Original Paper

Na⁺/H⁺ Exchanger Regulates Amino Acid-Mediated Autophagy in Intestinal Epithelial Cells

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Rong Lin^a Xiaohua Hou^a^aDepartment of Gastroenterology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, ^bWuhan Third Hospital, Wuhan, China**Key Words**Na⁺/H⁺ exchanger 3 (NHE3) • Autophagy • Alanine • Proline • Intestinal epithelial cells (IECs) • Inflammatory bowel diseases (IBDs)**Abstract**

Background/Aims: Dysfunctional autophagy has been reported to be associated with aberrant intestinal metabolism. Amino acids can regulate autophagic activity in intestinal epithelial cells (IECs). Na⁺/H⁺-exchanger 3 (NHE3) has been found to participate in the absorption of amino acids in the intestine, but whether NHE3 is involved in the regulation of autophagy in IECs is unclear. **Methods:** In the present study, an amino acid starvation-induced autophagic model was established. Then, the effects of alanine and proline with or without the NHE inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) were evaluated. Autophagy was examined based on the microtubule-associated light chain 3 (LC3) levels, transmission electron microscopy (TEM), tandem GFP-mCherry-LC3 construct, sequestosome-1 (SQSTM1, P62) mRNA and protein levels, and autophagy-related gene (ATG) 5, 7, and 12 expression levels. The autophagic flux was evaluated as the ratio of yellow (autophagosomes) to red (autolysosomes) LC3 puncta. **Results:** Following amino acid starvation, we found the LC3-II and ATG expression levels were enhanced in the IEC-18 cells. An increase in the number of autophagic vacuoles was concomitantly observed by TEM and confocal microscopy. Based on the results, supplementation with either alanine or proline depressed autophagy in the IEC-18 cells. Consistent with the elevated LC3-II levels, ATG expression increased upon NHE3 inhibition. Moreover, the mCherry-GFP-LC3 autophagic puncta representing both autophagosomes and autolysosomes per cell increased after EIPA treatment. **Conclusions:** These results demonstrate that NHE (most likely NHE3) may participate in the amino acid regulation of autophagy in IECs, which would aid in the design of better treatments for intestinal inflammation.

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Introduction

Autophagy is a multistep process involving nucleation, autophagosome formation, fusion of autophagosomes to lysosomes, and degradation in lysosomes [1], each of which is governed by multiple factors. Although the exact role of autophagy in disease remains the subject of debate, autophagy has been confirmed to be involved in tumour formation [2], neurodegeneration [3], epileptogenesis [4], cardiovascular disease [5, 6], inflammatory bowel diseases (IBDs) [7-9] and other diseases [10, 11]. Dysfunctional autophagy has been reported to be caused by genetic variation within Crohn's disease (CD) susceptibility genes, such as autophagy-related protein 16-like protein 1 (*ATG16L1*) and the immunity-related GTPase family M (*IRGM*), resulting in defective handling of invading bacteria in CD patients [12, 13].

The crosstalk between amino acids and autophagy was discussed as early as 1977 [14]. However, not all amino acids are involved in the regulation of this process. Recently, a growing body of evidence has indicated that proline and alanine each play an important role in the regulation of autophagy. As the only proteinogenic secondary amino acid, proline has special biological effects [15], serves as a regulator of all protein-protein interactions and responses to metabolic stress, and initiates a variety of downstream metabolic activities, including autophagy. In cancers, proline has been reported to act as a responder to nutrient and oxygen deprivation in autophagy [16]. Moreover, in the last few years, proline has been investigated for its distinctive metabolic functions in neuronal autophagy [17]. Although many reports have confirmed proline's unique autophagic functions, the utilization of this amino acid as a regulator of autophagy in the intestine remains unexplored.

Conversely, alanine has been shown to have a very specific coregulatory effect on autophagy [18]. The combination of alanine and leucine at physiological concentrations elicits the same inhibitory effect as a complete mixture of all 20 amino acids on autophagic proteolysis in rat hepatocytes [19]. This same study also showed that the metabolism of alanine, but not leucine, was required for its inhibitory effect on autophagy. Clearly, only certain amino acids are able to modulate autophagy, and their functions are highly cell-specific. However, the details of the relationship between certain amino acids and intestinal metabolism are unclear.

At the intestinal enterocyte brush border, amino acids are transported through H⁺-coupled amino acid transporter 1 PAT1 (SLC36A1) that symport H⁺ and amino acids into the cells and maintain the dynamic H⁺ electrochemical gradient [20, 21]. In particular, transport of both proline and alanine into intestinal cells has been shown to occur via the PAT1 [20, 22-24]. The transporters of amino acids in the intestine are critical for the supply of amino acids to all tissues and the regulation of the homeostatic plasma amino acid levels.

The driving force for PAT1 is the H⁺ electrochemical gradient across the intestinal epithelial membrane, which is primarily created by the exchange of Na⁺ and H⁺ via Na⁺/H⁺-exchanger (NHE) proteins, such as Na⁺/H⁺-exchanger 3 NHE3 (SLC9A3) [23, 24]. NHE3 is abundant in the Na absorptive cells of the mammalian small intestine and colon [25]. Thus, amino acid absorption via PAT1 indirectly depends upon NHE activity (specifically NHE3) in intestinal epithelial cells (IECs) [23, 24].

Herein, we investigated the function of NHE3 in amino acid-induced autophagy activation in the intestine. In particular, we examined the autophagic response following the administration of proline or alanine and ion transport in IEC-18 cells.

Materials and Methods

Establishment of autophagic cell models

For our autophagic cell model, the nontransformed rat small intestinal epithelial IEC-18 cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS;

Gibco Cell Culture, Melbourne, Australia) and 0.1 U/mL of bovine insulin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified 5% CO₂ atmosphere at 37 °C (Thermo Scientific, Waltham, MA, USA). For the amino acid starvation-induced autophagic cell model, the IEC-18 cells were incubated in Hank's Balanced Salt Solution (HBSS; Gibco/Invitrogen) as the culture medium for 6 h under the same atmospheric conditions. Then, alanine (1.0 mM final concentration; Sigma-Aldrich) [26, 27] or proline (0.5 mM final concentration, Sigma-Aldrich) [28] with or without the NHE3 inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA; 0.3 mM final concentration; A3085, Sigma-Aldrich) [29] was added to the culture medium and incubated with the cells for 6 h. To identify the optimal concentrations of amino acids (alanine and proline) and EIPA that elicited the best effect, various concentrations were tested (for all online supplementary material, see www.karger.com/doi/10.1159/000480184, Fig. S1). The effect of EIPA alone (without alanine or proline) was also examined in both control (DMEM cultured cells) and amino acid-starved cells. The cells were incubated for 6 h in DMEM containing 5% FBS and either 0 or 0.3 mM EIPA (labelled QNN and QNE, respectively), HBSS containing either 0 or 0.3 mM EIPA (labelled HNN and HNE, respectively), HBSS with 1.0 mM alanine (labelled HAN) or 0.5 mM proline (labelled HPN), HBSS with 1.0 mM alanine and 0.3 mM EIPA (labelled HAE), and HBSS with 0.5 mM proline and 0.3 mM EIPA (labelled HPE). To evaluate the autophagic activity, the autophagosome-lysosome fusion inhibitor chloroquine (CQ; 50 µM; Sigma-Aldrich) [30] was used. All grouping abbreviations were clarified in the Supplementary Material (see supplementary material).

Monomeric cherry (mCherry)–green fluorescent protein (GFP)–LC3 transfection and confocal microscopy

A plasmid-encoded tandem rat mCherry–GFP–LC3 construct was purchased for the quantification of autophagic maturation from Yingrun Biotechnology Co., Ltd. (Changsha, China). Transfection was performed with Lipofectamine 2000 (Invitrogen) in IEC-18 cells according to the manufacturer's instructions; then, the cells were cultured at 37 °C and 5% CO₂ for 6 h. The medium was replaced with fresh complete culture medium, and the cells were incubated for another 24 h. The cells were subsequently washed thrice with PBS for 5 min per wash and fixed with 4% paraformaldehyde for 10 min. The fixed cells were prepared for fluorescence scanning on glass slides with anti-fade mounting solution (Jackson ImmunoResearch Inc., PA, USA), and confocal microscopy was immediately performed using an A1Rsi/A1si Confocal Imaging System (Nikon, Tochigi, Japan) and the NIS-Element AR version 3.22 software (64-bit; Nikon).

Autophagic flux quantification

The autophagic flux was measured in the mCherry–GFP–LC3-transfected IEC-18 cells using an imaging-based assay as previously reported [31]. Confocal microscopy was used to count the number of yellow (autophagosome) and red (autolysosome) mCherry⁺ LC3 puncta per cell and the extent of the autophagic flux (loss of GFP fluorescence in mCherry⁺ puncta observed as a decrease in yellow puncta) [31]. Exposure to the acidic environment of the autolysosomes diminished the GFP fluorescence, whereas the mCherry⁺ signal remained stable. Hence, changes in the ratio of yellow to red LC3 puncta represent an autophagic flux.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from each cell sample using the TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using the MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed on a StepOne™ Real-Time PCR system (Applied Biosystems). The PCR products for autophagy-related gene (*ATG*) 5, 7, and 12, microtubule-associated light chain 3 (*LC3*), sequestosome-1/SQSTM1 (*P62*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were amplified. All samples were normalized to rat *GAPDH*. The primers used are listed in Table 1.

Table 1. Primers used to amplify the autophagy-related genes

Primer	Forward primer	Reverse primer
<i>ATG5</i>	ATACTCTGTCTCTGCTGTCCT	CAAGCTCAGCTAG
<i>ATG7</i>	TGGTGGAGCAGTGAAGACC	GATTCCACCAGAGACCGGAC
<i>ATG12</i>	GGCTACAGAGTAAGACTGTCA	GTGACCATGTTGTTCCACAGCA
<i>LC3</i>	CTTCGCCGACCGCTGTTA	ATCCGTCTTCATCCTTCTCTCTG
<i>P62</i>	CAACTGCTTTGACTGTCCTGGAT	GCTGTGGAGATGCTGTAGCT
<i>GAPDH</i>	TCTTCCAGGAGCGAGATCCC	TTCAGGTGAGCCCCAGCCTT

Western blotting

IEC-18 cells were seeded into 6-well plates and subjected to the respective treatments. After a 6-h incubation, the media were removed, and the cells were washed thrice with ice-cold PBS (pH 7.4). Adherent cells were collected with a cell scraper and lysed in radioimmunoprecipitation assay buffer containing 1% phosphatase inhibitors (Applygen Technologies Inc., Beijing, China) on ice for 30 min with intermittent vortexing (5-7 times, 30 s each, at 5-min intervals). Then, the cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C, and the supernatants were collected on ice. The protein concentration was measured using a Pierce BCA protein assay kit (Thermo Scientific), and the samples were stored at -80 °C until needed. Protein samples (30 µg/well) from each treatment group were separated by 12% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (0.45 µm). The membranes were blocked with 10% (w/v) skim milk in 1× Tris-buffered saline and Tween-20 (pH 7.4) at room temperature for 1 h and then probed separately with LC3B (1:1000; Cell Signaling Technology, Danvers, MA, USA) and P62 (1:1000; Cell Signaling Technology) antibodies at 4 °C overnight. The next day, the membranes were washed thrice for 10 min per wash and incubated with a goat anti-rabbit IgG secondary antibody (HRP) (1:5000; Jackson ImmunoResearch Inc.) at room temperature for 1 h. Then, the membranes were washed thrice as described above, and the antibody signals were detected by autoradiography using a Pierce™ ECL Western Blotting Substrate (Thermo Scientific). Detection of LC3-II was used to evaluate the level of activated LC3. Densitometric analysis of the blots was conducted using the FluorChem FC3 system (ProteinSimple, California, USA).

Transmission electron microscopy (TEM)

Cells were seeded into 6-well plates and subjected to the respective treatments. After 6 h of incubation, the media were removed, and the cells were washed twice with PBS (pH 7.4) prior to being trypsinized and collected. Then, the cells were fixed with 2.5% phosphate-buffered glutaraldehyde for 2 h and stored at 4 °C prior to embedding. After three washes with PBS (pH 7.4), the cells were post-fixed with 1% OsO₄ (GBC Biology, Guangzhou, China) for 30 min, dehydrated with an increasing gradient of ethanol and acetone, and embedded in Epon 812 (SPI Supplies, West Chester, PA, USA). Ultrathin sections (60-80 nm) were obtained with a diamond knife on a Leica Ultracut UCT (Leica Microsystems GmbH, Wetzlar, Germany), adhered to uncoated 200-mesh copper grids, stained with 2% uranyl acetate and lead citrate for 15 min each, and then observed under a Tecnai™ G2 12 TEM (FEI Co., Hillsboro, OR, USA) after drying overnight at room temperature.

Statistical analysis

The SPSS 21.0 statistical software was used for the data analysis. The data are expressed as the mean ± standard error or the standard deviation of at least three independent experiments. Differences between groups were analysed using one-way analysis of variance (ANOVA), followed by Student's t test for comparisons. *P* < 0.05 was considered significant.

Results

Alanine and proline reduce amino acid starvation-induced autophagy

To evaluate the role of alanine or proline in autophagy, autophagy-related gene mRNA expression, the LC3 and P62 protein levels were assessed by amino acid-starved IEC-18 cells. The alanine or proline addition decreased the LC3-II level in the presence/absence of the lysosomal inhibitor CQ (Fig. 1B and 1C) and reduced the P62 flux (Fig. 1B and 1C). The effect of the alanine and proline combination on the autophagic activity during starvation was examined by LC3 protein levels (see supplementary material, Fig. S2). LC3, P62, and ATG5, 7, and 12 expression was dramatically decreased in the HAN group compared with the HNN group (LC3: 0.01751±0.00358 vs 0.03755±0.00816, *P* < 0.05; P62: 0.00842±0.0019 vs 0.0176±0.00392, *P* < 0.05; ATG5: 0.0051±0.0015 vs 0.0555±0.0158, *P* < 0.05; ATG7: 0.0012±0.0001 vs 0.0268±0.0092, *P* < 0.01; ATG12: 0.0072±0.0015 vs 0.0236±0.0067, *P* < 0.05). Similar findings were observed in the HPN group (LC3: 0.01792±0.00186 vs 0.03755±0.00816, *P* < 0.05; P62: 0.00727±0.0022 vs 0.0176±0.00392, *P* < 0.05; ATG5: 0.0062

Fig. 1. Alanine and proline each reduce autophagy in nutrient-starved IEC-18 cells. (A) NHE3 in the intestinal epithelial cells (IECs). (B) IEC-18 cells were cultured under normal conditions (DMEM with 5% FBS; QNN group), HBSS (HNN group), HBSS with alanine (HAN group), or HBSS with proline (HPN group) for 6 h. The LC3 and P62 protein expression levels were detected by western blotting. The data are presented as the mean ± standard deviation (SD); **P* < 0.05; ***P* < 0.01. (C) IEC-18 cells were cultured with HBSS (HNN group), HBSS with alanine (HAN group), or HBSS with proline (HPN group) in the presence or absence of CQ for 6 h. Samples were collected and immunoblotted with an anti-LC3, anti-P62 or anti-GAPDH antibody. The data are presented as the mean ± standard error (SE) (*n* = 3 experiments); **P* < 0.05, ***P* < 0.01. (D) qRT-PCR was used to measure LC3, P62 and ATG5, 7, and 12 expression. Images are representative of more than three separate experiments. The data are presented as the mean ± SE; **P* < 0.05, ***P* < 0.01.

± 0.0026 vs 0.0555 ± 0.0158, *P* < 0.05; ATG7: 0.0014 ± 0.0002 vs 0.0268 ± 0.0092, *P* < 0.01; ATG12: 0.0079 ± 0.0034 vs 0.0236 ± 0.0067, *P* < 0.05). The NHE3 levels in the presence or

absence of HBSS, proline or alanine was also checked (see supplementary material, Fig. S3). All of the results indicated that alanine and proline reduced the autophagic activity induced by amino acid starvation.

Enhancement of LC3-II protein levels with EIPA treatment

To examine the role of NHE3 in the amino acid-dependent regulation of autophagy, we treated IEC-18 cells with the NHE3 inhibitor EIPA. The Western blotting results demonstrated

Fig. 2. EIPA enhances autophagy in IEC-18 cells. (A) IEC-18 cells were cultured with either alanine or proline with and without EIPA for 6 h. The effects of EIPA alone (without alanine or proline) were also tested in both control and nutrient-starved cells. The LC3 and P62 protein expression levels were determined by western blotting. The data are presented as the mean $\pm$ SE [n = 3 experiments]; *P<0.05, **P<0.01. (B) IEC-18 cells were cultured with alanine or proline with and without EIPA in the presence or absence of CQ for 6 h. The total LC3 and P62 density relative to the GAPDH levels was compared. The data are presented as the mean $\pm$ SE (n = 3 experiments); *P<0.05, **P<0.01; NS, not significant. (C) ATG5, 7, 12, and P62 expression was measured by qRT-PCR. The data are presented as the mean $\pm$ SE; *P<0.05, **P<0.01.

that the total LC3-II protein levels were markedly increased by EIPA treatment in the HAE and HPE groups compared with the HAN and HPN groups, respectively, and that this increase was more obvious in the presence of CQ in both the HAE and HPE groups (Fig. 2A and 2B). The P62 flux was also increased under EIPA treatment (Fig. 2A and 2B). Analysis of the LC3 flux and P62 flux in the presence or absence of a lysosomal inhibitor in QNN and QNE group was also conducted (see supplementary material, Fig. S4). These data demonstrated that EIPA reversed the reductions in autophagy upon supplementation with amino acids, suggesting that alanine and proline could each regulate IEC-18 cell autophagy through NHE3.

EIPA upregulates ATG expression

Compared with the HAN group, *ATG5*, 7, 12 and *P62* expression was increased in the IEC-18 cells treated with EIPA (HAE group) (Fig. 2C; *ATG5*: 0.0106 ± 0.0008 vs 0.0067 ± 0.0009 , $P < 0.05$; *ATG7*: 0.0051 ± 0.0012 vs 0.0029 ± 0.0005 , $P < 0.05$; *ATG12*: 0.0240 ± 0.0067 vs 0.0113 ± 0.0035 , $P < 0.05$; *P62*: 0.0102 ± 0.0021 vs 0.0036 ± 0.0005 , $P < 0.05$). Similarly, *ATG5*, 7, and 12 and *P62* expression was increased in the HPE versus HPN groups (*ATG5*: 0.016 ± 0.0025 vs 0.0066 ± 0.0009 , $P < 0.05$; *ATG7*: 0.0282 ± 0.0058 vs 0.0021 ± 0.0005 , $P < 0.05$; *ATG12*: 0.0633 ± 0.0191 vs 0.0437 ± 0.0160 , $P < 0.05$; *P62*: 0.0173 ± 0.0046 vs 0.0120 ± 0.0042 , $P < 0.05$). These data confirmed that both alanine and proline alone downregulated *ATG* expression through NHE3.

Fig. 3. Changes in autophagy-related structures observed via transmission electron microscopy in IEC-18 cells. (A) IEC-18 cells were cultured under normal condition (DMEM with 5% FBS; QNN group), HBSS (HNN group), HBSS with alanine (HAN group), HBSS with proline (HPN group), HBSS with alanine and EIPA (HAE group), or HBSS with proline and EIPA (HPE group) for 6 h. Autophagic vacuoles are denoted by black arrows. Scale bars: 1 or 2 μ m. The images are representative of three separate experiments. (B) The numbers of AVs in each group and the normalization counts per total cellular area are shown. The data are presented as the mean $\pm$ SE; * $P < 0.05$, ** $P < 0.01$. At least five cells in each group were counted.

EIPA treatment increases the number of autophagic vacuoles

As shown by TEM, amino acid deficiency increased the number of autophagic vacuoles in the HNN group compared with the QNN group ($P < 0.05$, Fig. 3A and 3B). The addition of either alanine or proline produced fewer autophagic vacuoles in the HAN and HPN groups than in the HNN group ($P < 0.05$, Fig. 3A and 3B). However, the number of autophagic vacuoles increased dramatically in the HAE and HPE groups after EIPA treatment compared with the HAN and HPN groups ($P < 0.01$, Fig. 3A and 3B).

EIPA regulates the initiation and maturation of autophagy

The EIPA-induced autophagic flux was investigated in the presence and absence of the autophagosome-lysosome fusion inhibitor CQ. As expected, more yellow (autophagosome) and red (autolysosome) LC3 puncta were observed in the HNN group than in the QNN group (Fig. 4A). Additionally, the numbers of yellow and red LC3 puncta were clearly reduced in both

Fig. 4. Transfection of the mCherry-GFP-LC3 plasmid into IEC-18 cells and quantification of the autophagic puncta by confocal microscopy. (A) IEC-18 cells were cultured under normal conditions (QNN group), HBSS (HNN group), HBSS with alanine (HAN group), HBSS with proline (HPN group), HBSS with alanine and EIPA (HAE group), or HBSS with proline and EIPA (HPE group) in the presence and absence of CQ for 6 h. Representative epifluorescence images show the cellular localization of mCherry-GFP-LC3 in the IEC-18 cells. (B-E) The mean number of autophagosomes and autolysosomes are represented by yellow and red dots, respectively, in the merged images. The results represent the means from at least three independent experiments; * $P < 0.05$, ** $P < 0.01$. At least five cells in each group were counted.

the HAN and HPN groups compared with the HNN group (Fig. 4A, 4B and 4C). Hence, both alanine and proline can individually reduce the amino acid starvation-induced activation of autophagy. The addition of EIPA markedly increased the number of yellow and red LC3 puncta in both the HAE and HPE groups compared with the HAN and HPN groups (Fig. 4A and 4D). When lysosomal degradation was inhibited by CQ, we observed an accumulation of yellow LC3 puncta in all groups (Fig. 4A and 4E). These data suggested that EIPA regulated the initiation and maturation of the autophagy associated with amino acids in IEC-18 cells.

Discussion

Recent advances have provided important new insights into several factors that are crucial for maintaining intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species generation, and autophagy [32, 33]. Moreover, dysfunctional autophagy has been shown to closely correlate with CD patients [12]. Historically, enteral nutrition, which is a supplemental nutritional therapy for CD patients with planned resection surgery, has been shown to produce unexpected anti-inflammatory activity in active CD patients [12]. Initially, amino acid-based elemental diets were administered to CD patients due to their lack of antigenic dietary proteins and oligopeptides, which were thought to be involved in the inflammatory process [34]. Furthermore, clinical trials [34] showed that amino acid-based elemental diets can induce remission of severely active CD. Thus, elucidating the exact mechanism whereby amino acids regulate autophagy will likely yield new therapeutic strategies for IBDs.

Autophagy, which plays a variety of physiological and pathophysiological roles, is thought to be a cell survival mechanism during nutrient deprivation conditions. Many amino acids have been clearly shown to be associated with autophagy. In particular, proline and alanine have been implicated in the regulation of this process [15, 16]. Interestingly, both proline and alanine can be transported into the intestine by the PAT transport systems [20, 22-24]. Considering the unique metabolic characteristics and functions of proline and alanine in autophagy, we examined the effects of these two amino acids on autophagy in IECs.

The present study demonstrated that amino acid deficiency could induce autophagy and that this autophagy could be relieved by supplementation with alanine or proline, which was in agreement with previous studies [35-37]. Proline and alanine can be transported into the intestine by PAT1 [20, 22-24]. However, the potential autophagy-regulating function of NHE3, which provides the direct driving force for PAT1, has been poorly characterized. Hence, we proposed that NHE3 might contribute to the regulation of autophagy under specific circumstances in IECs.

The PAT1 is a high-capacity amino acid carrier that is located at the luminal membrane of small intestine and mediates the uptake of nutrients and drugs in a pH gradient-dependent manner [20, 38]. NHE3, which continually traffics between cellular recycling compartments and the plasma membrane, is responsible for maintaining the Na^+ and H^+ balance in the intestine [25, 39]. The phenotype of *SLC9A3* knock-out mice includes induced mild diarrhoea, acidosis, an impaired acid-base balance, Na^+ -fluid volume homeostasis in the intestine and spontaneous distal colitis triggered by luminal bacteria [25].

One route for amino acid transport at the intestinal epithelial brush border is via PAT1, which is regulated by NHE3 maintenance of the H^+ electrochemical gradient across the membrane [20, 21]. The β -alanine can reportedly be transported by PAT1 across the intestinal epithelial brush border membrane. This activity can be modulated by caffeine, which is a type of phosphodiesterase inhibitor that indirectly inhibits NHE3 activity [20, 40]. Therefore, we inferred that NHE3 could similarly participate in the regulation of autophagy by modulating the amino acid absorption function of PAT1.

We believe our findings have important clinical implications. Elucidating the mechanism whereby NHE3 inhibition affects amino acid transport holds the potential to yield new strategies for IBD treatment. Our data show that the NHE3 inhibitor EIPA can reverse the

effects of alanine and proline on amino acid starvation-induced autophagy in IEC-18 cells, suggesting that NHE participates in the regulation of amino acid-mediated autophagic activity in IECs. We hypothesize that inhibition of NHE3 will result in an increase in the intracellular H^+ levels and suppression of the amino acid transporter activity, thereby resulting in the impaired absorption of amino acids associated with autophagy regulation in IECs.

Overall, our work emphasizes the significant role played by NHE in regulating amino acid-mediated autophagic activity in IECs, which further contributes to the maintenance of gut homeostasis. However, further studies are required to characterize the initiation of autophagy associated with NHE3 in the pathogenesis of intestinal inflammation. This thorough study of the mechanism by which NHE regulates autophagy in the intestine will provide important insights into the pathogenesis of IBDs and may yield new therapeutic approaches for IBD patients.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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