

## Letter to the Editor

**Green Tea Polyphenols Alleviate Autophagy Inhibition Induced by High Glucose in Endothelial Cells\***

ZHANG Pi Wei<sup>1,&</sup>, TIAN Chong<sup>2,&</sup>, XU Fang Yi<sup>1</sup>, CHEN Zhuo<sup>1</sup>, Raynard BURNSIDE<sup>1</sup>,  
YI Wei Jie<sup>1</sup>, XIANG Si Yun<sup>1</sup>, XIE Xiao<sup>1</sup>, WU Nan Nan<sup>1</sup>, YANG Hui<sup>3</sup>,  
ZHAO Na Na<sup>4</sup>, YE Xiao Lei<sup>4</sup>, and YING Chen Jiang<sup>1,5,#</sup>

**Bovine aortic endothelial cells (BAECs) were cultured with high glucose (33 mmol/L), 4 mg/L green tea polyphenols (GTPs) or 4 mg/L GTPs co-treatment with high glucose for 24 h in the presence or absence of Bafilomycin-A1 (BAF). We observed that high glucose increased the accumulation of LC3-II. Treatment with BAF did not further increase the accumulation of LC3-II. Results also showed an increased level of p62 and decreased Beclin-1. However, GTPs showed inversed trends of those proteins. Furthermore, GTPs co-treatment with high glucose decreased the level of LC3-II and a much higher accumulation of LC3-II was observed in the presence of BAF in comparison with high glucose alone. Results also showed a decreased p62 and increased Beclin-1. The results demonstrated that GTPs alleviated autophagy inhibition induced by high glucose, which may be involved in the endothelial protective effects of green tea against hyperglycemia.**

Autophagy is a lysosome degradation pathway that plays an important role in maintaining cell homeostasis. When autophagy is initiated, LC3-I (approximately 18 kD) is modified into the phosphatidylethanolamine (PE)-conjugated form, LC3-II (approximately 16 kD), and is incorporated into autophagic vacuoles (AVs) until degrades by lysosome. This make LC3-II to be a reliable protein marker, which is associated with completed autophagosome, for autophagy measurement by western blot. Nevertheless, autophagy is a dynamic process and reflection by a static measurement

facing difficulty. Therefore, a more accurate measurement of autophagy activity is autophagy flux. Autophagy flux is a measure of the number of autophagosomes that progress (from formation to degradation) through the autophagy pathway. Besides, Beclin-1, and SQSTM1/p62 are important autophagy activity indication proteins<sup>[1]</sup>. Beclin-1 participates in autophagy initiation by interaction with the class III type phosphoinositide 3-kinase (class III PI3K). SQSTM1/p62 is a protein that specifically targets ubiquitin binding molecules for autophagic degradation and inversely related to autophagic activity.

Impaired endothelial function is often seen in patients with diabetes and results in endothelial dysfunction. Endothelial dysfunction is an early pivotal event in the progress of atherosclerosis and chronic hyperglycemia was recognized as an independent risk factor for endothelial dysfunction. A proper level of autophagy is necessary for maintaining biological function in cardiovascular system. On the contrary, impairment of the autophagy results in serious dysfunction in cardiovascular system<sup>[2]</sup>. For example, fatty acids induced the impairment of autophagy in endothelial cells has been reported and restoration or enhancing of autophagy can attenuate the detrimental effects induced by fatty acids<sup>[3-4]</sup>. High glucose induced autophagy impairment was observed in endothelial cells also<sup>[5]</sup>. The beneficial health effects of GTPs on cardiovascular system have been well revealed with the mechanism being related to its antioxidant

doi: 10.3967/bes2016.069

\*This project was supported by grants (No. 81273060, 81302423, 81373007) from the National Natural Science Foundation of China.

1. Department of Nutrition and Food Hygiene, School of Public Health, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China; 2. School of Nursing, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China; 3. Kecheng People's Hospital, Quzhou 324000, Zhejiang, China; 4. School of Environmental Science and Public Health, Wenzhou Medical University, Wenzhou 325000, Zhejiang, China; 5. Key Laboratory of Environment and Health, Ministry of Education & Ministry of Environmental Protection, State Key Laboratory of Environmental Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China.

activity and anti-inflammatory effects, the ability to reduce free fatty acids and improve insulin sensitivity, and even down-regulation of caveolin-1<sup>[6]</sup>. Researchers has reported that epigallocatechin gallate (EGCG), the major component of GTPs, enhanced autophagy in endothelial cells (ECs)<sup>[4]</sup>. However, whether the protective effect of GTPs on endothelial dysfunction under hyperglycemia was mediated by autophagy is unknown.

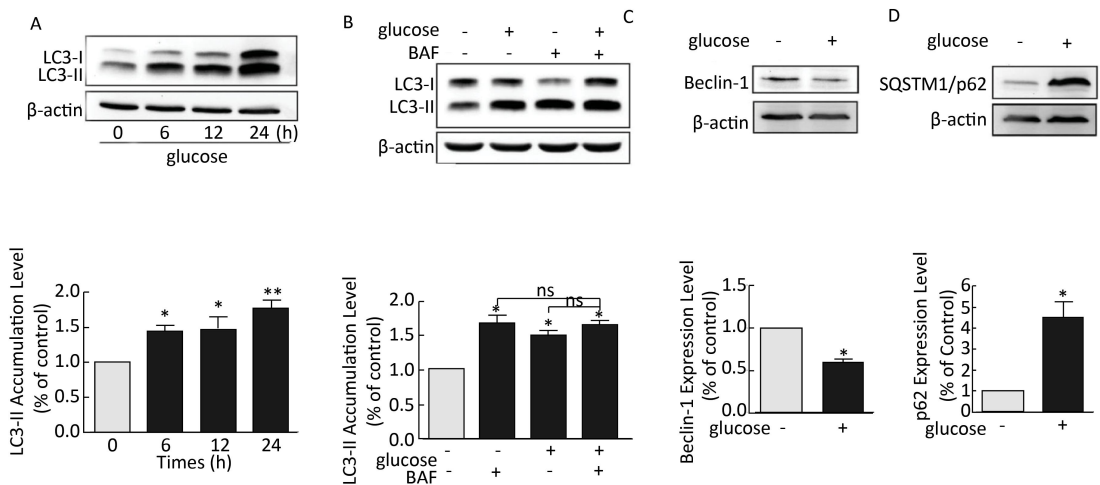
Bovine aortic endothelial cells (BAECs) were obtained from the Health Science Research Resources Bank (Osaka, Japan, no. C-003-5C). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 0.348% sodium bicarbonate, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C/5% CO<sub>2</sub>. The glucose level was 5.5 mmol/L in the normal condition or 33 mmol/L in the high-glucose condition. Before being treated, BAECs was cultured in medium containing 0.5% serum for 12 h. Then, as described in the figure legends, cells were treated with GTPs, high glucose and GTPs co-treatment with high glucose. As for autophagy flux experiment, 100 nmol/L BAF was added to the medium for 2 h before cell harvest.

After each treatment, confluent cells were washed thrice with warm PBS and scraped with RIPA extraction buffer and incubated in ice for 2 h. Then cells lysate were separated by centrifuging at 12,000 rpm for 15 min at 4 °C, and collected the supernatant.

Equal amounts of extracted proteins were separated by 12% or 15% SDS-PAGE and transferred to PVDF membrane. The primary antibodies used including anti-β-actin (Sigma, St. Louis, MO, USA); anti-LC3B (CST, Beverly, MA, USA), anti-SQSTM1/p62 (CST, Beverly, MA, USA), and anti-Beclin-1 (CST, Beverly, MA, USA). The dilution range and incubation time of primary and secondary antibodies were according to the specification. Proteins were visualized using an enhanced chemiluminescence western blotting luminal reagent (Amersham Biosciences, Little Chalfont, UK).

Values are expressed as the mean±SEM. The data among groups were compared using a one-way analysis of variation (ANOVA) followed by Bonferroni's post hoc test for multiple comparisons. The accepted level of significance was  $P < 0.05$ .

In the present study, treatment with high glucose significantly increased the accumulation of LC3-II in ECs from 0 to 24 h compared with those of normal dose glucose control (5.5 mmol/L) ( $P < 0.05$ ) (Figure 1A). Treatment with BAF did not further increase the accumulation of LC3-II under high glucose incubation ( $P > 0.05$ ) (Figure 1B). BAF is a lysosome function inhibitor that blocks the degradation of autophagic vacuoles. Meanwhile, decreased protein level of Beclin-1 (Figure 1C) ( $P < 0.05$ ) and increased SQSTM1/p62 (Figure 1D) ( $P < 0.05$ ) were observed in high glucose treated group



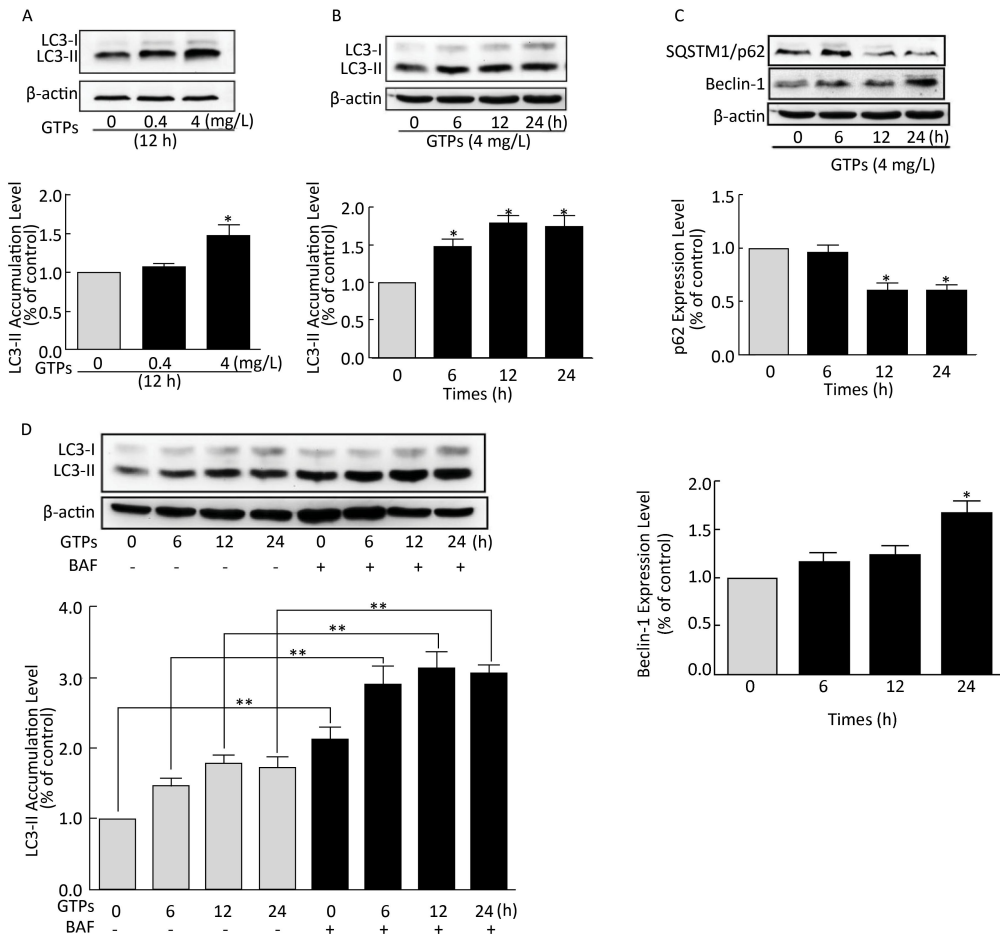
**Figure 1.** High glucose inhibits lysosome degradation of LC3-II. A, BAECs were treated with high glucose (33 mmol/L) for the indicated times. Western blots showing time course of LC3-II accumulation level under high glucose treatment. B, Western blots show protein levels of LC3-II in the presence of BAF (100 nmol/L) when BAECs were treated with high glucose for 24 h. C and D, Western blots show protein level of Beclin-1 and SQSTM1/p62 after high glucose treatment for 24 h. Mean ratio of the densities of LC3-II, p62 or Beclin-1 versus β-actin in the control (5.5 mmol/L glucose) was expressed as 1. Values are mean±SEM (n=3). \* $P < 0.05$  vs. control; \*\* $P < 0.01$  vs. control.

in comparison with the control group. These results indicated that high glucose impaired autophagy by inhibiting the degradation step of autophagy. The observed LC3-II accumulation was due to the blockage of autophagy degradation.

Impaired autophagy can be found in multiple tissues in metabolic syndrome animals and diabetes patients<sup>[7]</sup>. Hae-Suk Kim et al. showed that impaired autophagy degradation induced by palmitate was observed in ECs<sup>[4]</sup>. Liang X et al. showed impaired autophagy as indicated by reduced autophagosome formation in human umbilical vein cells (HUVECs) cultured in M199 medium containing 17 or 33 mmol/L glucose and lead to ECs injury<sup>[5]</sup>. This discrepancy with our results might be due to different cell lines and medium. And our results implied that

hyperglycemia induced endothelial dysfunction may result from autophagy impairment also. Impaired autophagy would limit the ability to remove damaged proteins and organelles induced by ROS under high glucose conditions and may responsible for endothelial dysfunction. In contrast, some ways of enhancing autophagy can promote endothelial function, such as exercise and resveratrol.

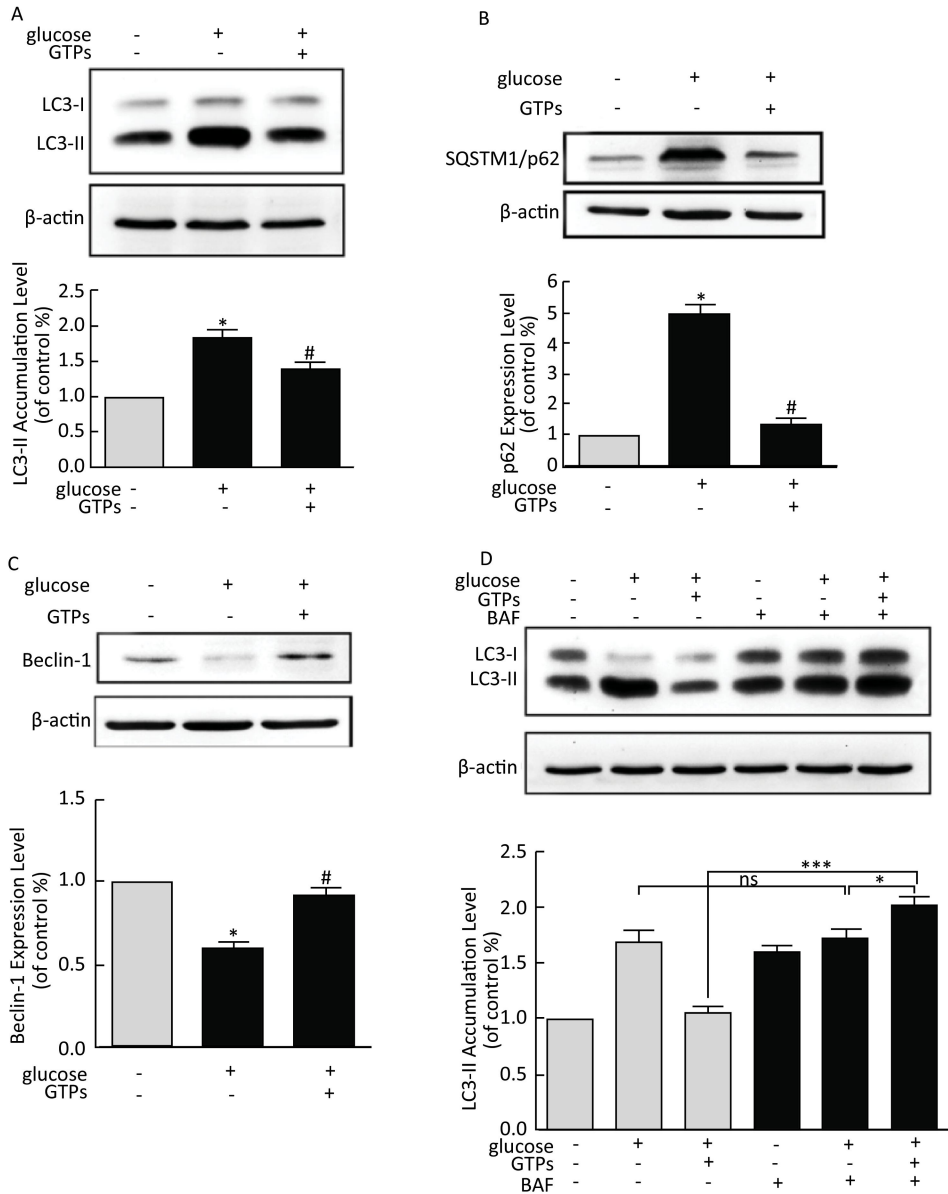
GTPs are beneficial to prevent non-communicable diseases, such as obesity, metabolic syndrome, diabetes and cardiovascular diseases. In the present study, GTPs treatment increased the accumulation of LC3-II in ECs dose-dependently with maximal inducing effects under 4 mg/L GTPs ( $P<0.05$ ) (Figure 2A). The time-dependent accumulations of LC3-II were also



**Figure 2.** Green tea polyphenols (GTPs) stimulates autophagy. BAECs were treated with green tea polyphenols (GTPs) for the indicated dosages and times. Cell lysates were analyzed by immunoblotting for LC3-II formation. A and B, dose and time course effects of GTPs on LC3-II formation. C, the protein level of p62 and Beclin-1 after GTPs treatment for indicated times. D, BAECs were treated with GTPs as indicated time in the absence or presence of BAF. Mean ratio of the densities of LC3-II versus  $\beta$ -actin in the control was expressed as 1. Values are mean $\pm$ SEM ( $n=3$ ). \* $P<0.05$  vs. control; \*\* $P<0.01$  vs. designated group in the figure.

observed by GTPs treatment from 0 to 24 h ( $P<0.05$ ) (Figure 2B). Meanwhile, decreased SQSTM1/p62 and increased Beclin-1 levels were observed after GTPs treatment for 24 h ( $P<0.05$ ) (Figure 2C). More

importantly, BAF treatment further elevated LC3-II accumulation induced by GTPs ( $P<0.01$ , GTPs+BAF vs. GTPs) (Figure 2D). Taken together, GTPs stimulated autophagy flux by enhancing both the formation and



**Figure 3.** GTPs alleviate high glucose induced autophagy inhibition. BAEC were treated with high glucose (33 mmol/L) or high glucose co-treatment with GTPs (4 mg/L) for 24 h with or without BAF. A, the accumulation of LC3-II was determined by Western blot. Mean ratio of the densities of LC3-II versus  $\beta$ -actin in the control was expressed as 1. Data are mean $\pm$ SEM ( $n=3$ ); \* $P<0.05$  vs. control; # $P<0.05$  vs. high glucose. B and C, the protein level of p62 and Beclin-1 were analyzed by immunoblotting. Mean ratio of the densities of p62 or Beclin-1 versus  $\beta$ -actin in the control was expressed as 1. Data are mean $\pm$ SEM ( $n=3$ ); \* $P<0.05$  vs. control; # $P<0.05$  vs. high glucose. D, the accumulation of LC3-II was measured by western blot with or without BAF. Mean ratio of the densities of LC3-II versus  $\beta$ -actin in the control was expressed as 1. Values are mean $\pm$ SEM ( $n=3$ ). \* $P<0.05$ ; \*\*\* $P<0.001$ ; ns: not significant.

degradation of autophagosome.

EGCG is the major component of GTPs and with the ability to stimulate autophagy<sup>[4]</sup>. Our results are in accordance with previous study. Another research reported that EGCG have the ability to protect the lysosomal membrane and stabilizes lysosomal enzymes<sup>[8]</sup>. This may be a mechanism of EGCG promotes autophagy degradation. We here observed increased Beclin-1 level after GTPs treatment and this may facilitate autophagy induction. The autophagy promoting effects of GTPs may be involved in the positive effects of GTPs on cardiovascular system.

As high glucose and GTPs showed inversed effects on autophagy, we speculated that GTPs may alleviate autophagy inhibition induced by high glucose.

To verify this, GTPs was co-treatment with high glucose for 24 h. We found that GTPs treatment significantly reduced the accumulation of LC3-II ( $P<0.05$ ) (Figure 3A) and the protein level of SQSTM1/p62 ( $P<0.05$ ) (Figure 3B) under high glucose conditions. Besides, GTPs treatment significantly increased the protein level of Beclin-1 under high glucose conditions ( $P<0.05$ ) (Figure 3C). More importantly, we found that the accumulation of LC3-II significantly increased ( $P<0.05$ ) after GTPs co-treatment with high glucose in the presence of BAF (Figure 3D). These data suggested that GTPs alleviated the inhibition effects of autophagy flux induced by high glucose.

Some researches indicated that enhanced autophagy can ameliorate myocardial oxidative stress injury and promote cardiac functions in diabetes animals<sup>[9]</sup>. The impairment of autophagy flux induced by linoleic acid was associated with endothelial cell senescence which can be prevented by enhancing autophagy<sup>[10]</sup>. Another phytochemicals -sulforaphane protects HUVECs from palmitic acid and stearic acid induced apoptosis by stimulating autophagy<sup>[3]</sup>. These data suggest that restore impaired autophagy in cardiovascular system may be a promising preventive approach. The effects of GTPs on restoration impaired autophagy induced by high glucose may be an important mechanisms of GTPs to promote endothelium function under hyperglycemia.

In summary, GTPs promote autophagy while high glucose blocks the degradation of autophagy and GTPs alleviate high glucose induced inhibition of autophagy. One limitation of this study is that the autophagic flux was determined in cells. Whether

our conclusion holds true requires further investigation using diabetic animal models. Although we still lack the data about the relationship between GTPs and diabetes cardiovascular complications, our results indicated that GTPs might still be a promising approach for prevention diabetes cardiovascular complications.

&These authors contributed equally to this work.

\*Correspondence should be addressed to Professor YING Chen Jiang, Tel: 86-27-83650523, Fax: 86-27-83693673, E-mail: yingcj@hust.edu.cn

Biographical notes for the first authors: ZHANG Pi Wei, male, born in 1988, Master of nutrition and food hygiene, majoring in nutrition, E-mail: zpwypidnfk@163.com; TIAN Chong, female, born in 1986, Lecture, majoring in nutrition and chronic disease, E-mail: tianchong0826@hust.edu.cn

Received: February 24, 2016;

Accepted: May 16, 2016

## REFERENCES

- Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell*, 2010; 140, 313-26.
- De Meyer GR, Martinet W. Autophagy in the cardiovascular system. *Biochim Biophys Acta*, 2009; 1793, 1485-95.
- Wang N, Zhao J, Liu Q, et al. Sulforaphane protects human umbilical vein cells against lipotoxicity by stimulating autophagy via an AMPK-mediated pathway. *Journal of Functional Foods*, 2015; 15, 23-34.
- Kim HS, Montana V, Jang HJ, et al. Epigallocatechin gallate (EGCG) stimulates autophagy in vascular endothelial cells: a potential role for reducing lipid accumulation. *J Biol Chem*, 2013; 288, 22693-705.
- Liang X, Zhang T, Shi L, et al. Ampelopsin protects endothelial cells from hyperglycemia-induced oxidative damage by inducing autophagy via the AMPK signaling pathway. *Biofactors*, 2015; 41, 463-75.
- Yang CS, Hong J. Prevention of chronic diseases by tea: possible mechanisms and human relevance. *Annu Rev Nutr*, 2013; 33, 161-81.
- Sciarretta S, Boppana VS, Umapathi M, et al. Boosting autophagy in the diabetic heart: a translational perspective. *Cardiovasc Diagn Ther*, 2015; 5, 394-402.
- Devika PT, Prince PS. Preventive effect of (-)epigallocatechin-gallate (EGCG) on lysosomal enzymes in heart and subcellular fractions in isoproterenol-induced myocardial infarcted Wistar rats. *Chem Biol Interact*, 2008; 172, 245-52.
- Wang B, Yang Q, Sun YY, et al. Resveratrol-enhanced autophagic flux ameliorates myocardial oxidative stress injury in diabetic mice. *J Cell Mol Med*, 2014; 18, 1599-611.
- Lee MJ, Kim EH, Lee SA, et al. Dehydroepiandrosterone prevents linoleic acid-induced endothelial cell senescence by increasing autophagy. *Metabolism*, 2015; 64, 1134-45.