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RESEARCH ARTICLE

Cadmium induces apoptosis and autophagy in swine small intestine by downregulating the PI3K/Akt pathway

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Abstract

Cadmium (Cd) is an environmental contaminant, which is potentially toxic. It is well known that Cd can accumulate in the liver and kidney and cause serious damage. However, few studies have investigated the mechanism of intestinal damage induced by Cd in swine. Here, we established Cd poisoning models in vivo and in vitro to explore the mechanism of intestinal injury induced by Cd in swine. The morphology of intestinal tissue cells was observed by TUNEL staining and electron microscopy, and the morphology of IPEC-J2 cells was observed by flow cytometry, Hoechst staining, and MDC staining. Cell morphological observations revealed that Cd treatment induced ileal apoptosis and autophagy. The effects of Cd on the PI3K/Akt pathway, as well as on apoptosis and autophagy-related protein expression in intestinal cells, were analyzed by western blot (WB) and the expression of mRNA was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The results showed that Cd induced autophagy by increasing the levels of autophagy markers Beclin1, Autophagy-associated gene 5 (ATG5), Autophagy-associated gene 16 (ATG16), and Microtubule-associated protein light chains 3–2 (LC3-II), and by reducing the expression levels of Mechanistic target of rapamycin kinase (mTOR) and Microtubule-associated protein light chains 3–1 (LC3-I). Cell apoptosis was induced by increasing the expression of apoptosis markers Bcl-2 associated X protein (Bax), Cysteinyl aspartate specific proteinase 9 (Caspase9), cleaved Caspase9, Cysteinyl aspartate specific proteinase 3 (Caspase3), and cleaved Caspase3, and by reducing the expression of B cell lymphoma/leukemia 2 (Bcl-2). At the same time, Cd decreased the expression of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), and their phosphorylation. We treated IPEC-J2 cells with the PI3K activator 740Y-P and analyzed the morphological changes as well as autophagy and apoptosis-related gene expression. The results showed that 740Y-P could reduce apoptosis and autophagy induced by Cd. In conclusion, our findings suggest that Cd induces intestinal apoptosis and autophagy in swine by inactivating the PI3K/Akt signaling pathway.

Keywords Environmental pollution · Cadmium exposure · Apoptosis · Autophagy · PI3K/Akt pathway · Swine small intestine

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Introduction

Cadmium (Cd) is highly toxic and it is considered to have no biological function in organisms. It is increasingly found in the environment due to industrial and agricultural practices(Ayres 1992). Human activities release Cd into the environment, causing pollution of the soil and water, which may lead to the gradual accumulation of Cd in vegetation and aquatic organisms(Chen et al. 2021; Gong et al. 2021). Moreover, once Cd pollutes the natural environment, it can accumulate in animals through food chain biomagnification(Skipper et al. 2016), which may lead to chronic poisoning and organ damage, including kidney injury, liver injury and heart injury, and in particular,

gastrointestinal injury. When Cd enters the body through the diet, only a small amount is systemically absorbed as most of the Cd is blocked by the gastrointestinal mucosa, where damage will occur(Breton et al. 2016). The small intestine can be seriously damaged by Cd (Ni et al. 2020). For a long time, it has been thought that heavy metals can damage the digestive system. Recent studies have shown that exposure to Cd destroys the structure of the intestinal tract, weakens its immune defense ability, and causes intestinal tract inflammation (Xie et al. 2019). Oral administration of Cd can cause intestinal injury and inflammation in rats (Ninkov et al. 2015). However, an understanding of its pathogenesis is still incomplete. Therefore, the mechanism of Cd-induced gastrointestinal toxicity needs to be further elucidated.

Apoptosis is an important mechanism regulated by a series of genes to maintain the stability of the body in the environment and allow the body to better adapt to the living environment (Li et al. 2021). However, excessive apoptosis is deleterious. Many environmental pollutants can lead to apoptosis. For instance, continuous H_2S intoxication triggers apoptosis, leading to immune injury in broilers (Hu et al. 2018) and nickel nanoparticles can induce apoptosis of germ cells in rat testes (Kong et al. 2019). In addition, Cd, as one of the most common environmental pollutants, can also cause apoptosis (Yuan et al. 2020; Zhang et al. 2020). For instance, Cd can induce osteoblast apoptosis through the p53 pathway (Zheng et al. 2020). Autophagy is a process in which cells degrade themselves through lysosomes (Allaire et al. 2019). Autophagy occurs in the body under normal circumstances, but it is maintained at a low level. However, adverse conditions, such as nutritional deficiencies, are also capable of inducing autophagy (Zheng et al. 2021). Under these environmental conditions, autophagy protects or destroys cells (Miao et al. 2021). Simultaneously, Cd can also induce autophagy. For instance, Cd can induce JNK-dependent autophagy in chicken kidney cells (Shi et al. 2019) and autophagy can also occur in duck kidney cells under the influence of Cd (Cz et al. 2021). Moreover, the PI3K/Akt signaling pathway is an important signal transduction pathway that has been proven to be involved in apoptosis and autophagy. For example, cancer cell apoptosis and autophagy are enhanced after the inhibition of the PI3K/Akt/mTOR pathway (Kumar et al. 2015). In addition, downregulation of the PI3K/Akt/mTOR pathway by capsaicin induces autophagy and apoptosis of cancer cells (Lin et al. 2017), and it also has been found that Cd can mediate cell injury through the PI3K/Akt signaling pathway. For example, the P2X7/PI3K/Akt pathway is involved in Cd-mediated differentiation of osteoblasts and osteoclasts, which leads to duck osteoporosis (Ma et al. 2021); Cd can also induce chicken hepatocyte apoptosis through PI3K/Akt pathway (Xiong et al. 2020). It is therefore clear that the PI3K/Akt signaling pathway plays an indispensable role in the cell damage induced by heavy metals.

In our study, IPEC-J2 cells were selected as an in vitro model and the findings were verified in vivo in porcine ileum to clarify whether Cd induces apoptosis and autophagy in the porcine small intestine through the PI3K/Akt pathway.

Materials and methods

Experimental animals and treatment

All procedures in this study were carried out in accordance with the European Community Council Directive (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11).

Ten 6-week-old, weaned piglets were randomly divided into two groups, Con (control group) and Cd (cadmium exposure group) (5 piglets/group). The piglets in the control group were fed with a basic diet and the piglets in the experimental group were fed with a basic diet containing 20 mg/kg $CdCl_2$. The concentration of cadmium chloride used in the experiment was based on our previous studies on Cd exposure in swine (Cai et al. 2021). On the 41st day of the experiment, the piglets were euthanized and their ileal tissues were extracted, washed with cold aseptic deionized water, and immediately frozen in liquid nitrogen until use.

Cell culture and treatment

The IPEC-J2 cell line, which is often used in the study of pig intestines, is a cell line isolated from the middle jejunum of newborn piglets. Eagle's medium (Gibco, New York, USA) was used as the IPEC-J2 cell culture medium, to which 1% penicillin–streptomycin (Gibco, New York, USA), and 10% fetal bovine serum (FBS; Gibco, New York, USA) were added. The medium was filtered with a 0.22-μm microporous filter. The cells were inoculated in a 6-well plate with an inoculation density of 3×10^5 and cultured in an incubator (37℃, 5% CO_2). The cells were divided into four groups: Con group (control group), Cd group (the concentration of Cd^{2+} was 5 μM), 740Y-P group (the concentration of 740Y-P was 20 μM), and 740Y-P + Cd group (co-treated with 740Y-P and Cd for 6 h). The challenge concentration of 740Y-P was formulated based on a recent study (Gu et al. 2019) and the concentration of cadmium chloride used in the experiment was based on our previous studies on Cd exposure in swine (Chen et al. 2021).

Sections for electron microscopy

The ileal tissue samples were cut to a size of 1.0 mm × 1.0 mm × 1.0 mm, and fixed in 2.5% glutaraldehyde

phosphate buffer (v/v, pH 7.2). 1% samarium tetroxide (v/v) was used for tissue fixation, and 4.8% uranyl acetate was used for dyeing. The tissue samples were dehydrated by different concentrations of ethanol, and after dehydration, the tissue samples were embedded in EPON. The samples were cut into thin slices (≤90 nm), affixed to a copper mesh, washed with propylene oxide, and then impregnated with epoxy resin; the impregnated samples were then stained with uranyl acetate and lead citrate. The micrographs were obtained by transmission electron microscope (GEM-1200ES, Japan).

Apoptosis assay

TUNEL staining

A TUNEL detection kit (Roche) was used to measure apoptosis in accordance with the manufacturer's instructions. The ileal tissue sections were embedded in paraffin, and the ileal tissues were treated with protease K and hydrogen peroxide. The ileal tissue sections were incubated at 37 °C for 1 h. Phosphate-buffered saline (PBS) was used to rinse tissue samples. The nuclei were labeled with horseradish peroxidase and diaminobenzidine; hematoxylin was used for counterstaining.

Hoechst staining

After rinsing once with PBS, the culture medium was removed and the IPEC-J2 cells were stained with Hoechst 33,258 fluorescent dye for 30 min and rinsed 3 times with PBS. The cells were then treated with fluorescence antiquenchers and a fluorescence microscope was used to observe the morphology of cells (Chi et al. 2021a).

Flow cytometry

IPEC-J2 cells were double stained with an Annexin V-FITC/PI apoptosis detection kit, according to the manufacturer's instructions. Annexin V-FITC (5 μL) and propidium iodide (PI) (5 μL) were added to the 1 mL binding buffer, and then, cells were stained with 30 min to detect apoptosis. After incubation, flow cytometry was used to measure apoptotic cells.

MDC staining

Autophagy staining was performed with an autophagy detection kit (Beijing Solar Biotechnology, Beijing, China). Ten-microliter MDC stain was added to the medium of IPEC-J2 cells and cultured at 37℃ for 45 min. After staining, the culture medium was aspirated with a liquid transfer gun, and the cells were washed 3 times with PBS. A fluorescence microscope was used to observe the morphology of the cells.

Determination of mRNA expression of PI3K/Akt pathway, apoptosis, and autophagy-related genes

The total RNA in vitro and in vivo was extracted using TRIzol (Invitrogen) reagent. The dried RNA pellets were re-suspended in 50μL of diethyl-pyrocarbonatetreated water and the total RNA concentration was determined by spectrophotometer. Following the manufacturer's instructions (Thermo Scientific, Massachusetts, USA), a kit was used to synthesize the first strand of complementary RNA with 3 μg total DNA. The cDNA was diluted by the addition of 9 times the volume of water and preserved at − 80 °C (Song et al. 2021; Liu et al. 2022). The sequences for PI3K, Akt, Bcl-2, Bax, Caspase9, Caspase3, mTOR, Beclin1, ATG5, ATG16, LC3-I, and LC3-II were obtained by GenBank (Table 1) and primers were synthesized by Sangon Biotech (Shanghai, China) Company. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference. The quantification process of relative mRNA abundance has been described in our previous studies (Chi et al. 2021b). The equation for this calculation method is relative expression $= 2^{-\Delta\Delta C_T}$. $\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$. $\Delta C_{T(test)} = \Delta C_{T(target,\ test)} - \Delta C_{T(reference,\ test)}$. $\Delta C_{T(calibrator)} = \Delta C_{T(target,\ calibrator)} - \Delta C_{T(reference,\ calibrator)}$.

Table 1 The primers used in the present study

Target gene	Primer sequence (5′—3′)
Bax	Forward 5′-AGCAGATCATGAAGACAGGGG-3′ Reverse 5′-CCAATGCGCTTGAGACACTC-3′
Bcl-2	Forward 5′-GCGCGTTAAGGGTCTGAGAT-3 Reverse 5′-ATCACAGTACGGTTGGCAGG-3
Caspase3	Forward 5′- GGATTGAGACGGACAGTGGG-3′ Reverse 5′-CCGTCCTTTGAATTTCGCCA-3′
Caspase9	Forward 5′-GTCTGTGGTCCAGACAGAGC-3′ Reverse 5′-GGCCTTGGCAGTCAGGTT-3′
mTOR	Forward 5′-CTGAATATGCCGCCAACA-3′ Reverse 5′-TCTCCTCTGTCTCTGGTTC-3′
Beclin1	Forward 5′-TCGTGTTACCATTCAGGAG-3′ Reverse 5′-TGTTCGCTGTCATCTTCAT-3′
ATG5	Forward 5′-TCACAAGCAACTCTGGATG-3′ Reverse 5′-GCTGATGGGTTTGCTTTT-3′
ATG16	Forward 5′-TCGCAGAAGCAGCAAAGGAACC-3′ Reverse 5′-TGATGGCTCGCACGGGAGAG-3′
LC3-I	Forward 5′-CCAGTCCTGGACAAGACCAAGTTC-3′ Reverse 5′-GGTTCACCAGCAGGAAGAAGGC-3′
LC3-II	Forward 5′-TTCCTGGTGCCTGATCATGTCAAC-3′ Reverse 5′-ACTCACCATGCTATGTCCGTTCAC-3′
PI3K	Forward 5′-ACGGCAATGTGGAGCAGATGAAG-3′ Reverse 5′-TGGTAGAGCAGGAGGAAGTGGTC-3′
Akt	Forward 5′-TCAAGAACGACGGCACCTTCATC-3′ Reverse 5′-CGCCACGGAGAAGTTGTTGAGG-3′
GAPDH	Forward 5′-GTGAACGGGTGAGTTAGGGG-3′ Reverse 5′-CGATGCGGCCAAATCTTGAG-3′

Determination of protein expression of PI3K/Akt pathway, apoptosis, and autophagy-related genes

Cell lysis buffer was added to ileal tissues and IPEC-J2 cells, and western blotting (WB) analysis was performed. Total protein was separated by 12% gel SDS-PAGE. The glycine buffer containing 20% methanol was prepared and the separated protein was transferred to a nitrocellulose membrane at 200 mA for 80 min. The nitrocellulose membrane was blocked with 5% bovine serum albumin (BSA) at 37 °C for 1 h and incubated with the following antibodies for 12 h: PI3K, phospho-PI3K (p-PI3K), Akt, phospho-Akt (p-Akt), Bcl-2, Bax, Caspase9, cleaved Caspase9, Caspase3, cleaved Caspase3, mTOR, Beclin1, ATG5, LC3, GAPDH. The primary antibodies were shown in Table 2. The membrane was then incubated with HRP-conjugated goat anti-mouse IgG. GAPDH was used as an internal reference gene (Shengchen et al. 2021).

Statistical analysis

All statistical analyses were performed in the GraphPad Prism 5.0 software using the *t* test. Where differences were apparent, the Bonferroni *t* test was used to compare the means; $P < 0.05$ was considered statistically significant (Zhao et al. 2021).

Results

Cadmium induced apoptosis in ileal histiocytes and IPEC-J2 cells

Transmission electron microscope (TEM) analysis showed that the ultrastructure of ileal cells in the control group was intact, normal and had no obvious changes. On the contrary, the typical characteristics of apoptosis were observed in the Cd group, including nuclear chromatin condensation and cytoplasmic vacuolization (blue arrows) (Fig. 1a). TUNEL analysis showed that there were few apoptotic cells in the control group, but the number of apoptotic cells increased significantly in the Cd treated group (identified by the number of green staining nuclei with DNA strand breaks) (orange arrows) (Fig. 1c).

In vitro, the results of flow cytometry analysis showed that compared to the control cells, the apoptosis rate of Cd-treated cells increased by 4.9% (Fig. 1b); this difference was statistically significant ($P < 0.05$). These results suggest that Cd can induce apoptosis of IPEC-J2 cells.

The effect of Cd treatment on the mRNA of genes related to apoptosis (Bax, Bcl-2, Caspase9, and Caspase3) is revealed in Fig. 2a, d. The qRT-PCR results showed that Bax, Caspase9, and Caspase3 were increased significantly ($P < 0.05$), and Bcl-2 was decreased in the Cd group in vitro (Fig. 2a) and in vivo (Fig. 2d) ($P < 0.05$). The protein expression levels were detected by WB analysis. Bax, Caspase9, cleaved Caspase9, Caspase3, and cleaved Caspase3 were increased significantly ($P < 0.05$), and Bcl-2 was decreased significantly at the protein level in the Cd groups in vitro (Fig. 2b, c) and in vivo (Fig. 2e, f) ($P < 0.05$). These results indicate that Cd induced apoptosis in porcine ileum in vitro and in vivo.

Table 2 The antibodies used in the present study

Antibody name	Dilution ratio
PI3K	1:1000
p-PI3K	1:500
Akt	1:1000
p-Akt	1:1000
Bcl-2	1:500
Bax	1:1000
Caspase9	1:1000
Caspase3	1:1000
mTOR	1:800
Beclin1	1:1000
ATG5	1:1000
LC3II	1:800
GAPDH	1:1000

Cadmium induced autophagy in ileal histiocytes and IPEC-J2 cells

In addition to the apoptosis observed by electron microscopy, we also observed typical features of autophagy, where the autophagosomes and vacuoles have been observed in the Cd group as well (orange arrows) (Fig. 1a).

The effect of Cd treatment on the mRNA of genes related to autophagy (mTOR, Beclin1, ATG5, ATG16, LC3-I, and LC3-II) is revealed in Fig. 3a, d. The results of the qRT-PCR showed that Beclin1, ATG5, ATG16, and LC3-II were significantly increased, and that LC3-I and mTOR were significantly decreased, in the Cd groups compared to the cells in the control groups in vitro (Fig. 3a) and in vivo (Fig. 3d) ($P < 0.05$). WB analysis was used to detect the protein expression of genes related to autophagy. Compared to the cells in the control groups, Beclin1, ATG5, and LC3-II/I were increased significantly ($P < 0.05$) and mTOR was decreased significantly ($P < 0.05$) at the protein level in the Cd groups both in vitro (Fig. 3b, c) and in vivo (Fig. 3e, f). The results indicate that Cd treatment induced autophagy in IPEC-J2 cells and swine ileum tissue.

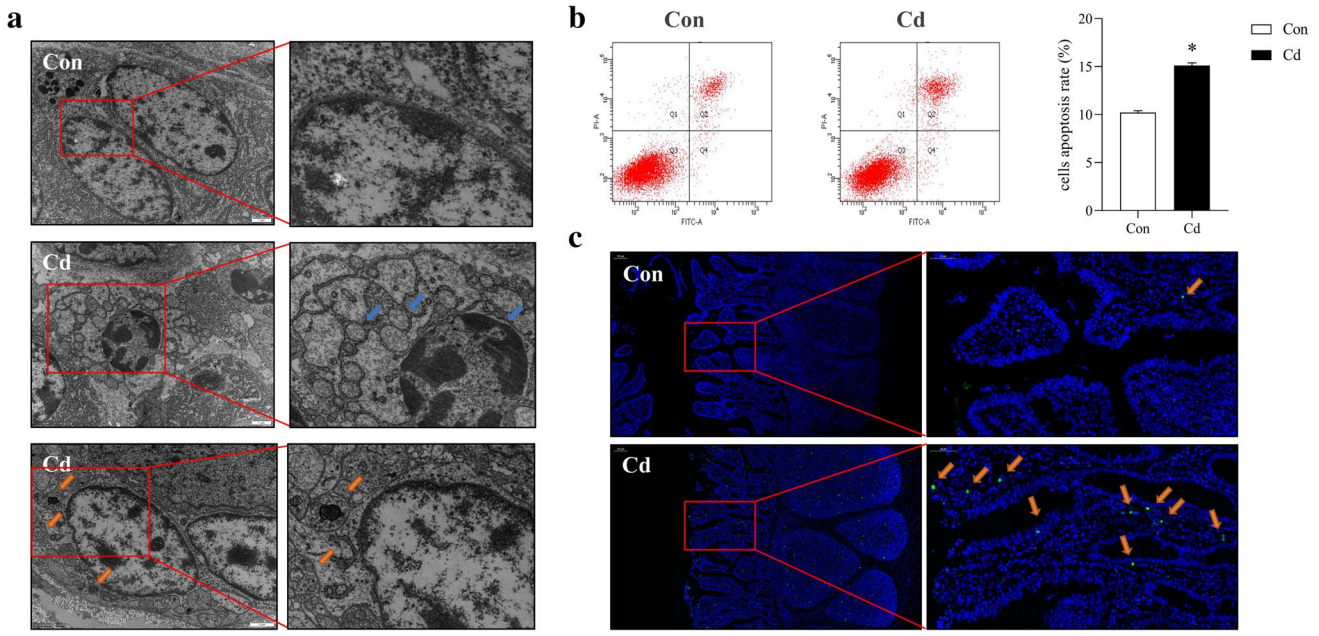

Fig. 1 Morphological observation of the effects of Cd on porcine ileum and IPEC-J2 cells. **a** The ultrastructure of ileal cells. The morphology of ileal tissue cells in the control group was normal and no ultrastructural changes were found. Nuclear chromatin condensation and cytoplasmic vacuolation were observed in the Cd group (blue arrows). Numerous autolysosomes (orange arrows) were observed in the Cd group. **b** The cells of the two groups were double stained with Annexin V-FITC and PI, and the staining results were analyzed by flow cytometry. Apoptotic cells were quantified, and the symbol * indicates a significant difference ($P < 0.05$) (mean ± SD, $n = 3$). **c** The results of TUNEL staining showed that the apoptosis rate increased in the Cd group. The green spots represent apoptotic cells (orange arrows). The blue spots represent normal cells

Protein and mRNA expression of the PI3K/Akt pathway-related genes in IPEC-J2 cells and swine ileum tissue

The effect of Cd treatment on the mRNA associated with the PI3K/Akt pathway (PI3K, Akt) is revealed in Fig. 4a, d. The qRT-PCR results indicated that PI3K and Akt mRNA levels were decreased significantly in the Cd groups in vitro (Fig. 4a) and in vivo (Fig. 4d) ($P < 0.05$). The results indicated that Cd exposure inhibited the PI3K/Akt pathway in IPEC-J2 cells and swine ileum tissue.

WB analysis was used to detect the protein expression of the genes associated with the PI3K/Akt pathway. The results showed the expression of PI3K, p-PI3K, Akt, and p-Akt was decreased significantly at the protein level in the Cd groups in vitro (Fig. 4b, c) and in vivo (Fig. 4e, f) ($P < 0.05$).

A PI3K pathway activator alleviated the morphological damage of the IPEC-J2 cells treated with cadmium

It has been previously demonstrated that the PI3K/Akt signaling pathway is involved in the regulation of apoptosis and autophagy (Lin et al. 2017; Liu and Fan 2018). To further understand the mechanism of Cd induced apoptosis and autophagy in IPEC-J2 cells, IPEC-J2 cells were co-treated with Cd and 740Y-P, an activator of PI3K, for 6 h. We observed the effects of Cd on cell viability and morphology, and preliminarily confirmed the mechanism of apoptosis and autophagy induced by Cd. The results of the Hoechst 33,258 staining showed that after 6 h of treatment with 740Y-P and Cd, the number of cells containing nuclei that were strongly stained was significantly less than observed in the Cd group (Fig. 5a). The results showed that the PI3K activator 740Y-P reduced the apoptosis induced by Cd. As demonstrated by MDC staining, after 6 h of co-treatment with 740Y-P and Cd, the number of dense green particles was significantly less than that of the Cd group (Fig. 5b). These results showed that the PI3K activator 740Y-P could reduce autophagy induced by Cd.

Effects of activating PI3K on apoptosis, autophagy, and PI3K/Akt pathway-related protein and mRNA expression

The qRT-PCR was used to detect the mRNA expression of genes related to apoptosis, autophagy, and the PI3K/Akt pathway (Fig. 6a). After treating IPEC-J2 cells with Cd for 6 h, the results showed that PI3K, Akt, Bcl-2, and LC3-I decreased in the Cd group compared to the Con group compared to the Cd group ($P < 0.05$). However, Bax, Caspase3, Beclin1, ATG5,

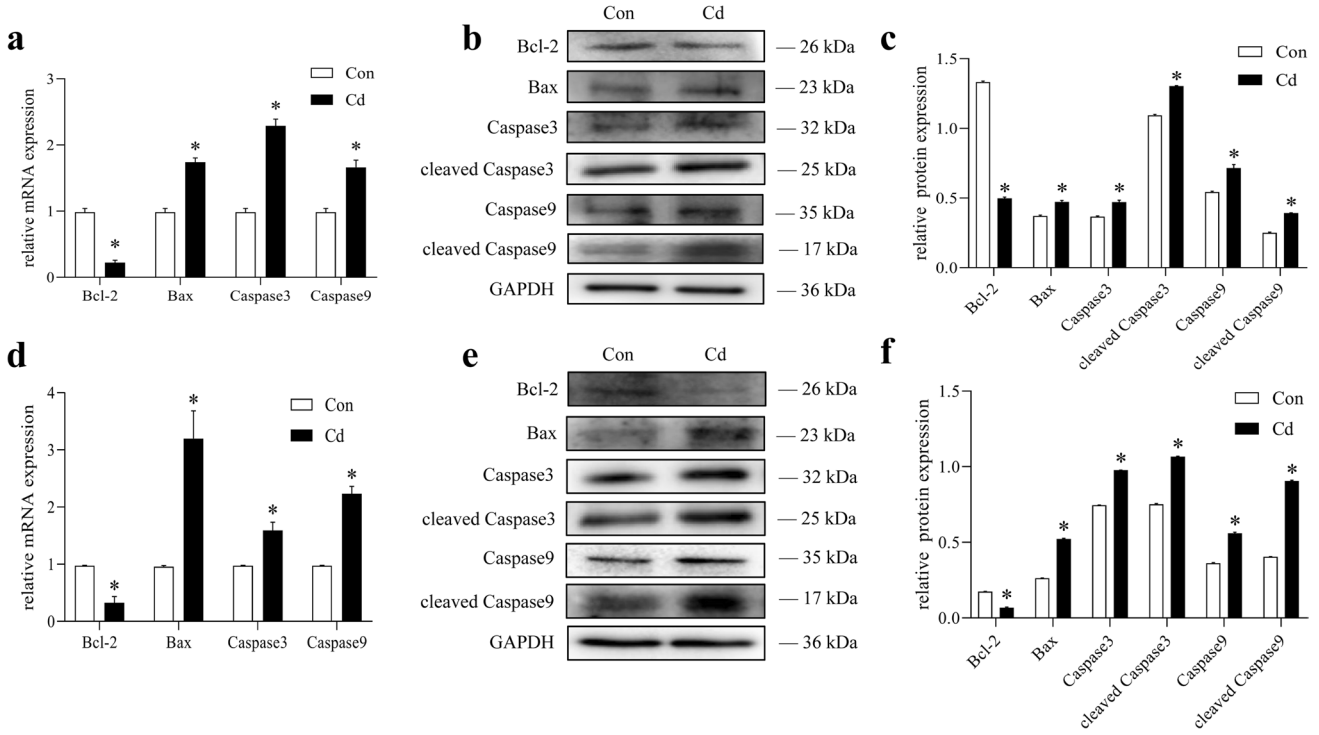

Fig. 2 Effects of Cd on the expression of apoptosis-related genes in porcine ileum and IPEC-J2 cells. **a** The mRNA levels of Bcl-2, Bax, Caspase9, and Caspase3 in the IPEC-J2 cells. **b**, **c** WB analysis of Bcl-2, Bax, Caspase9, cleaved Caspase9, Caspase3, and cleaved Caspase3 in the IPEC-J2 cells. **d** The mRNA levels of Bcl-2, Bax, Caspase9, and Caspase3 in the swine ileum tissue. **e**, **f** WB analysis of Bcl-2, Bax, Caspase9, cleaved Caspase9, Caspase3, and cleaved Caspase3 in the swine ileum tissue. The symbol * indicates a significant difference ($P < 0.05$). Results were expressed as mean ± SD ($n = 3$)

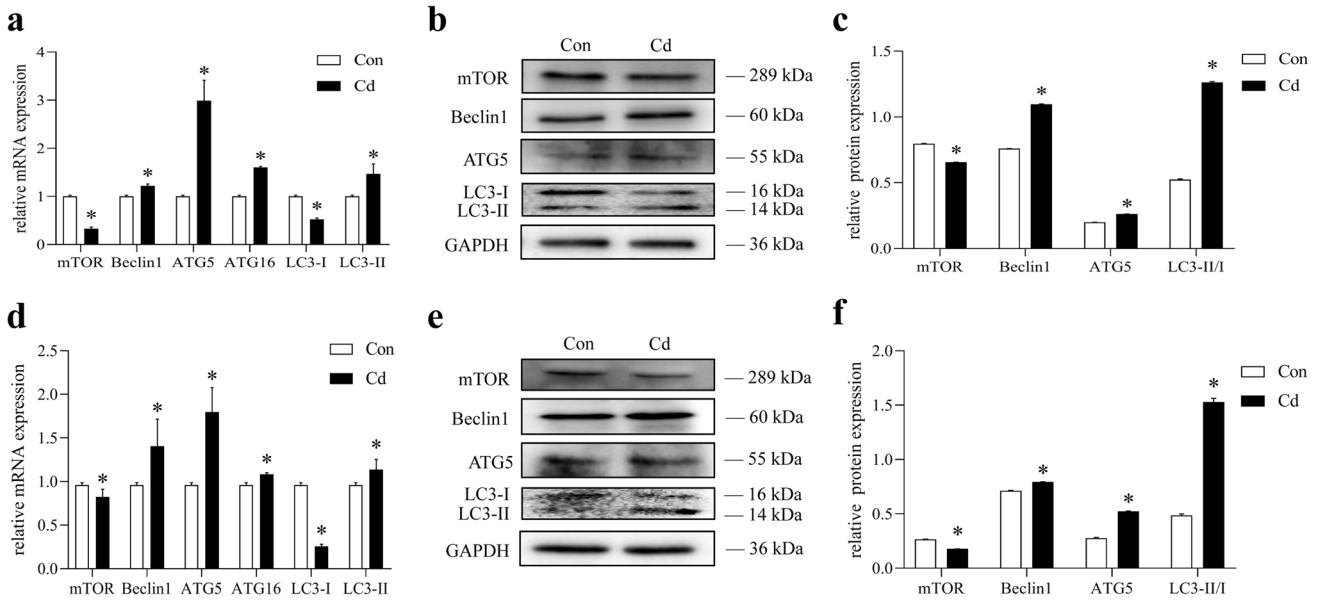

Fig. 3 Effects of Cd on the expression of autophagy-related genes in porcine ileum and IPEC-J2 cells. **a** The mRNA levels of mTOR, Beclin1, ATG5, ATG16, LC3-I, and LC3-II in the IPEC-J2 cells.,**b**, **c** WB analysis of mTOR, Beclin1, ATG5, and LC3-II/I in the IPEC-J2 cells. **d** The mRNA levels of mTOR, Beclin1, ATG5, ATG16, LC3-I, and LC3-II in the swine ileum tissue. **e**, **f** WB analysis of mTOR, Beclin1, ATG5, and LC3-II/I in the swine ileum tissue. The symbol * indicates a significant difference ($P < 0.05$). Results were expressed as mean ± SD ($n = 3$)

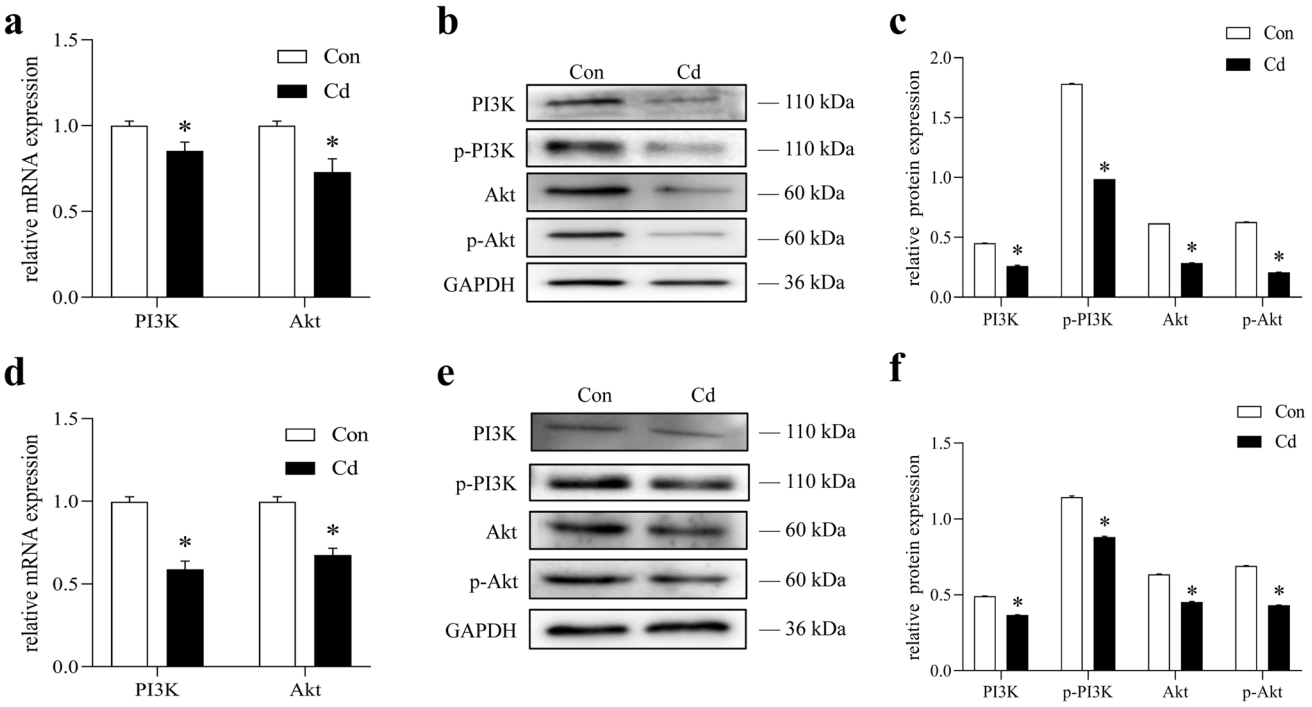

Fig. 4 Effects of Cd on the expression of genes related to the PI3K/Akt pathway in porcine ileum and IPEC-J2 cells. **a** The mRNA levels of PI3K and Akt in the IPEC-J2 cells. **b**, **c** WB analysis of PI3K, p-PI3K, Akt, and p-Akt in the IPEC-J2 cells. **d** The mRNA levels of PI3K and Akt in the swine ileum tissue. **e**, **f** WB analysis of PI3K, p-PI3K, Akt, and p-Akt in the swine ileum tissue. The symbol * indicates a significant difference ($P<0.05$). Results were expressed as mean ± SD ($n=3$)

and LC3-II increased in the Cd group compared to the Con group ($P<0.05$). In cells co-treated with Cd and 740Y-P for 6 h, PI3K, Akt, Bcl-2, and LC3-I were significantly increased ($P<0.05$). However, Bax, Caspase3, Beclin1, ATG5, and LC3-II were significantly decreased ($P<0.05$).

WB analysis was used to detect the protein expression of genes related to the PI3K/Akt pathway, apoptosis, and autophagy (Fig. 6b, c). IPEC-J2 cells were treated with Cd for 6 h, and compared to the Con group, PI3K, p-PI3K, Akt, p-Akt, and Bcl-2 decreased in the Cd group ($P<0.05$). However, Bax, Caspase3, cleaved Caspase3, Beclin1, ATG5, and LC3-II/I increased in the Cd group compared to the Con group ($P<0.05$). When cells were co-treated with Cd and 740Y-P for 6 h, PI3K, p-PI3k, Akt, p-Akt, and Bcl-2 were significantly increased compared to the Cd group ($P<0.05$). However, the protein expression of Bax, Caspase3, cleaved Caspase3, Beclin1, ATG5, and LC3-II/I was significantly downregulated ($P<0.05$). These results indicate that the PI3K/Akt pathway is involved in Cd-induced apoptosis and autophagy.

Discussion

Cd is a toxic metal with no known beneficial physiological effects. Cd poses a serious hazard to human health because of its long half-life and low excretion rate (Benoff et al. 2008). The gastrointestinal tract is essential for nutritional absorption in the body and plays an important role in immune function, whereas the intestinal epithelium plays an important role in maintaining the stability of intestinal physiological function (Nenci et al. 2007). The intestinal mucosa, while serving to protect the body against dietary Cd toxicity, is vulnerable to injury. Oral Cd can cause toxic gastroenteritis in mice, especially in the ileum and colon (Andersen et al. 1988). At the cellular level, Cd treatment of IPEC-J2 cells can cause DNA damage (Lynch et al. 2017). It has also been shown that Cd can cause apoptosis and autophagy. Cd can induce apoptosis and autophagy of rat proximal renal tubular cells (Bao et al. 2017; Liu et al. 2017), for example. In addition, Cd can also stop the cell cycle (Bork et al. 2010). However, there are few studies of the mechanisms of Cd-induced intestinal damage in swine. In this study, we treated porcine ileum and IPEC-J2 cells with Cd to further explore the mechanism of apoptosis and autophagy induced by Cd.

Apoptosis is a process in which normal cells are stimulated by adverse factors or die spontaneously. It is a process of active, highly ordered gene control and the participation of a series of enzymes (Liu et al. 2022). Many studies have demonstrated that Cd can induce apoptosis. For example, Cd can induce neuronal apoptosis in vitro (Chen et al. 2020). Cd inhibits the ROS/JNK/c-jun signaling pathway and induces apoptosis of mouse interstitial TM3 cells (Ren et al. 2020).

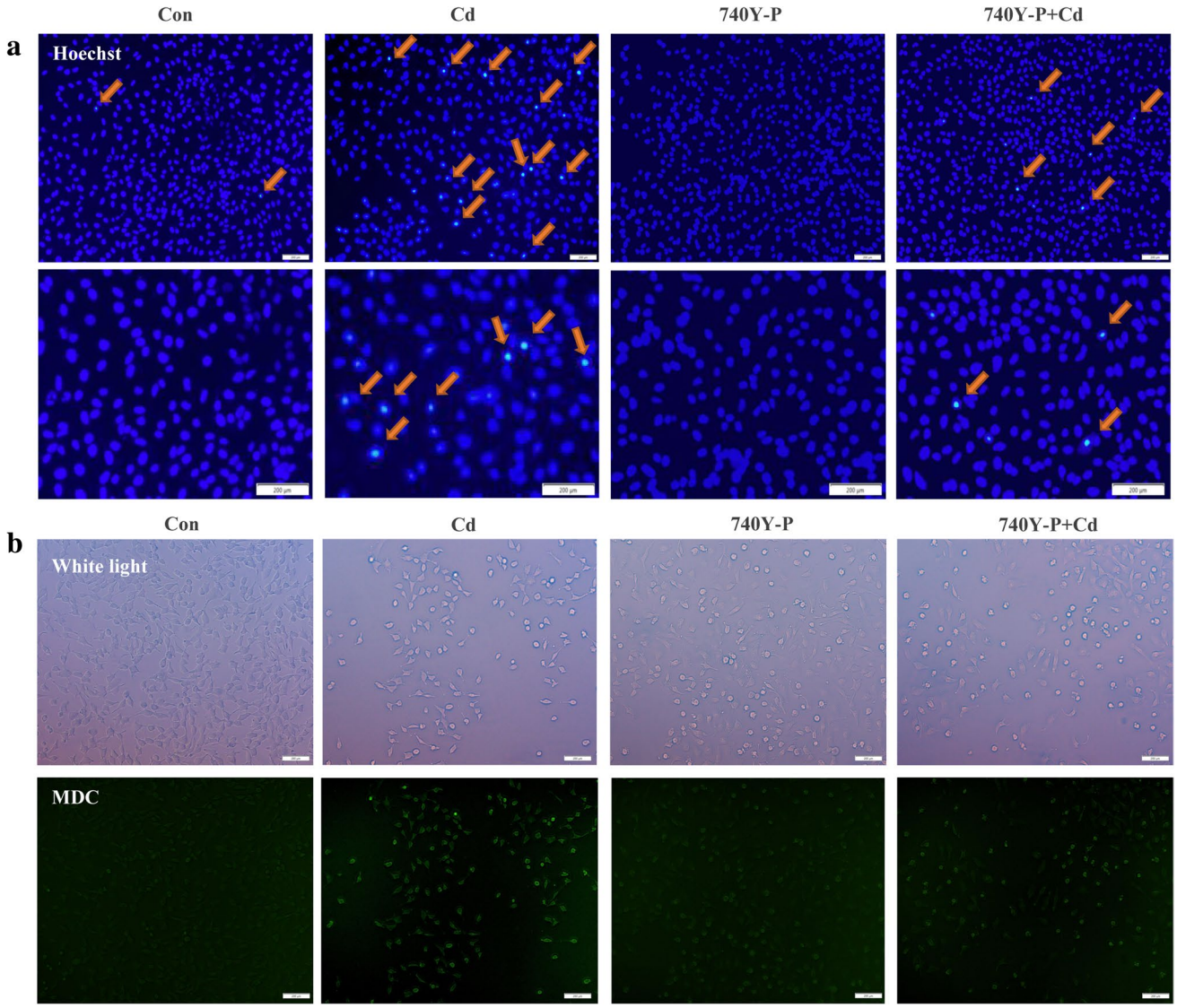

Fig. 5 Morphological observation of IPEC-J2 cells after activation of PI3K. **a** Results of Hoechst 33,258 staining. The nuclear fragmentation into oligo nucleosomes and chromatin condensation was observed under a fluorescence microscope. Arrows indicate apoptotic cells. **b** Results of MDC staining. The appearance of dense fluorescent green particles indicates the formation of autophagosomes

In our study, we used electron microscopy to detect apoptotic cells and observed obvious nuclear chromatin condensation and cytoplasmic vacuolization, which are typical features of apoptosis. Subsequently, we confirmed the occurrence of Cd-induced ileocyte apoptosis by other methods, i.e. the apoptosis of ileal cells induced by Cd was detected by the TUNEL method and apoptotic IPEC-J2 cells were detected by Annexin V-FITC/PI double staining and flow cytometry. These results showed that Cd treatment increased the number of apoptotic cells in porcine ileum. Based on the above data, we concluded that apoptosis occurred in Cd-treated IPEC-J2 cells and porcine ileum. This is consistent with the experimental results of H. J. Ni et al. which demonstrated that Cd can induce apoptosis of intestinal epithelial cells(Ni et al. 2020). The pathways of apoptosis are generally divided into exogenous and endogenous, of which the endogenous mitochondrial pathway is important (Cavalcante et al. 2019). The protein family closely related to the mitochondrial apoptosis pathway is the Bcl-2 family, which plays an important role in the regulation of the mitochondrial inner membrane (Kalkavan and Green 2018). The proteins in the Bcl-2 protein family have different functions, including the inhibition and promotion of apoptosis. Bcl-2 inhibits apoptosis and Bax promotes apoptosis (Popgeorgiev et al. 2018). Caspase9 is the promoter of the mitochondrial death pathway in the process of apoptosis in which Caspase3 is activated by Caspase9, and activated Caspase3 leads to apoptosis (Madan et al. 2008). The results of the present study show that the expression of

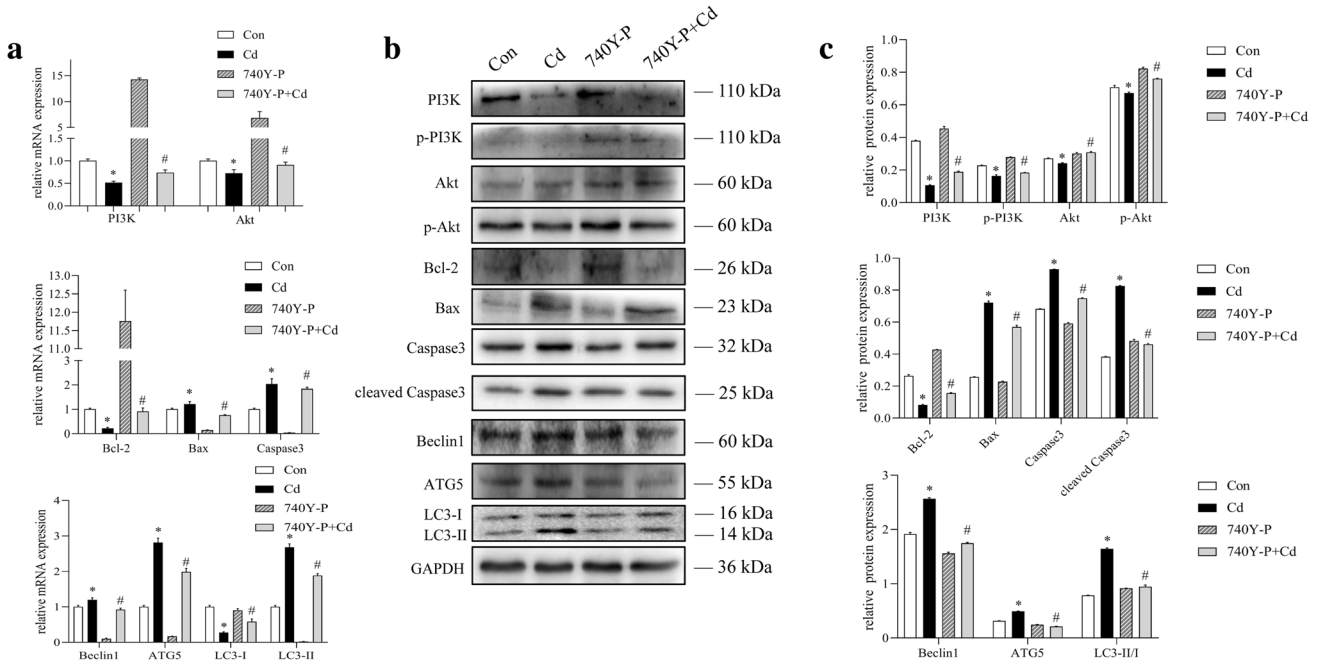

Fig. 6 The expression of mRNA and proteins in IPEC-J2 cells cultured with PI3k activator (740Y-P). **a** The mRNA expression levels of the PI3K pathway, apoptosis, and autophagy-related genes in IPEC-J2 cells. **b**, **c** Effects of the PI3K pathway activator, 740Y-P, on apoptosis, autophagy, and PI3K/Akt pathway-related protein expression levels. The symbol * indicates a significant difference ($P<0.05$) in the mean vs the control group; the symbol # indicates a significant difference ($P<0.05$) in the means vs the Cd group. Results were expressed as mean ± SD ($n=3$)

Bax, Caspase9, and Caspase3 was increased and Bcl-2 was decreased in IPEC-J2 cells treated with Cd. The protein levels of cleaved Caspase9 and cleaved Caspase3 also showed a similar trend. It is suggested that Cd treatment induced apoptosis in IPEC-J2 cells. This result was verified in the experimental model of porcine ileum treated with Cd. In addition, upon examination of the cells by electron microscopy, we also observed autophagy vacuoles and autophagosomes in the Cd group. Therefore, we performed further experiments to explore possible mechanisms.

Autophagy is the basic way for cells to degrade cytoplasmic components and maintain cell homeostasis with the help of lysosomes; autophagy is therefore important in many physiological and pathophysiological processes (Yang et al. 2021). Studies have shown that Cd exposure triggers autophagy in cells (Pesonen and Vähäkangas 2019). For example, autophagy occurs in duck renal tubular epithelial cells under the action of Cd (Wang et al. 2020a); oxidative stress and autophagy in rat testes can be induced by Cd (Wang et al. 2020b); and Cd can induce autophagy in chicken embryo fibroblasts (Shen et al. 2021). In consideration of the molecular mechanisms, autophagy is regulated by many genes and involves many pathways, among which the more common ones are the mTOR signal transduction pathway and the Beclin1 signal transduction pathway (Chu et al. 2018). Beclin1, ATG5, ATG7, ATG10, and ATG12 are the main genes that regulate the formation of autophagosomes. ATG12 and ATG5 combine with each other with the participation of ATG7 and ATG10, and the complex formed can combine with ATG16L to form the regulatory LC3 precursor (Otomo et al. 2013). The level of LC3-II/I plays a promoting role in the process of autophagy (Mizushima et al. 2010). mTOR is the main regulator of autophagy and activation of the mTOR signaling pathway can inhibit autophagy (Ahumada-Castro et al. 2019). Beclin1 can positively regulate autophagy; it is one of the main molecules of the class III phosphatidylinositol 3-kinase Vps34 complex and is necessary for phagocytic nucleation. Thus, the Beclin1-Vps34 complex plays an important role in the maturation of autophagosomes (Son et al. 2020). In our study, the results were consistent with our expectations. After Cd treatment, the expression of LC3-II/I, Beclin1, and ATG5 was upregulated, while the expression of mTOR was downregulated. Therefore, we conclude that Cd treatment also induced autophagy in small intestinal cells.

The PI3K/Akt pathway plays an important role in mediating cell proliferation, migration, apoptosis, and autophagy. Many heavy metals can affect the PI3K/Akt pathway and cause damage to the body; for example, the PI3K/Akt/mTOR signaling pathway is inhibited during arsenic induced autophagy in the cerebral cortex and hippocampus of mice (Manthari et al. 2018). The EGFR/PI3K/Akt signaling pathway is involved in the apoptosis of osteosarcoma

cells induced by Nimotuzuma (Liu et al. 2019). In addition, the P2X7/PI3K/Akt pathway is also involved in duck osteoporosis induced by Cd exposure (Ma et al. 2021). However, the role of the PI3K/Akt pathway in Cd-induced apoptosis and autophagy of small intestinal cells is still unclear. To explore the mechanisms of Cd-induced apoptosis and autophagy in porcine small intestinal cells, we measured the changes in cell morphology, apoptosis, and autophagy of IPEC-J2 cells treated with a PI3K activator (740Y-P) and Cd for 6 h. The results of Cd treatment alone were consistent with our previous findings. Through Hoechst 33,258 staining, it was observed that Cd treatment increased nuclear density staining, while the addition of the PI3K activator, 740Y-P, effectively restored cell morphology and inhibited Cd-induced apoptosis compared to the Cd group. In the process of autophagy, autophagosomes are formed, which are damaged organelles or aggregated proteins wrapped in bilayer membranes; these are transported to lysosomes for degradation to form autophagy lysosomes. The autophagosome is a kind of acidic vesicular organelle (AVO). Therefore, the presence of autophagosomes or AVOs is often used to detect the occurrence of autophagy in cells. MDC is a fluorescent pigment, which is usually used to detect AVOs (Gong et al. 2019). After MDC staining, we found that the number of AVOs in the Cd group was significantly higher than that of the control group, and there were more dense and green granules in the Cd group. The addition of the PI3K activator, 740Y-P, effectively reduced the number of AVOs and inhibited Cd-induced autophagy. We subsequently analyzed the expression of genes related to the PI3K/Akt pathway, apoptosis, and autophagy by qRT-PCR and WB. The results showed that 740Y-P increased the expression of PI3K/Akt-related genes, indicating that 740Y-P activated the PI3K/Akt pathway. In addition, compared to the Cd group, the addition of the PI3K activator, 740Y-P, significantly downregulated the expression of autophagy-related genes (Beclin1, ATG5, LC3-II/I) and apoptosis-related genes (Bax, Caspase3, cleaved Caspase3), and upregulated the expression of Bcl-2 in the 740Y-P + Cd group, indicating that 740Y-P can inhibit Cd-induced apoptosis and autophagy and reduce Cd-induced cell injury. These results suggest that the activation of PI3K could attenuate Cd-induced apoptosis and autophagy in IPEC-J2 cells, and that the PI3K/Akt pathway is involved in Cd-induced apoptosis and autophagy.

Conclusion

In summary, our study demonstrated that Cd exposure induced apoptosis and autophagy in porcine small intestinal cells. Further experiments showed that Cd could induce apoptosis and autophagy by inhibiting the PI3K/Akt signal pathway. These findings enhance our understanding of Cd-induced cytotoxicity and provide new concepts for the prevention and treatment of Cd-induced cytotoxicity.

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Author contribution Haoran Zhang: conceptualization, methodology, investigation, writing—original draft. Jiaqiang Huang: methodology, investigation, formal analysis. Jie Yang: investigation, formal analysis, methodology. Jingzeng Cai: software, visualization, formal analysis. Qi Liu: methodology, validation. Xintong Zhang: methodology, investigation. Jun Bao: conceptualization, investigation, methodology, writing—review and editing. Ziwei Zhang: conceptualization, methodology, writing—review and editing, funding acquisition.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

All authors have read the manuscript and agreed to submit it in its current form for consideration for publication in the Journal.

Declarations

Ethics approval and consent to participate All procedures in this study were carried out in accordance with the European Community Council Directive (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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