Biochemical and Biophysical Research Communications 438 (2013) 186–192

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Induction of cytoprotective autophagy in PC-12 cells by cadmium

Qiwen Wang [a,b,c,1], Jiaqiao Zhu [a,b,1], Kangbao Zhang [a,b], Chenyang Jiang [a,b], Yi Wang [a,b], Yan Yuan [a,b], Jianchun Bian [a,b], Xuezhong Liu [a,b], Jianhong Gu [a,b], Zongping Liu [a,b,*]

[a] College of Veterinary Medicine, Yangzhou University, Yangzhou 225009, PR China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, PR China
[c] Bijie Pilot Area Research Institute of Bijie University, Bijie 551700, PR China

ARTICLE INFO

Article history:
Received 8 July 2013
Available online 20 July 2013

Keywords:
Cadmium
Autophagy
Apoptosis
PC-12 cells
LC3

ABSTRACT

Laboratory data have demonstrated that cadmium (Cd) may induce neuronal apoptosis. However, little is known about the role of autophagy in neurons. In this study, cell viability decreased in a dose- and time-dependent manner after treatment with Cd in PC-12 cells. As cells were exposed to Cd, the levels of LC3-II proteins became elevated, specific punctate distribution of endogenous LC3-II increased, and numerous autophagosomes appeared, which suggest that Cd induced a high level of autophagy. In the late stages of autophagy, an increase in the apoptosis ratio was observed. Likewise, pre-treatment with chloroquine (an autophagic inhibitor) and rapamycin (an autophagic inducer) resulted in an increased and decreased percentage of apoptosis in contrast to other Cd-treated groups, respectively. The results indicate that autophagy delayed apoptosis in Cd-treated PC-12 cells. Furthermore, co-treatment of cells with chloroquine reduced autophagy and cell activity. However, rapamycin had an opposite effect on autophagy and cell activity. Moreover, class III PI3 K/beclin-1/Bcl-2 signaling pathways served a function in Cd-induced autophagy. The findings suggest that Cd can induce cytoprotective autophagy by activating class III PI3 K/beclin-1/Bcl-2 signaling pathways. In sum, this study strongly suggests that autophagy may serve a positive function in the reduction of Cd-induced cytotoxicity.

1. Introduction

Autophagy is a conserved evolutionary lysosomal pathway for degrading cytoplasmic proteins, macromolecules, and organelles [1,2]. As a highly ordered pathway, autophagy begins with the formation and expansion of an isolation membrane called "phagophore". The photosphere then fuses to form the autophagosomes that engulf portions of cytoplasm and/or organelles. This process is followed by the fusion of the autophagosome with a lysosome to promote luminal content degradation [3]. Depending on the environmental stress and cell type, autophagy pathway acts either as a survival or death safeguard mechanism. Autophagy can be induced when cells are subjected to environmental stress. This process helps maintain cellular homeostasis and prevents organism from damage and diseases [4]. However, a massive and persistent autophagy can also result in cell death known as autophagic cell death [5].

The crosstalk between autophagy and apoptosis is quite complex. Under different circumstances, autophagy may delay or promote the onset of apoptosis, which can also induce autophagy [6]. A recent report demonstrated that oxidative stress-induced autophagy is involved in apoptosis prevention in restrained mice [7] and human embryonic kidney cells [8]. However, autophagic cell death is independent of apoptosis in transformed and cancer cells [9]. Given the possibility of different sensitivity thresholds, the exact nature of the relationship between autophagy and apoptosis remains unknown.

Cadmium (Cd) is an occupationally and environmentally relevant toxic element. that is currently ranked seventh on the Priority list of Hazardous Substances by the Agency for Toxic Substances and Diseases Registry [10]. This heavy metal can enter into the brain parenchyma and neurons, leading to lower attention, hypernociception, olfactory dysfunction and memory deficits [11,12]. Furthermore, experimental data have shown that Cd can stimulate the phosphorylation of calcium/CaMKII and elevate Ca^{2+}_i, which induces ROS and activates MAPK and mTOR pathways, thus resulting in neuronal apoptosis [13–15], but the role of autophagy induced by Cd in neurons is still obscure. However, the role of Cd-induced autophagy in neurons remains obscure. In this study, we chose the PC-12 cell line as a neuronal model to survey whether Cd can induce autophagy and to investigate the effect and regulatory mechanisms of Cd-induced autophagy in PC-12 cells.

* Corresponding author. Address: 88, South University Ave., Yangzhou, Jiangsu 225009, PR China. Fax: +86 514 87972218.
E-mail address: liuzongping@yzu.edu.cn (Z. Liu).
[1] These authors contributed equally to this work.

0006-291X © 2013 The Authors. Published by Elsevier Inc. Open access under CC BY license.
http://dx.doi.org/10.1016/j.bbrc.2013.07.050

2. Materials and methods

2.1. Materials

The following chemicals and antibodies were used: a bicinchoninic acid (BCA) protein assay (Beyotime), Radio-immunoprecipitation assay (RIPA) lysis buffer (Solarbio), anti-LC3 (Sigma), anti-Beclin-1(Santa Cruz), anti-β-actin, anti-Class III PI3 K, anti-Bcl-2, horseradish peroxidase (HRP) and fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG were from Cell Signaling. Other reagents were from Sigma.

2.2. Cell cultures

The rat pheochromocytoma cell line PC-12 was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured at 37 °C and 5% CO_2 in antibiotic-free RPMI 1640 medium (Giboco) and supplemented with 10% heat-inactivated horse serum (Giboco) and 5% fetal bovine serum (Hyclone).

2.3. Immunofluorescence

PC-12 cells were treated with 0 and 20 μmol/L Cd for 4 h. The cells were fixed with 4% paraformaldehyde. The cells were then permeabilized with 0.5% Triton X-100 and blocked with 5% BSA. Cells were incubated with LC3 antibody in blocking solution for 2 h, and then stained with FITC conjugated anti-mouse IgG for 1 h. Thereafter, cell nuclei were stained by DAPI. Samples were examined under a fluorescence microscope (LEICA DMI 3000B, Germany).

2.4. Electron microscopy

For transmission electron microscopy (TEM) observation, after being treated with 0 and 20 μmol/L Cd for 4 h, the cells were collected and fixed in ice-cold glutaraldehyde (2.5% in 0.1 mol/L cacodylate buffer, pH 7.4) for 24 h and cells were postfixed in osmium tetroxide. After dehydration with a graded series of alcohol concentrations, the samples were rinsed in propylene oxide and impregnated with epoxy resins. The ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy. Electron micrographs were observed through a PHILIPS CM-120 transmission electron microscope.

2.5. Determination of apoptosis

Apoptosis was tested by detecting phosphatidylserine externalization as described in our previous report [16]. Briefly, after treatment, cells were collected and suspended in 100 μL of binding buffer containing 5 μL of FITC Annexin V and 5 μL of propidium iodide dye solution. After incubation in the dark at 25 °C for 15 min, the cells were analyzed by using an FACS Aria flow cytometer (Becton–Dickinson, San Jose, CA, USA). The apoptosis percentage was summed up from primary apoptosis (Annexin V^+/PI^-) and late apoptosis (Annexin V^+/PI^+).

2.6. Western blot analysis

After treatment, cells were then washed twice with cold PBS and then extracted into RIPA lysis buffer on ice. After being sonicated and centrifuged, the protein content was determined using a BCA protein assay kit according to manufacturer's instructions. Equivalent amounts of protein were separated on 15% SDS–polyacrylamide gel and transferred to NC membranes. After being blocked at room temperature for 2 h with 5% non-fat milk in TBS with 0.1% Tween-20, the membranes probed with LC3, Beclin-1, class III PI3 K, Bcl-2, β-actin and HRP conjugated IgG antibodies. The volumes of the bands were determined by standard scanning densitometry with normalization of densitometry measures to β-actin.

2.7. Measurement of cell viability

PC-12 cells were seeded at a density of 8×10^4 cells/mL in a flat bottomed 96-well plate. After treatment, the MTT solution was added to the culture medium (500 μg/mL). The reaction was completed by the addition of DMSO to the cell culture 4 h after the MTT addition. The absorbance was measured at 570/630 nm by a microplate reader (Sunrise, Austria).

2.8. Statistical analysis

Results were represented statistically as means ± SD. Significance was assessed by one-way ANOVA following appropriate transformation to normalized data and equalized variance where necessary. Statistical analysis was performed using SPSS statistics 18.0 (SPSS Inc., USA); $p < 0.05$ and $p < 0.01$ were considered to indicate significance and high significance, respectively.

3. Results

3.1. Cd triggers autophagy in PC-12 cells

First, the effect of Cd on the cell viability of PC-12 cells was investigated using an MTT assay. As shown in Fig. 1A and B, cell viability was decreased in a dose- and time-dependent manner after treatment with Cd. To determine whether this inhibitory effect of Cd on the cell activity of PC-12 was dependent on the induction of autophagy, the autophagy detection assay was applied to Cd-treated cells. The effect of Cd treatment on full-length LC3-I to LC3-II processing, a hallmark of autophagy, was determined. Immunoblot analysis was used to detect the extracted lysates from PC-12 cells treated with 0–20 μmol/L Cd for 4 h or with 20 μmol/L for 0 h to 24 h. As shown in Fig. 1C, the levels of LC3-II proteins increased after treatment with Cd (0–20 μmol/L). As cells were treated with 20 μmol/L Cd for 0–24 h, LC3-II proteins reached a peak for 4 h and then gradually decreased (Fig. 1D).

Moreover, the distribution of endogenous LC3-II in cells before and after Cd treatment for 4 h was monitored by indirect immunofluorescence staining. As revealed in Fig. 1E, specific punctate distribution of endogenous LC3-II was observed in the PC-12, given that more dots appeared in Cd-treated cells than in control cells ($p < 0.01$) (Fig. 1F).

To confirm further that autophagy is induced by Cd treatment, TEM was performed on both control and Cd-treated cells. As depicted in Fig. 1G, Cd-treated PC-12 cells exhibited several autophagosomes that were absent in control cells. In sum, these results indicate that Cd induces autophagy in PC-12 cells.

3.2. Autophagy delays apoptosis in Cd-treated PC-12 cells

To elucidate whether Cd-induced toxicity was accompanied by increased apoptosis, phosphatidylserine externalization was examined using flow cytometry with a two-color analysis of FITC labeled Annexin V/PI double-staining. As revealed in Fig. 2A, the percentage of apoptosis was slightly increased when PC-12 cells were treated with 20 μmol/L Cd for 4 h, but the ratio of apoptosis was increased from 4.5% to 45.8% for 24 h. Furthermore, after

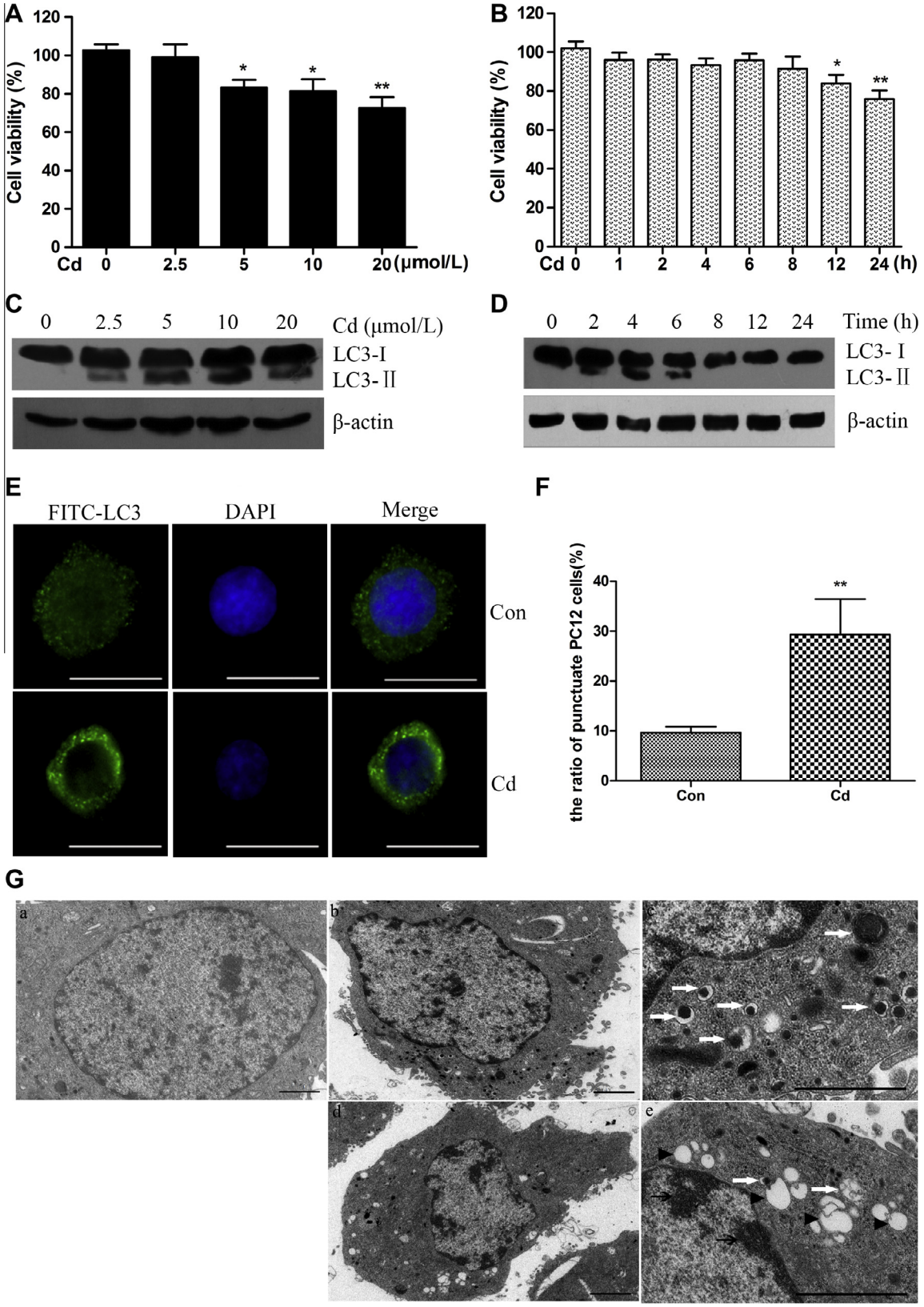

Fig. 1. Cd triggers autophagy in PC-12 cells. (A and B) PC-12 cells were treated with 0–20 μmol/L Cd for 24 h or with 20 μmol/L Cd for different times. Cell viability was measured by MTT assay. $n = 4–6$, Mean ± SD. (C and D) PC-12 cells were treated with 0–20 μmol/L Cd for 4 h or with 20 μmol/L Cd for different times, cell lysates were analyzed by western blot assay with a specific antibody to LC3. The β-actin protein level was used as a loading control. (E and F) PC-12 cells were treated with 20 μmol/L Cd for 4 h, and stained by indirect immunofluorescence. The distribution of endogenous LC3 was monitored using a fluorescent microscope. The number of cells with punctate FITC-LC3 is displayed as a histogram. Bar = 10 μm. (G) PC-12 cells treated without (a) or with 20 μmol/L Cd for 4 h (b, low power; c, high power) and 24 h (d, low power; e, high power) were subjected to TEM analysis. Autophagosomes are highlighted by white arrows in c and e, whereas apoptotic changes such as chromatin condensation (black arrows) and cytoplasmic vacuolization (black arrowheads) are highlighted in e. Bar = 1 μm. $^{*}p < 0.05$, $^{**}p < 0.01$ difference with control group.

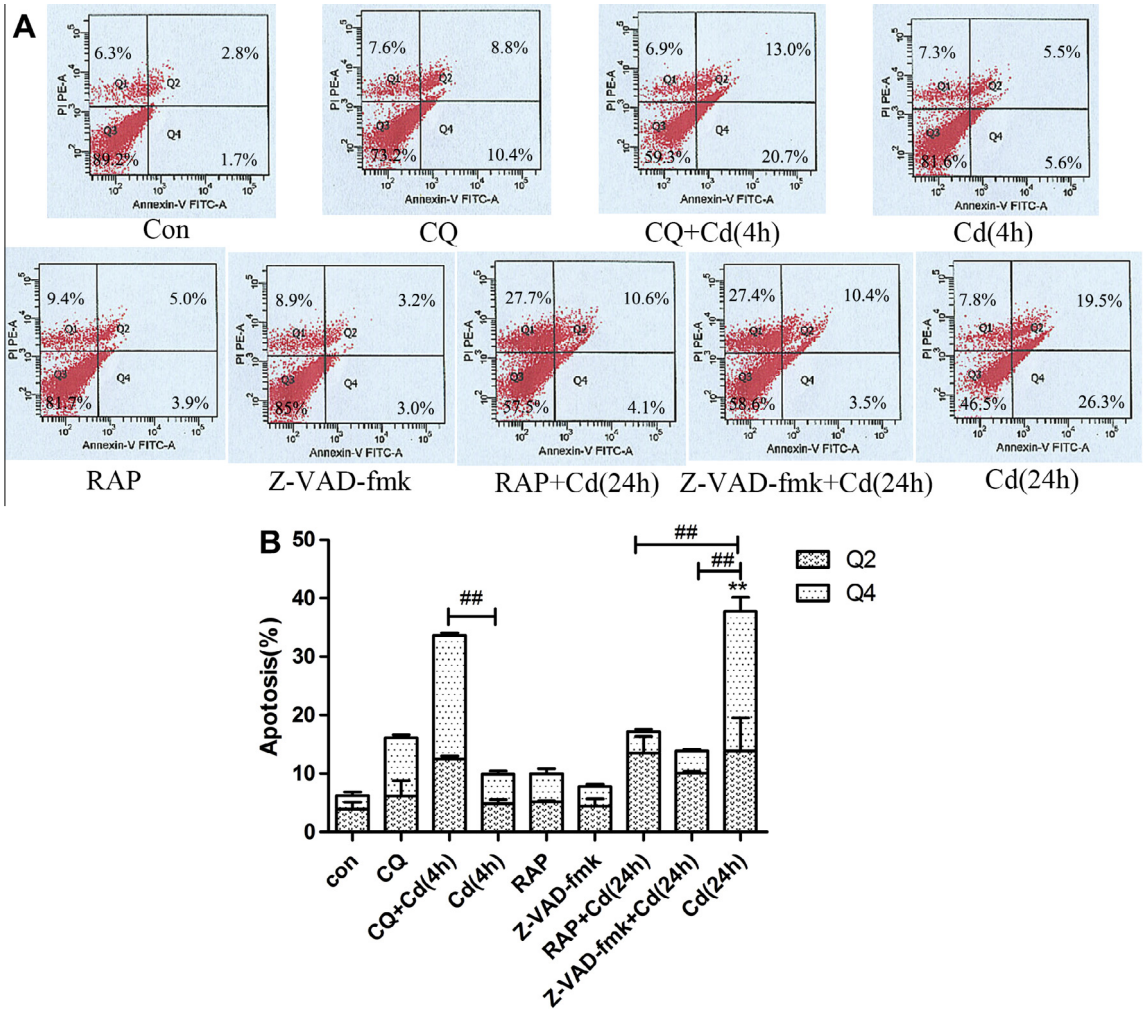

Fig. 2. Autophagy delays apoptosis in Cd-treated PC-12 cells. Cellular apoptosis was analyzed on a flow cytometer using Annexin V/PI staining methods. A representative of three independent experiments is shown (A). (A) PC-12 cells were harvested after 4 h treatment without/with 20 μmol/L Cd in the presence or absence of 5 μmol/L CQ (con, CQ, CQ + Cd, Cd). In the other five experiments, PC-12 cells were treated with 20 μmol/L Cd 24 h following pre-incubation with 100 nmol/L RAP for 24 h or 100 μmol/L Z-VAD-fmk for 1 h. Statistical results are presented in (B). $^{**}p < 0.01$ difference with control group. $^{\#\#}p < 0.01$ difference with respective group of Cd treatment.

24 h of Cd exposure, the percentage of apoptosis was effectively reduced by Z-VAD-fmk, a broad caspase spectrum inhibitor, thereby confirming the role of the caspase cascade in Cd-induced apoptosis. Electron microscopy hallmarks of apoptosis were further studied. As depicted in Fig. 1G, after 24 h of Cd treatment, PC-12 cells displayed typical nuclear apoptosis morphology, such as chromatin condensation and nuclear cytoplasmic vacuolization. Collectively, these results demonstrate that Cd can induce apoptosis in PC-12 cells.

As previously mentioned, Cd could induce both autophagy and apoptosis. To investigate the relationship between apoptosis and autophagy, the specific autophagic inhibitor CQ was introduced. The augmentation of apoptosis ratio resulting from 4 h of Cd exposure was reversed from 4.5% to 33.7%. Furthermore, co-treatment with 20 μmol/L Cd and RAP, an autophagic inducer, reduced the apoptosis ratio ($p < 0.01$, Fig. 2A and B), indicating that autophagy hinders apoptosis in Cd-treated PC-12 cells.

3.3. Cd-induced autophagy is cytoprotective

Many studies have demonstrated that autophagy can serve as a protective response to prevent heavy metal-induced cell death. Thus, CQ and RAP were employed to detect the effect of autophagy on Cd-induced cytotoxicity. We found that CQ and RAP markedly increased LC3-II production (Fig. 3A–D). Using an MTT assay to monitor the effects of CQ on cell viability, CQ or RAP alone failed to inhibit PC-12 cell growth, and CQ significantly augmented the growth inhibition induced by Cd (Fig. 3E). However, RAP markedly decreased Cd-induced injury (Fig. 3F), confirming that Cd-activated autophagy is cytoprotective.

3.4. Class III PI3K/Beclin-1/Bcl-2 pathway contributes to Cd-triggered autophagy

To determine the signaling pathway responsible for the induction of autophagy in Cd-treated PC-12 cells, class III PI3 K, Beclin-1, and Bcl-2 were examined by immunoblot analysis. Cd indeed resulted in a significant upregulation of class III PI3 K at the examined concentration points. Furthermore, Beclin-1 also increased after 2.5 mol/L Cd exposure. However, the expression of Bcl-2 was reduced in Cd-treated cells (Fig. 4), indicating the dependence of autophagy on the activation of class III PI3 K/Beclin-1/Bcl-2 signaling.

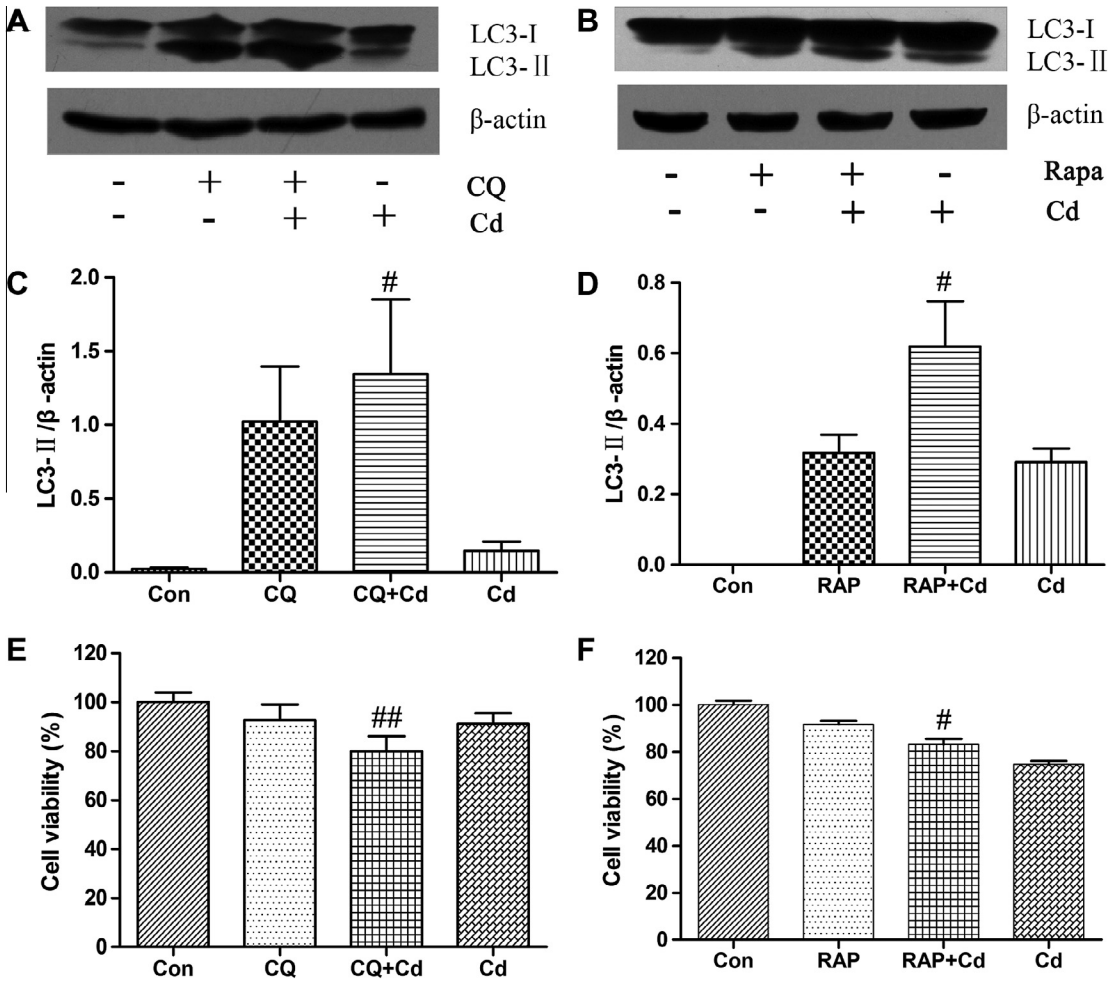

Fig. 3. Cd-induced autophagy is cytoprotective. PC-12 cells were pretreated with 5 μmol/L CQ for 0.5 h, followed by treatment with Cd for another 4 h, or pretreated with 100 nmol/L RAP for 24 h, followed by treatment with Cd for another 24 h. Western blot analysis was performed using LC3 antibody. The blots were probed for β-actin as a loading control (A and B). Blots for LC3 were semi-quantified using Image Lab (C and D). The respective cell viability using MTT assay is shown in (E and F). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ difference with Cd group.

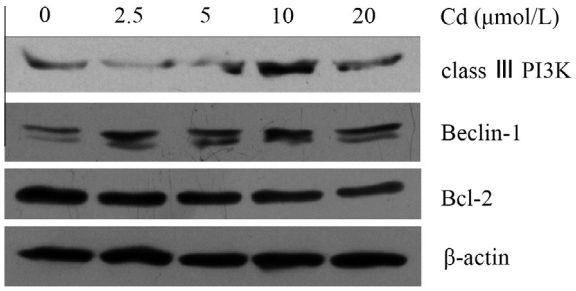

Fig. 4. The class III PI3 K/Beclin-1/Bcl-2 pathway contributes to Cd-triggered autophagy. PC-12 cells were treated with 0–20 μmol/L Cd for 4 h. Cell lysates were analyzed by western blot assay with indicative antibodies. The β-actin protein level was used as a loading control.

4. Discussion

The bioaccumulation of Cd has been proven toxic both for human and environmental health. Cd-induced adaptation to stress through the removal and recycling of damaged proteins and organelles may have been critical throughout evolution in biota that are naturally exposed to fluctuating levels of these toxicants [17]. The intracellular homeostasis disorder caused by Cd has recently been demonstrated to be a trigger for apoptosis in cortical neurons [18]. This study demonstrates that Cd can induce cytoprotective autophagy in PC-12 cells by delaying the occurrence of apoptosis and activating class III PI3 K/beclin-1/Bcl-2 signaling pathway.

Autophagy is morphologically characterized by the appearance of "double-membrane" vacuoles, that is, autophagosomes in the cytoplasm. In addition, LC3, the mammalian homologue of the yeast protein Atg8, was found to be a specific biochemical marker for autophagy. A newly synthesized LC3 termed LC3-I is evenly distributed throughout the cytoplasm. Upon the induction of autophagy, some LC3-I is converted into LC3-II which is tightly bound to autophagosomal membranes, thus forming ring-shaped structures in the cytosol. Cd affects a diverse range of cellular events such as proliferation, differentiation, and apoptosis. In this work, we initially demonstrate that Cd increased autophagy in PC-12 cells, as indicated by LC3 electrophoretic mobility shift, immunofluorescence for the autophagy protein LC3, and TEM.

In addition, Cd could induce apoptosis in PC-12 cells. Based on the EM results, the characteristics of apoptosis such as chromatin condensation and cytoplasmic vacuolization at the late stages of autophagy were determined. We thus postulated that autophagy activated by Cd in PC-12 cells was probably earlier than apoptosis. The results of flow cytometry also confirmed this hypothesis (Fig. 2B). It intrigues us to research the relationship between

autophagy and apoptosis. Some previous reports have proven that vascular endothelial cells treated with low concentrations (<10 μmol/L) of cadmium induced autophagy. As a consequence, apoptosis was inhibited [19]. However, autophagy in mesangial cells apparently has no significant effect on apoptosis [20]. This study suggested that Cd-induced autophagy can delay the occurrence of apoptosis in PC-12 cells. The above result is consistent with previous reports that autophagy preceded apoptosis under some stress conditions [21–23].

In mammalian cells, autophagy serves as a complement to the ubiquitin–proteasome system as two important intracellular pathways for protein degradation [24,25]. Autophagy may aid in cell survival by purging the toxic metabolites and intracellular pathogens from damaged cells. However, autophagy may also promote cell death through excessive self-digestion and degradation of essential cellular constituents. Dysregulation of autophagy contributes to age-related pathologies, neurodegenerative diseases, and carcinogenesis [3]. Given that the function of autophagy in cell survival or death is controversial and dependent not only on the duration and level of exposure, but also on other factors intrinsic to the cell itself, as well as its current metabolic state, the function of autophagy in Cd-exposed cells can differ. It was reported that autophagy did not protect cells from Cd-induced death such as mesangial cells [20]and skin epidermal cells [26], but a recent investigation supported the protective effect of autophagy on Cd-induced cell death in hematopoietic stem/progenitor cells [27], lung epithelial fibroblast cell [28] and proximal convoluted tubule cells [29], Our current findings suggest that autophagy involves cell survival mechanisms in Cd-exposed cells. This observation is supported by the results showing that CQ-mediated autophagy inhibition increases the levels of LC3-II proteins as the autophagy level is elevated by Cd treatment, whereas autophagy activators, such RAP, increase the levels of LC3-II proteins as the autophagy level is reduced by Cd treatment. In addition, Cd treatment decreased the viability of PC-12 cells in a dose- and time-dependent manner (Fig. 1A and B), which was significantly promoted by CQ treatment but inhibited by RAP treatment. Collectively, these results reveal that Cd-induced autophagy primarily acts as a protective mechanism in PC-12 cells. Additionally, autophagy did not completely protect cells from damage caused by Cd treatment. This condition, at least in part, may be contributed by other mechanisms, such as Cd toxicity on DNA repairing systems [30] and/or activation of P53, followed by cell cycle arrest and induction of cell death [31].

Among the direct activators of autophagy, Beclin-1, an essential autophagy protein that has been linked to diverse biological processes is well characterized. It was found that Beclin-1 and class III PI3 K can form a complex and are both required for autophagy [32,33]. However, under favorable physiological conditions, anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL interact with Beclin-1 (through the BH3 domain), thus inhibiting its pro-autophagic properties [34–36]. For Cd-stressed PC-12 cells, a drastic decrease in Bcl-2 expression was observed, which would enable Beclin-1 release, resulting in the combination of the Beclin-1-PI3 K III compound. These data are consistent with reports suggesting that during induced autophagy, the complex (Beclin-1-Bcl-2) is dissociated to enable Beclin-1 to join PI3 K III, thus initiating autophagosome formation [37].

In summary, our results demonstrate that Cd could induce viability inhibition as well as autophagy and apoptosis. At the early stages of Cd exposure, where autophagy acts as the principal, apoptosis becomes evident when the damage is irreversible. The findings further suggest that autophagy serves an important protective function against avoiding cytotoxicity through the class III PI3 K/beclin-1/Bcl-2 signaling pathways. Our findings provided new evidence for understanding the mechanism of Cd action in PC-12 cells.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31101866, 31172373), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the Specialized Research Fund for the Doctoral Program of Higher Education (20113250110003) and the Joint Foundation of Guizhou Province (LKB [2013]02). We are thankful to Dr Maozhi Hu for technical assistance in flow cytometry analysis.

References

[1] K.N. Dalby, I. Tekedereli, G. Lopez-Berestein, B. Ozpolat, Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer, Autophagy 6 (2010) 322–329.
[2] Y. Sheng, B. Sun, W.T. Guo, Y.H. Zhang, X. Liu, Y. Xing, D.L. Dong, 3-Methyladenine induces cell death and its interaction with chemotherapeutic drugs is independent of autophagy, Biochem. Biophys. Res. Commun. 432 (2013) 5–9.
[3] B. Levine, G. Kroemer, Autophagy in the pathogenesis of disease, Cell 132 (2008) 27–42.
[4] X. Yang, X. Zhong, J.L. Tanyi, J. Shen, C. Xu, P. Gao, T.M. Zheng, A.M. DeMichele, L. Zhang, Mir-30d regulates multiple genes in the autophagy pathway and impairs autophagy process in human cancer cells, Res. Commun. 431 (2013) 617–622.
[5] F. Scarlatti, R. Granata, A. Meijer, P. Codogno, Does autophagy have a license to kill mammalian cells?, Cell Death Differ 16 (2009) 12–20.
[6] N. Mizushima, B. Levine, A.M. Cuervo, D.J. Klionsky, Autophagy fights disease through cellular self-digestion, Nature 451 (2008) 1069–1075.
[7] W.J. Duan, F.L. Liu, R.R. He, W.L. Yuan, Y.F. Li, B. Tsoi, W.W. Su, X.S. Yao, H. Kurihara, Autophagy is involved in the effects of resveratrol on prevention of splenocyte apoptosis caused by oxidative stress in restrained mice, Mol. Nutr. Food Res. 57 (2013) 1145–1157.
[8] K. Ranjan, A. Sharma, A. Surolia, C. Pathak, Regulation of HA14-1 mediated oxidative stress, toxic response, and autophagy by curcumin to enhance apoptotic activity in human embryonic kidney cells, Biofactors (2013) [Epub ahead of print].
[9] Y. Chen, E. McMillan-Ward, J. Kong, S. Israels, S. Gibson, Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells, Cell Death Differ. 15 (2007) 171–182.
[10] J. Rennolds, S. Malireddy, F. Hassan, S. Tridandapani, N. Parinandi, P.N. Boyaka, E. Cormet-Boyaka, Curcumin regulates airway epithelial cell cytokine responses to the pollutant cadmium, Biochem. Biophys. Res. Commun. 417 (2012) 256–261.
[11] K. Łukawski, B. Nieradko, M. Sieklucka-Dziuba, Effects of cadmium on memory processes in mice exposed to transient cerebral oligemia, Neurotoxicol. Teratol. 27 (2005) 575–584.
[12] Y. Nishimura, J. Yamaguchi, A. Kanada, K. Horimoto, K. Kanemaru, M. Satoh, Y. Oyama, Increase in intracellular Cd^{2+} concentration of rat cerebellar granule neurons incubated with cadmium chloride: cadmium cytotoxicity under external Ca^{2+} free condition, Toxicol. In Vitro 20 (2006) 211–216.
[13] L. Chen, B. Xu, L. Liu, Y. Luo, H. Zhou, W. Chen, T. Shen, X. Han, C.D. Kontos, S. Huang, Cadmium induction of reactive oxygen species activates the mTOR pathway, leading to neuronal cell death, Free. Radic. Biol. Med. 50 (2011) 624–632.
[14] S. Chen, Y. Xu, B. Xu, M. Guo, Z. Zhang, L. Liu, H. Ma, Z. Chen, Y. Luo, S. Huang, CaMKII is involved in cadmium activation of MAPK and mTOR pathways leading to neuronal cell death, J. Neurochem. 119 (2011) 1108–1118.
[15] B. Xu, S. Chen, Y. Luo, Z. Chen, L. Liu, H. Zhou, W. Chen, T. Shen, X. Han, L. Chen, Calcium signaling is involved in cadmium-induced neuronal apoptosis via induction of reactive oxygen species and activation of MAPK/mTOR network, PLoS One 6 (2011) e19052.
[16] Y.R. Zhang, C.Y. Zhang, J.J. Wang, Y. Yan, J.H. Gu, J.C. Bian, X.Z. Liu, Z.P. Liu, Oxidative stress and mitogen-activated protein kinase pathways involved in cadmium-induced BRL 3A cell apoptosis, Oxid. Med. Cell. Longev., 2013 (in press).
[17] M. Di Gioacchino, C. Petrarca, A. Perrone, M. Farina, E. Sabbioni, T. Hartung, S. Martino, D.L. Esposito, L.V. Lotti, R. Mariani-Costantini, Autophagy as an ultrastructural marker of heavy metal toxicity in human cord blood hematopoietic stem cells, Sci. Total Environ. 392 (2008) 50–58.
[18] Y. Yan, J.C. Bian, L.X. Zhong, Y. Zhang, Y. Sun, Z.P. Liu, Oxidative stress and apoptotic changes of rat cerebral cortical neurons exposed to cadmium in vitro, Biomed. Environ. Sci. 25 (2012) 172–181.
[19] Z. Dong, L. Wang, J. Xu, Y. Li, Y. Zhang, S. Zhang, J. Miao, Promotion of autophagy and inhibition of apoptosis by low concentrations of cadmium in vascular endothelial cells, Toxicol. In Vitro 23 (2009) 105–110.

[20] S. Wang, Y. Shih, W. Ko, Y. Wei, C. Shih, Cadmium-induced autophagy and apoptosis are mediated by a calcium signaling pathway, Cell. Mol. Life. Sci. 65 (2008) 3640–3652.
[21] W. Han, J. Sun, L. Feng, K.F. Wang, D. Li, Q. Pan, Y. Chen, W. Jin, X. Wang, H. Pan, H. Jin, Autophagy inhibition enhances daunorubicin-induced apoptosis in K562 cells, PLoS One 6 (2011) e28491.
[22] M. Abedin, D. Wang, M. McDonnell, U. Lehmann, A. Kelekar, Autophagy delays apoptotic death in breast cancer cells following DNA damage, Cell Death Differ. 14 (2006) 500–510.
[23] Y. Cheng, F. Qiu, Y.C. Ye, Z.M. Guo, S.I. Tashiro, S. Onodera, T. Ikejima, Autophagy inhibits reactive oxygen species-mediated apoptosis via activating p38-nuclear factor-kappa B survival pathways in oridonin-treated murine fibrosarcoma L929 cells, FEBS J. 276 (2009) 1291–1306.
[24] N. Nedelsky, P.K. Todd, J.P. Taylor, Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection, Biochim. Biophys. Acta. 1782 (2008) 691–699.
[25] W.X. Ding, X.M. Yin, Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome, Autophagy 4 (2008) 141–150.
[26] Y.O. Son, X. Wang, J.A. Hitron, Z. Zhang, S. Cheng, A. Budhraja, S. Ding, J.C. Lee, X. Shi, Cadmium induces autophagy through ROS-dependent activation of the LKB1-AMPK signaling in skin epidermal cells, Toxicol. Appl. Pharmacol. 255 (2011) 287–296.
[27] M. Di Gioacchino, C. Petrarca, A. Perrone, S. Martino, D.L. Esposito, L. Vittoria, Autophagy in hematopoietic stem/progenitor cells exposed to heavy metals, Autophagy 4 (2008) 537–539.
[28] S.C. Lim, K.S. Hahm, S.-H. Lee, S.H. Oh, Autophagy involvement in cadmium resistance through induction of multidrug resistance-associated protein and counterbalance of endoplasmic reticulum stress WI38 lung epithelial fibroblast cells, Toxicology 276 (2010) 18–26.
[29] A. Chargui, S. Zekri, G. Jacquillet, I. Rubera, M. Ilie, A. Belaid, C. Duranton, M. Tauc, P. Hofman, P. Poujeol, Cadmium-induced autophagy in rat kidney: an early biomarker of subtoxic exposure, Toxicol. Sci. 121 (2011) 31–42.
[30] C. Giaginis, E. Gatzidou, S. Theocharis, DNA repair systems as targets of cadmium toxicity, Toxicol. Appl. Pharmacol. 213 (2006) 282–290.
[31] Z.M. Liu, G.G. Chen, A. Vlantis, G. Tse, C. Shum, C. Van Hasselt, Calcium-mediated activation of PI3K and p53 leads to apoptosis in thyroid carcinoma cells, Cell. Mol. Life. Sci. 64 (2007) 1428–1436.
[32] A. Simonsen, S.A. Tooze, Coordination of membrane events during autophagy by multiple class III PI3-kinase complexes, J. Cell Biol. 186 (2009) 773–782.
[33] B. Levine, S. Sinha, G. Kroemer, Bcl-2 family members: dual regulators of apoptosis and autophagy, Autophagy 4 (2008) 600–606.
[34] M.C. Maiuri, G. Le Toumelin, A. Criollo, J.C. Rain, F. Gautier, P. Juin, E. Tasdemir, G. Pierron, K. Troulinaki, N. Tavernarakis, Functional and physical interaction between Bcl-XL and a BH3-like domain in Beclin-1, EMBO J. 26 (2007) 2527–2539.
[35] C. He, B. Levine, The beclin 1 interactome, Curr. Opin. Cell Biol. 22 (2010) 140–149.
[36] R. Kang, H. Zeh, M. Lotze, D. Tang, The Beclin 1 network regulates autophagy and apoptosis, Cell Death Differ. 18 (2011) 571–580.
[37] S. Sinha, B. Levine, The autophagy effector Beclin 1: a novel BH3-only protein, Oncogene 27 (2008) S137–S148.