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Hydrocortisone Modulates Cholera Toxin-endocytosis by Regulating Immature Enterocytes Plasma Membrane Phospholipids

Lei Lu,

Developmental Gastroenterology Laboratory, Massachusetts General Hospital for Children, Boston, MA

Yuanwu Bao,

Glycobiology Laboratory, Massachusetts General Hospital for Children, Boston, MA

Abdullah Khan,

The Aga Khan University Hospital, Karachi, Pakistan

Allan M. Goldstein,

Pediatric Surgery, Massachusetts General Hospital for Children, Boston, MA

David S Newburg,

Glycobiology Laboratory, Massachusetts General Hospital for Children, Boston, MA

Andrea Quaroni,

Section of Physiology, Cornell University, Ithaca, NY

Dennis Brown, and

Program in Membrane Biology, Massachusetts General Hospital, Boston, MA

W. Allan Walker

Developmental Gastroenterology Laboratory, Massachusetts General Hospital for Children, Boston, MA

Abstract

Background and Aims—Diarrheal disease is a major cause of morbidity and mortality in infants and children worldwide. Evidence has indicated immature human enterocytes and their interaction with bacteria and enterotoxins may account for the noted increased susceptibility of neonates to diarrhea. Our aim is to characterize the developmental difference in (CT)-GM1-mediated endocytosis.

Methods—We used H4 cells (a fetal human small intestinal epithelial cell line), T84 cells, primary cultured mature human small intestinal epithelial cells and human fetal small intestine xenografts. In addition, hydrocortisone (HC) was used as a potent intestinal trophic factor to induce maturation of the human enterocytes.

Address correspondence to: Lei Lu, M.D. Developmental Gastroenterology Laboratory, Massachusetts General Hospital for Children, 114 16th Street (114-3503), Charlestown, MA 02129-4404, Tel: 617-726-7991, Fax: 617-726-4172, Email: lul@helix.mgh.harvard.edu.

“No conflicts of interest exist”

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Results—Here we demonstrated an increase in CT-caveolae and a decrease in CT-clathrin co-localization in H4/HC compared to H4 cells by EM. In T84 and hIECs, a significant amount of GM1 was partitioned into the lipid rafts. In contrast there was little CT-GM1/lipid raft association in H4 cells. However, hydrocortisone significantly increased GM1/lipid rafts association in H4 cells. Furthermore, we noted an increase in the level of phosphatidylcholine (PC), sphingomyelin (SPM) and the ratio of PC/PI (phosphatidylinositol) in mature compared to immature enterocytes and that hydrocortisone can accelerate this maturational process. Disruption of PITP α using siRNA demonstrated an increase in GM1/lipid raft association in H4 cells and resulted in a decreased CT-response.

Conclusions—Our studies suggest that the developmental change in CT-endocytosis is partially due to an increased GM1-lipid raft association through a maturational change of phospholipid composition on the cell surface of immature enterocytes.

INTRODUCTION

Diarrheal diseases including cholera are an important cause of childhood morbidity and mortality worldwide ¹. There has been increasing evidence that developmentally regulated features of the immature intestinal epithelial cell and its mucosal surface affect its interaction with bacteria and their exotoxins which may in part account for the increased susceptibility of neonates to diarrhea². .

During development, immature enterocytes are highly endocytic ^{3–7}. The existence of an enhanced endocytic capacity by the developing intestine undoubtedly plays a critical role in its growth and differentiation due to an increased capacity to engulf intact hormones and growth factors ^{8,9}. However, the immature intestine is not able to distinguish between those substances necessary for growth and development from those that can be potentially harmful. This developmental enterocyte feature may help to explain the increased susceptibility of newborn infants to enteric infections and to toxigenic diarrheas ^{10, 11}. However, amniotic fluid and breast milk contain trophic factors that can actively stimulate development of the enterocyte which enhances gut defenses to prevent or to attenuate the severity of these developmentally-associated disease states (reviewed in ¹²).

Among many biological mediators in breast milk, several hormones and growth factors have been shown to have trophic effects on the fetal and neonatal GI tract ^{13–15}. Hydrocortisone, epidermal growth factor and transforming growth factor have all been shown to promote growth and differentiation of intestinal epithelial cells ¹⁶. In previous publications, we have reported that hydrocortisone (a trophic factor that is abundant in human breast milk) reduces the CT-induced secretory response in human and rat immature enterocytes ^{2, 17, 18} in part by inducing an endocytic pathway change for cholera toxin in H4 cells from a clathrin-mediated to a caveolae-mediated process.

Several lines of evidence indicate that proteins that modify membrane lipids can function in the regulation of membrane and vesicle trafficking. One such protein family is the phosphatidylinositol/phosphatidylcholine transfer proteins (PITPs). Phosphatidylinositol transfer protein alpha (PITP α) plays an important role in regulating intracellular lipid and vesicular trafficking and in lipid-mediated-signal transduction pathways ^{19, 20}. *In vitro*, PITP α transfers phosphatidylinositol (PI) and phosphatidylcholine (PC) between membranes, exhibiting a 16-fold greater affinity for PI than for PC ²¹ and thereby may regulate the phospholipid composition in these membranes. The available data indicate a role for PITPs in regulating the specific interface between membrane lipid signaling and membrane trafficking. However, its mechanism remains unresolved.

While the effect of glucocorticoids on maturation of intestinal enzyme function has been intensely investigated, its effect on membrane phospholipids is largely unknown. In this study, we have begun to clarify the functional significance of hydrocortisone on endocytic development and on the biophysical properties of the developing human enterocyte cell membranes.

METHODS AND MATERIALS

Materials

Cholera toxin (CT) was purchased from Calbiochem (San Diego, CA). CTB-gold was obtained from List Biological Laboratories (Campbell, CA). Human epidermal growth factor (EGF) was obtained from Invitrogen (Carlsbad, CA). Anti-clathrin heavy chain antibody was from BD Transduction Laboratories (Palo Alto, CA). Anti-caveolin-1 was obtained from Cell Signaling Technology (Beverly, MA). Anti-CT antibodies were from Dr. Lencer (Children's Hospital, Boston, MA). HRP conjugated goat-anti-mouse and -anti-rabbit were from Bio-Rad (Hercules, CA). All tissue culture media and reagents are obtained from GIBCO. Collagenase type IV and all other chemicals unless specified were obtained from Sigma-Aldrich (St. Louis, MO).

Cell cultures

H4 (fetal) and T84 (adult) cells were used. H4 cells have been used as an immature intestine model in previous studies^{17, 22–24}. T84 is a well-characterized and highly differentiated human colon carcinoma cell line. H4 cells were routinely maintained in Dubelco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FBS) and human recombinant insulin (0.5unit/ml). In some experiments, H4 cells were preincubated in H4 media containing 1 μ M hydrocortisone (HC) for 5–7 days (H4/HC) before further treatment. T84 cells were maintained in DMEM-F12 Ham with 5% FBS. Cells seeded on 12mm (24-well) or 33mm (6-well) tissue culture plates were used before confluence was reached. CT was used at 20nM (dose range 0.2–20nM). All CT-stimulation experiments were done in serum-free Hanks Balanced Salt Solution (HBSS) supplemented with 0.25% bovine albumin.

Human intestinal samples

Surgically resected small intestinal specimens were obtained from patients undergoing intestinal resection for a variety of clinical indications. These specimens were used for isolation of primary intestinal epithelial cells. These studies were done according to the NIH guidelines and Partners Human Study Committee approval (IRB# 1999p003833). Written consent forms were signed and obtained from parents of the pediatric patients.

Epithelial cell isolation and culture

Isolation and culture of primary cultures of small intestine epithelia was modified from procedures described by Quaroni²⁵. Segments of discarded small intestine were obtained and incubated with a 1.25% trypsin-0.5 mM EDTA solution at room temperature for 10 min. Crypts were isolated and incubated with medium containing collagenase type IV (200unit/ml). The dissociated epithelial cells were collected and in OptiMEM supplemented with 20 ng/ml human epidermal growth factor, 10 μ g/ml insulin (human recombinant) and 4% FBS, plated and incubated with 5% CO₂ at 32°C. The detailed method and characterization of these cells is presented in the supplemental data (experimental procedure and Figures s1 and s2).

Fetal small intestinal xenograft transplant model

Human fetal small intestine xenografts were supplied by the Xenograft Core Facility in our laboratory (detailed in supplemental data and references^{26, 27}). Mice were injected

subcutaneously with hydrocortisone (50mg/kg body weight, n=3) or an equal volume of normal saline (n=3). Tissue was obtained 1 wk later by carefully removing all the serosal and muscle tissues surrounding the intestinal mucosa and homogenized with a hand held polytron.

SDS-PAGE and Western blot (immunoblot analysis)

After experiments were completed, cells were rinsed with HBSS. Surface bound toxin was removed with acidified HBSS (pH 2.5). Cells were lysed in tris-buffered solution (4°C) containing 1% triton X-100 (1% TTBS), 0.5% sodium dodecyl sulfate and protease inhibitors as described previously¹⁷. In some experiments, triton-soluble proteins were collected first and the insoluble fraction was then collected in 1% TTBS containing 0.5% SDS. Both fractions were analyzed by SDS-PAGE and western blotting as described before. Briefly, lysates were resolved on a 10–20% linear gradient polyacrylamide gel and transferred onto nitrocellulose membranes. After blocking with 5% nonfat milk solution, membranes were sequentially probed with appropriate dilution of primary and secondary antibodies and visualized by enhanced chemiluminescence (ECL, Biorad).

Sucrose equilibrium density centrifugation

Confluent monolayers of T84, H4 and H4/HC cells were used for isolation of detergent insoluble glycolipid/rafts (DIGs). All steps were completed at 4°C as described in detail elsewhere^{28, 29}. Briefly, cells were lysed in 1% triton 100-X (1% TBS) and homogenized with 15 strokes in a Dounce homogenizer on ice. The subsequent homogenates were adjusted to 40% sucrose with an equal volume of 80% sucrose in TBS, layered under a linear 5–30% sucrose gradient using a sucrose gradient maker, and centrifuged at 39,000 rpm for 16–20 h in a swinging bucket rotor (model SW 41). Sequential fractions were collected analyzed by SDS-PAGE and western blotting.

Electron microscopy and immunostaining

Cells were incubated with CT (20 nM) and fixed in 4% paraformaldehyde for 30 min then scraped, pelleted and embedded in 2% agarose. After infiltration with LR white resin, they were embedded in gelatin capsules and thin sections were cut and collected onto formvar-coated nickel grids. The grids were incubated with DAKO diluent (negative control), or primary antibody diluted 1:10 in DAKO diluent. Finally, the grids were stained for 5 min with aqueous uranyl acetate (EMS) and examined in a Philips CM 10 transmission electron microscope at 80 kV.

RNA interference (RNAi)

H4 and H4/HC cells were plated on 24- or 6-well plates at an optimal density the previous day and transfected with small interference RNA (siRNA) using lipofectamine (Invitrogen). The PITPα, GAPDH/control siRNA (at a dose range 0.1–100nM for initial study and optimal concentration afterwards) were used in various experiments. After transfection, cells were incubated for 48–72 hours before further study.

Enzyme immunoassay (EIA)

We used cAMP levels as the functional indicator for the CT-response in this study. EIA were used to measure cAMP levels as described previously².

Thin layer chromatography (TLC)

Confluent cells (H4, H4/HC and T84) or cell plasma membranes and xenograft small intestines were collected in normal saline and mixed overnight in chloroform and methanol (2:1). The monophasic homogenates were then diluted with 0.1% potassium chloride (KCl) to produce a

biphasic solution. The final ratio of chloroform:methanol:water was 8:4:3. The two phases were separated by centrifugation at 200g. The chloroform layer (lower phase containing purified lipids) was dried under nitrogen and resuspended in chloroform at 2 mg/ml.

Samples and phospholipid standards (Matreya Inc, Pleasant Gap, PA) were spotted on plates coated with a thin layer of silica gel G and developed in a solvent system of chloroform:methanol:ethyl acetate:n-propanol:0.1% KCl (28:10:25:25:7).

Determination of PC, PI, and SPM by liquid chromatography-mass spectrometry

The phospholipids were separated using a silica column (Adsorbosphere XL Silica 300A 3 U, Alltech Associates, Inc., Deerfield, IL). Mobile phase A was chloroform/methanol (90:10, v/v) containing 0.5% ammonium hydroxide and B was chloroform/methanol/water (60:34:6, v/v) containing 0.5% ammonium hydroxide. The protonated molecular ions, $[M+H]^+$, were used for quantification with the specific m/z at 760 for PC, 857 for PI, and 813 for SPM. Calibration graphs were constructed using linear regression of the peak area (Y) to the nominal concentration of the lipid (X, $\mu\text{g/mL}$).

RESULTS

Immunogold-electron microscopy of H4 cells

We previously showed that CT response in H4 cells is significantly diminished by drugs that interrupt clathrin coated vesicle formation such as CPZ but not by cholesterol depleting agents such as M β CD or Filipin. However, the CT-response in the mature IECs or H4 cells pretreated with HC was inhibited by M β CD but not by CPZ¹⁷.

To morphologically confirm our biochemical observations that a developmentally regulated endocytosis of CT occurs in immature cells via clathrin-coated pits and that this pathway is altered during enterocyte maturation, we used immunogold labeling and electron microscopy to examine H4 and H4/HC cells after exposure to CT. As expected, gold labeled CT was found co-localized with clathrin coated pits in H4 cells (Fig 1A). In contrast after HC treatment, H4 cells demonstrated a lack of co-localization of CT-clathrin coated pits but instead a co-localization of CT with caveolae (Fig 1B). In T84 cells pretreatment with HC did not induce any change in the CT-endocytosis pathways as shown in (suppl Fig 1). These results are consistent with our previous observations of a differential endocytic process for CT-uptake in the immature human enterocytes before and after HC treatment.

CT-GM1 cell surface interaction and membrane lipid composition

We have previously shown that there is little or no difference in CT binding between H4 and T84 cells. In addition, maturation of the immature enterocyte had no effect on CT binding as demonstrated by the little or no change of CT-GM1 binding in either mature human enterocytes (hIEC) or hydrocortisone treated H4 cells (H4/HC). However, there was considerable difference in the CT-uptake by these cells and this difference appeared to be developmentally regulated. In this study, we provide further evidence that mature enterocytes have an increased CT-GM1/lipid raft association as shown in Fig 2A. At 4°C a large portion of CT-GM1 complexes were found in the triton X-100 soluble (non-raft) membrane fraction of H4 cells. However pretreatment with HC resulted in a shift of CT-GM1 to the triton X-100 insoluble (lipid raft) fraction. CT-GM1 complexes in T84 as well as hIECs were also shown predominantly in the lipid raft fraction. To provide further evidence that an increase in GM1 association with lipid rafts occurs with the maturation of human enterocytes, we extracted DIGs from the plasma membranes of H4 or H4/HC cells on a discontinuous sucrose density gradient column. These membrane fractions were then compared to those obtained from T84 or hIEC cells. Pretreatment with HC resulted in a significantly increased level of GM1-lipid raft

association in H4 cells with similar DIGs distribution to that observed in T84 or hIEC cells (Fig 2B). However, this difference in GM1 distribution is not due to the lack of lipid raft in H4 cells. As shown in Fig 2C, the lipid raft fractions have a higher level of cholesterol (CHO) than the non-raft fractions in both cell types and the level is comparable in H4 vs T84. This result indicated that lipid rafts are present in both H4 and T84 cells. Since caveolin-1 is suggested not only as a lipid raft/caveolae marker but also stabilizes caveolae in adult cells, we next examined caveolin-1 distribution in H4 cells before and after HC treatment. As shown in Fig 2D, caveolin-1 is mostly in non-raft fractions in H4 cells. HC treatment of H4 cells induced a significant amount of caveolin-1 partitioned into lipid raft fractions. This is similar to our observation of caveolin-1 distribution in T84 and hIEC cells (Fig 2D). These data suggested that the immature enterocytes consist of a less stable caveolae/lipid raft organization and a different protein composition compared to the mature human enterocytes.

To provide insight into the mechanism by which HC induces a developmental change in the CT-response in human enterocytes, we utilized thin layer chromatography to analyze the total cellular and plasma membrane lipid composition of H4 cells pretreated with HC. The results were compared to immature H4 cells and T84 cells. Here, we provide evidence that HC elicits a change in the phospholipid composition of H4 cells by increasing the level of PC, sphingomyelin (SPM) and the ratio of PC/PI (Fig 3A). This effect is also seen in the plasma membrane, in which the change is more profound. This observed change in plasma membrane suggests that HC functions to induce a more mature pattern of phospholipid distribution in immature enterocytes similar to the pattern seen in T84 cells (Fig 3). The quantitative analysis of PC, PI and SPM extracted from plasma membrane of H4, H4/HC and T84 cells using a liquid chromatography-mass spectrometry system suggests that HC induces a significant increase in PC and SPM and a decrease in PI levels comparable to that seen in T84 cells (Fig 3B). Further studies are needed to confirm the role of HC in affecting the CT-response in part by regulating membrane phospholipids.

Next, we used a human fetal small intestinal xenograft model to validate our findings obtained from cell lines. SCID mice that carry fetal small intestine xenografts (for 20wks) were injected subcutaneously once with either HC (50mg/kg), or normal saline as a control. After 7 days, xenograft tissues were harvested and phospholipids extracted and measured using LC-mass spectrometry. As shown in Fig 3C, HC induces an increase in PC and SPM and a decrease in PI levels in the xenograft human small intestine tissue. This data confirm our cell line observations that the intestinal trophic factor, HC, can regulate cellular phospholipid composition.

Phosphatidylinositol transfer proteins (PITPs) affect CT-response in immature human enterocytes

Phosphatidylinositol transfer proteins (PITPs) belong to a family of phospholipid-binding proteins. They facilitate the exchange of phospholipids between membranes and participate in cellular phospholipid metabolism, signal transduction and vesicular trafficking *in vivo*. Therefore, we investigated whether PITP α , a ubiquitous and highly conserved phosphatidylinositol transfer protein, affects the CT-response in immature human enterocytes. Constitutive PITP α expression was measured using real-time PCR, as seen in Fig 4A. Compared to H4 cells, H4/HC and T84 cells had lower PITP α expression. To further characterize the role of PITP α in the CT- response in immature enterocytes, RNA interference using siRNAs targeting either PITP α or GAPDH was used in H4 cells. Knockdown of PITP α in H4 cells significantly diminished CT-induced cAMP accumulation in H4 cells as shown in Fig 4B. To investigate whether the reduction of PITP α resulted in a decrease in the cellular CT-response by induction of an increased GM1/lipid raft association we isolated DIGs on a discontinuous sucrose density gradient column from H4 cells transfected with siRNA

either targeting PITP α or GAPDH and compared results to those obtained from H4 cells. A decrease in the level of PITP α resulted in a significant increase of GM1-lipid raft association in H4 cells (Fig 4C) and had a similar DIG distribution to that observed in T84 or hIEC cells (Fig 2B). There is no change in GM1/lipid raft association in H4 cells transfected with GAPDH siRNA. We also isolated PI from H4 cells transfected with siRNAs either targeting PITP α or GAPDH. Note that knockdown of PITP α induces a change in PI composition in these cells relative to control cells. The PI extracted from these cells decreased significantly as shown in Fig 4D. The knockdown efficiency was measured by real-time PCR monitoring a decrease in PITP α mRNA expression in H4 cells (Fig 4E).

Collectively, these experiments suggested that cell membrane phospholipid composition and distribution are under developmental control and might play an important role in the developmental regulation of CT-endocytosis. However, further studies are needed to elucidate the precise cellular and molecular mechanism of this regulation in the immature human gut.

DISCUSSION

CT-endocytosis can occur by several mechanisms. In polarized mature epithelial cells, activation of signal transduction by CT requires endocytosis of the toxin-receptor complex into the apical endosome via a lipid raft/caveolar-mediated process, followed by retrograde transport into the Golgi cisternae/endoplasmic reticulum (ER) and finally translocation of the CT-A subunit to its site of action in the basolateral membrane^{30–32}.

In addition CT can also be taken up by a clathrin-dependent pathway^{17, 33, 34}. Our previous studies have demonstrated a developmentally different response to CT in immature vs. mature human enterocytes. This difference in response is largely due to differences in the endocytic process in immature and mature enterocytes¹⁷. However, the cellular and molecular mechanisms that regulate the maturation of endocytosis are not well known.

Epithelial development of the small intestine is controlled by many factors, among them genetic preprogramming, growth factors and hormones in amniotic fluid and breast milk, luminal factors such as gut flora, etc^{12–14}. Glucocorticoids are known to stimulate multiple functional maturations of the gastrointestinal epithelium including many enzymatic changes^{35, 36} and a drastic decrease in the capacity for endocytosis of proteins in *in vivo* animal studies. These studies have been reviewed by Henning and others^{36, 37}.

Although the majority of studies characterizing the effect of glucocorticoids on the development and maturation of enterocytes have been reported using animal models^{38–42}, numerous studies have also been done using cultured intestinal epithelial cells. These studies have demonstrated a direct effect of glucocorticoids on small intestinal crypt as well as villus cells without first affecting mesenchymal regulation^{43, 44}. Here, we report a direct influence of hydrocortisone on the developmental regulation of immature human enterocytes by altering CT-endocytosis, most likely due to a change in cell membrane lipid composition.

In this study we have investigated the effect of hydrocortisone in modulating the CT-response in immature human enterocytes and its possible mode of action. Previously, we have reported that immature human enterocytes (H4) use a predominantly clathrin-dependent endocytosis of CT in contrast to endocytosis of CT in mature human enterocytes (T84), which occurs largely via a lipid raft/caveolar-mediated pathway. In this study, we demonstrate that hydrocortisone modulates the CT-response in fetal small intestinal epithelial H4 cells by stimulating an increase in GM1 lipid raft association and switching to a caveolar-mediated CT-endocytosis similar to that seen in mature enterocytes (T84 cells) or primary cultured mature small intestinal crypt epithelial cells. Using freshly isolated mature human small intestinal crypt cells, we have also shown the CT-GM1 complexes in these mature IECs were in a detergent insoluble lipid

raft associated fraction. In addition, HC increases GM1 association with lipid rafts in the cell membrane in H4 cells, suggesting a cellular membrane mechanism for the shift to caveolar-endocytosis. Furthermore, HC has no effect on the CT-response in mature small intestinal epithelial cells. These observations collectively suggest a developmental basis for the CT-response in the immature human IECs that can be modified by HC through the induction of a maturational change in epithelial cell membrane phospholipid composition and association of certain receptors (GM1) and proteins (caveolin-1) with the lipid raft, leading to a shift in endocytosis.

The phospholipid content of cell membranes changes during development in drosophila and this change in phospholipids results in a change in endocytosis and signaling pathways⁴⁵. A study of the phospholipid profile in tracheal aspirates of preterm neonates suggested an elevated level of PI compared to term neonates. Corticosteroid treatment in preterm infants has been shown to lower the PI content, resulting in the maturation of surfactant⁴⁶. However, the importance of this change in phospholipid composition during maturation of human enterocytes in regulating the endocytic machinery is not well understood. It has been reported that PIs play a key role in regulation of endocytosis via clathrin-coated vesicles (reviewed in⁴⁷). In MDCK cells, antibody cross-linked to raft proteins has been shown to induce apical caveolae formation and these cross-linked raft proteins were internalized slowly via a caveolar-endocytosis. In contrast, a non-raft protein such as the LDL receptor when cross-linked was excluded from raft-caveolae and was rapidly internalized via clathrin-coated pits⁴⁸. Thus, maturation of membrane phospholipids and association of membrane proteins with lipid-rafts are key determinants of the mode of entry: either by a rapid internalization via clathrin-pathway or a slow and regulated caveolar-pathway. In this study, we have utilized a developmental human intestinal epithelial cell model to delineate the regulatory role of lipids in membrane endocytic dynamics and trafficking focused on the developmental differences in membrane lipid composition. Pretreatment of immature human IECs with a potent intestinal trophic factor, hydrocortisone, resulted in a change in the phospholipid composition in these cells, eg., from a fetal to an adult-like lipid content. As a consequence, these cells exhibit a mature type of CT-endocytosis and a diminished CT-response, suggesting that the lipid composition of the enterocyte surface membrane is a determinant in the endocytosis process.

These studies elucidate mechanisms by which trophic factors such as hydrocortisone can affect the cellular/molecular regulation of the CT-response in the developing gut. A potential limitation to this study is that by exclusively using an *in vitro* cell line system, we will only determine the direct effect of the hydrocortisone impact on immature enterocytes and might not be able to identify any additional physiologic effects of intestinal trophic factors on the developmental regulation of endocytosis and the secretory response. Since the maturation of immature enterocytes is also known to involve interactions between enterocytes and the extracellular matrix and/or mesenchyme, corticosteroids could have a direct impact on the epithelia or an indirect impact by first affecting the extracellular matrix, mesenchymal milieu as well as other organ systems. Further studies using an *ex vivo* xenograft model of human fetal small intestine should address this issue.

Numerous studies demonstrate glucocorticoid effects upon gastrointestinal function. These effects include changes in small intestine morphology, absorption of nutrients, stimulation of digestive enzyme activity and prevention of the excessive inflammation of necrotizing enterocolitis in the preterm infants^{26, 41, 44, 49, 50}. Thus glucocorticoids induce functional cell morphology changes and regulate intestinal physiological functions during gut maturation. This maturational change can be essential for the development of normal intestinal defense mechanisms against intestinal pathogens.

In summary, this study extends previous observations and suggests that an enhanced response to CT in immature enterocytes is largely due to an excessive uptake of CT by a developmentally regulated clathrin-endocytic pathway. Hydrocortisone can modulate the CT-uptake by inducing a maturation of endocytosis in immature human enterocytes. We conclude that this change in CT-endocytosis is partially due to a glucocorticoid-modulated CT-GM1 lipid raft association through a maturational change of the phospholipid composition on the cell surface of immature enterocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CT	cholera toxin
cAMP	cyclic adenosine monophosphate
hIEC	freshly isolated human small intestinal epithelial cell
T84	a human colonic cancer cell line
H4	a primary human fetal crypt small intestinal cell line
HBSS	Hanks balanced salt solution
DIGs	detergent insoluble glycolipids/rafts
PC	phosphatidylcholine
PI	phosphatidylinositol
SPM	sphingomyelin
PITPα	phosphatidylinositol transfer protein alpha
TTBS	tris-buffered solution with 1% triton 100-X
HC	hydrocortisone succinate

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Figure 1. Immunogold-electron microscopy of HC effect on CT-endocytosis in H4 cells
Arrows heads (▲) indicate clathrin-coated pits-1. **Block arrows (■)** indicate caveolar like structures. **Black arrow (→)** indicates CT (50nM gold-particle). The **bar** represents 500 nm in length. CT co-localizes with clathrin-coated vesicles in H4 cells (A). The figure is representative of all EM fields examined. Conversely, there is a lack of co-localization of CT with clathrin-coated pit in H4/HC cells but an increase in co-localization of CT-caveolae (B).

Figure 2. HC induces a shift in CT-GM1 membrane lipid raft association

(A). H4, H4/HC, T84 and hIECs were incubated with CT (20nM) for 60 min at 4°C. Triton X-100 soluble and insoluble fractions were isolated and subjected to western blotting with anti-CTB antisera. (B). The plasma membranes from H4, H4/HC and T84 cells were isolated using 30% percoll solution and DIGs were extracted. Lipid rafts (caveolae) are in the low-density sucrose fractions (fractions 3–7). Note that HC induces a shift of CT-GM1 from a non-raft fraction to a raft fraction compared to untreated H4 cells. The similar pattern of CT-GM1 lipid raft association can be seen in T84 cells as well as hIECs. (C). Concentrations of cholesterol (CHO, solid circle H4, solid triangle T84) and protein (open circle H4, open triangle T84)

in each sucrose gradient fractions. **(D)**. Western Blot depicting the expected ~ 22kD band probed with an anti-caveolin-1 antibody in each sucrose gradient fractions.

Figure 3. Effect of HC on cellular levels of PC/PI/SPM in H4 cells using thin layer chromatograph (TLC) analysis

(A). Thin layer chromatograph of total cellular and plasma membrane phospholipids in H4 and T84 cells. There is a difference in phospholipid composition in these cells with regard to the level of phosphatidylcholine (PC), phosphatidylinositol (PI) and sphingomyelin (SPM). HC increases SPM as well as PC content and increases the ratio of PC/PI in H4 cells. (B). The level of PC, PI and SPM in H4, H4/HC and T84 cells was measured by LC Mass spectrometry and expressed as µg/ml. (C). The level of PC, PI and SPM in xenograft small intestinal tissues was measured by LC Mass spectrometry and normalized with tissue weight and expressed as µg/g.

Figure 4. Down regulation of PITP α induces a shift in CT-GM1 membrane lipid raft association (A) PITP α expression in H4, H4/HC and T84 cells were measured using real-time PCR and expressed as relative level of mRNA compared to H4. (B). The effect of PITP α gene knockdown on CT-induced cAMP response in H4 cells and the result is presented as picomoles per milligram total cellular protein (pmol/mg). (C). H4 cells, transfected with either PITP α or GAPDH siRNA were lysed in 1% triton X-100 and applied onto a sucrose gradient (5–30%) column and 12 fractions were probed with anti-CTB antibody. Lipid rafts (caveolae) are in low-density sucrose fractions (fractions 3–7). (D). PI level in H4 cells was measured by LS Mass spectrometry and expressed as μ g/ml. (E). Gene knockdown was monitored by the down regulation of target gene mRNA expression in the cell.