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Ethanolamine enhances the proliferation of intestinal epithelial cells via the mTOR signaling pathway and mitochondrial function

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Abstract Ethanolamine (Etn), which is the base constituent of phosphatidylethanolamine, a major phospholipid in animal cell membranes, is required for the proliferation of many types of mammalian epithelial cells. However, it is not clear whether the proliferation of intestinal epithelial cells requires Etn. The present study was conducted to examine the effects of Etn on the proliferation of intestinal epithelial cells and to elucidate the underlying mechanisms. The addition of Etn at 100 or 200 μM was found to enhance the proliferation of IPEC-1 cells. The expression of cell cycle-related proteins CDK4, RB3, cyclin A, and PCNA was also enhanced by Etn. Moreover, the expression or phosphorylation levels of the mammalian target of rapamycin (mTOR) signaling pathway protein and the expression of proteins related to mitochondrial function were also affected by Etn in IPEC-1 cells. These results indicate that Etn promotes the proliferation of intestinal epithelial cells by exerting effects on mTOR signaling pathway and mitochondrial function.

Keywords Ethanolamine · Intestine · Epithelial cells · Proliferation · mTOR signaling pathway

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Introduction

Ethanolamine (Etn) is the base constituent of phosphatidylethanolamine, which is a major phospholipid in animal cell membranes (Kent 1995; Shiao and Vance 1995). The proliferation of many types of epithelial cells, such as mammary, esophageal, bronchial, lung, and prostatic epithelial cells, and hepatocytes requires Etn (Kano-Sueoka and Errick 1981; Peehl and Stamey 1986; Sasaki et al. 1997; Kano-Sueoka et al. 2001; Kume and Sasaki 2006). Phosphoethanolamine and phosphatidylethanolamine also have similar effects on promoting cell proliferation (Kano-Sueoka and Errick 1981; Kano-Sueoka et al. 2001). Cells cultured in Etn-free medium have been reported to show alteration in the composition of membrane phospholipids, with phosphatidylethanolamine content reduced by about 50% and phosphatidylcholine content increased by about 30% (Kano-Sueoka et al. 1983; Kano-Sueoka and King 1987). Additionally, the functions of transmembrane proteins (e.g., epidermal growth factor (EGF) receptor), membrane-associated cytosolic proteins (e.g., protein kinase C), and cell junction complexes were also reported to be impaired, and cell proliferation was demonstrated to cease (Kano-Sueoka et al. 2001). DNA synthesis in hepatocytes and liver weight gain was enhanced by Etn in rats after partial hepatectomy (Kume and Sasaki 2006). These findings suggest that Etn may be an important factor for the proliferation of

epithelial cells in vitro and in vivo. However, the effect of Etn on the proliferation of intestinal epithelial cells remains unknown. In the present study, we hypothesized that Etn is also involved in promoting intestinal epithelial cell proliferation. Therefore, the objectives of the present study were to investigate the effects of Etn on the proliferation of intestinal epithelial cells and to elucidate the underlying mechanism.

Materials and Methods

All chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

Cell culture and cell proliferation Intestinal porcine epithelial cell lines (IPEC-1) were maintained in Dulbecco's Modified Eagle Medium/Ham's F-12 medium (DMEM/F12; Hyclone, Logan, UT) supplemented with 5% fetal bovine serum (Hyclone). Cells were incubated at 37°C, in humidified air with 5% CO_2, and subcultured every 2 d (Tan et al. 2010). In order to examine the effect of Etn on IPEC-1 cell proliferation, cells were seeded in 96-well plates and cultured in serum-free DMEM/F12 medium supplemented with 0, 100, 200, or 500 μM Etn for 24 or 48 h. For mechanistic assays, the cells were incubated in serum-free DMEM/F12 medium with 0 or 200 μM Etn (based on the results of proliferation assay) after about 80% confluence was achieved. Cell proliferation was assayed using the cell counting kit CCK-8 (Dojindo, Osaka, Japan), according to the manufacturer's instructions. An ELISA plate reader (Bio-Tek, Winooski, VT) was used for detection. The results are expressed as optical density (OD) values.

iTRAQ The harvested cells were re-suspended and disrupted in lysis buffer composed of 7 M urea, 2 M thiourea, 4% *w/v* CHAPS, 20 mM TBP, 0.2% Bio-lyte (pH 3–10), and a protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, Germany). DNAse I and RNAse A were added to the lysate at final concentrations of 1 and 0.25 mg/mL, respectively. After cell disruption, the protein solution was separated from the cell debris by centrifugation (12000×*g*, 5 min, 4°C). The crude protein extracts were further purified using the Ready Prep 2-D Cleanup Kit (Bio-Rad Laboratories, Hercules, CA) and then subjected to reductive alkylation. The protein concentration was determined using a 2-D Quant Kit (GE Healthcare, Piscataway, NJ). Trypsin digestion and iTRAQ labeling were performed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Labeled peptides were fractionated using Ultremex strong cation exchange (SCX) chromatography containing 5-μm particles (Phenomenex, Torrance, CA) and then by Strata X C18 chromatography (Phenomenex). A split-less nanoACQuity (Waters, Milford, MA, USA) system coupled to Triple TOF was used for analytical separation. Mascot software (version 2.3.02, Matrix Science) was used to simultaneously identify and quantify proteins. Searches were made against the NCBI non-redundant database consisting of mammalian proteins. In order to select differentially expressed proteins, we used the following criteria: (1) the proteins must contain at least two unique high-scoring peptides (peptide confidence >95 %), and (2) proteins must have ratios higher than 1.2 or lower than 0.8, with the relative quantified *p* values below 0.05.

Western blotting The harvested cells were lysed in RIPA buffer composed of 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, with a protease inhibitor cocktail (Roche, Shanghai, China) and phosphatase inhibitors (Thermo Scientific, Bremen, Germany). Soluble proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were then blocked with 5% nonfat milk in TBS with 0.05% Tween-20 for 1 h, incubated overnight with the primary antibodies, and then probed with horseradish peroxidase-linked secondary antibodies. The bound antibodies were subjected to enhanced chemiluminescence (Applygen Technologies Inc., Beijing, China) for detection. AlphaImager 2200 software (Alpha Innotech Corporation, San Leandro, CA) was used to determine the density of the protein bands.

Statistical analyses All data are expressed as mean ± SEM. Where appropriate, data were analyzed by ANOVA and Tukey's tests or two-sided, non-paired, Student's *t* test using the SAS version 9.2 Program. *P* values < 0.05 were considered statistically significant.

Results

Cell proliferation Proliferation of IPEC-1 cells was measured by CCK-8 assay, the results of which indicated that Etn enhances IPEC-1 cell proliferation in a dose-dependent manner. Etn at 100 μM enhanced ($P < 0.05$) IPEC-1 cell proliferation 24 h after treatment. Etn at 100 and 200 μM concentrations promoted ($P < 0.05$) cell proliferation 48 h after treatment (Fig. 1). The expression of cell cycle-related proteins CDK4, RB3, cyclin A, and PCNA was also enhanced ($P < 0.05$) by supplementing the cell culture medium with Etn (Fig. 2).

iTRAQ and mTOR The effects of Etn on the expression of proteins in IPEC-1 cells were measured by iTRAQ. The expression of proteins involved in regulating DNA replication (histone deacetylase 10 and DNA replication licensing factor MCM5) and protein translation (putative elongation factor 1-alpha-like 3) was affected ($P < 0.05$) by including Etn in cell culture medium (Table 1). The expression of proteins involved

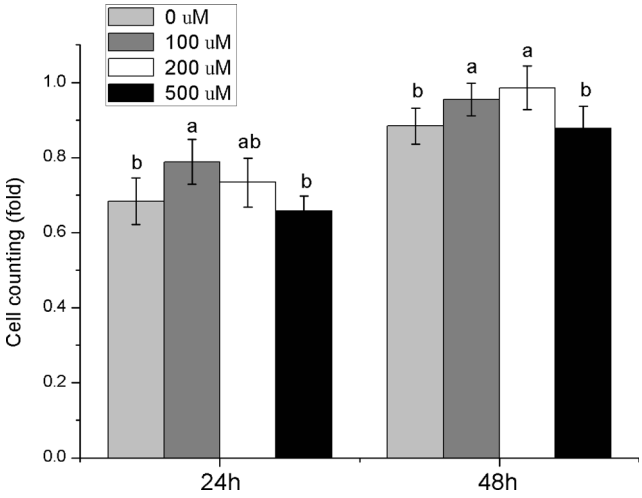

Figure 1. Effects of Etn on the proliferation of IPEC-1 cells. Cells were cultured in Etn-supplemented serum-free DMEM/F12 media for 24 or 48 h and cell proliferation was evaluated with the CCK-8 assay. Data are expressed as means ± SEM, $n = 6$. $P < 0.05$.

in regulating mitochondrial function (mitochondrial import receptor subunit TOM70, mitochondrial cytochrome c oxidase subunit 4 isoform 1, and mitochondrial ATP synthase subunit delta) was also regulated ($P < 0.05$) by supplementing cell culture medium with Etn (Table 1). Moreover, Etn reduced ($P < 0.05$) the expression of ménage à trois 1 and hypoxia upregulated protein 1 (Table 1). The levels of mTOR signaling pathway proteins p-AKT, mTOR, p-4E-BP1, 4E-BP1, p-S6K, S6K, and S6K were enhanced ($P < 0.05$) by Etn in IPEC-1 cells (Fig. 3).

Discussion

The proliferation of several types of mammalian epithelial cells, such as mammary, esophageal, bronchial, lung, and prostatic epithelial cells, and hepatocytes requires Etn, as de novo PE synthesis (through decarboxylation of phosphatidylserine) is not sufficient unless Etn supplied in cell culture or present in blood is utilized to synthesize PE (Kano-Sueoka and King 1987). Membrane phospholipids play an important role in membrane-associated functions, and cells possess unique compositions of membrane phospholipids adapted to cell-specific membrane-associated functions (Kano-Sueoka et al. 1990). Therefore, abnormal membrane-associated functions, and reduced or ceased cell proliferation, is observed in cells starved of Etn. The results of the present experiment showed that the inclusion of 100 or 200-μM Etn in the serum-free medium significantly increased the proliferation of IPEC-1 cells, which indicates that the proliferation of intestinal epithelial cells requires Etn, and de novo PE synthesis in intestinal epithelial cells is not sufficient for cell proliferation. However, the proliferation of IPEC-1 cells decreased when the level of Etn in the medium reached 500 μM, which may be because Etn is toxic and a high dose of Etn damaged the cells. Therefore, an appropriate dose is required for the proliferation of intestinal epithelial cells, and a dose of 200 μM was selected for the following experiment. The proliferation of intestinal epithelial cells requires EGF, and the number of EGF receptors has been shown to be 22–30% lower in cells cultured without Etn as compared with those cultured in the presence of Etn (Blay and Brown 1985; Kume and Sasaki 2006). This suggests that the effects of Etn on the proliferation of intestinal epithelial cells may via be attributable to its effects on the functions of EGF receptors. The mTOR protein, an evolutionarily conserved checkpoint protein kinase, is involved in sensing intracellular and extracellular signals such as nutrients, growth factors, energy status, oxygen levels, and stress (Sengupta et al. 2010). The mTOR signaling pathway plays an important role in controlling critical cellular processes, such as cell proliferation, growth, differentiation, and metabolism, by integrating intracellular and extracellular cues (Ekim et al. 2011; Xiang et al. 2011). The mTOR signaling pathway is activated by EGF via EGF receptors (Nomura et al. 2003; Chiu et al. 2005; Sobolewska et al. 2009). It was surmised that Etn regulates the functions of EGF receptors, thereby affecting the activity of the mTOR signaling pathway and the proliferation of IPEC-1 cells, by enhancing the levels of expression or phosphorylation of mTOR signaling pathway proteins.

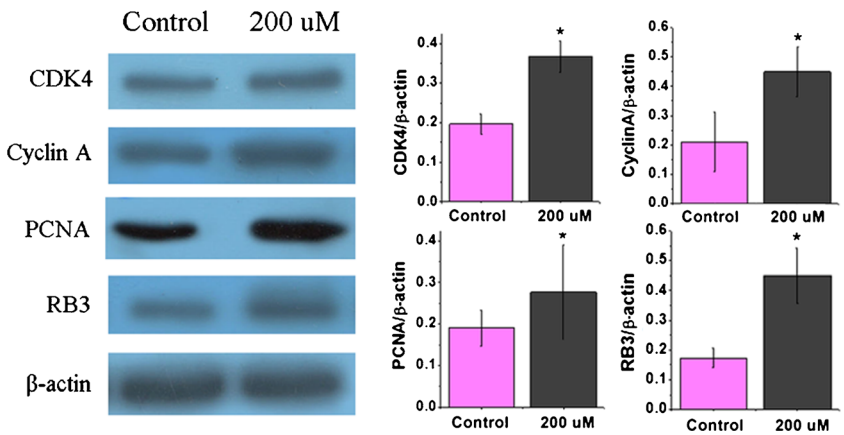

Figure 2. Effects of Etn on the expression of proteins related to the cell cycle in IPEC-1 cells. IPEC-1 cells were treated with Etn (200 μM) for 48 h, and the expression of DCK4, cyclin A, PCNA, and RB3 was determined by western blotting. Data are expressed as means ± SEM, $n = 3$. $P < 0.05$.

Table 1. Effects of Etn on the expression of proteins related to cell proliferation and mitochondrial function in IPEC-1 cells

Protein name	0 μM	200 μM	*P* value
Histone deacetylase 10	0.95 ± 0.07	0.76 ± 0.06	0.02
DNA replication licensing factor MCM5	0.94 ± 0.09	1.20 ± 0.04	0.01
Putative elongation factor 1-alpha-like 3	1.03 ± 0.04	1.26 ± 0.13	0.05
Mitochondrial import receptor subunit TOM70	0.89 ± 0.11	1.14 ± 0.07	0.03
Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	0.89 ± 0.11	0.66 ± 0.08	0.04
ATP synthase subunit delta, mitochondrial	0.88 ± 0.13	0.60 ± 0.11	0.04
Ménage à trois 1	1.14 ± 0.13	0.87 ± 0.05	0.03
Hypoxia up-regulated protein 1	0.89 ± 0.10	1.13 ± 0.07	0.03

Data are expressed as means ± SEM, $n = 3$

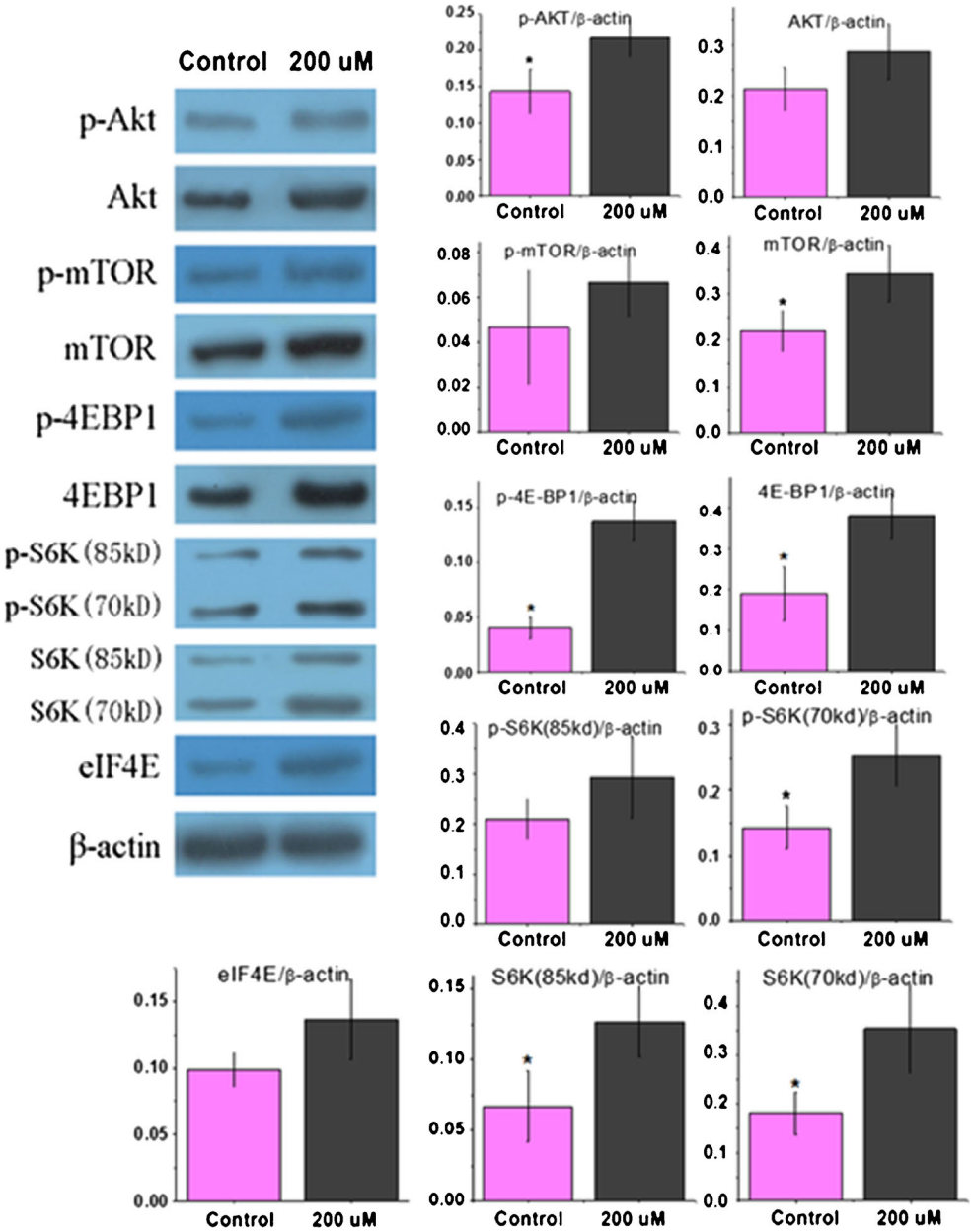

Figure 3 Effects of Etn on the expression or phosphorylation of proteins in the mTOR signaling pathway in IPEC-1 cells. IPEC-1 cells were treated with Etn (200 μM) for 48 h, and the expression or phosphorylation of proteins in the mTOR signaling pathway was determined by western blotting. Data are expressed as means ± SEM, $n = 3$. $P < 0.05$.

It is known that CDK4, cyclin A, PCNA, and RB3 are involved in regulating the progression of the cell cycle from early to late G1 and through G2, thereby regulating mitosis, DNA synthesis, and the G1/S transition. The expression of CDK4, cyclin A, PCNA, and RB3 was found to be reduced in IPEC-1 cells, suggesting that inclusion of Etn in cell culture medium affects the cell cycle of IPEC-1 cells (Stoll et al. 2000). Moreover, the expression of genes that function in modulating cell cycle G1 exit (Ménage à trois 1) was decreased by Etn (Buchman et al. 1995). Histone deacetylases play an important role in regulating DNA replication and transcription by changing chromatin structure (Davie and Spencer 1999; Krude and Keller 2001). Inclusion of Etn in cell culture medium affected the expression of histone deacetylase 10 in IPEC-1 cells, which suggests that Etn may, by affecting chromatin structure, enhance DNA replication and transcription and further regulate cell cycle and proliferation of IPEC-1 cells. Moreover, the expression of DNA replication licensing factor MCM5, an essential component for "once per cell cycle" DNA replication (Chong and Blow 1996), was enhanced by Etn, which further indicated that Etn may elicit an increase in DNA replication in, and proliferation of, IPEC-1 cells. The expression of proteins involved in importing nuclear-encoded protein into mitochondria (mitochondrial import receptor subunit TOM70) (Young et al. 2003) and energy metabolism (mitochondrial cytochrome c oxidase subunit 4 isoform 1 and mitochondrial ATP synthase subunit delta) was regulated by inclusion of Etn in cell culture medium. The phospholipid compositions of mitochondria in the liver of rats were altered by dietary supplementation with ethanolamine, suggesting that Etn, by affecting mitochondrial membrane phospholipid composition, may also affect the function of mitochondria (Shimada et al. 2003). These data suggest that Etn may affect IPEC-1 cell proliferation partly by regulating mitochondrial function, as mitochondrial function is closely linked to cell proliferation (Heerdt et al. 1997; Merkwirth and Langer 2009; Mandal et al. 2011).

Conclusion

The results of the present study indicate that Etn is involved in regulating the proliferation of intestinal epithelial cells. Additionally, it was demonstrated that Etn may affect DNA replication and the cell cycle in intestinal epithelial cells by exerting effects on the mTOR signaling pathway and mitochondrial function.

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