 1.0 μM $CdCl_2$ for 24 h. Moreover, the signals in cells treated with both 1.0 μM $CdCl_2$ and 10 μM salubrinal were higher than in cells treated with $CdCl_2$ only (Fig. 5B). These results suggest that salubrinal did not interfere $CdCl_2$ induced lysosomal alkalization but induced excessive autophagosome formation in SH-SY5Y cells treated with $CdCl_2$.

Monitoring of autophagic flux by immunoblotting analysis of LC3B-II

As methods for monitoring autophagic activity are complex, flux assay of autophagy is essential for the determination of autophagic impairment or autophagic activation [21, 23]. Several interpretations have been described for measuring autophagic flux in cultured cells [22]. Bafilomycin A1 is active against for autophagic flux because it is known as inhibitor for lysosomal acidification and fusion of autophagosome and lysosome [43]. Cadmium-induced LC3B-II was not interfered by treatment with bafilomycin A1 (Fig. 6A). However, treatment with both salubrinal and bafilomycin A1 induced a significant increase in the level of LC3B-II compared with cells treated with bafilomycin A1 only (Fig. 6B). These results indicate that cadmium inhibits the autophagic flux, whereas salubrinal activates the autophagic flux.

Discussion

The present study shows that salubrinal affects the PERK signaling pathway of ER stress and autophagic flux, leading to suppression of cadmium-induced cell death in SH-SY5Y cells. In the PERK signaling pathway associated with ER stress, activated PERK phosphorylates Ser51 of eIF2α and blocks the binding of the initiator Met-tRNA. As the frequency of recognizing the AUG initiation codon declines, general translation is attenuated [14]. Increased phosphorylation of eIF2α diminishes its translational activity and results in reduced global protein synthesis and subsequent reduction in ER activities such as protein folding, maturation, quality control, and trafficking [44]. Cadmium exposure induces phosphorylation of eIF2α through activation of the PERK signaling pathway during ER stress [7]. In contrast, salubrinal induces an increase in phosphorylation of eIF2α by functioning as a selective inhibitor of eIF2α dephosphorylation both *in vitro* and *in vivo* [1, 45]. Salubrinal induces phosphorylation of eIF2α by inhibiting the function of the GADD34/PP1 protein complex, which consists of the general cellular serine/threonine phosphatase PP1 and non-enzymatic cofactor GADD34 [1]. Therefore, cadmium and salubrinal can increase phosphorylation of eIF2α in SH-SY5Y cells through distinct mechanisms, such as phosphorylation of eIF2α through PERK and inhibition of eIF2α dephosphorylation through GADD34/PP1, respectively.

In the present study, the expression of GRP78 and CHOP, well-known downstream targets of ATF4, changed following treatment with cadmium and salubrinal. However, the level of ATF4 expression was not affected by treatment with either cadmium or salubrinal or both. The expression of both GRP78 and CHOP is reportedly controlled by not only ATF4 but also ATF6 [14, 15]. In the case of rotenone-mediated ER stress, salubrinal was shown to affect not only the PERK signaling pathway but also the ATF6 and IRE1 signaling pathways in Neuro-2a mouse neuronal cells [46]. Although the mechanism underlying the fluctuation in GRP78 and CHOP expression was not elucidated in the present study, the expression levels of GRP78 and CHOP may be individually regulated by independent of ATF4 expression and cross-talk among signaling pathways. Induction of GRP78 expression suppresses inhibits apoptosis [47–49]. In the present study, GRP78 expression increased in cadmium-exposed SH-SY5Y cells, and salubrinal further enhanced GRP78 up-regulation in these cells. These results are consistent with the previous findings

demonstrating that GRP78 expression exerts a cytoprotective effect against cadmium toxicity [50–52]. Excessive ER stress is also related to apoptosis because CHOP, known as pro-apoptotic protein, promotes the expression of other apoptosis-related proteins [14, 53]. Cadmium-induced up-regulation of CHOP expression plays a critical role in triggering apoptotic cell death [3]. In the present study, CHOP mRNA levels were increased in cadmium-exposed SH-SY5Y cells. On the other hand, salubrinal treatment suppressed the up-regulation of CHOP expression in cadmium-exposed SH-SY5Y cells. It has been reported that inhibiting of CHOP expression suppress an apoptotic effect by cadmium [54]. Collectively, our findings suggest that the induction of GRP78 expression and attenuation of CHOP expression suppress cadmium-induced cytotoxicity.

TFEB, a basic helix-loop-helix leucine zipper, plays an important role in the regulation of autophagy and lysosomal function by activating the transcription of lysosomal target genes [55]. It has been reported that exposure to cadmium inhibits TFEB expression and affects lysosomal pH in Neuro-2a mouse neuroblastoma cells [56, 57] and rat primary proximal tubular cells [58]. In the present study, we observed a decrease in TFEB protein levels and lysosomal alkalization in SH-SY5Y cells following exposure to cadmium. Unexpectedly, in SH-SY5Y cells treated with both cadmium and salubrinal, the levels of TFEB were significantly lower than in cells treated with cadmium only. In contrast, cadmium-mediated lysosomal alkalization was not affected by salubrinal treatment. These results suggest that salubrinal additively contributes to cadmium-mediated changes in lysosome function. Additional studies are needed to elucidate the details regarding how salubrinal affects the expression of TFEB.

The autophagy-related proteins p62 and LC3B-II are localized on both the outer and inner membranes of autophagosomes. Upon fusion with lysosomes, the space between the outer and inner autophagosomal membranes becomes acidified, which is followed by degradation of p62 and LC3B-II and the formation of autolysosomes [21]. Cadmium-induced disruption of lysosomal function and autophagic flux leads to the accumulation of p62 and LC3B-II [24, 59, 60]. What could be the trigger for the increased accumulation of p62 and LC3B-II in SH-SY5Y cells treated with both cadmium and salubrinal? To answer this question, “autophagic flux assays” could be used to distinguish whether the accumulation of autophagy-related proteins is due to autophagy induction or instead to a block in the downstream steps. We performed the flux assay by using LC3B-II because p62 did not correlate with autophagic activities in this method [61]. The results of autophagic flux assays indicated that cadmium exposure inhibits autophagic flux and that salubrinal treatment activates autophagic flux. It was reported that inhibition of mammalian target of rapamycin complex 1 (mTORC1) exerts cytoprotective effects against cadmium toxicity by enhancing autophagic flux [59]. Inhibition of mTORC1 is the most effective enhancer of autophagy [62]. A well-established upstream regulator of mTORC1 is the phosphatidylinositol 3-kinase/protein kinase B (AKT) signaling pathway, which is thought to activate mTORC1 [63–66]. Salubrinal reportedly decreases the level of phosphorylated AKT in mouse cholangiocarcinoma cells [67]. Therefore, in the case of cadmium-mediated autophagic impairment in SH-SY5Y cells, salubrinal may activate autophagic flux and promote cell survival via mTORC1 inhibition.

Conclusions

we investigated the effect of salubrinal on the ER stress/autophagy axis in cadmium-exposed SH-SY5Y human neuroblastoma cells. Our data suggest that salubrinal plays a role as an effector of PERK signaling and autophagic flux. These results indicate that salubrinal modulates both cadmium-induced ER stress and autophagic impairment, thereby protecting SH-SY5Y cells from apoptotic cell death.

Declarations

Ethics approval and consent to participate

There are no ethical objections to the conduct of the study.

Consent for publication

Not applicable because there are no participants in this study.

Availability of data and materials

The all data and materials are available from the corresponding author upon reasonable request.

Competing interests

The authors have no conflicts of interest to disclose.

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Authors' contributions

TM and MM designed the study and interpreted data. TM carried out the experiments and data analysis. TM made the figures and drafted the manuscript. TM and MM edited and completed the manuscript to the final version.

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Figures

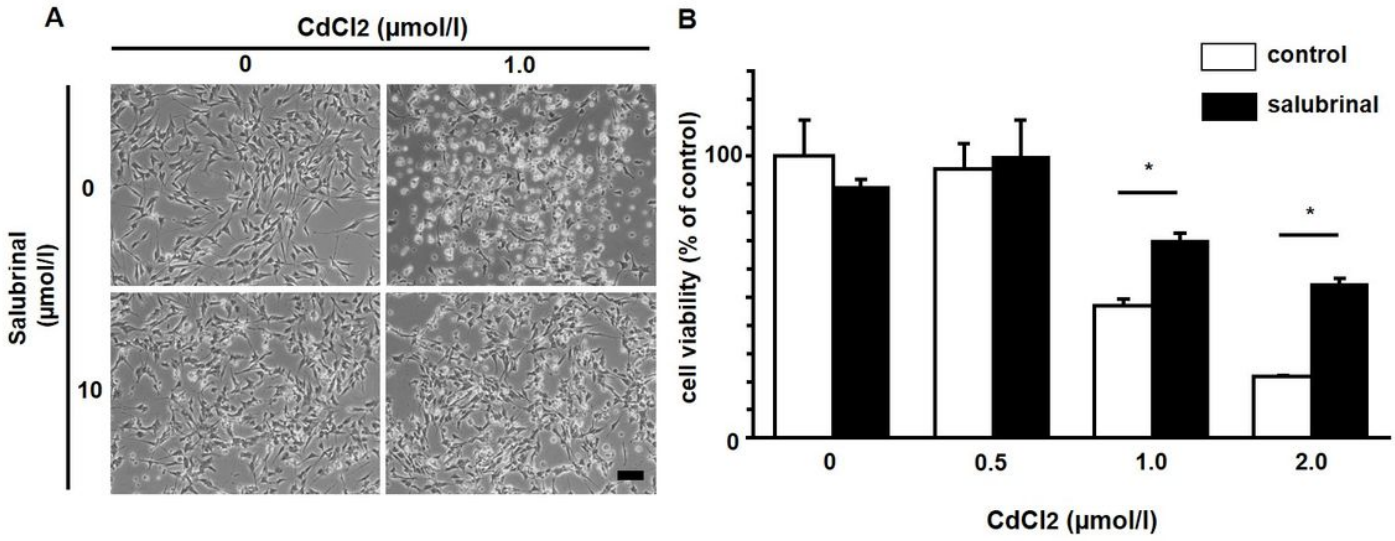

Figure 1

Effects of salubrinal on cadmium-induced cytotoxicity in SH-SY5Y cells. (A) Phase-contrast micrographs. Cells were treated with $CdCl_2$ (1.0 μM) and/or salubrinal (10 μM) for 24 h. Scale bar, 100 μm. (B) SH-SY5Y cells were treated with $CdCl_2$ (0-2.0 μM) and/or salubrinal (10 μM) for 24 h. Cell viability was determined using a WST-8 assay. Each value (mean ± SEM, n = 4) represents percent survival relative to untreated control cells (0 μM $CdCl_2$). *P<0.05 compared to individual $CdCl_2$ concentration groups.

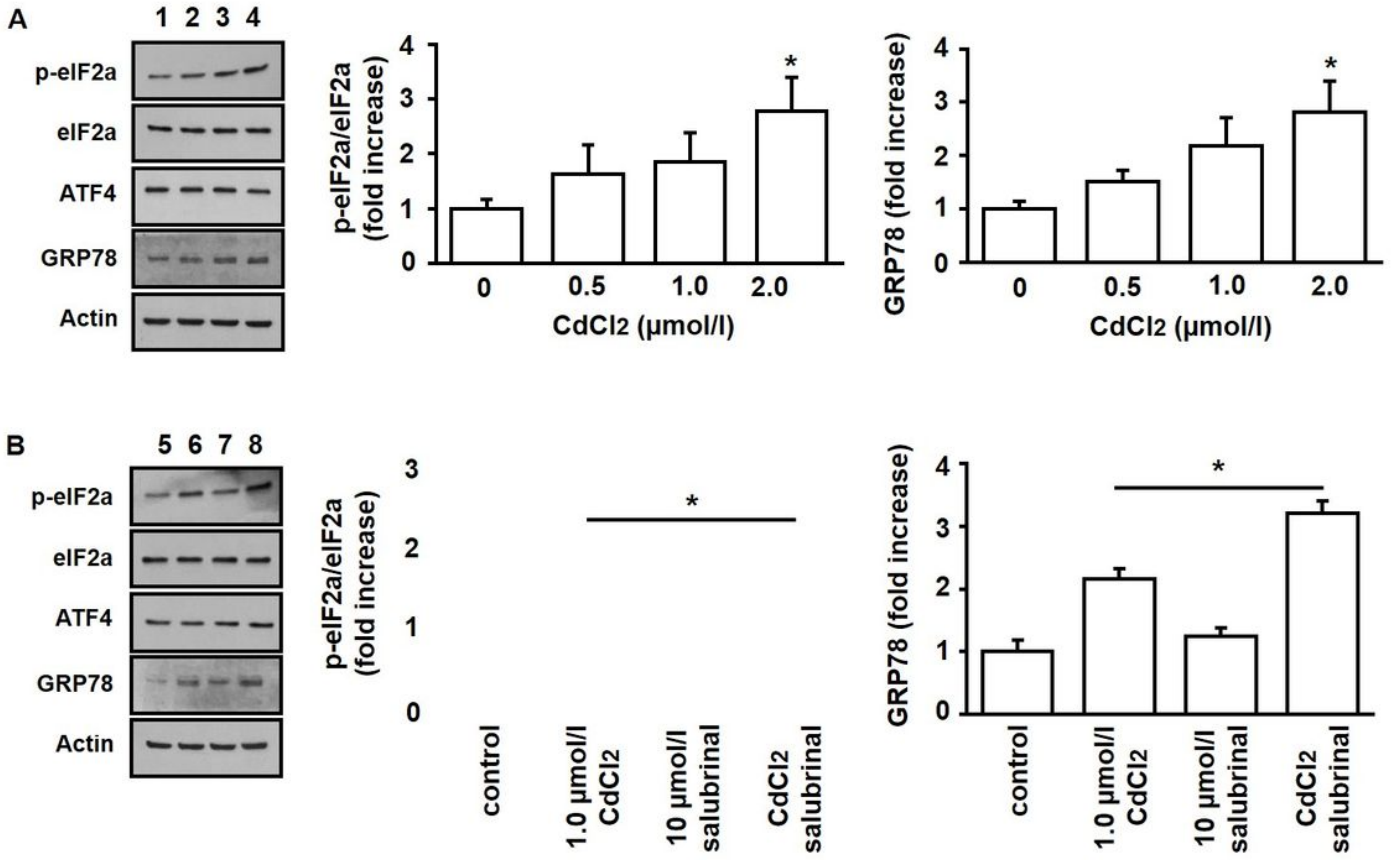

Figure 2

Effects of $CdCl_2$ and salubrinal on the expression of phosphorylated eIF2α, ATF4, and GRP78 proteins in SH-SY5Y cells. (A) Cells were treated with $CdCl_2$ (0–2.0 μM) for 24 h. (B) Cells were treated with 0.1% DMSO or 10 μM salubrinal for 2 h and then treated with or without 1.0 μM $CdCl_2$ for 24 h. Cell lysates were subjected to Western immunoblotting using the indicated antibodies. Representative immunoblots and results of densitometric analyses are shown. Each value (fold-increase, mean ± SEM, n = 3) was normalized to that of eIF2α or actin, and the control value (0 μM $CdCl_2$) was set to 1. *P<0.05 compared to 0 μM $CdCl_2$ (A) or control (B).

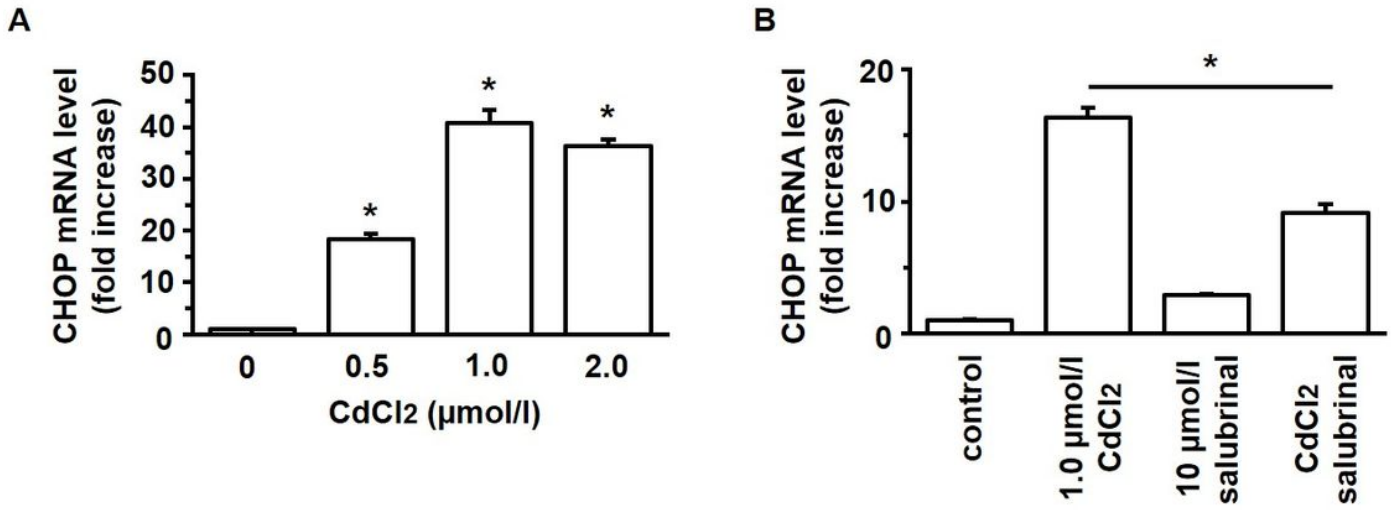

Figure 3

Effects of $CdCl_2$ and salubrinal on the expression of CHOP in SH-SY5Y cells. (A) Cells were treated with $CdCl_2$ (0–2.0 μM) for 24 h. (B) Cells were treated with 0.1% DMSO or 10 μM salubrinal for 2 h and then treated with or without 1.0 μM $CdCl_2$ for 24 h. Total RNA was isolated and subjected to real-time PCR analysis. Each value (fold-increase, mean ± SEM, n = 3) was normalized to that of GAPDH, and the control value (0 μM $CdCl_2$) was set to 1. *P<0.05 compared to 0 μM $CdCl_2$ (A) or control (B).

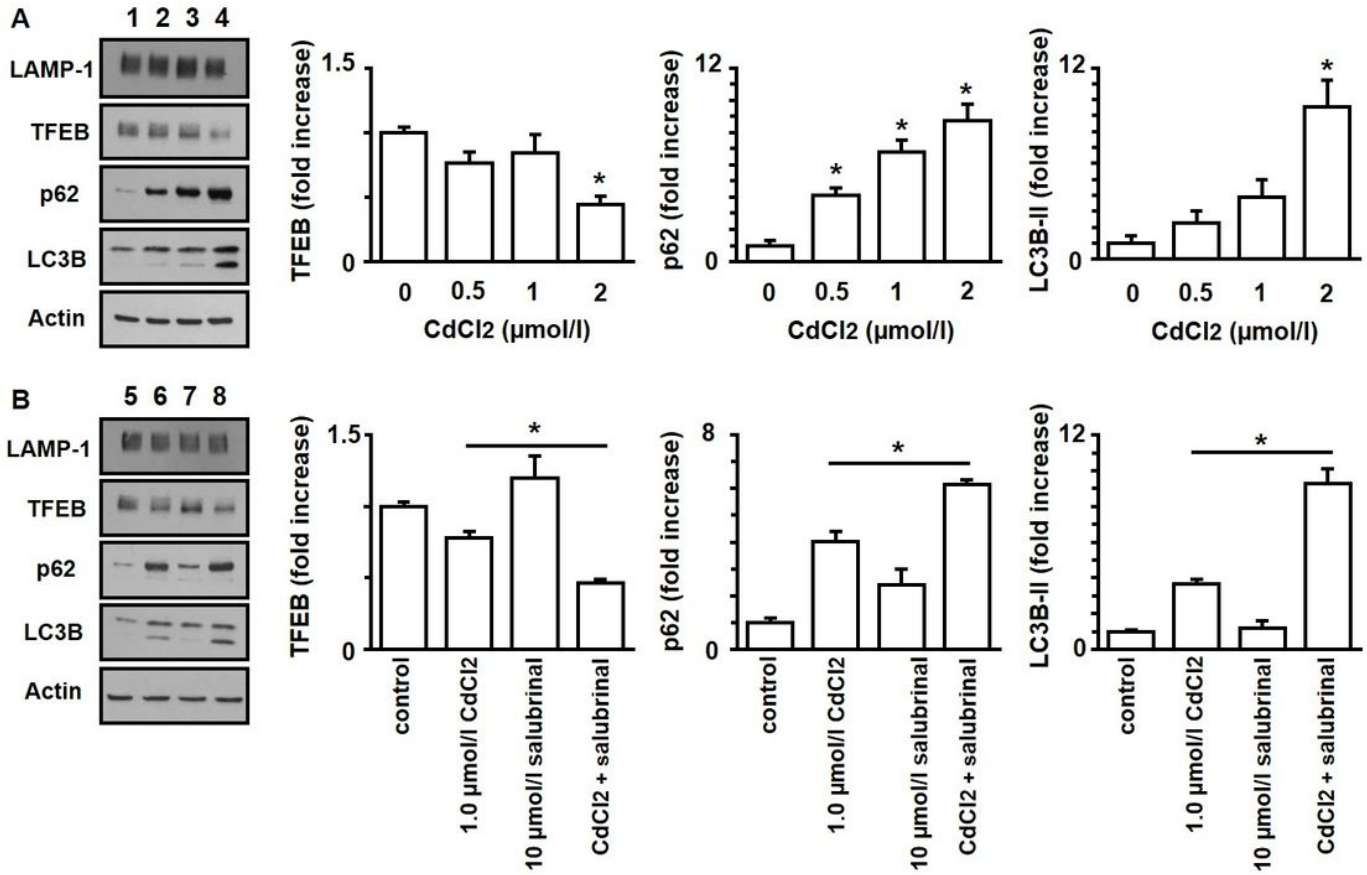

Figure 4

Effects of $CdCl_2$ and salubrinal on the accumulation of autophagy-related proteins. (A) Cells were treated with $CdCl_2$ (0–2.0 μM) for 24 h. (B) Cells were treated with 0.1% DMSO or 10 μM salubrinal for 2 h and then treated with or without 1.0 μM $CdCl_2$ for 24 h. Cell lysates were subjected to Western immunoblotting using the indicated antibodies. Representative immunoblots and results of densitometric analyses are shown. Each value (fold-increase, mean ± SEM, n = 3) was normalized to that of actin, and the control value (0 μM $CdCl_2$) was set to 1. *P<0.05 compared to 0 μM $CdCl_2$ (A) or control (B).

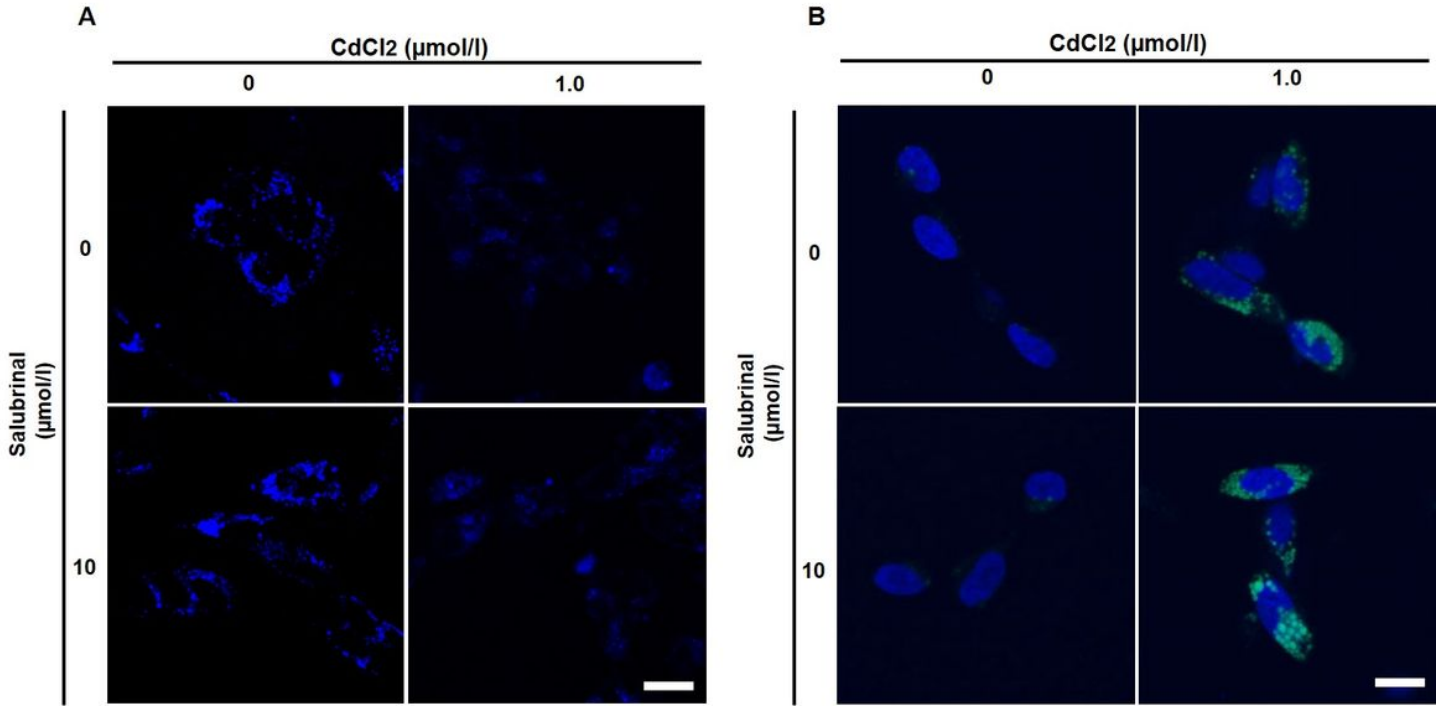

Figure 5

Effects of $CdCl_2$ and salubrinal on lysosomal alkalization and autophagosome formation. Cells were treated with 0.1% DMSO or 10 μM salubrinal for 2 h and then treated with or without 1.0 μM $CdCl_2$ for 24 h. The cells were then incubated with LysoTracker Blue DND-22 (A) or Cyto-ID with Hoechst 33342 (B) for 30 min. Scale bars, 10 μm.

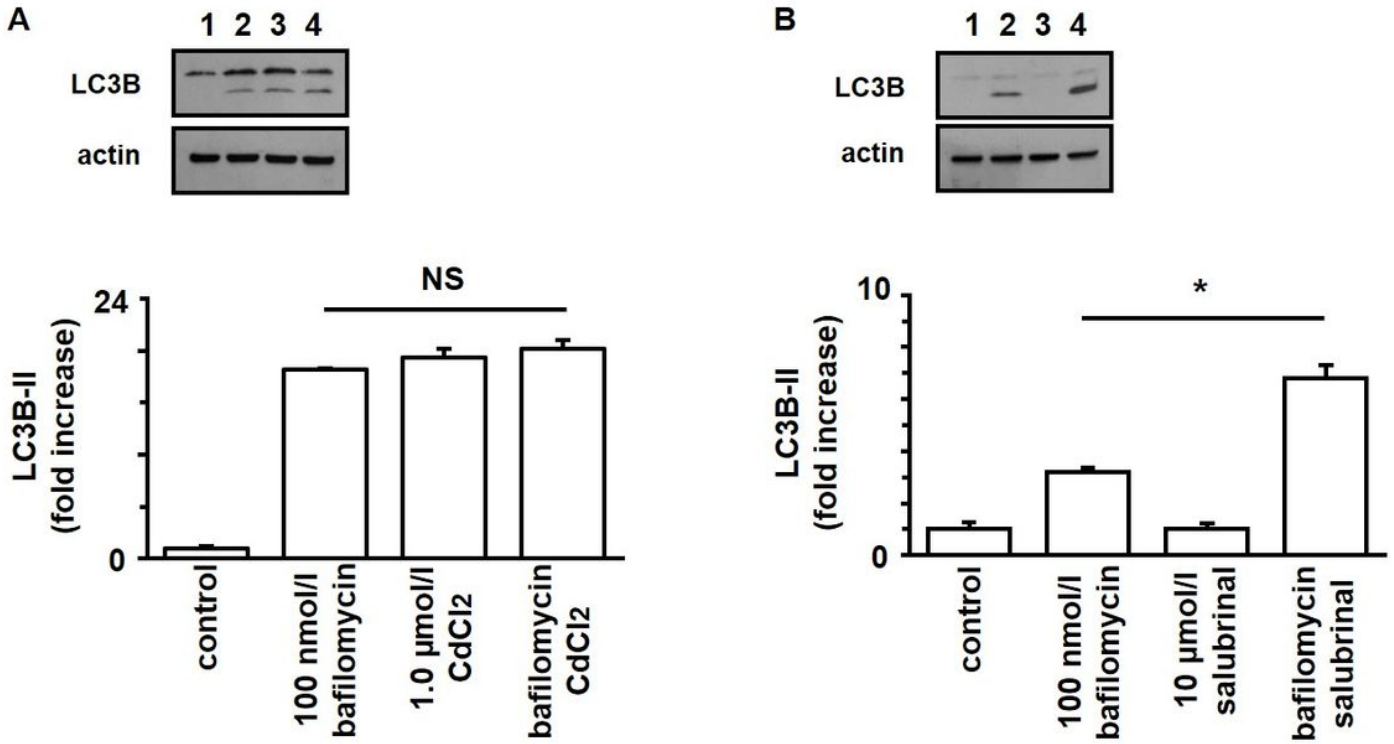

Figure 6

Effects of bafilomycin A1 treatment on LC3B-II accumulation in cells treated with $CdCl_2$ or salubrinal. Cells were treated with or without 100 nM bafilomycin A1 for 24 h before treatment with or without 1.0 μM $CdCl_2$ (A) or with or without 10 μM salubrinal (B) for an additional 24 h. Cell lysates were subjected to Western immunoblotting using antibodies against LC3B and actin. Representative immunoblots and results of densitometric analyses are shown. Each value (fold-increase, mean ± SEM, n = 3) was normalized to that of actin, and the control value (cells without bafilomycin A1 and $CdCl_2$ [A] or cells without salubrinal [B]) was set to 1. *P<0.05 compared to cells treated with bafilomycin A1 only. NS, not significant.

Supplementary Files

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