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ORIGINAL ARTICLE

Methionine deficiency reduces autophagy and accelerates death in intestinal epithelial cells infected with enterotoxigenic *Escherichia coli*

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Abstract Infections by enterotoxigenic *Escherichia coli* (ETEC) result in large economic losses to the swine industry worldwide. Dietary supplementation with amino acids has been considered as a potential mechanism to improve host defenses against infection. The goal of this study was to determine whether methionine deprivation alters ETEC interactions with porcine intestinal epithelial cells. IPEC-1 cells were cultured in media with or without L-methionine. Methionine deprivation resulted in enhanced ETEC adhesion and increased both the cytotoxicity and apoptotic responses of IPEC-1 cells infected with ETEC. Methionine deprivation inhibited IPEC-1 cell autophagic responses, suggesting that the increased cytotoxicity of ETEC to methionine-deprived IPEC-1 cells might be due to defects in autophagy.

Keywords Apoptosis · Autophagy · Cytotoxicity · Enterotoxigenic *Escherichia coli* · IPEC-1 · Methionine

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC) causes porcine post-weaning diarrhea (Turner et al. 2006), resulting in enormous economic losses to the swine industry (Fairbrother et al. 2005). ETEC adherence to intestinal epithelial cells and disruption of host cell functions by protein enterotoxins are both considered essential to the disease process (Fleckenstein et al. 2010).

Amino acid deficiency can affect immune responses (Sakkas et al. 2013) and thus dietary supplementation of specific amino acids in animal feed has been considered as a potential production strategy (Ren et al. 2013). However, there is a general lack of understanding of how specific amino acids might modulate the functions of intestinal epithelial cells.

Methionine is a sulfur-containing amino acid with numerous biological functions, including protein metabolism, oxidative stress, and methylation (Metayer et al. 2008; Tesseraud et al. 2009). Methionine deficiency results in reduced protein synthesis, alters the mTOR/S6K1 signaling pathway (Metayer-Coustard et al. 2010), and may impact the oxidative status of the cell.

Autophagy involves the sequestration of cytoplasmic proteins in double-membrane autophagosomes and their subsequent delivery to lysosomes for degradation (Mizushima et al. 2010). Autophagy serves as a cell survival mechanism during periods of nutrient deprivation and microbial infection (Deretic and Levine 2009). Glutamine and arginine concentrations acids regulate autophagy (Sakiyama et al. 2009; Yan et al. 2012). However, the effects of methionine on gut function remain poorly documented. Thus, the aims of the present study were to investigate the role of methionine on ETEC interactions with intestinal epithelial cells and to test the hypothesis that methionine

deficiency would affect host apoptotic and autophagic responses.

Materials and methods

Bacterial strains

The ETEC strain SEC470 (F12, LT+, STa+ and STb+) was originally isolated from the anus of a piglet afflicted with diarrheal disease. It was cultured in Luria–Bertani broth or on agar at 37 °C (Chen et al. 2014a, b).

Cell culture and transfection

A custom-prepared DMEM-F12 medium lacking methionine was prepared by the manufacturer (Invitrogen). For experiments involving methionine supplementation, methionine (1.724 mg/ml) was diluted 100-fold into the DMEM-F12 media. IPEC-1 intestinal epithelial cells were grown in DMEM-F12 with or without 17.24 mg/l methionine and supplemented with 5 % fetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml) (ITS, ScienCell), and 5 ng/ml of epidermal growth factor (EGF; Sigma). For methionine-deprivation assays, IPEC-1 cells were cultured to 90–95 % confluence, and then cells were incubated for 24 h in IPEC medium without methionine. IPEC/EGFP-LC3 cells that stably express EGFP-LC3 were used for autophagy experiments (Tang et al. 2014). All cells were incubated at 37 °C and 5 % CO_2.

Bacterial infection

IPEC-1 or IPEC/EGFP-LC3 cells were grown in 6-well tissue culture plates. ETEC SEC470 was cultured in LB for 6 h at 37 °C, harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), and resuspended in fresh DMEM. Confluent monolayers of IPEC-1 or IPEC/EGFP-LC3 cells were infected with ETEC SEC470 at a multiplicity of infection (MOI) of 1:100.

Confocal microscopy

IPEC/EGFP-LC3 cells were seeded on coverslips (Fisher) in 6-well tissue culture plates for overnight culture at 37 °C. For the detection of autophagosomes, cells cultured in medium with or without L-methionine were infected with ETEC or treated with 0.5 μM rapamycin (a positive control for autophagy experiments) for indicated times. Cells were washed with PBS, fixed and permeabilized with 80 % cold acetone at –20 °C for 20 min, and washed again with PBS. Cells were then counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Life Technologies). EGFP-LC3 fluorescence was observed using a laser scanning confocal microscope and the average number of EGFP-LC3 punctae per cell from at least 60–80 cells per sample was counted.

Cell proliferation assays

Proliferation of IPEC-1 cells was evaluated using a CCK-8 assay (Beyotime Institute of Biotechnology, China), based on the cleavage of the tetrazolium salt WST-8 by mitochondrial dehydrogenase in viable cells. Five thousand cells/well were cultured for 1–4 h before 10 μl CCK-8 (5 mg/ml) was added to 100 μl culture medium in 96-multiwell plates. After 1 h incubation at 37 °C, the absorbance at 450 nm of each well was measured using a Thermomax microplate reader. Each experiment was repeated three times, and the data are expressed as the mean and standard deviation.

Bacterial adherence assays

IPEC-1 cells were split into 24-well plates (approximately 5×10^5 cells/well), cultured to 90–95 % confluence, and then maintained in DMEM with or without L-methionine for 24 h. ETEC strains were grown in LB medium for 6 h at 37 °C, harvested by centrifugation, and washed three times in sterile PBS. Adherence assays were performed by inoculating the confluent cell monolayers with $\sim 5 \times 10^6$ CFU/ml ETEC. The plates were incubated for 1 h at 37 °C and 5 % CO_2. The monolayers were then washed with PBS, trypsinized, and disrupted by repeated pipetting. Serial dilutions of the cell lysates were plated onto LB agar plates and incubated overnight at 37 °C for bacterial enumeration.

Cytotoxicity assays

IPEC-1 cells cultured in DMEM with or without L-methionine were split into 24-well plates (approximately 5×10^5 cells/well). ETEC was added to the wells and incubated at 37 °C for 2 h. Cytotoxicity due to ETEC infection was assayed by quantifying lactate dehydrogenase (LDH) release using the CytoTox96 kit (Roche). Absorbance was measured at 490 nm using a Thermomax microplate reader.

Hoechst and TUNEL staining

Cell morphology was assessed using Hoechst 33342 (10 ng/ml) staining for 20 min at 4 °C in the dark. Condensed or fragmented nuclei of apoptotic cells were observed using fluorescence microscopy. Terminal

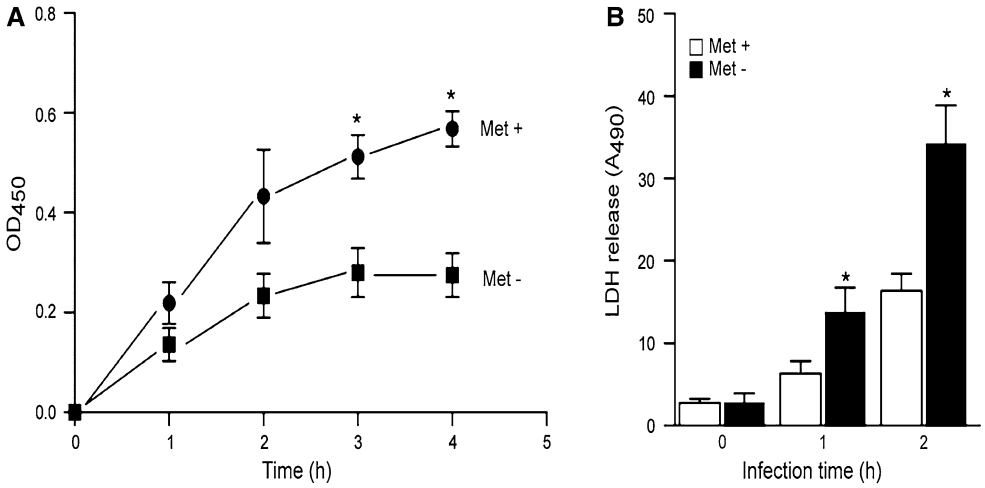

Fig. 1 IPEC-1 growth and cytotoxicity. **a** IPEC-1 growth curves of cells cultured in DMEM with (Met+) or without L-methionine (Met–). **b** LDH release from IPEC-1 cells grown in Met+ vs. Met– DMEM

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed using a one step TUNEL kit (Beyotime Institute of Biotechnology, China). Cells were permeabilized with 0.1 % Triton X-100 for 2 min on ice and stained with TUNEL for 1 h at 37 °C. FITC-labeled TUNEL-positive cells were observed using fluorescence microscopy.

Protein extraction, SDS-PAGE, and immunoblotting

Immunoblotting assays were performed as described previously (Tang et al. 2014) using rabbit polyclonal anti-LC3B (Sigma), Bcl-2 (Cell Signaling), cleaved caspase-3 (Cell Signaling), and β-actin (Cell Signaling) primary antibodies. The blots were revealed using the ECL Plus detection system (Thermo, Marina, CA, USA). The signals were detected on Fujifilm LAS-3,000 (Tokyo, Japan), and the densities of the protein bands were normalized to β-actin signals and quantified using Quantity One software.

Statistics

All data were expressed as mean ± standard deviation from at least three independent experiments. Data were analyzed using a two-tailed Student's *t* test. *P* values <0.05 were considered statistically significant.

Results

Methionine deprivation inhibits IPEC-1 growth and increases ETEC adhesion and cellular cytotoxicity

Methionine deprivation significantly reduced IPEC-1 cell growth as compared with cells grown in DMEM, reaching statistical significance at 3–4 days post-methionine deprivation (Fig. 1a). Methionine deprivation significantly increased ETEC adhesion to IPEC-1 cells, as compared with ETEC adherence to IPEC-1 cells grown in regular medium, with 8.6 ± 0.9 vs. 3.1 ± 0.7 % of bacterial inocula adhering to IPEC-1 cells, respectively.

ETEC cytotoxicity to IPEC-1 cells was assayed by quantifying lactate dehydrogenase (LDH) release. Methionine deprivation did not affect LDH release in the absence of ETEC infection (Fig. 1b). By contrast, cells infected with ETEC displayed significantly more cytotoxicity when cultured in the absence of methionine, as compared to the cytotoxicity observed when these cells were grown in DMEM supplemented with methionine (Fig. 1b).

Methionine deprivation promotes apoptosis in ETEC-infected IPEC-1 cells

IPEC-1 cells grown in DMEM lacking methionine had a significant increase in the number of Hoechst-positive apoptotic cells after a 2 h ETEC infection, as compared with cells grown in control DMEM (Fig. 2a, b). TUNEL-positive cell numbers were also increased in ETEC-infected cells grown in DMEM lacking methionine (Fig. 2c, d). Consistent with these indications of cellular apoptosis, Caspase-3 cleavage was enhanced and Bcl-2 expression was decreased after ETEC infection of cells grown in media lacking methionine, as compared with control cells (Fig. 2e, f).

Methionine deprivation reduces IPEC-1 autophagy during ETEC infection

We transfected cells with eGFP-LC3 for fluorescence analysis to detect autophagosomes (Zhou et al. 2012). While methionine deprivation triggered a modest amount of autophagosome formation in uninfected cells, (Fig. 3a, b),

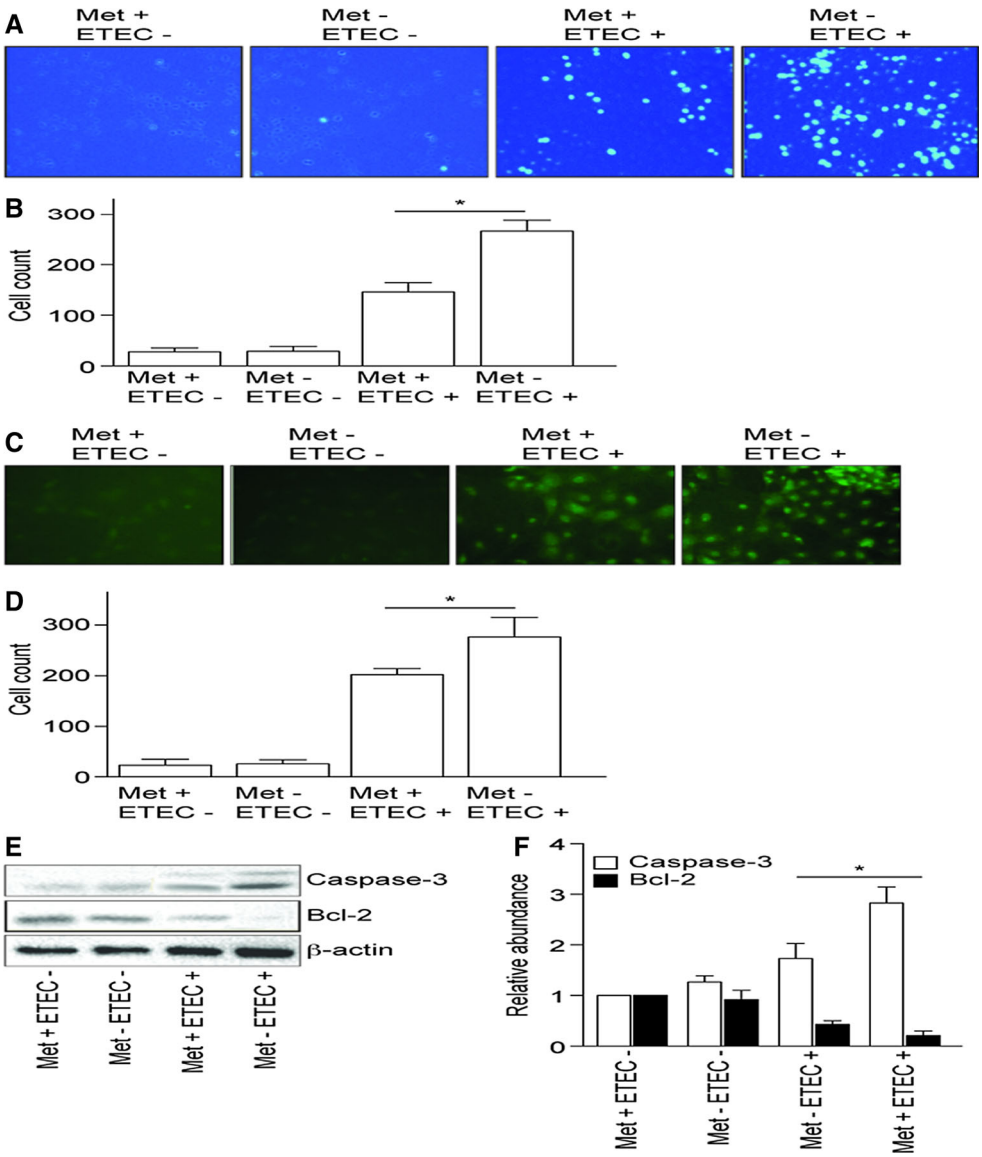

Fig. 2 Apoptosis. **a** Representative photomicrographs of IPEC-1 cells stained with Hoechst 33,342 after growth in DMEM with (Met+) or without L-methionine (Met–) and ETEC infection. **b** Quantitative analysis of apoptotic cells characterized as possessing condensed or fragmented nuclei. **c** Representative photomicrographs of TUNEL-stained IPEC-1 cells. **d** Quantitative analysis of TUNEL-positive cells. **e** Caspase-3 and Bcl-2 immunoblotting after methionine deprivation and ETEC infection. **f** Densitometry of panel E

methionine deficiency ultimately inhibited the further autophagic ability of cells infected with ETEC (Fig. 3a, b). During autophagy, cytosolic LC3-I is linked to phosphatidylethanolamine to form LC3-II, which remains bound to the autophagosomal membranes (Mizushima et al. 2010). LC3-II formation was examined using immunoblotting. ETEC-infected cells grown in DMEM had a higher relative abundance of LC3-II, as compared with infected cells grown in DMEM lacking methionine (Fig. 3c, d).

Discussion

We showed previously that inhibiting autophagy affected IPEC-1 cell viability during ETEC infection, suggesting the cellular autophagic machinery might be beneficial to IPEC-1 cells (Tang et al. 2014). Amino acid availability can affect the susceptibility of humans and animals to infectious disease (Li et al. 2007; Newsholme et al. 2011). Here we characterized the role of methionine deprivation on intestinal epithelial cell responses to ETEC infection. Methionine deprivation negatively affected the proliferation of IPEC-1 cells and enhanced ETEC adhesion.

Previous studies showed that dietary methionine supplementation maintains the barrier function of the small-intestinal mucosa in post-weaning piglets (Bauchart-Thevert et al. 2009; Chen et al. 2014a, b). Here we found that methionine deprivation led to the increased cytotoxicity of ETEC to IPEC-1 cells infected with ETEC. Both human and animal intestinal epithelial cells exhibit apoptosis during ETEC infection (Tamayo et al. 2009). Methionine

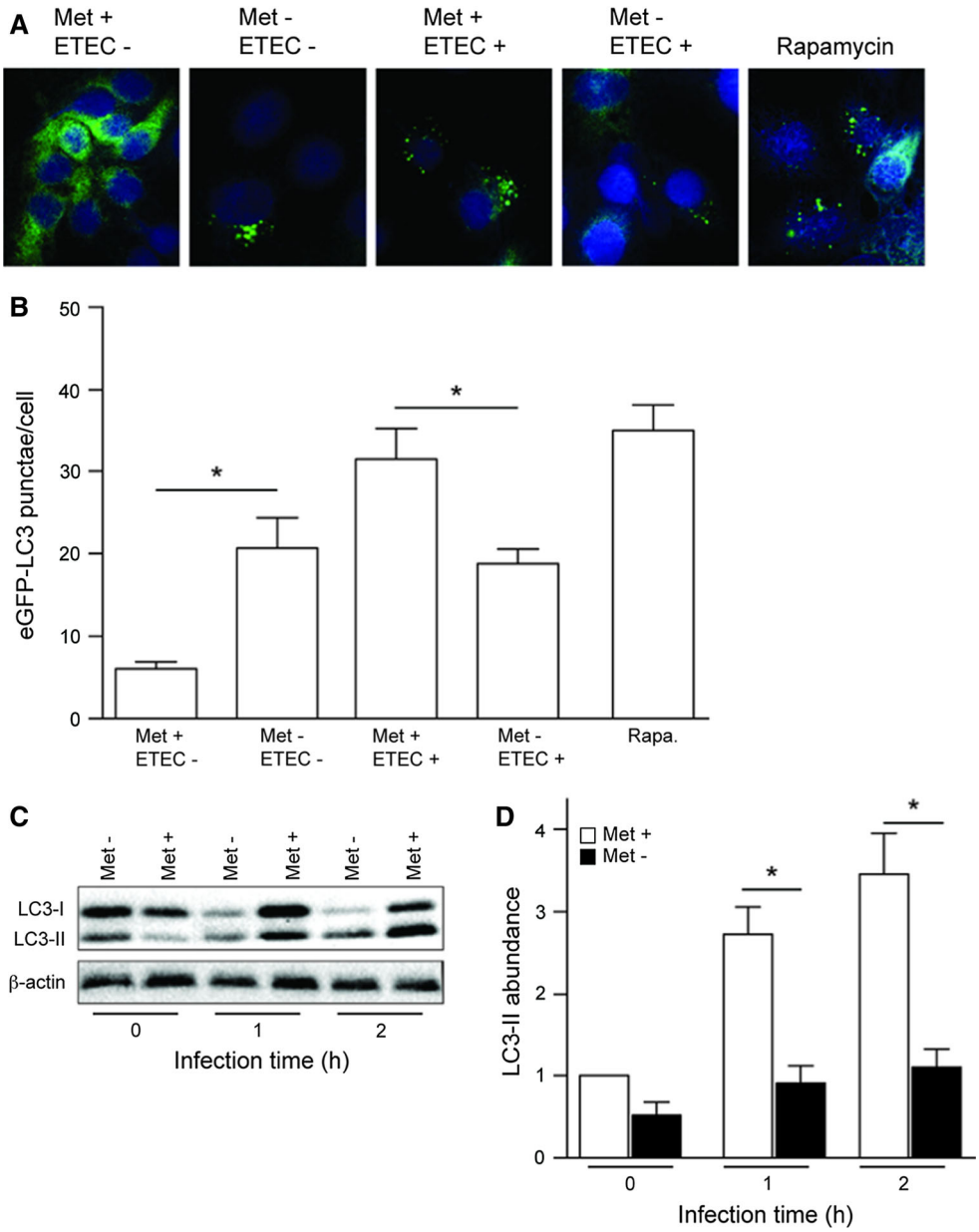

Fig. 3 Autophagy. **a** eGFP-LC3 expression in IPEC-1 cells grown in the presence or absence of methionine. Where indicated, cells were infected with ETEC or treated with 0.5 μM rapamycin (positive control). **b** Quantification of eGFP-LC3 punctae formation as a function of treatment condition ($n = 60$–80 cells/treatment). **c** Immunoblotting of LC3-I and LC3-II in ETEC infected IPEC-1 cells. **d** Densitometry of panel C

deprivation led to greater IPEC-1 cell apoptosis after ETEC infection, as compared with IPEC-1 cells grown in control media.

Autophagy allows cells to respond to changes in nutrient availability and provides a defense against microbial infection (Russell et al. 2014). Amino acids can serve as signaling molecules to regulate autophagy. For example, the leucine-mediated autophagic response is intensively studied (Yan et al. 2012) and methionine levels can contribute to autophagy regulation in yeast (Sutter et al. 2013; Laxman et al. 2014). Here we found that methionine deprivation led to autophagosome formation in IPEC-1 cells. We previously observed both that ETEC infection can induce intestinal epithelial cell autophagy and that inhibiting autophagy enhanced cell death during ETEC infection (Tang et al. 2014). Overall, it appears that methionine deprivation leads to reduced autophagy responses to ETEC infection and thus, increased IPEC-1 cell death.

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

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