Acidic bile salts modulate the squamous epithelial barrier function by modulating tight junction proteins

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Submitted 8 March 2011; accepted in final form 21 May 2011

Chen X, Oshima T, Tomita T, Fukui H, Watari J, Matsumoto T, Miwa H. Acidic bile salts modulate the squamous epithelial barrier function by modulating tight junction proteins. Am J Physiol Gastrointest Liver Physiol 301: G203–G209, 2011. First published May 26, 2011; doi:10.1152/ajpgi.00096.2011.—Experimental models for esophageal epithelium in vitro either suffer from poor differentiation or complicated culture systems. An air-liquid interface system with normal human bronchial epithelial cells can serve as a model of esophageal-like squamous epithelial cell layers. Here, we explore the influence of bile acids on barrier function and tight junction (TJ) proteins. The cells were treated with taurocholic acid (TCA), glycocholic acid (GCA), or deoxycholic acid (DCA) at different pH values, or with pepsin. Barrier function was measured by transepithelial electrical resistance (TEER) and the diffusion of paracellular tracers (permeability). The expression of TJ proteins, including claudin-1 and claudin-4, was examined by Western blotting of 1% Nonidet P-40-soluble and -insoluble fractions. TCA and GCA dose-dependently decreased TEER and increased paracellular permeability at pH 3 after 1 h. TCA (4 mM) or GCA (4 mM) did not change TEER and permeability at pH 7.4 or pH 4. The combination of TCA and GCA at pH 3 significantly decreased TEER and increased permeability at lower concentrations (2 mM). Pepsin (4 mg/ml, pH 3) did not have any effect on barrier function. DCA significantly decreased the TEER and increased permeability at pH 6, a weakly acidic condition. TCA (4 mM) and GCA (4 mM) significantly decreased the insoluble fractions of claudin-1 and claudin-4 at pH 3. In conclusion, acidic bile salts disrupted the squamous epithelial barrier function partly by modulating the amounts of claudin-1 and claudin-4. These results provide new insights for understanding the role of TJ proteins in esophagitis.

stratified epithelium; claudin; air-liquid interface

REFLUX ESOPHAGITIS IS INDUCED by caustic, chemical injury from agents such as gastric acid, bile acids, and pepsin. It starts at the luminal surface of the squamous epithelium and progresses through the epithelium and lamina propria into the submucosa (23, 30). One of the primary functions of the esophageal epithelium is to protect the underlying tissue from mechanical and chemical damage. The epithelial layers of the distal esophagus need to withstand reflux from the stomach and its contents (30). When the epithelium fails to protect the underlying tissue from this damage, it leads to erosions, esophagitis, and may lead to Barrett’s esophagus.

Tight junctions (TJs) are the most apical component of the intercellular junctional complexes, which form the paracellular seal between epithelial cells (17, 24, 34). TJs separate the apical cell surface domains from the basolateral cell surface domains to establish cell polarity and provide a barrier, defending against foreign insults and injuries. TJs consist of occludin, claudins, junctional adhesion molecule (JAM), and scaffold proteins, which together are known as the zonula occludens (ZO). Among these TJ molecules, claudins are the major integral membrane proteins of TJ strands (2, 7, 8, 10, 21, 27).

We recently established esophageal-like nonkeratinized stratified epithelium using normal human bronchial epithelial (NHBE) cells in vitro, which formed a stratified multilayer of squamous epithelial phenotype and showed tight barriers such as in esophageal biopsy specimens (26). This in vitro cell model has a more similar gene expression profile to esophageal epithelial specimens of healthy volunteers than any other esophageal cell lines. With respect to morphology, molecular markers, barrier properties, and acid resistance, this model presents a new way to investigate the barrier properties and possible effects of different agents on human “esophageal-like” epithelium. In our previous study, we found that the air-liquid interface (ALI)-cultured NHBE cells were damaged by acid (26). Bile may also be implicated in the pathogenesis of reflux disease. Bile acids and gastric acid may synergistically cause greater inflammatory injury to the mucosa and loss of esophageal function than either gastric acid or bile alone (16, 25, 36). However, the involvement of bile in the damage of esophageal epithelial barrier function has not been fully elucidated. Therefore, the aim of this study was to examine the influence of acidic conditions and bile acids on the barrier function of esophageal-like stratified epithelial cells. Furthermore, we evaluated the influence of acidic bile acids on TJ protein expression.

MATERIALS AND METHODS

Cell culture. NHBE cells were purchased from Lonza Walkersville (Walkersville, MD) at passage 1. Transwell-Clear inserts (1.12 cm2, 0.4-μm pore size; Costar, Cambridge, MA) were coated with collagen (0.033 mg/ml), human fibronectin (0.01 mg/ml), and BSA (0.03 mg/ml) in PBS. The cells were seeded in bronchial epithelial growth media (BEGM; Lonza Walkersville) and subcultured until reaching ~80% confluence. The cells were harvested by 0.05% trypsin-EDTA solution and stocked in cell banker-2 (serum free type; Nippon Zenyaku Kogyo, Fukushima, Japan) solution.

NHBE cells at passages 4 to 7 were used for the experiments. Before the cells were seeded in Transwell plates, the inserts were coated as above for 1 h at room temperature. The coating mixture was...
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4 days). changed the day after seeding and then every other day until the cells

ml of BEGM was used in the basal chamber. The medium was

cells/ml in BEGM, and 0.5 ml was seeded in each insert. A total of 1.5

incubator, and then removed. NHBE cells were diluted to 80,000

apically and 0.3 ml basolaterally) for 20 min in a 37°C, 5% CO2

removed, and the inserts were equilibrated using BEGM (0.2 ml

apically and 0.3 ml basolaterally) for 20 min in a 37°C, 5% CO2

incubator, and then removed. NHBE cells were diluted to 80,000

cells/ml in BEGM, and 0.5 ml was seeded in each insert. A total of 1.5

ml of BEGM was used in the basal chamber. The medium was

changed the day after seeding and then every other day until the cells

reached confluency (~4 days).

ALI culture was initiated by removing the apical medium and
decreasing the basal chamber medium volume to 600 µl. The ALI

medium consisted of a mixture of BEGM and DMEM (1:1) as
described previously (26). The ALI medium was changed every day,

and medium that leaked through to the apical chamber was removed
carefully.

**Reagents and antibodies.** Rabbit anti-claudin-1, mouse anti-clau-
din-4, and Alexa 488-conjugated anti-mouse IgG antibodies were

purchased from Invitrogen (Carlsbad, CA). Biotinylated donkey anti-
rabbit, anti-mouse, and Cy3-conjugated mouse anti-rabbit IgG antibi-
yodies were purchased from Jackson ImmunoResearch Laboratories

(West Grove, PA). Taurocholic acid (TCA), glycocholic acid (GCA),

and ursodeoxycholic acid (UDCA) were purchased from Calbiochem

(Milan, Italy), and deoxycholic acid (DCA) was purchased from

Wako Pure Chemical Industries (Tokyo, Japan). All reagents were

purchased from Sigma unless stated otherwise.

**Bile acid and pepsin exposure.** In the bile acid or pepsin exposure

experiments, ALI media at pH 7.4 was added to the apical chambers,

and TEER values were recorded. The plates were subsequently placed

in a 5% CO2 incubator at 37°C for 1 h. At 0 h, TEER values were

recorded again as above and used as a control value for each well. The

apical media was then changed to ALI media with bile acids at pH 3,

4, or 7.4 or with pepsin at 1, 2, or 4 mg/ml at pH 3.

**Measurement of transepithelial electrical resistance.** The resistance

across the stratified epithelium was measured using MILLICELL-ERS

(Millipore, Bedford, MA) with “chopstick” electrodes. The value

obtained from a blank insert was subtracted to give the net resistance,

which was multiplied by the membrane area to give the resistance in

area-corrected units (Ω·cm2). Transepithelial electrical resistance

(TEER) values were recorded at 5, 15, 30, and 60 min after stimula-

tion.

**Epithelial solute permeability.** Epithelial paracellular permeability

was measured in response to the treatments using fluorescein-5-(and-

6-)sulfonic acid (FSA) (relative molecular mass 478 Da; Invitrogen)
as a permeable tracer that passes across the stratified epithelial layers.
The cells were grown on the surface of 0.4-µm-pore size tissue culture

inserts. The insert was placed on a 12-well plate with 0.7 ml of ALI

medium in the basal chamber. A 300-µl aliquot of medium containing

0.2 mg/ml FSA was added to the luminal chamber, and then the

apparatus was placed in a 5% CO2 incubator at 37°C. After incubation

for 60 min, a 100-µl sample was taken from the basal chamber, and

the fluorescence intensity of FSA was determined at 485-nm excita-

tion and 538-nm emission using a spectrophotometer (Fluoroskan

Ascent; Thermo Fisher Scientific, Waltham, MA). The results were

expressed as the permeability ratio compared with the control (pH 7.4)

condition.

**Western blot analysis of cell lysates.** Cells were collected after the

stimulation. Protein was extracted by Nonidet P-40 (NP-40) lysis

buffer (25 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA,

25 mM NaF, 1% NP-40, 1 mM Na3VO4, and protease inhibitors) or

SDS lysis buffer (25 mM HEPES/NaOH, pH 7.5, 4 mM EDTA, 25

mM NaF, 1% SDS, and 1 mM Na3VO4) for the soluble or insoluble


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fraction, respectively. Protein concentrations were determined using the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions.

Equal quantities of protein were separated by electrophoresis on 12% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and blocked with 5% milk powder in PBS for 1 h at room temperature. The membrane was washed three times at 5 min/wash with wash buffer (0.1% milk powder in PBS) and then incubated with the appropriate primary antibody overnight at 4°C. The membrane was washed again in 0.1% milk-PBS for three times at 5 min/wash and then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody for 1 h at room temperature. Finally, the membrane was washed three times at 5 min/wash with wash buffer and detected with an ECL-plus kit (ECL-Plus Western Blotting Detection system; GE Healthcare) according to the manufacturer’s instructions. Blots were stripped (1% SDS, 0.5% 2-mercaptoethanol, and PBS) for 1 h at 40°C and then rinsed three times in distilled water. Stripped membranes were re-blocked for 1 h in 5% milk-PBS before going through the normal Western procedure. Finally, chemiluminescence was detected by an ImageQuant Imager 350 system (GE Healthcare Life Sciences). All experiments were reproduced three times. The results of a typical experiment are shown. The Western blot bands were analyzed by ImageJ software.

Statistical analysis. All values are expressed as means ± SE. Data were analyzed using one-way ANOVA followed by Fisher’s protected least-significant difference or Scheffe’s F-test for multiple comparisons. Significance was accepted at P < 0.05.

RESULTS

Influence of acidic bile salts on ALI-cultured NHBE cells.

ALI-cultured NHBE cells (10 days in ALI culture) were exposed to acid with or without bile acids on the apical side for 60 min. TEER values were recorded at 0, 5, 15, 30, and 60 min after stimulation, and the permeability values were recorded at 60 min.

When ALI-cultured cells were exposed to acidic conditions, pH 1.5 but not pH 3 significantly decreased the TEER and increased the permeability. When ALI-cultured NHBE cells were exposed to TCA (4 mM) at pH 7.4, neither TEER nor permeability was affected (Fig. 1A). TCA (4 mM) significantly decreased TEER and increased permeability at pH 3, but not at pH 4. These data indicate that TCA itself does not influence the TEER or permeability of ALI-cultured NHBE cells. Although neither TEER nor permeability was affected at pH 3, TCA at pH 3 dose-dependently and significantly decreased TEER and increased permeability after 60-min stimulation (Fig. 1B).

When ALI-cultured NHBE cells were exposed to GCA (4 mM) at pH 7.4, neither TEER nor permeability was affected.

Fig. 3. Combined influence of TCA and GCA on ALI-cultured NHBE cells. A combination of GCA (2 mM) and TCA (2 mM) at pH 3 significantly decreased the TEER compared with the control or pH 3 conditions (A) and significantly increased the permeability compared with the control or pH 3 (B) (*P < 0.001 vs. control, &P < 0.001 vs. pH 3, n = 4). The combined influence of TCA (2 mM) and GCA (2 mM) was similar to GCA (4 mM, pH 3) (not significant (ns)) but significantly decreased the TEER compared with TCA (4 mM, pH 3) (A) (&P < 0.01 vs. 4 mM TCA, pH 3, n = 4). Data are expressed as means ± SE.

Fig. 4. Influence of ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), and pepsin on ALI-cultured NHBE cells. A: UDCA (200 μM) at pH 3 or pH 7.4 did not change the TEER [ns, UDCA (200 μM, pH 7.4) vs. pH 7.4 medium; P > 0.5, UDCA (200 μM, pH 3) vs. pH 3 medium, n = 3]. B: weakly acidic conditions (pH 6) with DCA (2 mM) significantly decreased the TEER and significantly increased the permeability (*P < 0.001 vs. control, n = 3). C: different concentrations of pepsin (1–4 mg/ml) in pH 3 medium did not decrease the TEER compared with pH 3 medium alone (ns, n = 3). Data are expressed as means ± SE.
GCA (4 mM, pH 3) significantly decreased TEER and increased permeability at pH 3, but not at pH 4. These data indicate that GCA itself does not influence the TEER or permeability of ALI-cultured NHBE cells. GCA at pH 3 dose-dependently and significantly decreased TEER and increased permeability after 60 min of stimulation (Fig. 2B).

Combined effects were induced when GCA and TCA were mixed at acidic conditions at pH 3. The combination of TCA (2 mM) and GCA (2 mM) at pH 3 significantly decreased the TEER and significantly increased the permeability compared with the control or at pH 3 alone (P < 0.001) (Fig. 3). The influence of this combination on the TEER and FSA permeability was similar to that of GCA (4 mM, pH 3) but significantly decreased compared with TCA (4 mM, pH 3) (P < 0.01). In ALI-cultured NHBE cells were stained with hematoxylin and eosin. The morphology showed stratified squamous epithelial cells forming esophageal epithelial cell layers, and the layers were not affected by TCA (4 mM, pH 3) or by GCA (4 mM, pH 3). Claudin-1 was predominantly detected at the cell-cell contacts in the suprabasal and intermediate cell layer in a continuous linear pattern, and not in the basal cell layer, and was faint in the superficial layers (Fig. 7, D–F). Claudin-4 was detected in the suprabasal to superficial layers. It was detected in dots or whisker-like lines in the superficial layers and had relatively broad cell surface staining in the suprabasal cell layer in ALI conditions. Claudin-1 and claudin-4 were still detected at junctions after 1 h stimulation with TCA (4 mM, pH 3) or with GCA (4 mM, pH 3). TCA or GCA stimulation did not change the staining patterns of claudin-1 and claudin-4 compared with the control condition.

Influence of UDCA, DCA, and pepsin on ALI-cultured NHBE cells. NHBE cells were treated with 200 μM UDCA at pH 7.4 and pH 3 for 60 min. UDCA in different pH conditions did not change the TEER (Fig. 4A). UDCA was not soluble at more than 200 μM.

DCA (2 mM) at weakly acidic (pH 6) conditions significantly decreased the TEER or significantly increased the permeability (Fig. 4B). DCA (2 mM, pH 7.4) or medium at pH 6 did not decrease the TEER or permeability.

Different concentrations of pepsin (1–4 mg/ml) at pH 3 did not change the TEER compared with medium at pH 3 (Fig. 4C).

TJ proteins changed by bile acid stimulation. To determine the expression patterns of TJ proteins, Western blotting was performed with ALI-cultured NHBE cells. Stimulation at pH 3 did not change the amount of NP-40-soluble and -insoluble fractions of claudin-1 and claudin-4 proteins. Soluble fractions of claudin-1 and claudin-4 were not changed by TCA (4 mM, pH 3). The insoluble fraction of claudin-1 significantly decreased with TCA (4 mM, pH 3) (P < 0.05 vs. pH 3 and P < 0.01 vs. control). The insoluble fraction of claudin-4 also significantly decreased with TCA (4 mM, pH 3) (P < 0.05 vs. control) (Fig. 5).

GCA (4 mM, pH 3) significantly increased NP-40-soluble fractions of claudin-1 and claudin-4 compared with the control condition. On the other hand, GCA (4 mM, pH 3) significantly decreased NP-40-insoluble fractions of claudin-1 and claudin-4 (Fig. 6).

Fig. 5. Influence of TCA on the Nonidet P-40 (NP-40) solubility of tight junction proteins. TCA (4 mM, pH 3) or pH 3 alone did not change the NP-40-soluble fractions of claudin-1 and claudin-4. pH 3 alone did not change the NP-40-insoluble fractions of claudin-1 and claudin-4. However, TCA (4 mM, pH 3) significantly decreased the NP-40-insoluble fractions of claudin-1 and claudin-4 (**P < 0.05 vs. pH 3 and ***P < 0.01 vs. control, n = 5). Data are expressed as means ± SE.

Fig. 6. Influence of GCA on the NP-40 solubility of tight junction proteins. pH 3 alone did not change the NP-40-soluble fractions of claudin-1 and claudin-4. In contrast, GCA (4 mM, pH 3) significantly increased both (***P < 0.05 vs. control, n = 5) (A and B). pH 3 alone did not change the NP-40-insoluble fractions of claudin-1 and claudin-4. However, GCA (4 mM, pH 3) significantly decreased the NP-40-insoluble fractions of claudin-1 and claudin-4 (ns vs. pH 3 and ***P < 0.05 vs. control, n = 5). Data are expressed as means ± SE.
DISCUSSION

The human esophagus is lined by a nonkeratinizing stratified squamous epithelium, which produces an effective barrier against the influx of luminal content. Histologically, the esophageal epithelium consists of three layers, the basal, suprabasal, and superficial layer (13). Although many esophageal cell lines have been used in in vitro studies, such as the SV40 T-antigen immortalized normal human esophageal cell line (Het-1A), or esophageal carcinoma cell lines (11), the mechanisms by which different stressors damage the esophageal epithelial cell layers and cause damage are still not clear. Het-1A did not grow like a stratified epithelial cell layer in ALI conditions and did not express TJ proteins as does the human esophagus (data not shown). We therefore used NHBE cells, because tracheobronchial epithelium can be transformed into stratified squamous epithelium-like esophageal epithelial cells in an ALI culture system (15, 19, 26).

Acid is a major factor in the development of esophagitis. An animal study suggested that only strong acid (pH 1.0–1.3) alone could injure the esophageal epithelium (36). In our previous study, ALI-cultured NHBE cells were stimulated with acid at pH 1, the TEER was significantly decreased, and FSA permeability was significantly increased (26). However, as previously reported, the refluxate includes not only gastric acid but also bile acid and pepsin. Recently, it has become widely accepted that mixed (gastric and bile acid) reflux is the dominant pattern of reflux in patients with severe GERD (5, 6, 23, 32). Gastric acid combined with pepsin or bile salts seems to be more harmful to the esophageal epithelial layers than gastric acid alone (12, 16, 18, 22, 23). Different concentrations of bile salts refluxed with acid have been reported to range from ~200 μM to 6 mM in esophagitis patients (23). The main bile acids present in the patients’ refluxate are TCA and GCA (12, 23).
Therefore, we used TCA, GCA, and a combination of both in this study. Although an acidic medium of pH 3 did not change the barrier function in this study, TCA and GCA at pH 3 significantly decreased TEER and increased paracellular permeability. These data are consistent in part with a previous report using esophageal mucosal sheets of rabbits (6) and indicate that only acidic TCA and GCA can damage the barrier function. In low pH conditions, conjugated bile acids enter the mucosal cells in a nonionized form, which tends to diffuse through the mucosa more efficiently than the ionized form (4, 23). These refluxed bile acids can cause intracellular damage by the dissolution of cell membranes and TJ.s (23, 30).

The reflux often consists of mixed fluids in vivo. Therefore, we combined TCA and GCA in acidic medium at pH 3. This caused impaired barrier function at low concentrations. Although pepsin has been used in the background in a previous in vitro study (6), pepsin in acidic conditions did not impair the squamous barrier function, at least in this short time exposure, indicating a less damaging effect of pepsin compared with bile acids. However, the influence of pepsin should be examined at different pH values and durations in future studies.

Reflux has the esophagus in patients with acid suppression therapy is different from those in patients without. Higher levels of secondary bile acids like DCA are detected in patients with acid suppression therapy (14). Here, we showed that DCA in weakly acidic conditions affected the barrier function, indicating that, even if acid suppression is successful, weakly acidic reflux with DCA can damage the esophagus. While bile acids, including GCA, TCA, and DCA, are known to damage barrier function (22, 30) or to affect colon tumor promoters (20), UDCA is thought to be chemopreventive (1). Although the solubility of UDCA was low in the culture media, we examined the influence of UDCA at maximum concentration at pH 3, and it did not affect the barrier function. This may be related to the pH. The influence of UDCA in weakly acidic conditions should be explored in future studies.

Several previous reports have indicated that the disruption of barrier function is related to dislocalization and loss of TJ complexes between epithelial cells (2, 7, 8, 10, 21, 27). Several distinct types of claudins have been reported to colocalize in various types of cells (8, 35), and several reports including ours have indicated that claudin-1, claudin-4, claudin-7, JAM-A, occludin, and ZO-1 are expressed in the human esophagus (9, 26, 29). Because the functional TJ proteins are insoluble in nonionic detergents (28, 31, 33, 37) and related to the regulation of paracellular permeability in epithelial cells (24), proteins from ALI-cultured cells were divided into nonionic detergent (NP-40)-soluble and -insoluble fractions, and we measured the levels of claudin-1 and claudin-4, which play a major role in esophageal barrier function, in each fraction. The decrease of TEER and increase of permeability were related to the decrease of insoluble fractions of claudin-1 and claudin-4. In contrast, Asaoka et al. (3) reported that the total amount of claudin-1 and claudin-4 proteins increased in a chronic acid reflux esophagitis rat model. This may be related to the different fractions of claudins. Species differences may also play a role because claudin-3 is detected in rat esophagus but not in human esophagus.

Previous studies indicated that the dislocalization of TJ proteins can be detected both by detergent solubility of TJ proteins and by immunofluorescent staining (27, 28). Although the detergent solubility of claudin-1 and claudin-4 was affected by TCA or GCA in this study, we could not detect dislocalization of claudin by immunofluorescent staining. The reason for this is unclear. However, immunofluorescent staining may not be as sensitive as detergent solubility for detecting the dislocalization of TJ proteins from TJ strands.

In conclusion, based on the esophageal-like squamous epithelial cell layers of the ALL system, we found that acidic bile salts disrupted the squamous epithelial barrier function partly by modulating the amounts of claudin-1 and claudin-4. Our findings demonstrate a new method for the study of stratified epithelial barrier function and provide new insights for understanding the role of TJ proteins in esophagitis. This can help us understand how chemical factors cause epithelial injury. In future studies, we plan to investigate the influence of other bile acids or cytokines on the barrier function of ALI-cultured cells and on TJ protein expression patterns. Once the mechanisms of the maintenance of barrier function become clear, we may be able to find ways to strengthen the esophageal barrier.

ACKNOWLEDGMENTS

We thank Noriko Kamiya and Masayo Togawa for excellent technical assistance.

DISCLOSURES

The authors have no competing interests.

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