Pediatric Surgery International (2019) 35:1389–1394
https://doi.org/10.1007/s00383-019-04566-2

ORIGINAL ARTICLE

The role of autophagy in intestinal epithelial injury

Masaya Yamoto[1,2] · Carol Lee[1] · Sinobol Chusilp[1,3] · Yuta Yazaki[1] · Mashriq Alganabi[1] · Bo Li[1] · Agostino Pierro[1,4]

Accepted: 12 September 2019 / Published online: 25 September 2019

Abstract

Purpose Autophagy is a natural mechanism aimed to degrade and recycle cellular components within cells. Previous studies reported that autophagy in the intestinal epithelium can be activated and that excessive autophagy can have negative consequences. However, the mechanism by which autophagy is regulated during intestinal epithelial injury remains unclear. This study aimed to investigate the mechanism of autophagy regulation during intestinal epithelial cells (IEC) injury.

Methods Rat IEC18 were exposed to hypoxia and Lipopolysaccharide (LPS) (200 µg/ml) to induce injury. IEC18 were treated with autophagy initiation inhibitor, Wortmannin or with autophagy degradation inhibitor, Bafilomycin A1 were added for 24 h. We assessed the number and diameter of autophagic vacuoles, Cell viability, inflammation and apoptosis.

Results Hypoxia and LPS administration increased the number and diameter of autophagic vacuoles in IEC18. Wortmannin administration reduced the number and diameter of autophagic vacuoles. On the contrary, Bafilomycin A1 administration increased the number of autophagic vacuoles. Cell viability increased following administration of Wortmannin and decreased following administration of Bafilomycin A1.

Conclusions We found that accumulation of autophagic vacuoles which characterize excessive or incomplete autophagy was detrimental to cell survival. This was shown by an increase in the number and size of the autophagic vacuoles with Bafilomycin A1treatment after hypoxia and LPS stressors relative to hypoxia and LPS alone. Conversely, there was a decrease in the number of autophagic vacuoles with Wortmannin treatment after hypoxia and LPS stressors relative to hypoxia and LPS alone. Therefore, reducing autophagosomes accumulation may represent a novel therapeutic strategy for intestinal injury.

Keywords Autophagy · Intestinal injury · Wortmannin · Bafilomycin A1

Introduction

Autophagy is an intracellular degradation process that mediates the bulk clearance of long-lived and injured proteins, mitochondria, defective organelles and certain pathogens. Autophagy also provides a source of nutrition during periods of stress to promote healthy cell homeostasis and boost survival [1].

Autophagy proceeds through the sequential nucleation and elongation of a double-membraned vesicle called an autophagosome, encapsulating a portion of cytoplasm in the process. Then, autophagosomes fuse to lysosomes and the vacuoles form autolysosomes (Fig. 1a). The acidic pH and enzymatic action of hydrolases within the lysosome lead to the breakdown of the internal membranes of autophagosomes as well as their contents [2]. Each stage of autophagy has its own regulations. The master controller of the initiator of autophagy is the phosphoinositide 3-kinase (PI3K)–protein kinase B (AKT), a target of the Rapamycin complex in mammals (mTOR) pathway, which represses autophagosome biogenesis and autophagy under basal and stimulation conditions. Upon the onset of certain stressors, mTOR becomes deactivated which leads to the initiation of autophagosome biogenesis and autophagy [3].

✉ Agostino Pierro
agostino.pierro@sickkids.ca

1 Division of General and Thoracic Surgery, Translational Medicine Program, The Hospital for Sick Children, 1526-555 University Ave, Toronto, ON M5G 1X8, Canada

2 Department of Pediatric Surgery, Shizuoka Children's Hospital, Shizuoka, Japan

3 Division of Pediatric Surgery, Department of Surgery, Khon Kaen University, Khon Kaen, Thailand

4 Division Head, Pediatric Surgery, Robert M. Filler Professor of Surgery, University of Toronto, Toronto, Canada

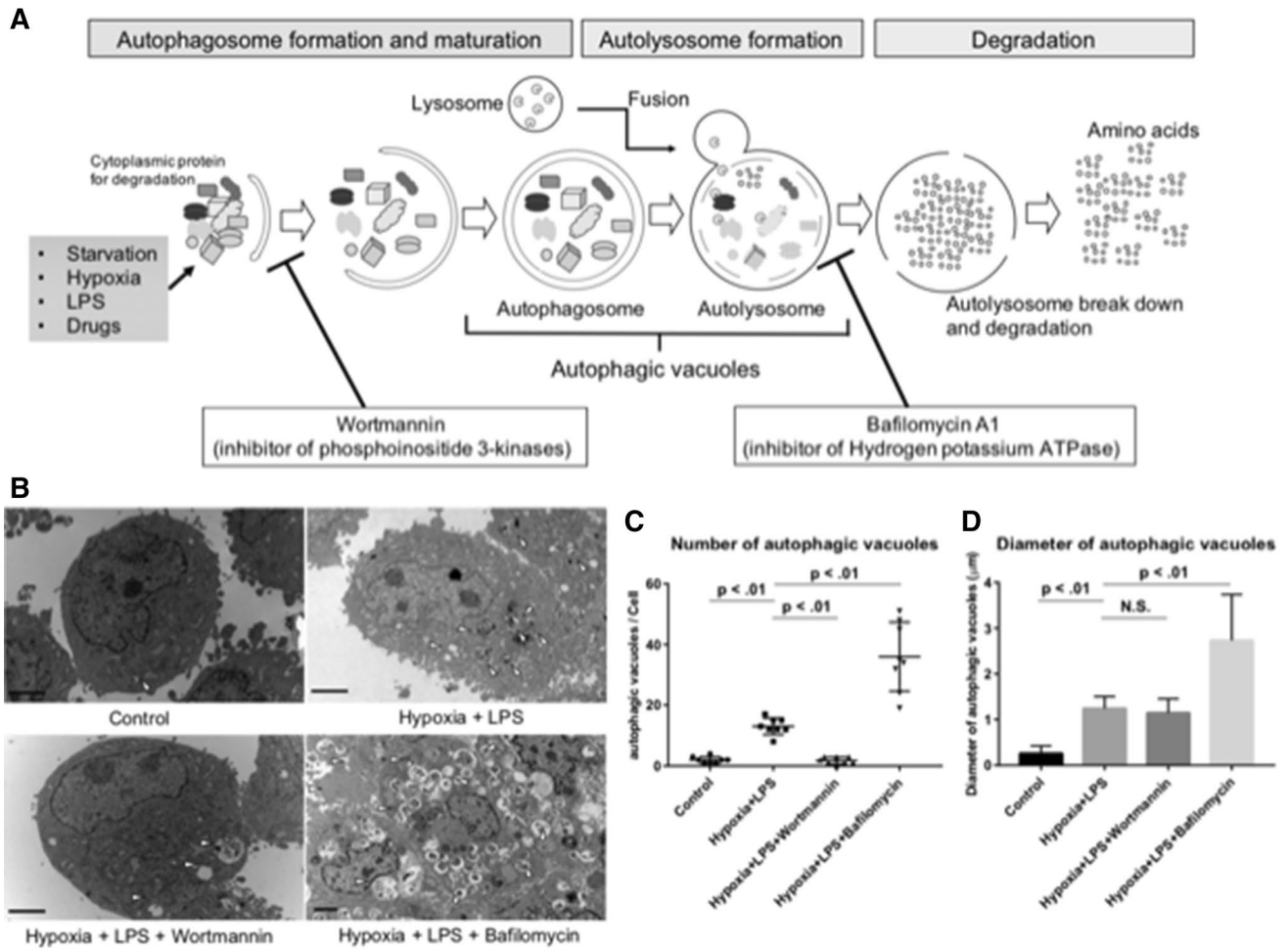

Fig. 1 Autophagic pathway and effects of autophagic inhibitor. **a** The autophagic pathway proceeds through several phases, including initiation (formation of a pre-autophagosomal structure leading to an isolation membrane by phagophore), vesicle elongation, autophagosome maturation and cargo sequestration, and autophagosome–lysosome fusion. Finally, autophagosomal contents are degraded by lysosomal acid hydrolases and the contents of the autolysosome are released for metabolic recycling. Wortmannin can inhibit initiation of autophagy via inhibiting autophagic sequestration. Bafilomycin A1 prevents cargo degradation and functional autophagic flux. Autophagic vacuoles include autophagosomes and autolysosomes. **b** Transmission electron micrographs showing autophagic vacuoles within IEC 18 after following administration of hypoxia and LPS together with Wortmannin or Bafilomycin A1. White triangles show autophagic vacuoles including autophagosomes and autolysosomes. Number (**c**) and diameter (**d**) of autophagic vacuoles of IEC 18 following administration of hypoxia and LPS with Wortmannin or Bafilomycin A1 measured using transmission electron microscopy. Data are presented as means ± SD from four independent experiments and differences between mean values were assessed by one-way ANOVA. $p < 0.05$ was considered significant. *N.S.* non-significance. Scale bars 2 μm

Currently autophagy is largely believed to function as a pro-survival process due to its critical role in cellular energy and nutrition homeostasis. In addition, autophagy protects cells from excessive inflammation by directly suppressing pro-inflammatory complexes or indirectly facilitating the efficient clearance of damaged organelles [4, 5]. However, various stress conditions such as hypoxia, starvation, Lipopolysaccharide (LPS), and reactive oxygen species (ROS) [6], and/or certain drugs such as Rapamycin [7], Imatinib [8], and Everolimus [9] induce excessive autophagy. Furthermore, excessive autophagy often compromises autolysosomal activity and causes cell death such as apoptosis and autophagy-associated cell death [4, 10]. Previous studies reported that autophagy in the intestinal epithelium can be activated and that excessive autophagy can have negative consequences [11]. However, the mechanism by which autophagy is regulated in the intestinal epithelial during injury remains unclear.

In this study, we investigated chemical autophagy during intestinal epithelial injury to discern the effects of decreased autophagosome formation or lysosomal dysfunction on cell viability.

Materials and methods

Epithelial cells and experimental groups

Rat small-intestinal epithelial cells (IEC 18) were purchased from the American-Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's modified Eagle's medium containing high glucose (DMEM, Gibco BRL), supplemented with 10% fetal bovine serum (FBS, GibcoBRL) and 100 mg/ml penicillin and streptomycin (Sigma-Aldrich). Cells were cultured at 37 °C in a humidified atmosphere composed of 5% CO_2.

Three experimental groups were utilized in this study: (1) control: IEC 18 were untreated; (2) hypoxia and LPS to mimic injury: IEC 18 were exposed to hypoxia (5% O_2) and LPS (200 µg/ml) for 24 h; (3) hypoxia and LPS (injury) together with Wortmannin administration: IEC 18 were exposed to hypoxia (5% O_2) and LPS (200 µg/ml) to mimic injury and concomitantly exposed to the inhibitor of autophagy initiation, Wortmannin (10 µM) (inhibitor of phosphoinositide 3-kinases: Sigma-Aldrich, W1628) for 24 h; (4) hypoxia and LPS (injury) together with Bafilomycin A1 administration: IEC 18 were exposed to hypoxia (5% O_2) and LPS (200 µg/ml) to mimic injury and concomitantly exposed to the inhibitor of autophagic flux, Bafilomycin A1 (10 nM) (inhibitor of vacuolar-type Hydrogen potassium ATPase) (Sigma-Aldrich, B1793) for 24 h.

Electron microscopy

Cells were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer at a pH of 7.3 for 2 h at room temperature. Then cells were post fixed with 1% osmium tetraoxide and dehydrated through a graded ethanol series from 50 to 100%. Samples were infiltrated with propylene oxide and embedded in spur resin. Thin sections of 70 nm were cut by diamond knife and put on formvar cupper grid. Sections were viewed by Tecnai FEI T20 at 120 kV.

Cell viability (MTT assay)

Cell viability was tested utilizing a colorimetric assay that measures formazan, which is a species generated by the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into formazan by the mitochondrial enzyme succinate dehydrogenase. After the cells were treated with hypoxia and LPS, Wortmannin (group 3) or Bafilomycin A1 (group 4), 0.5 mg/ml MTT (ATCC, Manassas, VA) was added to the media. Following 3 h of incubation, the insoluble formazan crystals within the cells were extracted using DMSO, and the absorbance was measured using a microplate reader (Molecular Devices SpectraMax Gemini EM) at a wavelength of 570 nm.

Quantitative gene expression

Total RNA was isolated from IEC 18 using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA (1 µg) were reverse transcribed using qScript cDNA supermix (Quanta Biosciences, Gaithersburg) and SYBR green-based RT-qPCR was performed with advanced qPCR Supermix (Wisent Inc., Quebec, Canada). Gene expression levels were quantified using Bio-Rad CFX Manager Software (Hercules, USA). Expression levels were calculated using the ΔΔCt method, and data were normalized to reference housekeeping genes GAPDH and RPLO. Inflammation (IL-6 and TNFα) and cellular apoptosis (caspase3) were analyzed. Sequence of primer in each gene is shown in Table 1.

Statistical analysis

GraphPad Prism6 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Results are presented as mean ± SD, as data were normally distributed (Kolmogorov–Smirnov test). Groups were compared using Student's *t* test or one-way ANOVA with Bonferroni correction as appropriate. p values of < 0.05 were considered significant. Data were quoted as mean ± standard deviation unless indicated otherwise.

Table 1 Primer genes used in running quantitative reverse transcription polychain reaction (qRT-PCR) along with their forward and reverse sequences

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
IL6	CCAATTTCCATTGCTCTCCT	ACCACAGTGAGGAATGTCCA
TNFα	TTCCGAATTCACTGGAGCCTCGAA	TGCACCTCAGGGAAGAATCTGGAA
Caspase 3	CCTCAGACATTCATGG	GCAGTAGTCGCCTCTGAAGA
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
RPLO	GGCGACCTGGAAGTCCAACT	CCATCAGCACCACAGCCTTC

IL6 Interleukin 6, *TNFα* tumor necrosis factor-alpha, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *RPLO* ribosomal protein LO

Results

Effects of Wortmannin and Bafilomycin A1 on autophagic vacuole number and diameter

Following administration of hypoxia, LPS, and Wortmannin or Bafilomycin A1, the number of autophagic vacuoles including autophagosomes and autolysosomes were measured using Electron microscopy (Fig. 1b).

We observed a significant increase the number of autophagic vacuoles 24 h post-administration of hypoxia + LPS compared to control (control: 2.2 ± 1.07, hypoxia + LPS: 13.4 ± 2.72, $p < 0.01$) (Fig. 1c). Wortmannin administration with hypoxia and LPS had a significant decrease in the number of autophagic vacuoles and Bafilomycin A1 administration with hypoxia and LPS had a significant increase in the number of autophagic vacuoles in comparison to of hypoxia + LPS alone (Wortmannin administration with hypoxia and LPS: 1.75 ± 1.16, $p < 0.01$; Bafilomycin A1 administration with hypoxia and LPS: 47.25 ± 11.36, $p < 0.01$).

There was a significantly larger diameter of autophagic vacuoles 24 h post-administration of LPS + hypoxia compared to control (control: 0.26 ± 0.16 µm, hypoxia + LPS: 1.24 ± 0.26 µm, $p < 0.01$) (Fig. 1d). Wortmannin administration with hypoxia and LPS did not show a significant difference in the diameter of autophagic vacuoles relative to hypoxia and LPS. By contrast, Bafilomycin A1 administration with hypoxia and LPS had a significantly larger autophagic vacuoles compared to administration of hypoxia + LPS (Wortmannin administration with hypoxia and LPS: 1.15 ± 0.31 µm, $p = 0.35$; Bafilomycin A1 administration with hypoxia and LPS: 2.73 ± 1.01 µm, $p < 0.01$).

Wortmannin increased cell viability while Bafilomycin A1 decreased cell viability relative to hypoxia and LPS alone

Following administration of hypoxia, LPS, Wortmannin, or Bafilomycin A1, cell viability was measured using an MTT assay after 24 h (Fig. 2a). Compared to control, we observed a significant decrease in cell viability 24 h post-administration of hypoxia + LPS (control: 0.59 ± 0.14, LPS + hypoxia: 0.43 ± 0.05, $p < 0.01$). Compared to administration of hypoxia + LPS alone, Wortmannin administration with hypoxia and LPS increased cell viability while Bafilomycin A1 administration with hypoxia and LPS decreased cell viability (Wortmannin administration with hypoxia and LPS: 0.49 ± 0.03, $p = 0.03$; Bafilomycin A1 administration with hypoxia and LPS: 0.34 ± 0.06, $p < 0.01$).

Wortmannin and Bafilomycin A1 both did not significantly affect inflammation relative to the hypoxia and LPS treated groups

The mRNA expressions of inflammatory cytokines were significantly higher in the groups of cells receiving hypoxia and LPS compared to control (IL6: $p < 0.01$, TNFα: $p < 0.01$, Fig. 2b, c). There was no significant difference in IL6 and TNFα among the three groups receiving stress factors (hypoxia and LPS) indicating that the administration of Wortmannin or Bafilomycin A1 did not affect inflammation (Fig. 2b, c).

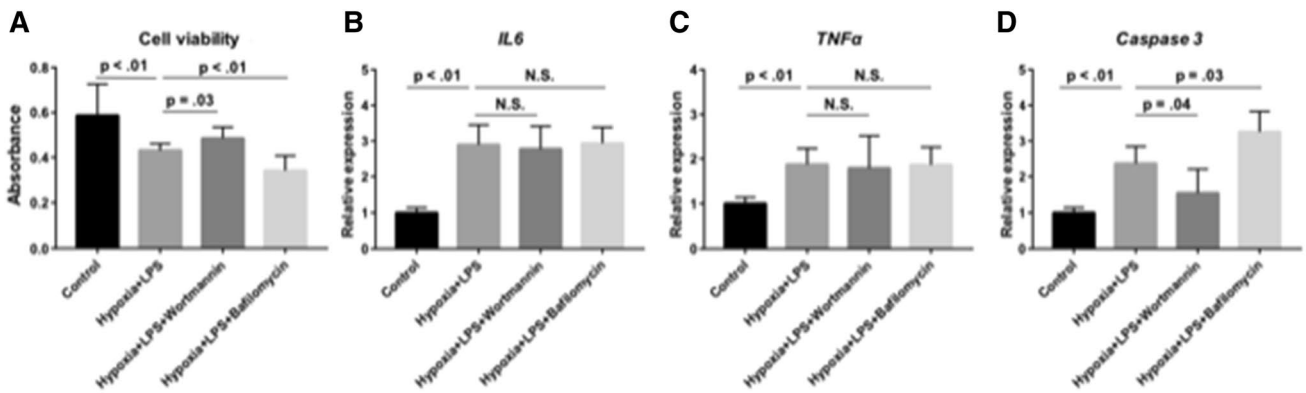

Fig. 2 Effect of inhibitor of autophagy flux. **a** Cell viability of IEC-18 following administration of hypoxia and LPS with Wortmannin or Bafilomycin A1 measured using an MTT colorimetric assay. **b**–**d** Relative expression of IL6 and TNFα inflammatory markers and caspase 3 apoptotic marker in IEC 18. IL6 (**b**), TNFα (**c**) and caspase 3 (**d**) relative mRNA expression levels were measured using qPCR. Data are presented as means ± SD from four independent experiments and differences between mean values were assessed by one-way ANOVA. $p < 0.05$ was considered significant. *N.S.* non-significance

Caspase 3 (mediator of apoptosis) was decreased by Wortmannin and increased by Bafilomycin A1

The mRNA expressions of caspase 3 were significantly higher in the hypoxia and LPS group relative to control ($p < 0.01$, Fig. 2d). However, during stress factors exposure (hypoxia and LPS), Wortmannin administration decreased the expression of caspase 3 while Bafilomycin A1 increased this expression, compared to administration of hypoxia + LPS alone (Wortmannin administration with hypoxia and LPS: $p = 0.04$; Bafilomycin A1 administration with hypoxia and LPS: $p = 0.04$, Fig. 2d).

Discussion

In this study, we demonstrated that accumulation of autophagic vacuoles was related to cellular toxicity. Our data show that stimulation of autophagosome synthesis such as that seen in hypoxia and LPS administration can be catastrophic when autophagy flux is defective.

Wortmannin is a selective inhibitor of phosphatidylinositol 3-kinase (PI3K) which is required for autophagy [12]. Inhibition of PI3K with Wortmannin can inhibit initiation of autophagy via inhibiting autophagic sequestration [13]. Bafilomycin A1 inhibits autophagic flux through two independent targets. By inhibiting the lysosomal proton pump V-ATPase, Bafilomycin A1 prevents autolysosome acidification and lysosomal enzyme activation. Additionally, Bafilomycin A1-dependent inhibition of the endoplasmic reticulum calcium (2+)-ATPase induces a defect in autophagosome–lysosome fusion. Bafilomycin A1 prevents cargo degradation and functional autophagic flux by acting on the autophagic process [14].

We demonstrated that the hypoxia and LPS-stimulated IEC 18 showed a significantly decreased cell viability compared with the control cells. Moreover, we found that inhibition of autophagy initiation by Wortmannin significantly abolished the hypoxia and LPS-mediated decrease in cell viability and increased apoptosis of IEC 18. In contrast, inhibition of autophagy flux by Bafilomycin A1 significantly worsened the hypoxia and LPS-mediated cell viability and increased apoptosis of IEC 18.These effects on cell viability and apoptosis can be attributed to the regulation of autophagy by Wortmannin and Bafilomycin A1 and highlight the important role that appropriately regulated autophagy plays in maintaining cells.

Autophagy plays an important role in cellular homeostasis and clearance of toxic substrates and damaged organelles. Moreover, autophagy is an important source of energy in times of stress, and a defect of protein degradation will eliminate this lifeline [4]. However, excessive autophagosome synthesis negatively impacts autophagy and leads to energy and nutrient depletion, which leads to cellular toxicity. During periods of stress, autophagosome synthesis is promoted. However, if autophagosome–lysosome fusion is rendered dysfunctional by an inhibitor of autophagy flux like Bafilomycin A1, further non-fused autophagosome synthesis becomes futile to the cell because autophagy cannot be completed. Also, excessive stress may cause a feedback loop to induce more autophagosome formation [15]. Therefore, the synthesis of non-fused autophagosomes is detrimental to cell survival by causing more strain on energy levels as well as a failure to clear potentially harmful toxins and promoting cell death [16]. Based on our study, reducing accumulation of autophagosomes may ameliorates cell viability of enterocytes.

Autophagy is induced by multiple stress pathways which may impact the intestinal barrier such as necrotizing enterocolitis (NEC) [11, 17] ischemic reperfusion (I/R) [18] and bacterial infection [19]. Neal et al. stated that the development of NEC in both mouse and human was associated with increased enterocyte autophagy; furthermore, they suspected that the induction of autophagy was not merely a consequence of NEC but a cause of NEC [8]. Moreover, Yu et al. demonstrated that autophagy and apoptosis were both rapidly upregulated in the rat experimental NEC model, where autophagy preceded the onset of apoptosis in the intestine [20]. These reports collectively have also suggested some benefit of depleting autophagy in the development of NEC [11, 20]. In the intestinal I/R model, intestinal injury was further deteriorated, and autophagy was further increased when pretreated with rapamycin (inhibition of mTOR and promoter of autophagy initiation). These results indicated that activation of autophagy by rapamycin aggravated intestinal injury induced by I/R [18]. In the same study, Li et al. also described that it showed that inhibition of autophagy could reduce intestinal injury induced by I/R [18]. Therefore, our model of toxicity resulting from accumulation of autophagosomes is consistent with previous studies and provides mechanistic insight into this phenomenon. Moreover, stimulation of autophagosome synthesis such as that induced by hypoxia and LPS can be worse when autophagy flux is defective. It is important to note that the most promising strategies for manipulating excessive autophagy in intestinal disease treatment have been those geared toward restoring flux, rather than solely inhibiting autophagosome synthesis.

Finally, our findings suggest that lowering the accumulation of autophagosomes may have a therapeutic value for intestinal disorders. Future in vitro as well as in vivo investigations into the toxicity of autophagosome accumulation in enterocytes may offer a new treatment option for these conditions.

Acknowledgements AP is supported by a Canadian Institutes of Health Research (CIHR) Foundation Grant 353857 and is the Robert M. Filler Chair of Surgery, The Hospital for Sick Children (HSC).

Author contributions MY: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. CL, SC, YY, MA, BL: collection and/or assembly of data, data analysis and interpretation, final approval of manuscript. AP: conception and design, financial support, final approval of manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

1. Choi AM, Ryter SW, Levine B (2013) Autophagy in human health and disease. N Engl J Med 368:651–662
2. Mizushima N, Noda T, Yoshimori T et al (1998) A protein conjugation system essential for autophagy. Nature 395:395–398
3. Munson MJ, Ganley IG (2015) MTOR, PIK3C3, and autophagy: Signaling the beginning from the end. Autophagy 11:2375–2376
4. Boya P, González-Polo RA, Casares N et al (2005) Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol 25:1025–1040
5. Song S, Tan J, Miao Y et al (2017) Crosstalk of autophagy and apoptosis: Involvement of the dual role of autophagy under ER stress. J Cell Physiol 232:2977–2984
6. Hosokawa N, Hara T, Kaizuka T et al (2009) Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell 20:1981–1991
7. Takeuchi H, Kondo Y, Fujiwara K et al (2005) Synergistic augmentation of rapamycin-induced autophagy in malignant glioma cells by phosphatidylinositol 3-kinase/protein kinase B inhibitors. Cancer Res 65:3336–3346
8. Ertmer A, Huber V, Gilch S et al (2007) The anticancer drug imatinib induces cellular autophagy. Leukemia 21:936–942
9. Crazzolara R, Cisterne A, Thien M et al (2009) Potentiating effects of RAD001 (Everolimus) on vincristine therapy in childhood acute lymphoblastic leukemia. Blood 113:3297–3306
10. Hotchkiss RS, Strasser A, McDunn JE et al (2009) Cell death in disease: mechanisms and emerging therapeutic concepts. N Engl J Med 361:1570–1583
11. Neal M, Sodhi C, Dyer M et al (2013) A critical role for TLR4 induction of autophagy in the regulation of enterocyte migration and the pathogenesis of necrotizing enterocolitis. J Immunol 190:3541–3551
12. Arcaro A, Wymann MP (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J 296:297–301
13. Blommaart EF, Krause U, Schellens JP et al (1997) The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem 243:240–246
14. Mauvezin C, Neufeld TP (2015) Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome–lysosome fusion. Autophagy 11:1437–1438
15. Button RW, Luo S (2017) The formation of autophagosomes during lysosomal defect: a new source of cytotoxicity. Autophagy 13:1797–1798
16. Button RW, Roberts SL, Willis TL et al (2017) Accumulation of autophagosomes confers cytotoxicity. J Biol Chem 292:13599–13614
17. Yu Y, Shiou S, Guo Y et al (2013) Erythropoietin protects epithelial cells from excessive autophagy and apoptosis in experimental neonatal necrotizing enterocolitis. PLoS One 8:e69620
18. Li B, Yao X, Luo Y et al (2019) Inhibition of autophagy attenuated intestinal injury after intestinal I/R via mTOR signaling. J Surg Res 243:363–370
19. Conway KL, Kuballa P, Song JH et al (2013) Atg16l1 is required for autophagy in intestinal epithelial cells and protection of mice from Salmonella infection. Gastroenterology 145:1347–1357
20. Maynard A, Dvorak K, Khailova L et al (2010) Epidermal growth factor reduces autophagy in intestinal epithelium and in the rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 299:614–622