2,3,7,8-tetrachlorodibenzo-p-dioxin inhibits autophagy via Akt/ mTOR signaling pathway in RAW 264.7 macrophages

Jing Lu, Meitong Liu, Haochen Zheng, Yong Fan, Qian Zhang, Shuang Guan
(College of Food Science and Engineering, Jilin University, 130062)

Abstract: 2,3,7,8-tetrachlorodibenzodioxine (TCDD) was a highly toxic contamination and detected in the environment and food. It can be bio-magnified through food chain and bio-accumulate in human bodies, then cause various adverse health effects. Autophagy was a self-regulating process that degraded proteins and organelles in cells. The disruptions of autophagy balance have been bound up with multiple diseases and metabolic processes. In this paper, we detected the effects of TCDD on autophagy in RAW 264.7 cells and traced the molecular mechanism. The results showed that TCDD dose-dependently inhibited autophagy with the range from 0 to 10nM. Further, we found that this inhibition of autophagy was due to the decrease of p-Akt/Akt and increase of p-mTOR/mTOR expression. In addition, the autophagy inhibition caused by TCDD was resumed by using reversible mTOR inhibitor (rapamycin) and a selective Akt inhibitor (LY294002). This evidence showed that Akt/mTOR signaling pathway plays an important role in the autophagy induced by TCDD in RAW 264.7 cells. Our study confirmed the autophagy injury of TCDD on RAW 264.7 macrophages and the mechanism of TCDD toxicity was supplemented at the molecular level.

Key words: 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD); Autophagy; Macrophages; Akt/mTOR

0 Introduction

Autophagy is an essential component of growth regulation and homeostasis in multicellular organisms. Long-lived proteins and cytoplasmic organelles are engulfed by a double membrane autophagosome, then the autophagosomes fuse with lysosomes and then degrade them.[1] Defective autophagy has been bound up with multiple diseases such as alzheimer's disease, metabolic disorder and some forms of cancer. [2,3] Multiple signaling pathways involved in the regulation of cell autophagy. Among the signal pathway, the kinase mammalian target of rapamycin (mTOR) is a significant modulator of autophagy.[4] Kinase mTOR is the downstream target of kinase Akt pathway. Mounting evidence has shown that the Akt-mTOR energy and nutrient sensing pathway mediates autophagy.[5]

Dioxins are a class of persistent organic pollutants (POPs) which are carcinogenic, mutagenic and genotoxic.[6] 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic homologue of dioxins. Its toxic mechanism has been widely studied in recent years.[7] Waste incineration, chemical processing and the use of some herbicides are the main known sources of pollution for dioxins.[8] Through the food chain, TCDD is incorporated into naturally fat-rich animal-derived products such as milk, cheese, meat, seafood, and drinking water.[9] TCDD has multiple toxicities, mainly including carcinogenic toxicity,[10] reproductive toxicity,[11] immunotoxicity. Several studies have confirmed that the immune system is one of the most susceptible targets for TCDD.[12,13] In immune cells, macrophages play a vital role in the innate immune system, which is capable of clearing out pathogens, triggering inflammatory signals and engulfing dead cells.[14] Thus, macrophages play a significant role in maintaining homeostasis and resisting infection and are involved in the development of many diseases such as inflammatory related diseases, arteriosclerosis, and cancer.[15] Experimental studies have demonstrated that exposure to TCDD affects autophagy in different cell lines. However, the effects of TCDD on autophagy in immune

Foundations: National Natural Science Foundation of China (31540049); National College Students Innovation and Entrepreneurship Training Program of China (201910183315)
Brief author introduction:Jing Lu(1973-),Femal
Correspondance author: Shuang Guan(1973-), Male. E-mail: 907761215@qq.com

cells have not been studied.

In this paper, we evaluated the effect of TCDD on autophagy in RAW 264.7 macrophages and the molecular mechanisms involved. Our works were helpful to supplement new insights for the immunotoxicity mechanism of TCDD.

1 Materials and methods

1.1 Reagents and antibodies

TCDD standard was purchased from Accu Standard (New Haven，USA). Stock solution of 10 μM TCDD was prepared by Dulbecco's Modified Eagle's Medium（DMEM）from HyClone. Rapamycin (Rapa), LY294002 were acquired from Selleck.cn (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LC3I/II, p62, GAPDH were purchased from Protein Tech Inc (Chicago, USA) and secondary antibodies were also purchased from Protein Tech Inc (Chicago, USA) too. p-mTOR and p-AKT were bought from Cell Signaling Technololgy (CST, Boston, USA)

1.2 Cell culture

RAW 264.7 cells (ATCC) were cultured in 5% CO_2 medium with 10% fatal bovine serum (FBS) from Clark biosciences and 1% penicillin / streptomycin (37 °c) in DMEM medium (HyClone, USA). When cells grew to 75% confluence, 0.25% trypsin (w/v) and 0.52 mM Ethylene Diamine Tetraacetie Acid (EDTA) were dispersed to separate cells.

1.3 Cell viability assays

Cell viability evaluated by MTT assay. Cells (1×10^4 cells/well) were seeded in 96-well plates. After overnight incubation, TCDD was added to the cells for 44h and then added MTT solution (5 mg /mL in 1% PBS) to each well for 4h in cell incubator. Next, the supernatant was discarded and after that, added 100μL DMSO to each well. The absorbance value was detected at 570 nm on a microplate reader (Bio Tek, American).

1.4 MDC staining

Monodansylcada-verine (MDC) was an eosinophilic fluorescent stain that was commonly used to detect specific markers of autophagy formation. Cells were cultured in 24-well plates for 12 h. Then TCDD was added to the cells for 48 h. Next, the supernatant was discarded. The wells were washed twice with scrub solution. Next, the cells were stained with 300μL of MDC solution (0.05mM) at 37 °C for 45 min. After that, the cells were rinsed twice with a scrub solution and observed by the fluorescence microscope (BX53, Olympus, Japan).

1.5 Transmission electron microscope

Cells were treated with TCDD for 6h. Then cells were immobilized with 2.5 % glutaraldehyde in 0.05 M sodium cacodylate buffer at pH 7.2. Subsequently, cells were double-dyed with uranyl acetate and lead citrate. Then observed by transmission electron microscope (H7650, Hitachi, Japan). For analysis, a minimum of five random fields per cell were taken at ×1200 and ×2500 magnification.

1.6 Western blot analysis

RAW 264.7 cells were treated with different concentrations of TCDD for 6 h. Cell pellets were lysed on ice with lysis buffer (RIPA: PMSF = 100:1) The protein content in the supernatant was determined by the BCA protein assay kit (Beyotime Institute of Biotechnology, China). The same amounts of protein (60 μg) were electrophoresed on SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gels). The proteins on gels were then transferred onto PVDF (polyvinylidene fluoride) membranes and blocked with 5 % BSA for 2 h at room temperature. PVDF membranes were incubated overnight at 4 ℃ with antibodies and then incubated with the secondary antibodies. The protein level was detected by ECL (electrochemiluminescence) plus (Azure c300, America).

1.7 Statistical analysis

All data were analyzed with SPSS 19.0 software, by using one-way analysis of variance (ANOVA) or Tukey's test to analyze the differences among different groups. For each control group, $P < 0.05$ was considered statistically significant after passing the Dunnett's test.

2 First-order headline

2.1 Cell viability

THP-1 macrophages were treated with different concentrations of TCDD (0-80nM) for 48 h and measured by MTT assay. The data showed TCDD had no significant effect on the activity of macrophages in the range of 0-20nM (Fig.1). In this study, we selected TCDD at no-observed effect concentration (NOEC) in subsequent autophagy experiments.

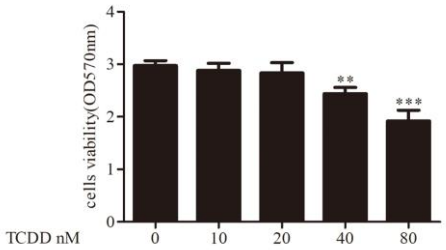

Fig. 1 Effects of TCDD on various concentrations by MTT (48 h). Data were shown as mean ± SEM. (n = 6). **P < 0.01 vs. control or ***P < 0.001 vs. control

2.2 TCDD inhibited autophagosomes formation in RAW 264.7 macrophages

To determine whether TCDD can inhibit the autophagy of RAW 264.7 cells, we explored the expression of LC3II/I and p62 protein. We found that the expression of LC3II/I was decreased at 3 h, 6 h, 12 h, with the rock bottom at 6 h (Fig.2A). Next, we treated the cells with various concentrations of TCDD (2.5, 5 and 10nM) for 6 h. The levels of LC3II/I were reduced in a dose-dependent manner, but the levels of the p62 were dose-dependently increased (Fig.2B and C). These results suggested that TCDD decreased autophagosome formation.

MDC is an autofluorescent compound that is an eosinophilic dye and is commonly used to detect autophagy formation. So, it was used to detect autophagy processes. As shown in figure 3, our results showed that TCDD dose-dependently decreased the fluorescence expression of MDC in RAW 264.7 macrophages.

Observed by TEM was another standard way to measure autophagy. Electron microscopic

analysis also detected that the number of autophagosomes decreased as the concentration of TCDD increased (Fig. 4).

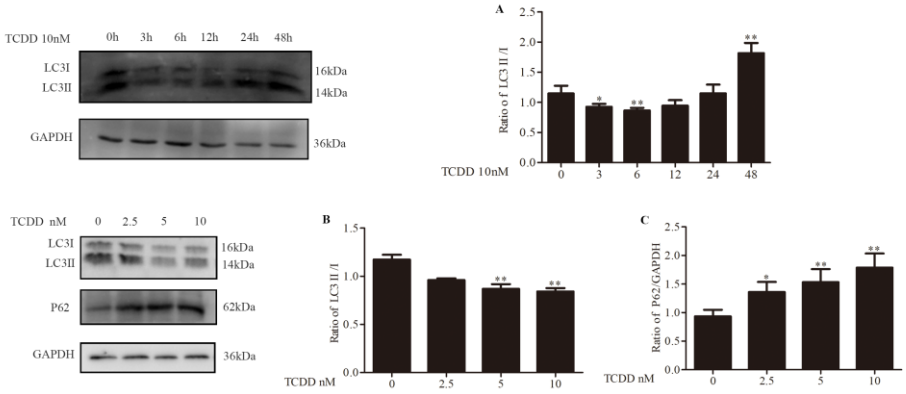

Fig. 2 (A) Western blotting analysis of LC3Ⅱ/Ⅰ for various times (0, 3, 6, 12, 24 and 48 h). (B and C) Western blotting analysis of LC3Ⅱ/Ⅰ and p62 at 6 h. Data were shown as mean ± SEM. (n = 3). *P < 0.05 vs. control. or **P < 0.01 vs. control.

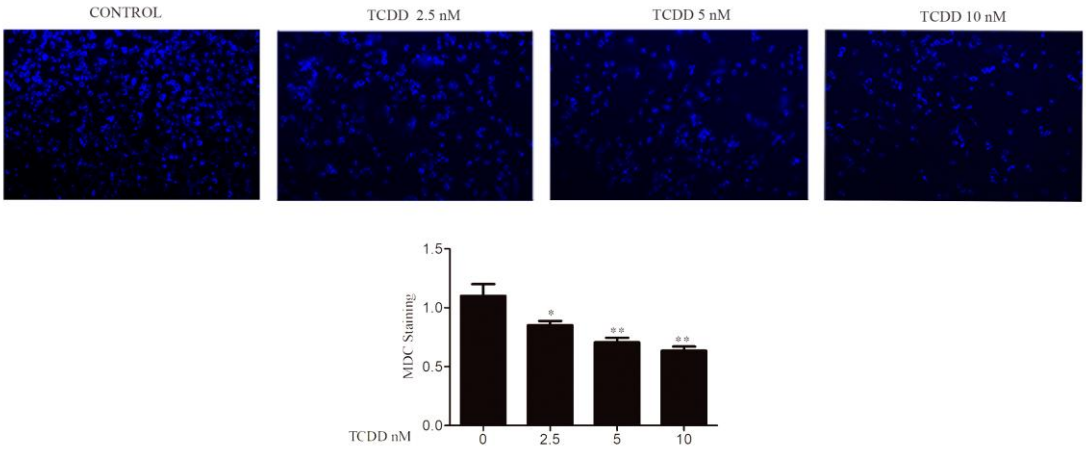

Fig.3 After treatment with TCDD for 6 h, RAW 264.7 macrophages were stained by MDC, and observed with a fluorescence microscope (magnification × 200). All data were presented as the mean SEM (n=3). *P < 0.05 vs. control. or **P < 0.01 vs. control.

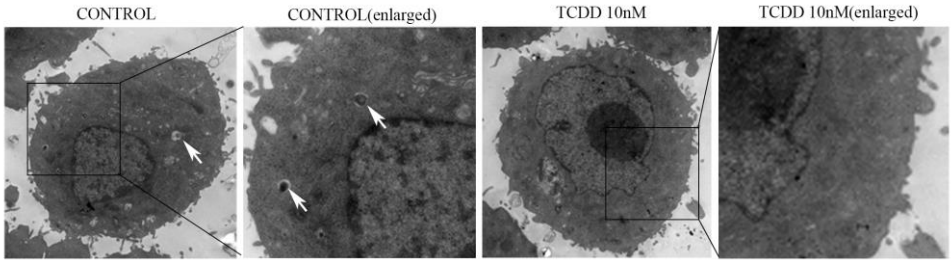

Fig.4 Transmission electron microscope images (TEM). The thick arrows represented the characteristic autophagosomes (magnification × 1200 and magnification × 2500). RAW 264.7 macrophages were treated with 10nM TCDD for 6 h. N, nucleus.

2.3 TCDD inhibited LC3II/I through mTOR–dependent phosphorylation

Firstly, after treatment with different dose of TCDD (2.5, 5 and 10nM) we found that the ratio of p-mTOR/mTOR was increased in a dose-dependent manner (Fig.5A). To further

investigate the role of mTOR on TCDD-mediated autophagy. RAW 264.7 cells were pretreated with a specific mTOR inhibitor (Rapa) for 1 h. Rapamycin reduced the ratio of p-mTOR/mTOR which was upregulated due to TCDD exposure (Fig. 5C). As expected, rapamycin increased the level of LC3 II/I which was decreased by TCDD exposure (Fig. 5B)

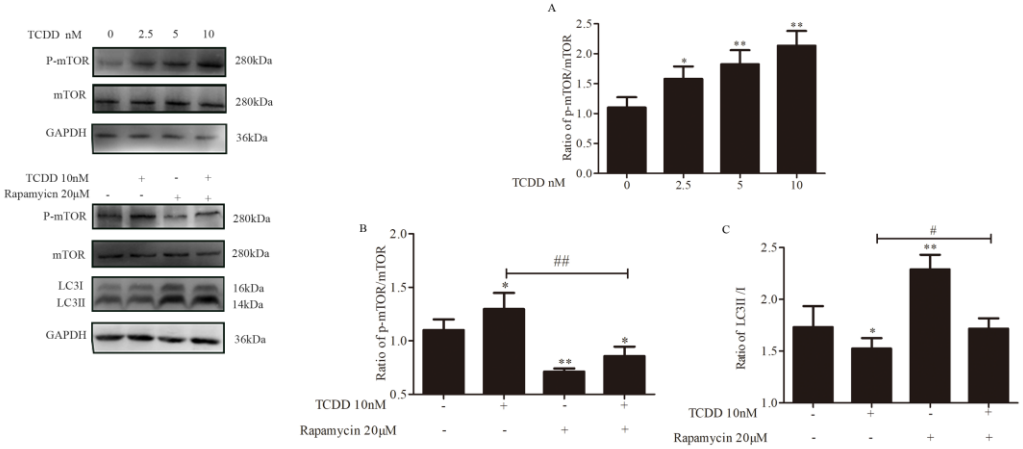

Fig.5 (A)Western blotting analysis of p-mTOR/mTOR under increasing TCDD in RAW 264.7 macrophages at 6 h. (B and C) Western blotting analysis of p-mTOR/mTOR and LC3 at 6 h. All data were presented as the mean ± SEM (n=3). *P < 0.05 vs. control. or **P < 0.01 vs. control. ##P < 0.01 vs. treatment of 10nM TCDD.

2.4 TCDD activated mTOR phosphorylation through Akt-dependent phosphorylation

TCDD induced a high level of p-Akt/ Akt expression (Fig.6A). To further confirm the effect of AKT on autophagy mediated by TCDD, RAW 264.7cells were preincubated for 1 h with LY294002 (Akt inhibitor). Pretreatment with LY294002 decreased the level of p-Akt/Akt which was increased by TCDD treatment (Fig.6B). As expected, LY294002 pretreatment suppressed the ratio of p-mTOR/mTOR which was upregulated by TCDD exposure (Fig.6C). LY294002 pretreatment promoted the level of LC3 II/I which was reduced by TCDD exposure (Fig.6D). Remarkably, the Akt/mTOR signaling pathway may be involved in TCDD-mediated autophagy in RAW 264.7 cells.

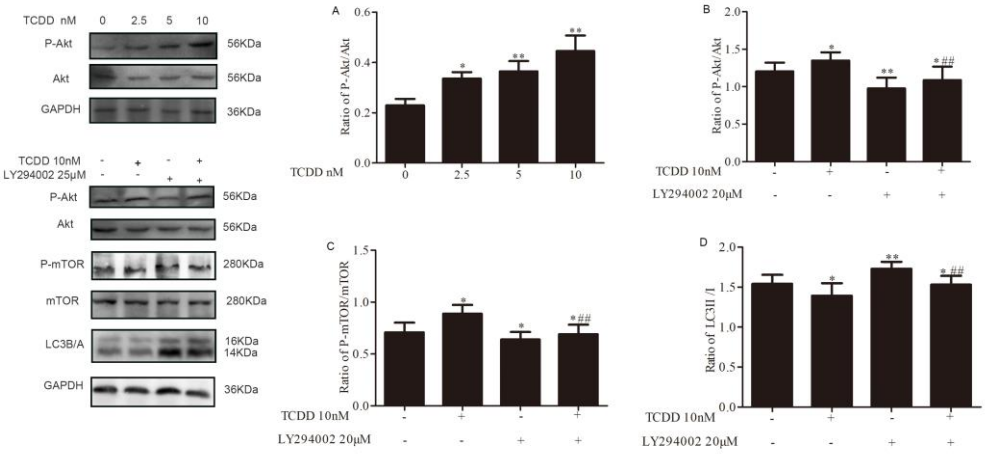

Fig.6 (A)Western blotting analysis of p-Akt/Akt under increasing TCDD in RAW 264.7cells at 6 h. (B, C and D) Western blotting analysis of p-Akt/Akt, p-mTOR/mTOR and LC3 at 6 h. All data were presented as the mean ± SEM (n=3). *P < 0.05 vs. control. or **P < 0.01 vs. control. ##P < 0.01 vs. treatment of 10nM TCDD.

3 Discussion

TCDD is absorbed by the body through the enrichment of the food chain. In this process it is essential to emphasize that the half-life of TCDD in humans is very long, estimated to be between 7.1[16] and 11.3[17] years. Most studies showed that TCDD-exposure has been to cause significant and sustained suppression of both cell-mediated and humoral immunity.[18]

Macrophages are the cellular components of innate immune system, found in almost all tissues, and contribute to immunity, repair, and homeostasis.[15] However, the effect of TCDD on autophagy of macrophages and its related mechanism was not clear. This study aimed to explore the effects of TCDD on autophagy in RAW 264.7 macrophages.

During the process of autophagy, LC3-I, a cytoplasmic form of LC3, binds to the phosphatidylethanolamine to form LC3-Phosphatidylethanolamine conjugate LC3-II which is incorporated into the autophagic membrane.[19] Thus, lipidated LC3 is a molecular marker for the autophagosome.[20] Another widely used autophagy marker is p62, known as the sequestosome 1 SQSTM1.[21] p62 was a multifunctional protein that bound to LC3 and degraded within the autolysosome. So is a marker of autophagy inhibition.[22,23] In this study, TCDD reduced the ratio of LC3II/I and increased p62 protein levels. The results indicated that TCDD inhibited autophagy in RAW 264.7 cells. MDC assay and TEM also proved that. Similarly, Filomena Fiorito et al. proved that higher doses of TCDD apparently induced both autophagy (LC3II protein accumulation) and cell death. However, they did not detect the level of p62. There were two conditions for the ratio of LC3II/I increased: promote the increase of autophagosomes or inhibited the degradation of autophagosomes. We used different doses probably because we use different cell lines.

The kinase mammalian target of rapamycin (mTOR), is a primary regulator of autophagy , regulated by hunger, growth factors, and cellular stress factors.[24] Furthermore，mTOR is an important negative regulator of autophagy and also controls the expression of autophagy-related genes.[25] Abundant researches have shown that Akt activated downstream signal transduction and induced autophagy.[26] Additionally, Akt phosphorylated can relate substrates that activate mTORC1.[27] In this study, we hypothesized that TCDD affected autophagy through the Akt/mTOR signaling pathway. As expected, pretreatment rapamycin and LY294002 abolished the ability of TCDD to inhibit autophagy. Therefore, our results suggested that TCDD inhibits autophagy via the Akt/mTOR signaling pathway.

In this paper we demonstrated that TCDD at NOEC inhibited the induction of autophagy and a potential molecular mechanism in RAW264.7 macrophages. The Akt/mTOR signaling pathway may involve in the regulation of TCDD-inhibited autophagy. It may contribute to explain the molecular mechanism of immunotoxicity caused by TCDD and provide evidence for health risk assessment of TCDD. In the following experiments, we will further explore the specific effects of TCDD on macrophages function through autophagy.

References

[1] Kelekar, A. Autophagy[J]. Ann. N. Y. Acad. Sci, 2006, 1066, 259-271.
[2] Song D, Chen Y, Wang B, Li D, et al. Bisphenol A Inhibits Autophagosome-Lysosome Fusion and Lipid Droplet Degradation. Ecotoxicol[J]. Environ. Saf, 2019, 15, 183.
[3] Cuyàs E, Corominas-Faja B, Joven J, et al. A. Cell Cycle Regulation by the Nutrient-Sensing Mammalian Target of Rapamycin (MTOR) Pathway[J]. Methods Mol Biol, 2014, 113-44.
[4] Heras-Sandoval D, Pérez-Rojas J. M, Hernández-Damián J, et al. The Role of PI3K/AKT/MTOR Pathway in the Modulation of Autophagy and the Clearance of Protein Aggregates in Neurodegeneration[J]. Cell. Signal, 2014, 26(12): 2694-2701.

[5] Luo Y, Xu T, Xie H Q, et al. Effects of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin on Spontaneous Movement of Human Neuroblastoma Cells[J]. Sci. Total Environ, 2020, 715.
[6] Choi E M, Suh K S, Park S Y et al. Orientin reduces the inhibitory effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on adipogenic differentiation and insulin signaling pathway in murine 3T3-L1 adipocytes[J]. Chemico-Biological Interactions, 2020, 318: 108978.
[7] Chen C C, Wu K Y, Chang-Chien G P. Point source identification using a simple permutation test: a case study of elevated PCDD/F levels in ambient air and soil and their relation to the distance to a local municipal solid waste incinerator[J]. Stochastic environmental research and risk assessment, 2012, 26(2): 225-233.
[8] Fiorito F, Santamaria R, Irace C, et al. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin and the viral infection[J]. Environmental research, 2017, 153: 27-34.
[9] Yang X, Ku T, Sun Z, et al. Assessment of the carcinogenic effect of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin using mouse embryonic stem cells to form teratoma in vivo[J]. Toxicology letters, 2019, 312: 139-147.
[10] Bruner-Tran K L, Gnecco J, Ding T, et al. Exposure to the environmental endocrine disruptor TCDD and human reproductive dysfunction: translating lessons from murine models[J]. Reproductive Toxicology, 2017, 68: 59-71.
[11] Phadnis-Moghe A S, Chen W, Li J, et al. Immunological characterization of the aryl hydrocarbon receptor (AHR) knockout rat in the presence and absence of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)[J]. Toxicology, 2016, 368: 172-182.
[12] Ilbäck N G, Friman G. Interactions among infections, nutrients and xenobiotics[J]. Critical reviews in food science and nutrition, 2007, 47(5): 499-519.
[13] Wu M Y, Lu J H. Autophagy and macrophage functions: inflammatory response and phagocytosis[J]. Cells, 2020, 9(1): 70.
[14] McWhorter F Y, Davis C T, Liu W F. Physical and mechanical regulation of macrophage phenotype and function[J]. Cellular and Molecular Life Sciences, 2015, 72(7): 1303-1316.
[15] Gentek R, Molawi K, Sieweke M H. Tissue macrophage identity and self‐renewal[J]. Immunological reviews, 2014, 262(1): 56-73.
[16] Pirkle J L, Wolfe W H, Patterson D G, et al. Estimates of the half‐life of 2, 3, 7, 8‐tetrachlorodibenzo‐p‐dioxin in Vietnam veterans of Operation Ranch Hand[J]. Journal of Toxicology and Environmental Health, Part A Current Issues, 1989, 27(2): 165-171.
[17] Wolfe W H, Michalek J E, Miner J C, et al. Determinants of TCDD half‐life in veterans of operation ranch hand[J]. Journal of Toxicology and Environmental Health, Part A Current Issues, 1994, 41(4): 481-488.
[18] Kerkvliet N I. TCDD: an environmental immunotoxicant reveals a novel pathway of immunoregulation-a 30-year odyssey[J]. Toxicologic pathology, 2012, 40(2): 138-142.
[19] Kim Y J, Hwang S H, Jia Y, et al. Barley sprout extracts reduce hepatic lipid accumulation in ethanol-fed mice by activating hepatic AMP-activated protein kinase[J]. Food Research International, 2017, 101: 209-217.
[20] Bjørkøy G, Lamark T, Brech A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death[J]. The Journal of cell biology, 2005, 171(4): 603-614.
[21] Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Methods Mol Biol.[J]. 2008, 445: 77-88.
[22] Lane J D, Korolchuk V I, Murray J T, et al. Regulation of selective autophagy: the p62/SQSTM1 paradigm[J]. Essays in biochemistry, 2017, 61(6): 609-624.
[23] Zhou X, Yan B. Induction of mTOR-dependent autophagy by WS 2 nanosheets from both inside and outside of human cells[J]. Nanoscale, 2019, 11(22): 10684-10694.
[24] Fiorito F, Ciarcia R, Granato G E, et al. 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induced autophagy in a bovine kidney cell line[J]. Toxicology, 2011, 290(2-3): 258-270.
[25] Hsu Y J, Hsu S C, Huang S M, et al. Hyperphosphatemia induces protective autophagy in endothelial cells through the inhibition of Akt/mTOR signaling[J]. Journal of vascular surgery, 2015, 62(1): 210-221.
[26] Feng F B, Qiu H Y. Effects of Artesunate on chondrocyte proliferation, apoptosis and autophagy through the PI3K/AKT/mTOR signaling pathway in rat models with rheumatoid arthritis[J]. Biomedicine & Pharmacotherapy, 2018, 102: 1209-1220.
[27] Li Y, Zhang Y, Ji G, et al. Autophagy Triggered by Oxidative Stress Appears to Be Mediated by the AKT/mTOR Signaling Pathway in the Liver of Sleep-Deprived Rats[J]. Oxidative Medicine and Cellular Longevity, 2020, 2020.

2,3,7,8-四氯二苯并对二恶英通过 Akt/mTOR 信号通路抑制 RAW 264.7 巨噬细胞的自噬

卢静，刘美彤，郑浩沉，范勇，张倩，关爽
（食品科学与工程学院，吉林大学，130062）

摘要：目的：以 2,3,7,8-四氯二苯并对二恶英（TCDD）刺激 RAW264.7 巨噬细胞，研究 TCDD 对巨噬细胞自噬的影响及其背后的分子机制。方法：使用 MTT 法检测 TCDD 对巨噬细胞的

毒性；细胞自噬染色检测试剂盒(MDC 法)以及透射电镜从形态学上检测自噬小体形成情况；蛋白免疫印迹法（Western blot）检测自噬标志蛋白 LC3、p62 以及自噬相关信号通路 p-mTOR、mTOR、p-Akt、Akt 表达情况。结果：在 TCDD 刺激的巨噬细胞中 LC3Ⅱ/LC3Ⅰ水平下降,p62 水平升高，自噬小体形成减少，p-Akt/Akt、p-mTOR/mTOR 水平升高。mTOR 抑制剂雷帕霉素显著降低 p-mTOR/mTOR 水平，Akt 抑制剂 LY294002 显著下降 p-Akt/Akt 水平，同时 TCDD 显著缓解了雷帕霉素和 LY294002 引起的 p-mTOR/mTOR 和 p-Akt/Akt 表达下降。结论：TCDD 通过影响 Akt/mTOR 信号通路抑制巨噬细胞 RAW264.7 自噬。

关键词：2,3,7,8-四氯二苯并对二恶英(TCDD)；自噬；巨噬细胞；Akt/mTOR
中图分类号：Q28