Inhibition of autophagy enhanced cobalt chloride-induced apoptosis in rat alveolar type II epithelial cells

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Abstract. Hypoxia is a type of cellular stress that may result in apoptosis and autophagy. The molecular mechanisms underlying the association between autophagy and apoptosis remain unclear, particularly in hypoxic conditions. Transmission electron microscope, AO-PI staining, flow cytometry and western blot were used to examine the crosstalk between autophagy and apoptosis in hypoxic conditions. Rat alveolar type II epithelial RLE-6TN cells were cultured in a long-term hypoxic environment established by cobalt (II) chloride. It was demonstrated that autophagy and apoptosis occurred in RLE-6TN cells under hypoxic conditions. Treatment of RLE-6TN cells with the autophagy inhibitor 3-methyladenine increased the generation of reactive oxygen species, mitochondrial damage and hypoxia-induced apoptosis. The expression of caspases, particularly caspase-9, increased and may have participated in these processes. The data indicated that the inhibition of autophagy enhanced apoptosis through the mitochondria-mediated intrinsic pathway. These findings provide important insight into the molecular mechanism of autophagy and apoptosis crosstalk. This may provide new insights into pulmonary disease surveillance, diagnosis and treatment.

Introduction

Hypoxia results in pathophysiological damage to various cells and tissues. Cells possess a powerful regulatory system involving hypoxia-inducible factors (HIFs) that respond to hypoxic stress (1-3). HIF-1α regulates metabolic adaptation to hypoxia and activates multiple target genes, including vascular endothelial growth factor, erythropoietin and nuclear factor-kB under hypoxic conditions (4). Furthermore, HIF-1α has been implicated as a co-regulator of autophagy activation and also participates in apoptosis (5).

Autophagy protects cells from various damaging factors, including hypoxia or starvation and maintains intracellular stability (6). It has been demonstrated that autophagy is closely associated with apoptosis under hypoxic condition (5). Apoptosis is a type of programmed cell death which is triggered by intrinsic or extrinsic signals (7). Hypoxia and cobalt (II) chloride (CoCl₂) treatment activate autophagy through the target genes induced by HIF, such as mechanistic target of rapamycin (2) and correlate with the expression of certain pro-apoptotic factors, including caspase-9 and caspase-3 (8,9). These results suggest that autophagy and apoptosis often occur in parallel following CoCl₂ treatment, with autophagy typically occurring prior to apoptosis. The important role of autophagy may be associated with the regulation of apoptosis. It has been reported that autophagy attenuates cellular injury by inhibiting the induction of apoptosis (10).

Autophagy and apoptosis may serve pivotal roles in neurodegenerative disease (11). A previous report revealed that autophagy serves a dual role, by exhibiting a protective role for cell survival, but autophagy may also promote apoptosis associated with various lung diseases, including chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis and sepsis (12). However, the molecular mechanism of crosstalk between autophagy and apoptosis remains unclear. In the present study, the association between autophagy and apoptosis induced by long-term CoCl₂ treatment in RLE-6TN cells was investigated. The results suggested that autophagy provided a survival strategy for RLE-6TN cells via apoptosis inhibition. The present study may aid in elucidating the underlying molecular mechanism of autophagy and apoptosis interaction in a hypoxic environment, which may contribute to the development of novel treatments for the therapy of lung diseases.

Materials and methods

Reagents and antibodies. CoCl₂, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), Tris, glycine, Tween-20, SDS, acridine orange (AO), propidium iodide (PI), anti-microtubule associated...
proteins 1A/1B light chain 3B (LC3/II; cat. no. L7543) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Trypsin-EDTA was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Trypsin solution without EDTA was purchased from Beyotime Institute of Biotechnology (Hangzhou, China). The Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). Primary antibodies were purchased from CST Biological Reagents, Co., Ltd. (Shanghai, China) or Abcam (Cambridge, UK). Secondary antibodies were purchased from OriGene Technologies, Inc. (Beijing, China). All other chemicals were obtained from Sigma-Aldrich (Merck KGaA). Radioimmunoprecipitation assay (RIPA) lysis buffer (1 ml RIPA; 10 µl PMSF) for 30 min. Protein concentration was determined with a bicinchoninic acid protein assay kit and proteins (50 µg/lane) were separated by 12 and 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked at room temperature for 2 h with 5% skimmed milk in Tris-buffered saline with 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; ZB2301; OriGene Technologies, Inc.) antibody for 1 h at room temperature. Proteins were visualized with an enhanced chemiluminescence substrate kit (Clixn Science Instruments Co., Ltd., Shanghai, China) and standard X-ray film development (ChemiScope 3,300 mini; Clinx Science Instruments Co., Ltd.). Results were quantified with ImageJ software v1.48 (National Institutes of Health, Bethesda, MD, USA) and processed using Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA, USA).

Results

Hypoxia induces organelle impairment and autophagosome generation. Previous studies have demonstrated that cancer cells adapt to hypoxia or nutrient deprivation through autophagy activation (15,16). In the present study, TEM was used to observe the ultrastructure following CoCl₂ treatment.

Statistical analysis. All experiments were performed at least three times. The data were expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance followed by Fisher’s least significant difference. P<0.05 was considered to indicate a statistically significant difference.
in RLE-6TN cells (Fig. 1). Compared with untreated cells (Fig. 1A), numerous autophagosomes consisting of double membranes were observed in the CoCl$_2$-treated RLE-6TN cells on days 5 and 7 (Fig. 1B and C, respectively). No notable autophagosomes were observed after 1 and 3 days following treatment with CoCl$_2$ (data not shown). Cytoplasmic material and/or membrane vesicles were encapsulated in the autophagosomes. Furthermore, swollen endoplasmic reticuli and damaged mitochondria were also observed following CoCl$_2$ treatment (Fig. 1B and C). These results indicated that 100 µm CoCl$_2$ treatment resulted in severe organelle impairment and induced autophagy generation.

**3-MA enhances apoptosis in hypoxic conditions.** Apoptosis is characterized by distinct morphological features and energy-dependent biochemical mechanisms. Cell pyknosis, cell shrinkage and chromatin condensation typically occur in early apoptosis (17,18). Budding in late apoptosis is a result of extensive blebbing of the plasma membrane to form apoptotic bodies containing tightly packed organelles (19). In the present study, fluorescence microscopy was used to detect the morphological changes in RLE-6TN cells following CoCl$_2$ and/or 3-MA treatment for 1, 3, 5 and 7 days (Fig. 2A-D). CoCl$_2$ treatment resulted in a reddish-orange color in nucleus due to the binding of PI to denatured DNA. Lysosomes in the cytoplasm were stained orange by PI in Fig. 2. This abundance of apoptotic cells, as indicated by arrows, was more prominent as the incubation time increased. 3-MA markedly increased the number reddish-orange stained nuclei in 100 µm CoCl$_2$-treated cells (Fig. 2D). These results indicated that autophagy was associated with apoptosis in RLE-6TN cells in hypoxic conditions and that autophagy may have a protective role in a hypoxic environment.

The apoptotic effect induced by CoCl$_2$ was further confirmed by flow cytometry (Fig. 3A). The results demonstrated that CoCl$_2$ treatment significantly increased in the percentage of cells in upper right and lower right quadrants (early and late apoptotic cells, respectively) after 3 days of treatment. 3-MA significantly increased CoCl$_2$-induced apoptosis in RLE-6TN cells at days 5 and 7 (P<0.05; Fig. 3B).

Taken together, these results suggested that the inhibition of autophagy strongly accelerated the process of apoptosis. 

**Inhibiting autophagy increases mitochondrial damage.** Mitochondria have a double-membrane structure and are key organelles for the production of energy (20). In addition, mitochondria regulate cellular redox signaling pathways and programmed cell death (20). In order to explore the effect of 3-MA in CoCl$_2$-treated mitochondria, TEM was used to examine the mitochondrial ultrastructure. As presented in Fig. 4, swollen mitochondria were observed following CoCl$_2$ treatment at day 7, compared with the control. Mitochondrial damage was not notably observed on day 3; however, more notable damage was reported on day 5 (data not shown). Notably, more marked
mitochondrial damage was observed in the CoCl$_2$ + 3-MA group, including severely swollen mitochondria, double-membrane destruction and loss of normal morphology, suggesting that the inhibition of autophagy increased the extent of mitochondrial damage in RLE-6TN cells under hypoxic conditions.

Inhibiting autophagy increases the production of ROS. ROS are critical regulators in various cellular processes, including autophagy and apoptosis (21, 22). Mitochondria are a major source of ROS in cells (23). A fluorescent probe, DCFH-DA, was used to detect the generation of ROS. As presented in Fig. 5, DCFH-DA fluorescence was markedly increased following CoCl$_2$ and 3-MA treatment, compared with CoCl$_2$ treatment alone. The results revealed that the inhibition of autophagy with 3-MA increased ROS generation and increased the rate of apoptosis in RLE-6TN cells. These results indicated that autophagy may prevent cell impairment in a hypoxic environment.

3-MA upregulates the expression of caspases in hypoxic conditions. Autophagy and apoptosis are two important catabolic processes (24) and the relationship between them remains unclear. In order to explore the complex crosstalk between autophagy and apoptosis, the expression of several proteins associated with these processes in response to hypoxia was investigated. As presented in Fig. 6, HIF-1α expression was significantly upregulated on days 2, 3, 5 and 7 after treatment with CoCl$_2$. This data indicated that the hypoxia model was successfully conducted in our study. In addition, cleaved-caspase-9, cleaved-caspase-8, cleaved-caspase-3, LC3II/I exhibited increasing trend; the expression levels were significantly higher on days 5 and 7 compared with in the control. Notably, 3 days following CoCl$_2$ treatment, cleaved-caspase-8 also significantly increased. Following 3-MA treatment (Fig. 7), the expression levels of LC3II/LC1, key proteins in autophagy, did not exhibit significant alterations under hypoxic conditions compared with in the control. By contrast, expression levels of cleaved caspase-9 and cleaved caspase-3 were significantly increased, particularly on days 5 and 7. Compared with cleaved-caspase-9 and cleaved-caspase-3, the expression level of cleaved caspase-8 appeared to be upregulated to a lesser degree. These results indicated that autophagy and apoptosis occurred in RLE-6TN cells under hypoxic conditions. Inhibition of autophagy may have accelerated apoptosis, predominantly through caspase-9-mediated intrinsic pathways in RLE-6TN cells in a chronic hypoxic environment.

Discussion

Cellular stress stimuli, such as hypoxia, may induce cells to exhibit their dual role (25). Cells may activate cytoprotective pathways, such as autophagy, to favor survival until the stress is resolved. Conversely, cells also may undergo programmed cell
death. CoCl₂ has been extensively used as a reagent to establish hypoxia in vitro (26-29). In the present study, autophagy and apoptosis occurred in RLE-6TN cells following exposure to CoCl₂. The association between these two processes is complex.

Autophagy serves an essential role in cellular catabolism (30). Autophagy is a process which involves the engulfment of cytoplasmic materials and intracellular organelles within autophagosomes, which are subsequently delivered to lysosomes (31). The materials within autophagolysosomes provide energy in order to maintain cell metabolism. Cell survival may be impeded if autophagy is interrupted (32). By contrast, excessive autophagy may lead to a type of cell death known as autophagic apoptosis (33). Apoptosis is a highly controlled genetic program of cell death, which also serves a critical role...
in determining cell fate. Apoptosis is triggered by multiple signaling pathways, including the extrinsic and intrinsic signaling pathways (34). Zhang et al (35) demonstrated that the inhibition of autophagy with 3-MA increases apoptosis in a mouse model of middle cerebral artery occlusion (MCAO). In addition, hypoxia-induced autophagy decreases the production of cytochrome c and the activation of caspase-mediated apoptotic pathways (35). Therefore, the ischemia-induced neuronal injury is depressed in an MCAO model following treatment with 3-MA. Yan et al (36) demonstrated that chronic ischemia induces autophagy in the heart, and the areas of damaged heart have fewer apoptotic cells due to an increase in autophagy. By contrast, Cheng et al (14) revealed that pre-treatment of human malignant glioma U87-MG cells in hypoxic conditions with autophagy inhibitors suppresses cell apoptosis and caspase-3 activation. These studies were performed in different experimental models and suggest that there is a complex interaction between autophagy and apoptosis. Examining the potential underlying mechanisms of interaction in various environments of cellular stress will aid in understanding the relationship between autophagy and apoptosis.

Evidence suggests that mitochondria are the main intracellular source of ROS in cells (37,38). ROS participate in various cellular processes, including autophagy and apoptosis (39). Accumulation of ROS impairs mitochondrial function (40). In response to diverse pathological conditions, including oxidative stress, autophagy serves a dual role by exhibiting protective and harmful effects, which promote cell survival and apoptosis, respectively (12,41). Dewaele et al (42) demonstrated that ROS-dependent accumulation of LC3-PE facilitates autophagosome formation. Previous studies have demonstrated that autophagy may inhibit apoptosis in hypoxic conditions through ROS removal and inhibition of caspase activation (43,44). Chien et al (45) revealed that following ischemia/reperfusion in the kidney, autophagy serves a central role in ROS clearance and prevents apoptosis. The results of the present study were consistent with this finding: An increase in mitochondrial damage and dysfunction was observed following autophagy inhibition with 3-MA, resulting in increased ROS production. In addition, the expression levels of apoptosis-associated proteins were increased following autophagy inhibition, including caspase-9, caspase-8 and caspase-3. Furthermore, the number apoptotic cells increased. It is well established that the intrinsic apoptotic pathway is predominantly dependent on caspase-9, and the extrinsic apoptotic pathway is predominantly mediated by caspase-8 (14). Notably, the expression level of caspase-9 may have increased faster and to a greater degree compared with caspase-8 following 3-MA treatment. Therefore, the results of the current study demonstrated that 3-MA treatment in hypoxic conditions increased the production of ROS and the apoptotic rate, primarily through the caspase-9-dependent intrinsic apoptotic pathway. Furthermore, these results suggested that autophagy may serve a protective role in the early stages of lung damage in a hypoxic environment.

In conclusion, autophagy served a protective role in RLE-6TN cells, via inhibition of ROS production and prevention of apoptosis in hypoxic conditions. The present study only investigated the effects of one inhibitor in one cell line. Therefore, further research is required to provide further
evidence, in order to explore the potential molecular mechanisms between hypoxia-induced autophagy and apoptosis. This may provide new insights into pulmonary disease surveillance, diagnosis and treatment.
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Availability of data and materials

Not applicable.

Author’s contributions

YY made substantial contributions to the conception and design of the present study. WL conducted the AO-PI staining, western blotting, ROS detection and drafted the manuscript. LR conducted western blotting, analyzed the data and critically revised the manuscript for important intellectual content. CY conducted TEM. DL and XH made substantial contributions to the conception and western blotting. YS conducted flow cytometry. CL CY conducted TEM. DL and XH made substantial contributions to the conception and author's contributions.

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