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ORIGINAL ARTICLE

Amino acid deprivation disrupts barrier function and induces protective autophagy in intestinal porcine epithelial cells

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Abstract The integrity of intestinal barrier is essential for the absorption of nutrients and health in humans and animals. Dysfunction of the mucosal barrier is associated with increased gut permeability and development of various gastrointestinal diseases. Aside from serving as substrates for protein biosynthesis, amino acids also maintain the health of intestinal mucosal barrier. However, the underlying mechanisms remain unclear. We aimed to determine the effect and mechanism of non-essential amino acid (NEAA) deprivation on intestinal tight junction permeability using porcine intestinal epithelial cells as a model. We found that NEAA deprivation led to an impairment of barrier function as evidenced by increased permeability, decreased trans-epithelial resistance, and decreased expression of tight junction proteins claudin-1 and ZO-1. Importantly, NEAA deprivation induced both apoptosis and autophagy as shown by caspase-3 activation, and poly ADP-ribose polymerase cleavage; and LC3II lipidation and p62 degradation, hallmarks of apoptosis and autophagy, respectively. Importantly, we showed that the autophagy induced by NEAA deprivation counteracts apoptosis. Abrogation of autophagy by 3-methyladenine enhanced NEAA deprivation-induced barrier dysfunction and apoptosis; whereas, activation of autophagy by rapamycin partially rescued NEAA deprivation-induced barrier dysfunction and apoptosis. Taken together, our results demonstrate a critical role of NEAA on the mucosal integrity by regulating cell death and survival signaling pathways.

Keywords Amino acid deprivation · Intestinal barrier function · Apoptosis · Autophagy

Abbreviations

FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
IPEC	Intestinal porcine epithelial cells
MTT	4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
NEAA	Non-essential amino acids
mTORC	Mammalian target of rapamycin complex
PARP	Poly ADP-ribose polymerase
PI3K	Phosphatidylinositol 3-kinase
PKB/Akt	Protein kinase B
PMSF	Phenylmethylsulfonyl fluoride
TER	Trans-epithelial electrical resistance
ZO-1	Zonula occludens protein 1

Introduction

The single layer of epithelial cells and the junctional complexes between adjacent enterocytes constitute a selective barrier allowing the transport of essential dietary nutrients, electrolytes, and water from the intestinal lumen into the circulation and preventing the passage of harmful or unwanted substances from entering the internal environment, thereby maintaining the intracellular homeostasis (Arrieta et al. 2006; Jacobi and Odle 2012; Wang et al. 2014). Loss of intestinal barrier integrity, characterized by

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increased intestinal permeability, is associated with multiple gastrointestinal disorders (Arrieta et al. 2006; Blikslager et al. 2007; Camilleri et al. 2012; Groschwitz and Hogan 2009). Among the four types of junctional complexes, tight junctions located at the most apical region of the paracellular space between adjacent epithelial cells are the key regulators of epithelial paracellular permeability (Oswald 2006; Wang et al. 2014). In the intestine, tight junctions are assembled from at least three transmembrane proteins, including occludin, the claudins, and the junctional adhesion molecules, which are anchored to the cytoskeleton via peripheral membrane proteins, such as zonula occludens-1 (ZO-1), ZO-2, and ZO-3 (Furuse and Tsukita 2006; Schneeberger and Lynch 2004; Wang et al. 2014). Regulation of the assembly, disassembly, and maintenance of tight junction structures are influenced by various physiological and pathological stimuli, including oxidative stresses, cytokines, bacterial or viral infection, growth factors, hormones and nutrient status (Bojarski et al. 2000; Mizushima and Komatsu 2011; Singletary and Milner 2008; Ulluwishewa et al. 2011). For example, glutamine deprivation has been reported to disrupt barrier function and reduced tight junction proteins, claudin-1 and occludin in Caco-2 cells (Bojarski et al. 2000; DeMarco et al. 2003; Li et al. 2004) which can be rescued by glutamine addition (DeMarco et al. 2003), suggesting an important role for amino acid in barrier function.

The epithelium is consistently exposed to microbiota (commensal bacteria or pathogenic bacteria), toxin, damaged cells, proinflammatory cytokines, and nutrient metabolites, with the potential to induce apoptotic cell death, thus resulting in abnormal structure and function of the gut (Camilleri et al. 2012; Haynes et al. 2009; Mates et al. 2006). To function appropriately, new enterocytes are produced to replace the damaged cells and maintain the intestinal homeostasis (Bojarski et al. 2000; Camilleri et al. 2012; Parlesak et al. 2000). Breakdown of the balance between cell proliferation and apoptosis will lead to dysfunction of intestinal barrier and the development of intestinal diseases (Radtke and Clevers 2005; Schulzke et al. 2006). Interestingly, more and more recent studies demonstrated that apoptosis and autophagy are interconnected and contribute to diverse pathogenesis, such as intestinal disease, cardiovascular disease, and metabolic disease (Levine and Kroemer 2008; Mizushima et al. 2008; Patel et al. 2013; Wittkopf et al. 2012). It is unknown whether the crosstalk between apoptosis and autophagy exists and implicates in intestinal barrier function in response to non-essential amino acid (NEAA) deprivation. In this study, porcine intestinal epithelial cells (IPEC-1) were subjected to NEAA deprivation, and the trans-epithelial intestinal resistance, paracellular permeability, the expression tight junction proteins, apoptosis and autophagy were determined to explore the cellular response under NEAA deficiency.

Materials and Methods

Reagents

Insulin, rapamycin, propidium iodide, fluorescein isothiocyanate (FITC)-labeled dextran 4 kDa (FITC-D4), and 3-methyladenine were obtained from Sigma (St. Louis, MO). Antibodies against occludin, claudin-1, claudin-3, and ZO-1 were purchased from Zymed Laboratories (San Francisco, CA). Antibodies against LC-3, cleaved caspase-3, cleaved poly ADP-ribose polymerase (PARP), p62, β-actin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Immobilon-P PVDF transfer membrane was obtained from Millipore (Billerica, MA).

Cell culture and treatment

Cell culture medium (DMEM/F12), trypsin, phosphate-buffered saline (PBS), fetal calf serum (FCS), penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). IPEC-1 were grown in DMEM/F12 media supplemented with 2 mM glutamine, 10 mM HEPES, 100 units/mL penicillin G, 100 μg/mL streptomycin, 5 % FBS, epidermal growth factor (5 μg/L), insulin (5 μg/mL), transferrin (5 μg/mL), selenium (5 ng/mL) at 37 °C in an atmosphere of 95 % air and 5 % CO_2. Experiment for amino acids deprivation was performed as described (Tato et al. 2011). Briefly, cells were deprived of serum overnight and then were incubated for the indicated time periods with DMEM in the presence or absence of NEAA. In some experiments, cells were pretreated with specific inhibitors for 1 h before the exposure to NEAA deprivation at a final concentration of 20 nM rapamycin (Rapa) or 10 mM 3-methyladenine (3-MA).

Cell viability assay

Cell viability was determined by MTT assay as described (Martinet et al. 2005). Briefly, cells were plated in 96-well plates at a density of 5,000 cells/well 24 h before treatment. Then, the cells were incubated in the presence (served as control) or absence of NEAA for indicated time points. Cells were incubated with fresh MTT solution for 2 h and the absorbance at 570 nm was measured according to the manufacturer's protocol. Each value was normalized to that of control and expressed as mean ± SEM. Each experiment was repeated three times.

Determination of trans-epithelial electrical resistance

Cells were seeded at a density of 3×10^5 cells/cm^2 on collagen-coated permeable polycarbonate filters with a surface area of 0.33 cm^2 and 0.4 μm pore size (Corning, NY). The medium was changed every other day until a steady transepithelial electrical resistance (TER) was observed. The medium in both the apical and basolateral chambers was carefully replaced with fresh medium in the presence (served as control) or absence of non-essential amino acid. The monolayers were incubated at 37 °C in 5 % CO_2 for indicated time points before measuring the TER using an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) with a pair of chopstick electrodes. Data were collected from triplicate inserts per treatment in three experiments and expressed as percentage of basal TER obtained before treatment.

Measurement of paracellular permeability

Paracellular permeability was measured according to the method previously described with minor modification (Le Bacquer et al. 2003). In brief, monolayer cells were grown on Transwell plates and treated as described above. By the end of TER measurements, FITC-D4 was added to the apical compartment at a concentration of 0.2 g/L and incubated at 37 °C for indicated time points. Then, 50-μL aliquots of the medium from the apical and basolateral compartments, respectively, were collected and transferred into 96-well plates. FITC-D4 fluorescence was measured spectrophotometrically at an excitation wavelength of 492 nm and an emission wavelength of 540 nm. Monolayer permeability was qualified as percentage of FITC-dextran permeating from the apical to the basolateral compartment.

DNA fragmentation assay

Cells seeded at a density of 1×10^5/well in 24-well plates were incubated in the presence (served as control) or in the absence of NEAA for the indicated time periods. DNA fragmentation was determined as previously described (Larson et al. 2007). Briefly, both adherent and floating cells were collected by centrifugation and lysed, and the cell lysate was added to streptavidin-coated 96-well plates. DNA fragmentation (a measure of apoptosis) was quantitated by examination of cytoplasmic histone-associated DNA fragments using a Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol.

Caspase-3 colorimetric assay

Caspase-3 colorimetric assay was conducted as previously described (Larson et al. 2007). Cells were seeded in 60-mm plates at a density of 1×10^6/plate for 24 h before treatment. Cells were maintained in the presence (served as control) or absence of non-essential amino acid for indicated time points. Both adherent and floating cells were collected, washed with PBS, and lysed. The cell lysate was then analyzed for caspase-3 activity by the addition of a caspase-specific peptide conjugated with the color reporter molecule *p*-nitroaniline. Caspase-3 activity was quantitated spectrophotometrically at a wavelength of 405 nm according to the manufacturer's protocol. Results were expressed as fold change compared with control cells.

Flow cytometric analysis

Cells were treated as in caspase-3 activity assay and cell death was determined be the using of flow cytometric analysis. Briefly, cells were collected and washed with cold PBS two times by centrifugation at 1,000*g* for 3 min at room temperature. Cells fixed with 70 % (vol/vol) cold ethanol were then stained with propidium iodide (50 μg/mL). Nuclear DNA fragmentation was quantified by flow cytometric determination of hypodiploid DNA. Data were collected and analyzed by the using of FACScan flow cytometer (Becton-Dickinson).

Western blot analysis

Cells were lysed with ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 % Triton X-100, 100 μM NaF, 100 μM Na_3VO_4, 1 mM PMSF, 1 % sodium deoxycholate, 0.1 % SDS, and 1 mM protease inhibitor cocktail from Roche (Alameda, CA). Cell lysates were centrifuged for 15 min at 12,000*g* to remove cellular debris. Supernatants were collected and protein concentration was measured using a Bio-Rad Protein Assay kit. Equal amounts of protein were separated on SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were incubated at room temperature for 1 h in blocking solution (5 % skimmed milk), and then were incubated with indicated primary antibodies overnight at 4 °C. The bound primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and determined by the using of the ECL detection system (Amersham Biosciences, Piscataway, NJ). Blots were stripped and re-probed with anti-β-actin antibody to demonstrate equal loading.

Statistical analysis

Values are expressed as mean ± SEM. Statistical significance of differences between mean values were assessed with Student's *t* test or one-way ANOVA followed by the Turkey pairwise multiple comparison test (SPSS for

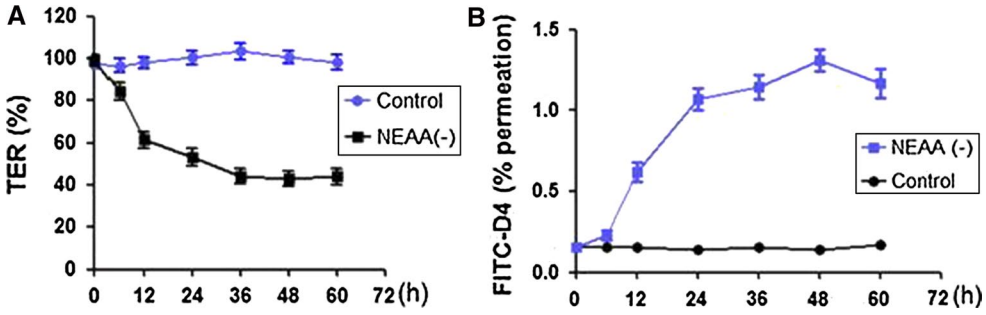

Fig. 1 Effect of NEAA deprivation on barrier functions in intestinal epithelial cells. IPEC-1 cells were incubated in the presence (served as control) or in the absence of NEAA, labeled as NEAA (−), for indicated time points. **a** Trans-epithelial electrical resistance (TER) and **b** paracellular permeability were determined. The data shown were mean ± SEM for three independent experiments

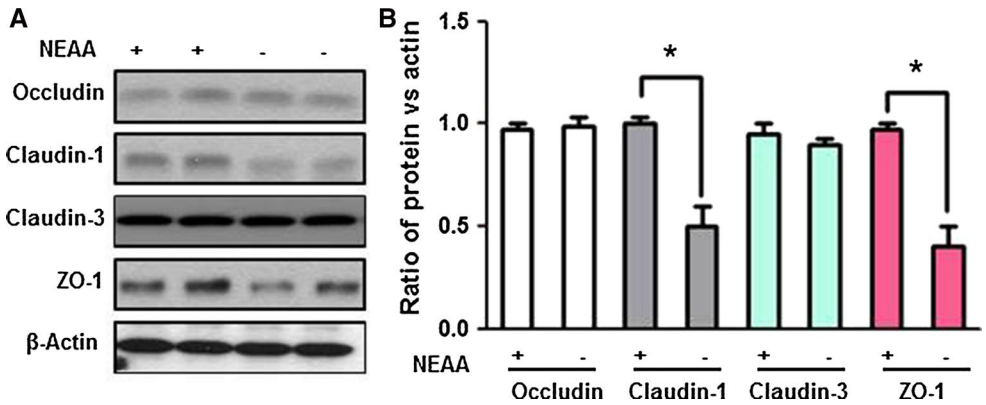

Fig. 2 Effect of NEAA deprivation on the expression of tight junction proteins. IPEC-1 cells were incubated in the presence or absence of NEAA for 24 h. **a** The protein levels of occludin, claudin-1, claudin-3, and ZO-1 were analyzed by Western blot (WB). β-actin was used as a loading control. **b** Densitometric analysis of each band was carried out using Image J software, and normalized against β-actin. Results were represented as mean ± SEM ($n = 3$), $^*P < 0.05$

Windows, version 17.0, Chicago, IL). A value of $P < 0.05$ was considered significant.

Results

NEAA deprivation impairs barrier function in intestinal porcine epithelial cells

To determine the consequence of NEAA deprivation on the barrier function, IPEC-1 cells were seeded in transwells and incubated in the presence or absence of NEAA. TER and tight junction permeability were determined. As shown in Fig. 1a, NEAA deprivation produced a time-dependent decrease of TER which was observed as early as 6 h post-treatment. The maximal decrease (60 % compared with that of control) of TER occured at 36 h post-treatment and lasted until 60 h. Consistent with the decreased TER, a time-dependent increase in paracellular permeability to FITC-D4 was observed 6 h post-treatment and the maximal increase was reached by 48 h post-treatment (Fig. 1b). The inverse relationship between TER and paracellular permeability (Fig. 1a, b) suggested an important role for NEAA on the maintenance of barrier integrity.

NEAA deprivation downregulates the expression of tight junction proteins

Tight junction proteins are located adjacent to epithelial cells and responsible for preventing lumen content and toxin from entering the internal environment by sealing the intracellular space (Arrieta et al. 2006; Wang et al. 2014). We next examined the effect of NEAA deprivation on the expression of tight junction proteins. Western blot results demonstrated that NEAA deprivation for 24 h led to a significant decrease of claudin-1 and ZO-1 (Fig. 2a, b). In contrast, the protein levels of occludin and claudin-3 were not affected by NEAA deprivation. This result suggested that the impaired barrier function by NEAA deprivation might be due to the declined expression of claudin-1 and ZO-1.

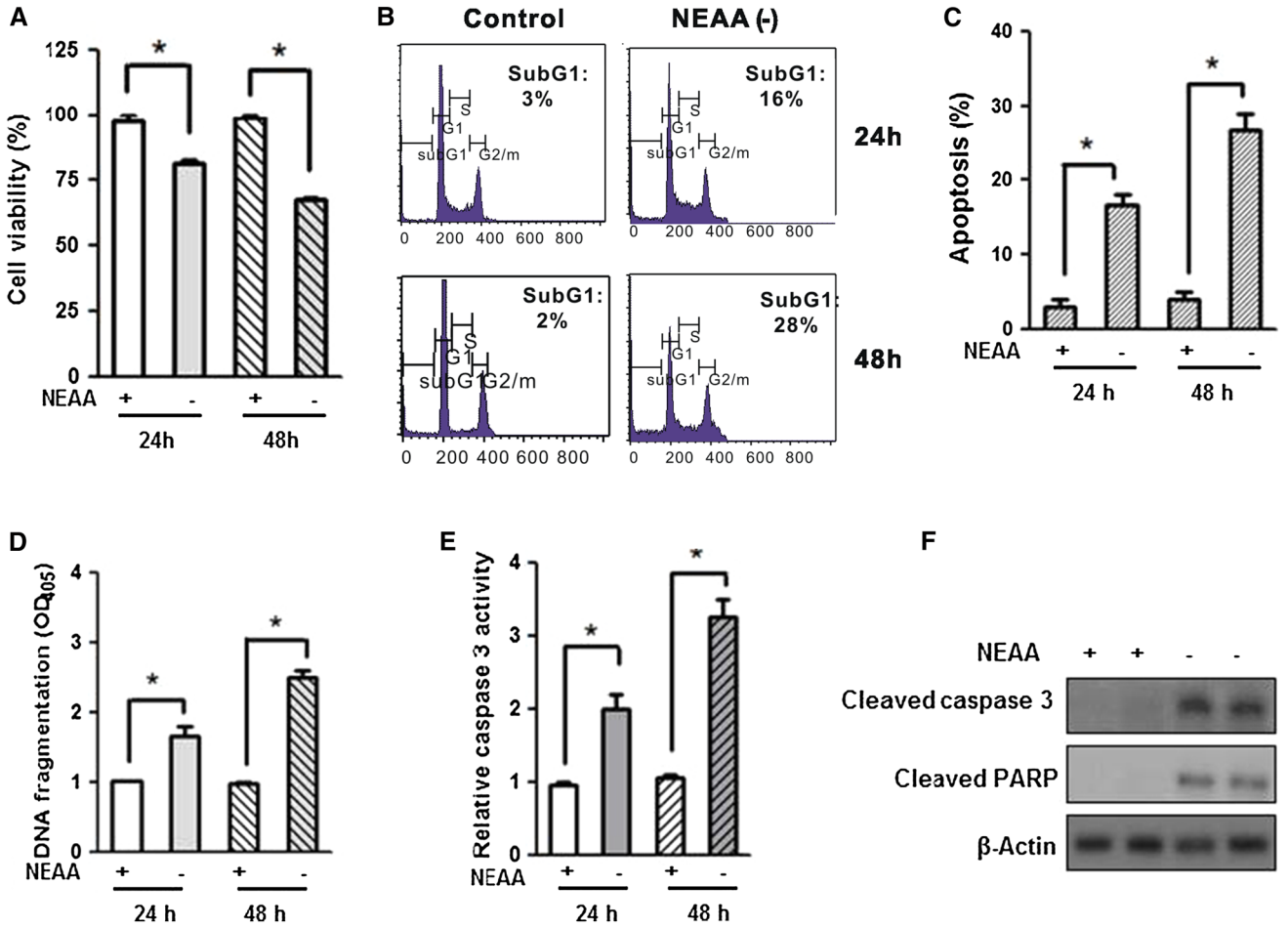

Fig. 3 Effect of NEAA deprivation on apoptotic cell death. Indicated assays were done using the cells incubated in the presence or in the absence of NEAA for 24 or 48 h. **a** Cell viability; **b** flow cytometric assay. **c** Quantitative analysis of apoptotic cell death in **b**; **d** DNA fragmentation; **e** caspase-3 activation; and **f** WB of protein expression of cleaved caspase-3 and cleaved PARP. Representative data from three separate experiments were shown here, $*P < 0.05$

NEAA deprivation induces apoptotic cell death

Cell death has been proposed as a mechanism that is associated with increased paracellular permeability in epithelial cells in response to stresses (Bojarski et al. 2000; Demehri et al. 2013; Schulzke et al. 2006). Amino acid deprivation can induce cell death in C2C12 muscle cells (Martinet et al. 2005) and colonic adenocarcinoma cells (Mates et al. 2006; Paquette et al. 2005). We next investigated the possibility of apoptosis in response to NEAA deprivation in IPEC-1. MTT assay showed that deprivation of NEAA resulted in decreased cell viability at both 24 and 48 h (Fig. 3a). Flow cytometric analysis showed that the decreased cell viability was caused by apoptotic cell death, as demonstrated by increased sub-G1 cells (Fig. 3b, c), not by the alteration in cell cycle progression (Fig. 3b). Consistently, the apoptotic effect was further confirmed by increased DNA fragmentation (Fig. 3d) and increased caspase-3 activity (Fig. 3e). Western blot analysis showed that deprivation of NEAA resulted in the activation of caspase-3 and PARP cleavage (Fig. 3f), two classic characteristics of apoptosis.

Autophagy is involved in NEAA deprivation

As amino acid limitation is a well-known stimulus of autophagy, an intracellular response to nutrient stress (Mizushima and Komatsu 2011), we next analyzed whether autophagy was induced. As shown, NEAA deprivation for 6 h led to a marked accumulation of lipidated LC3 and degradation of p62 (Fig. 4a, b), two of the autophagy markers (Fimia et al. 2013; Kroemer et al. 2010). Interestingly, autophagy inhibitor 3-MA (10 mM) treatment abolished NEAA deprivation-induced LC3 accumulation and p62 degradation, and enhanced the protein level of cleaved PARP (Fig. 4a). In agreement with this observation, NEAA deprivation-induced apoptosis was further enhanced in the presence of 3-MA as indicated by increased sub-G1 cells (Fig. 4b) and elevated caspase-3 activity (Fig. 4c).

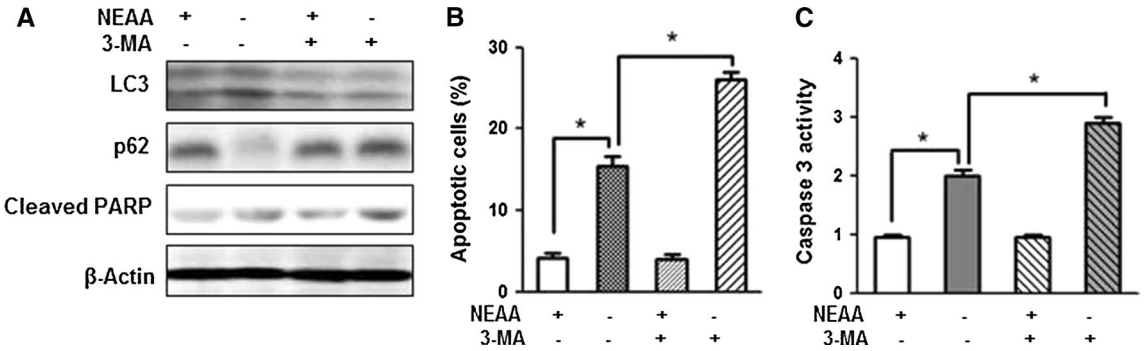

Fig. 4 NEAA deprivation induces protective autophagy that counteracts apoptosis. Cells were first pretreated with 3-MA (10 mM) for 1 h and then incubated in the presence or in the absence of NEAA for 6 h. **a** WB analysis determined the protein levels of LC3, p62 and cleaved PARP. β-actin was used as a loading control. **b** Apoptotic cell death was determined by flow cytometric analysis. **c** Caspase-3 activity was determined. Representative data from three separate experiments were shown here, $*P < 0.05$

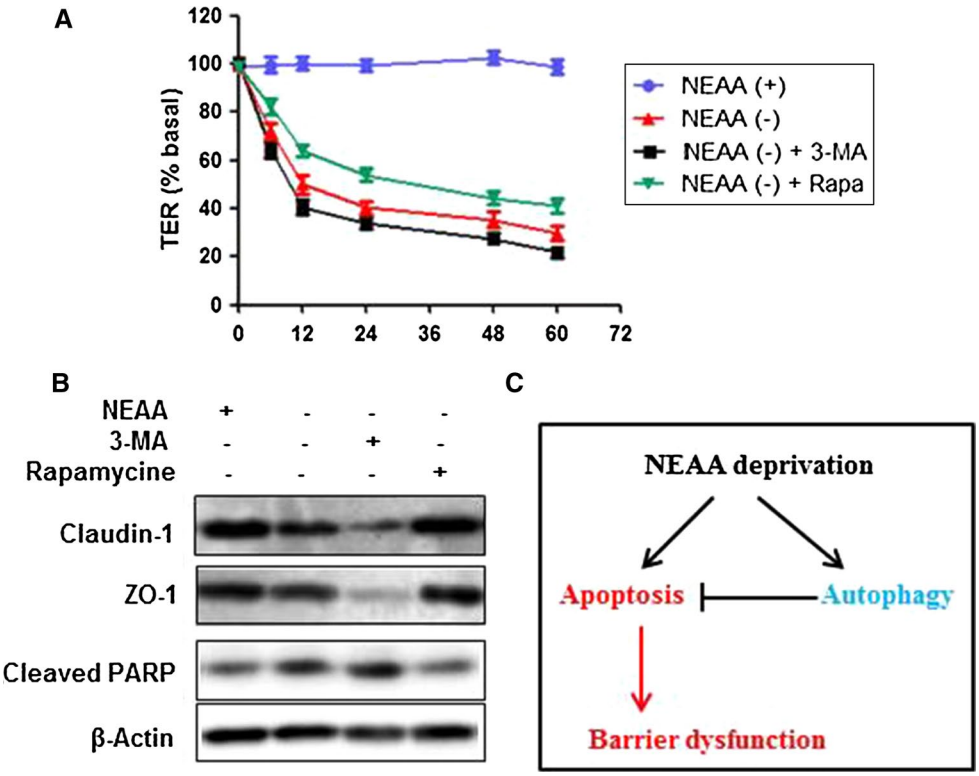

Fig. 5 NEAA depletion-induced autophagy is implicated in barrier dysfunction. Cells were pretreated with 3-MA (10 mM) or Rapa (20 nM) for 1 h and then incubated in the absence of NEAA for indicated time points. **a** TER was determined as described. The data shown were mean ± SEM for three independent experiments. **b** Protein levels of claudin-1, ZO-1, and cleaved PARP were determined by Western blot analysis at 24 h time point. Representative data from three separate experiments were shown. **c** Hypothetical model of the effect of NEAA deprivation in barrier dysfunction in the context of apoptosis an autophagy

NEAA deprivation-induced autophagy

To explore the functional role for autophagy on intestinal barrier integrity, cells pretreated with autophagy inhibitor 3-MA (10 mM, 1 h) or Rapa (20 nM, 1 h), an autophagy activator, were incubated in the presence or absence of NEAA and then TER was evaluated. Our result showed that abrogation of autophagy with 3-MA resulted in a further decrease of TER compared with NEAA-deprived cells (Fig. 5a); whereas, autophagy activator Rapa treatment partially reversed NEAA deprivation-induced TER drop (Fig. 5a). Consistently, NEAA deprivation-induced downregulation of tight junction proteins claudin-1 and ZO-1 was further enhanced or abolished by 3-MA or Rapa, respectively (Fig. 5b). In contrast, NEAA deprivation-induced PARP cleavage was further enhanced by 3-MA, but diminished by Rapa (Fig. 5b). These results indicated that autophagy induced by NEAA deprivation is a negative regulator of apoptosis.

Discussion

In the present study, we demonstrate that the deprivation of NEAA resulted in barrier function disruption as shown by decreased TER and increased paracellular permeability. The impairment of barrier function is accompanied by decreased tight junction protein expression, which includes claudin-1 and ZO-1. Mechanistically, the impairment of barrier function is mediated by the induction of both apoptosis and autophagy. One of the novel findings in the present study is that NEAA deprivation induced a protective autophagy because abrogation of autophagy by 3-MA further enhanced apoptosis and breakdown of barrier function, which can be partially rescued by Rapa, a negative regulator of mTOR.

IPEC-1 have been widely used in research on amino acid metabolism, nutrients absorption, protein biosynthesis and barrier function in vitro (Oswald 2006; Xi et al. 2012). In this study, we found that IPEC cells developed TER gradually and reached a comparative level, which is consistent with the observation in human epithelial cells previously reported (Le Bacquer et al. 2003). However, deprivation of NEAA in the culture medium led to decline of TER and increased tight junction permeability (Fig. 1). Among the critical components of junction proteins analyzed in our study, the protein levels of claudin-1 and ZO-1, instead of claudin-3 and occludin, were downregulated upon NEAA deprivation. The decreased protein level of claudin-1 was consistent with that observed in glutamine-deprived Caco-2 cells (DeMarco et al. 2003; Li et al. 2004). It is of note that the decreased claudin-1 expression in our system was not mediated by PI3K/AKT signaling as previously reported in Caco-2 cells (Li and Neu 2009), because PI3K or AKT inhibitors did not affect the protein level of claudin-1 and ZO-1 in NEAA-deprived cells (data not shown). Moreover, we did not observe the effect of NEAA deprivation on the expression of occludin, which was identified in another study (Noth et al. 2013). One possibility for this discrepancy is that the colonic adenocarcinoma cells used in Noth's study are different from the IPEC-1 cell line, which originated from a non-cancerous intestinal epithelium. It is known that cancer cells and normal epithelial cells differ in genetic or epigenetic background which might affect the gene or protein expression profiles (Meadows et al. 2008; Wang et al. 2012). The precise role for tight junction proteins in the modulation of intestinal barrier function remains unclear and warrants further investigation. In addition, numerous studies have demonstrated that increased expression of tight junction proteins is correlated with an enhanced barrier function (DeMarco et al. 2003; Li et al. 2004), whereas others have indicated an opposite correlationship between increased tight junction proteins and a decreased barrier function (Findley and Koval 2009). Thus, further investigation is required to resolve this discrepancy.

The epithelium of the intestine undergoes rapid renewal and turnover which is distinct from other types of cells in the body (Camilleri et al. 2012; Wang et al. 2014). This process is well-controlled by the balance between cell proliferation, survival, and apoptosis (Bach et al. 2000; Jacobi and Odle 2012). An increased epithelial apoptosis is thought to contribute to the impairment of intestinal barrier function in multiple gastrointestinal disorders (Strater et al. 1997; Zeissig et al. 2004). Consistently, inhibition of apoptosis has been reported to ameliorate barrier dysfunction induced by diverse stimuli (Gitter et al. 2000). In our study, NEAA deprivation led to decreased cell viability and the effect is due to an increased apoptosis instead of altered cell proliferation, as the S-phase cells (Fig. 3b) and BRDU staining analysis (data not shown) failed to detect significant difference between NEAA-depleted and control cells. The anti-apoptotic effect of NEAA might be mediated by down-regulating pro-apoptotic genes such as Bax and Bak, or by upregulating anti-apoptotic Bcl-2 family proteins Bcl-2 and Bcl-XL (Haynes et al. 2009; Mates et al. 2006; Takayama et al. 2009). Importantly, we showed that NEAA deprivation activated autophagy, an intracellular "self-eating" pathway, as shown by increased LC3 lipidation and p62 degradation. Our result is in agreement with a recent study showing that amino acid deprivation induced both apoptosis and autophagy in muscle cells (Martinet et al. 2005). Activation of autophagic survival mechanism by NEAA deprivation in our study is mediated by mTOR, a central regulator of cell growth and survival, and a negative regulator of autophagy (Singletary and Milner 2008). Under normal condition, mTOR is activated by amino acid to promote protein translation, and thus repressing autophagy. When extracellular amino acids are limited, mTOR is inactivated thus inducing autophagy to generate nutrients and energy through degradation of macromolecules and organelles in order to provide survival advantage under amino acid limitation conditions (Marino et al. 2014; Nicklin et al. 2009).

In conclusion, the data presented here demonstrated that NEAA deprivation disrupted the tight junction integrity and led to increased paracellular permeability. The breakdown of barrier function was mediated, at least in part, through the induction of apoptosis. The activation of autophagy is protective to NEAA deprivation. Our results suggest a critical role of NEAA on the mucosal integrity.

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Conflict of interest The authors declare no conflicts of interest.

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