

β -Conglycinin enhances autophagy in porcine enterocytes

Dan Yi¹ · Yongqing Hou¹ · Huimin Mei¹ · Lei Wang¹ · Chien-An Andy Hu^{1,2} · Guoyao Wu^{1,3}

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Abstract β -Conglycinin (β -CG) is well known for inducing intestinal allergies and dysfunction in neonates and young pigs. However, the underlying mechanisms are largely unknown. In this study, to clarify the role of autophagy in β -CG-induced cytotoxicity, we investigated the effects of β -CG on cell viability and autophagy activity in porcine enterocytes (IPEC-1 cells). The results indicated that the cell viability was decreased with the increasing levels of β -CG. β -CG treatment enhanced the eGFP-LC3 puncta per cells and LC3-II/LC3-I, and the latter was further increased in IPEC-1 cells cultured with bafilomycin A1. We conclude that β -CG enhances autophagy activity in enterocytes.

Keywords β -Conglycinin · Autophagy · Microtubule-associated protein light chain 3 · IPEC-1 cells

Abbreviations

β -CG	β -Conglycinin
Baf A1	Bafilomycin A1
IPEC-1	Intestinal porcine epithelial cell 1
LC3	Microtubule-associated protein light chain 3
mTOR	Mammalian target of rapamycin

✉ Yongqing Hou
houyq@aliyun.com

¹ Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China

² Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA

³ Department of Animal Science, Texas A&M University, College Station, TX 77843, USA

Introduction

Soy products are high-quality protein sources for human and animals, but they are well known for the induction of allergies in neonates and young pigs (Chen et al. 2011). The major allergic protein in soy products is β -conglycinin (β -CG), which was reported to induce intestinal inflammation and dysfunction, and consequent vomiting and diarrhea in infants and piglets (Chen et al. 2011; Zhao et al. 2014). More specifically, β -CG caused an abnormality of intestinal morphology, and impaired absorption and barrier function in the intestines of piglets, mice, and fish (Chen et al. 2011; Zhang et al. 2013; Zhao et al. 2014). Although the effects of β -CG on the small intestine are extensively reported, the underlying mechanisms remain largely unknown.

Autophagy is a cellular pathway involved in protein and organelle degradation, with an astonishing number of connections to disease and physiology (Mizushima et al. 2008). Autophagy can be either protective or harmful to cells, depending on the biological context (Patel and Stappenbeck 2013). The protective effect of autophagy can be seen under the conditions of nutrient deprivation, stress, microbial infection, and diseases, so that intracellular protein degradation provides amino acids for gluconeogenesis and anti-oxidative defense (Wang et al. 2015; Wu 2009). In contrast, the harmful effect of autophagy can manifest in cancer (Mizushima et al. 2008). Several methods are reported in mammalian autophagy research, such as fluorescence measurement of the GFP-LC3 puncta, the assay of microtubule-associated protein light chain 3 (LC3) turnover, monitoring autophagic flux, and the electron microscopy of autophagic compartments (Mizushima et al. 2010). Among these methods, the LC3 turnover assay is the most widely used, since LC3 is the hallmark of autophagosomes. In

the autophagy process, nascent LC3 becomes LC3-I, which is subsequently conjugated with phosphatidylethanolamine to become LC3-II. In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome. After fusion with the lysosome, LC3-II is degraded in autolysosomes. Thus, the measurement of LC3 turnover is regarded as a reliable indicator of autophagic flux (Mizushima et al. 2010).

Since autophagy has been implicated in many physiological and pathological processes (Meijer et al. 2015), we hypothesized that autophagy is associated with the effects of β -CG on intestinal function. To test this hypothesis, we conducted the present study to investigate the effects of β -CG on cell viability and autophagy activity in porcine enterocytes (IPEC-1 cells).

Materials and methods

Cell viability

IPEC-1 cells were provided by Dr. Guoyao Wu at Texas A&M University (Haynes et al. 2009). They were cultured in the presence of DMEM-F12 containing 17.5 mM D-glucose, 2 mM, 0.7 mM arginine, 15 mM HEPES (pH = 7.4), 5 % FBS, epidermal growth factor (5 μ g/L), insulin (5 μ g/mL), transferrin (5 μ g/mL), selenium (5 μ g/mL), penicillin (50 μ g/mL), streptomycin (4 μ g/mL) and 0.25 μ g/mL amphotericin B (Fungizone®) (Yi et al. 2016). At confluence, cells were trypsinized, washed thoroughly with DMEM-F12, and then seeded in 96-well cell culture plates with approximately 5×10^3 cells per well and maintained at 37 °C in a 5 % CO₂ incubator. After overnight culture, the cells were washed twice with phosphate buffer saline (PBS) and then cultured in 200 μ L custom DMEM medium containing 5 % FBS, 5 mM D-glucose, amino acids of physiological concentrations, 0.1 mM sodium pyruvate (Xi et al. 2012), and serial levels of β -CG (0, 0.28, 0.56, 1.12, 2.24, or 4.48 mg/ml) (Sigma–Aldrich). The medium was changed every day. Cell viability was determined by Cell Counting-8 Kit (CCK-8) (Beyotime Inst. Biotech, China) after a 3-day culture period.

Cell autophagy

Autophagosomes were determined by fluorescence analysis through the transfection of eGFP-LC3 to IPEC-1 cells (Tang et al. 2014). In the present study, the stably expressing eGFP-LC3 cells (IPEC-1/eGFP-LC3 cells) were kindly donated by Dr. Tang (Institute of Subtropical Agriculture, Chinese Academy of Sciences). The cells were seeded on coverslips in 6-well culture plates (Corning Inc. NY, USA) and incubated with 1.12 mg/ml β -CG for 6, 12, 24, 48, or

72 h. Cells were then washed, fixed, and permeabilized with 80 % cold acetone. Fluorescence of eGFP-LC3 was observed by an Olympus BX41 fluorescence microscope (Tokyo, Japan). The average number of EGFP-LC3 puncta per cell from at least 60 cells per sample was counted (Tang et al. 2014).

After a 3-day period of culture with 1.12 mg/ml β -CG and 8.9 nM bafilomycin A1 (Baf A1, Sigma–Aldrich), LC3 in IPEC-1 cells were analyzed using western blotting following our previous procedure (Yi et al. 2016). Briefly, cells' proteins were separated by electrophoresis on a polyacrylamide gel, and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with primary antibodies: LC3B (Sigma–Aldrich, 1:1000) or β -actin (Sigma–Aldrich, 1:5000). Blots on the membrane were developed using an ECL-plus system (Amersham Biosciences, Sweden) and quantified using an imaging system (Alpha Innotech FluorChem FC2, CA, USA) (Yi et al. 2015). Abundances of LC3 were normalized to those for β -actin.

Results and discussion

β -CG, an allergic protein in the soybean food (feed), can induce diarrhea in infants and piglets, and cause health risks and agricultural economic loss. To better understand the underlying mechanisms of the effects of β -CG on intestinal dysfunction, we first investigated the effects of β -CG on the viability of IPEC-1 cells, a well-established intestinal cell line (Haynes et al. 2009; Zhu et al. 2015). After a 3-day incubation, the viability of IPEC-1 cells in treatment groups with different levels of β -CG had decreased in comparison with that of the control group (without β -CG) (Fig. 2a), indicating that β -CG inhibits enterocyte proliferation. More specifically, addition of 1.12 mg/ml β -CG reduced the cell viability by about 50 %, compared with the control group; therefore, the β -CG level of 1.12 mg/ml was used in other assays. Consistently, previous studies have reported the adverse effects of β -CG on intestinal cells in pigs (Chen et al. 2011; Zhao et al. 2014), mice (Xu 2014), and fish (Zhang et al. 2013). The adverse effects of β -CG are summarized as follows: causing inflammatory disorders via promoting the secretion of inflammatory cytokines (e.g., IL-8) (Xu et al. 2010), decreasing barrier function (Zhao et al. 2014) and absorptive function (Xu 2014), altering the cell morphology (Chen et al. 2011), inducing cell oxidative stress (Zhang et al. 2013), and increasing the caspase-3 protein abundance and cell apoptosis (Chen et al. 2011).

Given that autophagy is involved in many physiological and pathological processes, we determined the autophagy activity in IPEC-1 cells after β -CG treatment using fluorescence measurement of GFP-LC3 puncta, LC3 turnover

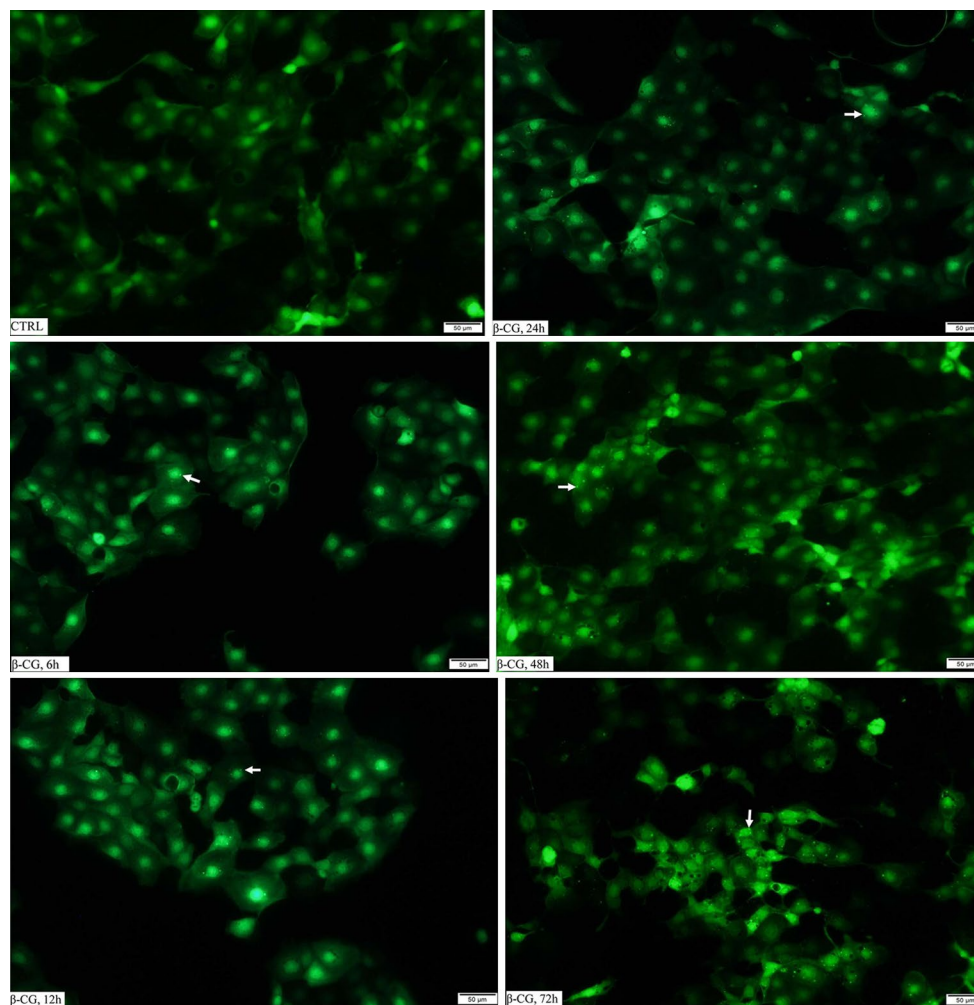


Fig. 1 Fluorescence determination of autophagosome formation of IPEC-1/eGFP-LC3 cells. IPEC-1 cells were treated with 1.12 mg/ml β -CG for 0 (CTRL, the control), 6, 12, 24, 48, and 72 h

assay, and monitoring autophagic flux. Results of fluorescence measurement in IPEC/eGFP-LC3 cells showed that the eGFP-LC3 puncta per cell was increased with the incubation times (Fig. 1). Western blot results also demonstrated that LC3 and the ratio of LC3-II to LC3-I were significantly elevated after a 3-day period of incubation with β -CG, as compared with the control group (Fig. 2b, c). These results implied that cell autophagy activation was enhanced by β -CG administration. Moreover, addition of both Baf A1 (an autophagy inhibitor) and β -CG to the culture medium further increased the ratio of LC3-II to LC3-I. Although Baf A1 inhibits the fusion of the lysosome with autophagosome in the autophagy process, the forward steps of autophagy, such as LC3-I being transformed to LC3-II, were normally processed and not altered, and consequently resulted in an accumulation of LC3-II (Mizushima et al. 2010). Therefore, the finding that a higher LC3-II/LC3-I ratio in the Baf A1 + β -CG group than in the β -CG group

further substantiated the enhanced autophagy in IPEC-1 cells by β -CG.

To our best knowledge, this is the first report demonstrating how β -CG could enhance autophagy activation in enterocytes. Though the information on the association between β -CG and autophagy is scarce, the β -CG-induced cellular immune response may play an important role in autophagy activation (Xu and Eissa 2010). It is worth noting that β -CG was reported to reduce the intestinal expression of TOR (Zhang et al. 2013), which can inhibit autophagy (Wu et al. 2015). Accordingly, further studies are warranted to investigate the effects of β -CG on innate immunity, mTOR expression, and protein turnover in IPEC-1 cells. Moreover, it is imperative to study the effects of β -CG-induced autophagy activation on the degradation of impaired organelles and β -CG itself.

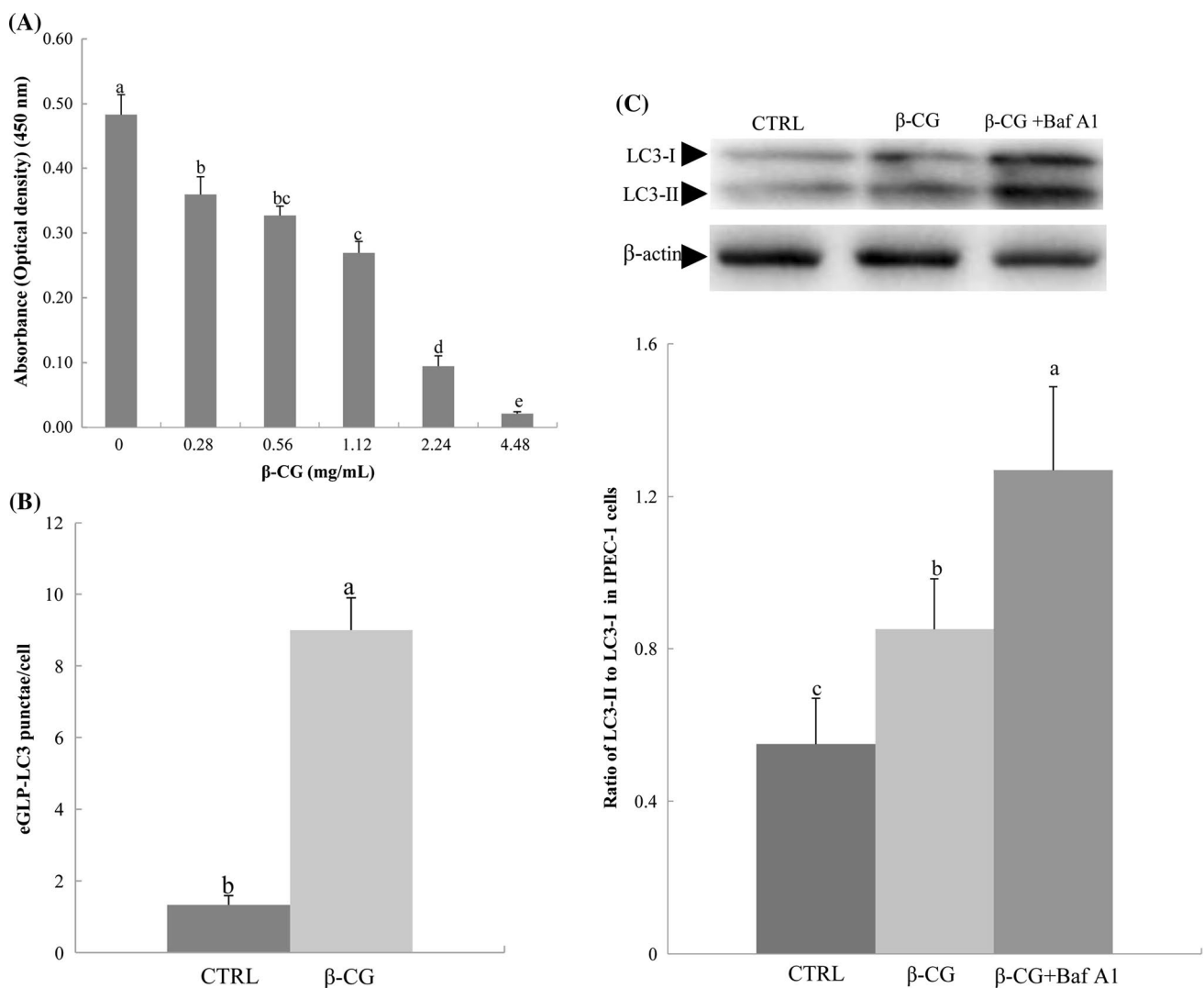


Fig. 2 β -CG reduces cell viability and enhances autophagy activity in IPEC-1 cells. **a** IPEC-1 cells were cultured with 0, 0.28, 0.56, 1.12, 2.24, and 4.48 mg/ml β -CG for 72 h, and cell viability was measured by CCK-8 kit. **b** Average numbers of the eGFP-LC3 puncta in each cell ($n \geq 60$). IPEC-1/eGFP-LC3 cells treated with 0 (CTRL, the con-

trol) or 1.12 mg/ml β -CG for 72 h. **c** Immunoblotting of LC3-I and LC3-II in IPEC-1 cells treated with 0 (CTRL), 1.12 mg/ml β -CG, or 1.12 mg/ml β -CG + 8.9 nM Baf A1 for 72 h. **a–c** means sharing different letters differ ($P < 0.05$)

Taken together, β -CG inhibited proliferation and enhanced autophagy activity in enterocytes. The results of the present study provide an important biochemical mechanism responsible for the actions of a soybean-derived allergic protein on inducing intestinal dysfunction in human infants and piglets.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement This study involved an established porcine intestinal cell line and did not require the approval of animal use.

Informed consent This study did not involve informed consent.

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