Deoxycholic acid impairs glycosylation and fucosylation processes in esophageal epithelial cells

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It is generally accepted that esophageal adenocarcinoma arises from a Barrett’s metaplastic lesion. Altered glyco-protein expression has been demonstrated in tissue from patients with Barrett’s esophagus and esophageal cancer but the mechanisms regarding such changes are unknown. The bile acid deoxycholic acid (DCA) alters many cell signaling pathways and is implicated in esophageal cancer progression. We have demonstrated that DCA disrupts Golgi structure and affects protein secretion and glycosylation processes in cell lines derived from normal squamous epithelium (HET-1A) and Barrett’s metaplastic epithelium (QH). Cell surface expression of glycans was identified using carbohydrate-specific probes (wheat germ agglutinin, concanavalin A, peanut agglutinin, lithocholic acid and Ulex europaeus agglutinin) that monitored N-glycosylation, O-glycosylation and core fucosylation in resting and DCA-treated cells. DCA altered intracellular localization and reduced cell surface expression of N-acetyl-D-glucosamine, α-methyl-mannopyranoside (Man/Glc) and fucose in both cell lines. Furthermore, DCA reduced the expression of epithelial growth factor receptor and E-cadherin in a manner analogous to treatment of cells with the N-glycan biosynthesis inhibitor tunicamycin. This is the first study to identify an altered Golgi structure and glycomic profile in response to DCA in esophageal epithelial cells, a process which could potentially contribute to metaplasia, dysplasia and cancer of the esophagus.

Keywords: Barrett’s esophagus / bile acid / E-cadherin / glycosylation / esophageal cancer

Introduction

A proportion of patients with gastroesophageal reflux disease (GORD) develop Barrett’s esophagus (BO) where normal squamous epithelium undergoes metaplasia to an intestinal columnar-type epithelium (Atherfold and Jankowski 2006). Patients with BO are at increased risk for developing esophageal cancer as unstable epithelial or stem cells from the premalignant Barrett’s lesion can undergo metaplasia, dysplasia and carcinogenesis (Jankowski et al. 1999). Altered expression of complex glycoproteins have been observed in patients with reflux disease, BO and esophageal cancer indicating an impairment of glycoprotein processing (Shimamoto et al. 1987; Neumann et al. 2008).

Glycosylation is crucial for protein processing and therefore expression and function of cell surface receptors (Fan et al. 1997; Lee et al. 2003; Liwosz et al. 2006; Gu et al. 2009). Abnormal glycosylation is a hallmark of inflammation and cancer but the mechanisms behind disruption of glycan processing are unknown (Crocker and Redelinghuys 2008). Impaired glycosylation has been demonstrated in many cancer cell types and impacts multiple cell physiological processes such as proliferation, differentiation and cell–cell interactions as the terminal oligosaccharide units act as recognition molecules for a variety of proteins involved in cell signaling (Paulson 1989). One of the main functions of the Golgi is to modify synthesized proteins through the addition of simple sugars. This process is tightly regulated by glycosyltransferases. The two main types of glycosylation processes are N- and O-linked glycosylation, N-linked glycosylation occurs when sugar chains are added to the protein backbone at asparagine residues within the sequence Asn-Xaa-Ser/Thr (Xaa can be any amino acid except proline). N-Glycans are highly branched structures which play key roles in cell-to-cell contact, cell–extracellular matrix interactions and thus adhesion. Disruption of these processes are associated with cancer metastasis (Yanyang et al. 2008). Many receptors require N-glycosylation including epithelial growth factor receptor (EGFR), E-cadherin, platelet-derived growth factor, insulin-like growth factor and fibroblast growth factor and impaired glycosylation can result in aberrant localization and altered signaling processes (Dennis et al. 2009). EGFR and E-cadherin specifically have been shown to be dysregulated in Barrett’s and cancer tissue (Gong et al. 2009; Takahashi et al. 2009). O-glycosylation involves the addition of N-acetylgalactosamine (GalNAc) to Ser and Thr residues and is mediated by GalNAc transferases. O-Glycosylation creates binding sites for glycoproteins such as galectins and selectins which mediate binding of leukocytes to endothelial cells (Hang and Bertozzi 2005).

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Bile acids have been implicated in the pathogenesis of GORD, BO and esophageal cancer progression, and clinical studies have detected the presence of unconjugated bile acids in refluxate from BO patients (Nehra et al. 1999). Evidence from animal models demonstrates that bile acids are more injurious than gastric acid. A jejunoesophageal anastomosis animal model demonstrated that jejunal contents (bile acids) could induce esophageal adenocarcinoma independently of gastric acid (Fein et al. 1998). The bile acid deoxycholic acid (DCA) is pro-tumourigenic and associated with metaplasia and cancer. In vitro experiments using epithelial cells demonstrate that DCA activates several transcription factors and signaling pathways associated with inflammation and carcinogenesis including activator protein-1 (AP-1), nuclear factor kappa-B (NFKB), mitogen-activated protein kinase (MAPK) and protein kinase C (PKC; Looby et al. 2005; Shah et al. 2006). In a colonic cell model, we demonstrated that DCA has a specific effect on the protein secretory pathway where it induces Golgi fragmentation by over-activation of the membrane fission process in a PKC-η/PKD-dependant manner (Byrne et al. 2010). Fragmentation of the Golgi has profound implications for protein processing (including secretion and glycosylation) and consequently affects cell processes such as adhesion, migration and inflammation.

We hypothesized the bile acid DCA, present in the refluxate could induce Golgi fragmentation in esophageal cells and impair protein processing, glycosylation and secretion. This may represent an important novel mechanism of bile acid-induced carcinogenesis in the esophagus.

**Results**

**DCA induced Golgi fragmentation in HET-1A and QH cells**

In this study, the effect of bile acids on Golgi morphology was examined in esophageal epithelial cells. We looked at models of normal squamous epithelium (HET-1A) and Barrett's metaplasia (QH). Barrett's metaplastic cells are more resistant to bile acids in the context of disease (Aldulaimi and Jankowski 1999), but given that adenocarcinoma arises from Barrett's metaplastic cells, it is of interest to identify whether bile acids alter their glycoprofiles. In order to determine the impact of DCA on Golgi structure in normal and BO cell lines, cells were treated with various doses of DCA (0–300 µM) for 6 h. Brefeldin-A (1 µg/mL) was used as a positive control for Golgi fragmentation. Visually, by immunofluorescence, Golgi structure was altered at concentrations as low as 10 µM in the HET-1A cells, whereas it was more evident at higher concentrations of 50 µM in the QH cell line, suggesting the HET-1A cells are more sensitive to DCA in terms of effects on the Golgi structure (Figure 1A). Quantification of complete Golgi fragmentation was undertaken objectively by high-content analysis using the Incell Analyser-1000 and analysis software. Golgi were completely fragmented in 51.7% of HET-1A cells at 1 h and 68.4% of HET-1A cells at 6 h in

![Fig. 1. DCA induced Golgi fragmentation in HET-1A and QH cell lines.](https://academic.oup.com/glycob/article-abstract/22/5/638/1988659)
DCA impaired protein secretion in HET-1A and QH cells

Disruption of Golgi structure may lead to impaired protein secretion. To test this hypothesis, we transfected cells with a Gaussia luciferase (GLuc) construct then exposed the HET-1A and QH cells to DCA. GLuc follows the classic secretory pathway and has been used to monitor effects on the protein secretory pathway and ER stress (Badr et al. 2007). Compounds that interfere with protein trafficking both within the Golgi (monensin) and between the ER-Golgi (brefeldin-A and nocodazole) cause a decrease in secretion of GLuc (Badr et al. 2007). The amount of luciferase secreted into the supernatant is monitored by luminescence. There was an initial increase in secretion in HET-1A cells treated with 100 µM DCA at the 1 h timepoint indicating an initial hyper-secretion of GLuc. A significant decrease in secretion was then observed at 300 µM DCA at 1 h and for 200 and 300 µM DCA at the 6 h timepoint. DCA significantly decreased secretion at all doses and timepoints in the QH cell line (P < 0.05, Figure 2). In this cell line, the secretion of GLuc is impaired in response to DCA prior to complete breakdown of the Golgi structure (Figure 1). This may be due to mis-localization of Golgi resident enzymes, incorrect targeting of carrier vesicles at the trans-Golgi-network or effects of DCA on ER-Golgi-plasma membrane transport.

DCA alters glycan expression and intracellular localization in HET-1A and QH cells

A panel of lectins was used to identify glycan products expressed by the HET-1A and QH cell lines (Supplementary data, Table S1). Core substitutions, branching and sialylation are the key functional aspects that we hypothesized to be affected by DCA disruption of the Golgi structure and are the processes typically observed in malignant transformation. Wheat germ agglutinin (WGA) detects N-acetylglucosamine (GlcNAc) with preferential binding to dimers (GlcNAcβ1,4GlcNAc) or trimers (GlcNAcβ1,4GlcNAcβ1,4GlcNAc) of this sugar but also binds to terminal sialic acid moieties. Concanavalin A (ConA) and lithocholic acid (LCA) bind mannose-containing glycans. Fucosylation was monitored using Ulex europaeus agglutinin-1 (UEA-1) and LCA. UEA-1 preferentially binds α-2 fucose (Fuc) and LCA binds α-1-6 Fuc. Peanut agglutinin (PNA) binds to the Galβ1-3GalNAc residue on the cancer-associated Tn oncofetal antigen. The lectin profile of both cell lines was determined by flow cytometry using various concentrations of FITC-labeled lectins. Optimal concentrations were determined for subsequent studies as indicated (Supplementary data, Figure S1). In order to investigate the effect of DCA on cell surface glycan expression, we treated cells at the timepoint and concentrations shown to induce Golgi fragmentation in both cell lines (100 or 300 µM DCA for 6 h, Figure 1). Cells were fixed and incubated with FITC-conjugated lectins. The mean fluorescence intensity and the percentage positivity were measured by flow cytometry (Figure 3). Subcellular localization of glycan expression in resting and DCA-treated cells was assessed by immunofluorescence microscopy. Pre-incubation with haptenic sugars (H.S) indicate specificity of lectin binding.

There was a significant decrease in WGA, ConA and LCA lectin binding in HET-1A normal esophageal cells, indicating
a decrease in cell surface GlcNAc, Man/Glc and Fuc containing oligosaccharides \((P < 0.05, \text{Figure 3})\). By examining the intracellular localization of these lectins, WGA, ConA and LCA were found to be localized to the peri-nuclear region in untreated HET-1A cells and distributed throughout the cytoplasm when treated with DCA (Figure 4, arrows).

In the QH cell line, there was a significant decrease in cell surface binding of WGA in response to 100 µM DCA \((P < 0.05)\) but not when treated with 300 µM DCA. However, when we examined the intracellular localization of this lectin, there was a clear redistribution from the peri-nuclear region in cells treated with either 100 or 300 µM DCA (Figure 4, arrows).

**Fig. 3.** DCA alters lectin profiles for HET-1A and QH cells. Cells were treated with either 100 or 300 µM DCA for 6 h. The glycan expression profile was assessed using FITC-labeled WGA, ConA, PNA, UEA-1 or LCA. Specificity of lectin binding was demonstrated by the pre-incubation of the lectin with its haptenic sugar prior to incubation with the cells (H.S). Histograms represent the % positivity and mean fluorescence intensity (bold) respectively. Unstained cells are represented (shaded). Histograms are a representative of \(n = 3\) independent experiments.
A greater decrease in cell surface binding of ConA was observed by flow cytometry in QH cells treated with 100 µM DCA compared with 300 µM (P < 0.05, Figure 3). However, again immunofluorescence analysis demonstrated clear alterations in intracellular expression and localization at both concentrations of DCA (Figure 4, arrows). There was an apparent decrease in the fluorescence intensity by immunofluorescence for both concentrations of DCA which may be due to dispersal of the glycan expression throughout the cytoplasm, as opposed to being expressed as the discrete entity observed in untreated cells.

A decrease in cell surface binding of UEA-1 and LCA to both cell lines indicates a decrease in α1,2- and α1,6-Fuc (Figure 3), which are present on both N- and O-linked glycoproteins (Varki et al. 1999). Apparent UEA-1 binding was weak in HET-1A cells by immunofluorescence (again potentially due to the dispersal of α1,2-fucosylated structures); therefore, we could not determine the effects of DCA on intracellular localization (data not shown). UEA-1 binding was variable in QH cells but in the majority of cells it was pronounced at the peri-nuclear region; however, DCA did not alter the localization of UEA-1 (Supplementary data, Figure S2, arrows). DCA disrupted the peri-nuclear localized LCA lectin causing redistribution throughout the cytoplasm (Figure 4). PNA did not bind to HET-1A cells (data not shown) suggesting the lack of Tn antigen in these cells but...
did bind to a subset of QH cells. DCA did not alter the expression pattern (Supplementary data, Figure S2). Pre-incubation with haptenic sugars indicate specificity of lectin binding (Supplementary data, Figure S3).

Taken together, these results indicate that DCA causes a redistribution of GlcNAc, Man/Glc and Fuc-containing glycans in normal esophageal epithelial cells. DCA affects glycosylation by impairing both GlcNAc and trimannoside core processing in HET-1A and QH cells as indicated by alterations in WGA and ConA binding, respectively. The decrease in UEA-1 and LCA binding to the cell surface suggests that DCA affects core fucosylation. The cancer-associated Tn antigen, which is recognized by PNA, was absent in normal esophageal cells (HET-1A) but expressed in a subset of QH Barrett’s esophageal cells. DCA did not alter Tn expression, suggesting that it does not affect Gal-1,3-GalNAc.

**DCA alters EGFR and E-cadherin expression in QH Barrett’s esophageal cell line**

EGFR and E-cadherin are associated with carcinogenesis in many cell types. EGFR plays a role in cell proliferation and differentiation. Loss of E-cadherin at adheren junctions is associated with the epithelial-mesenchymal transition and invasion (Curto et al. 2007). EGFR has 11 potential N-linked glycosylation sites and E-cadherin has 4, and since DCA had a profound effect on N-linked glycosylation as demonstrated by lectin binding above, we investigated the effect of DCA on both of these receptors. To establish whether DCA affects N-glycosylation of EGFR and E-cadherin, we used a gel mobility shift assay. A loss of complex carbohydrates results in lower molecular weight protein which is observed as an increase in mobility on the gel. QH cells were treated with 300 μM DCA for 6 h and expression of EGFR and E-cadherin was examined by western blot.

To demonstrate the mobility shift is due to impaired glycosylation, we used tunicamycin and PNGase-F. Tunicamycin is an inhibitor of N-glycan biosynthesis, whereas PNGase-F cleaves high-mannose, hybrid and complex N-glycans at asparagine residues. QH cells were treated with tunicamycin for the same timepoints as DCA, and following cell lysis, electrophoretic shift was examined by western blot. When probed with antibodies to EGFR and E-cadherin, lower molecular weight bands were observed (Figure 5A). PNGase-F treatment of resting cell lysates indicated the increase in mobility when all N-glycans are cleaved (160 kDa for EGFR and 110 kDa for E-cadherin, Figure 5A). Appearance of bands with lower molecular weight was observed in response to DCA for both EGFR (~167 kDa) and E-cadherin (~115 kDa), suggesting that DCA impairs N-glycan biosynthesis (Figure 5A). DCA did not increase the mobility of EGFR or E-cadherin to the same extent as PNGase treatment, indicating that DCA did not cause cleavage of all high-mannose, hybrid and complex N-linked glycoproteins. Overall expression levels of EGFR and E-cadherin were decreased in response to DCA consistent with a study, which demonstrated that altered N-glycosylation of E-cadherin at Asn-633 impaired folding and trafficking of E-cadherin resulting in degradation by the endoplasmic reticulum-associated degradation pathway (Zhou et al. 2008).

DCA has also been shown to cause autoactivation of EGFR in the absence of EGF in Caco2 colon cancer cells (Raimondi et al. 2008). To determine whether EGFR signaling is involved in DCA-mediated effects on E-cadherin expression, we pre-treated the QH cells with the EGFR-specific tyrosine kinase inhibitor erlotinib. Pre-treatment of cells with the EGFR tyrosine kinase inhibitor erlotinib further reduced the glycosylation of EGFR. Furthermore, treatment of cells with erlotinib further potentiated DCA-stimulated reduction in E-cadherin expression. This result would suggest that DCA-mediated down-regulation of E-cadherin expression is not entirely dependent on EGFR activity.

Localization of E-cadherin at cell–cell contacts is important for adherence junction formation (Jovov et al. 2011). Impaired glycosylation of E-cadherin causes a loss of membrane localization, breakdown of adherens junctions and loss of epithelial barrier function (Takahashi et al. 2009). Since we demonstrated DCA impaired N-glycosylation processes and altered expression of E-cadherin, we investigated the cellular localization of E-cadherin in response to DCA. We demonstrate reduced membrane localization of E-cadherin and loss of cell-cell contact in the QH cell line (Figure 5B, arrows). An overall reduction in E-cadherin staining was observed, which correlates with the expression levels observed by western blot in response to DCA (Figure 5A).

**Discussion**

In this study, we demonstrate that DCA induced fragmentation of the Golgi, impaired protein secretion and altered GlcNAc, Man/Glc and Fuc intracellular localization and cell surface expression. We demonstrate impaired glycosylation of two heavily N-linked glycosylated proteins, EGFR and E-cadherin in cell line models of normal esophageal squamous epithelium (HET-1A) and Barrett’s metaplasia (QH).

Global alterations to glycosylation processes are frequently observed in malignant cells resulting in the expression of shorter, less highly branched carbohydrate structures. The underlying cause behind this disruption to protein processing is still poorly understood. Both genomic and non-genomic mechanisms have been proposed. At a genetic level, decreased expression of Golgi-resident enzymes involved in glycoprotein synthesis (glycosyl-, sialyl and sulfotransferases) is observed in colon cancer, but correlates poorly with altered carbohydrate expression (Yang et al. 1994). At an epigenetic level, DNA hypermethylation of multiple glycogenes such as B4GALNT2 and ST3GAL6 are observed in gastric cancer cells and associated with impaired glycosylation (Kawamura et al. 2008). At a post-transcriptional level, mis-localization of Golgi resident proteins and enzymes are thought to play a significant role (Rivinoja et al. 2009). Altering Golgi pH caused fragmentation and expression of the Tn antigen in non-malignant NRK and COS7 cells (Kellokumpu et al. 2002). A number of colon cancer cell lines have inherently fragmented Golgi and consequently express the short-chained cancer-associated Tn antigen. Thus, Golgi integrity is crucial for glycosylation processes.

In the esophagus, glycosylation is disrupted in high-grade dysplastic and esophageal cancer tissue and progression from...
normal to metaplasia, dysplasia and esophageal adenocarcinoma is associated with deletion of terminal glycosylation structures (Shimamoto et al. 1987). The glycan profile of serum from patients with Barrett’s and adenocarcinoma identified distinct glycoproteins (Mechref et al. 2009); however, the underlying cause of impaired glycosylation in this context has not previously been defined. Cell surface proteins with altered glycosylation can be shed into the blood circulation and could account for the altered glycomic profile. However, the possibility of inflammatory cytokines such as IL-6 or IL-1β triggering glycosylation changes in acute phase proteins cannot be ruled out.

In this study, DCA decreased UEA-1 and LCA cell surface lectin binding in both normal and Barrett’s esophageal cell lines demonstrating altered α1,2- and α1,6-Fuc, respectively. A decrease in UEA-1 binding has been observed in high-grade dysplasia and esophageal adenocarcinoma tissue (Shimamoto et al. 1987). A decrease in fucosylation was also observed in serum from patients with BO and esophageal cancer compared with serum from control patients (Mechref et al. 2009). We propose that DCA disruption to the protein secretory process may be a mechanism for the observed decrease in core fucosylation in esophageal disease. Core fucosylation affects many properties observed in cancer cells. It is required for binding of EGF to its receptor and downstream extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) activation (Liu et al. 2007). A decrease in core fucosylation of E-cadherin alters cell-to-cell and cell-to-extracellular matrix interaction (Osumi et al. 2009). Fucosylation deficiencies play a role in evasion of tumor immune surveillance allowing escape from NK cell-mediated apoptosis via impaired tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling (Moriwaki et al. 2009).

Alterations in GlcNAc and Man/Glc-linked glycoprotein expression and cellular localization were observed by WGA and ConA binding, in response to DCA. E-cadherin is synthesized in the ER as a precursor polypeptide where it is
glycosylated then further processed in the Golgi complex to form the fully mature 120 kDa protein (Carpenter et al. 2002). E-cadherin has four potential N-glycosylation sites located in the extracellular domain. N-Glycans specifically at Asn-633 are essential for folding, trafficking and expression of E-cadherin (Zhou et al. 2008). In this study, the expression of lower molecular weight forms of the E-cadherin protein suggests impaired N-linked glycosylation in response to DCA, and consequently, a decrease in expression and cell membrane localization of E-cadherin was observed. E-cadherin expression is implicated in the pathogenesis of many gastro-intestinal diseases. In gastric adenocarcinoma tissue, E-cadherin expression is lost at the cell membrane but observed in the Golgi implying a defect in transport to the membrane (Carpenter et al. 2002). E-cadherin expression is reduced along the esophageal metaplasia-dysplasia-adenocarcinoma sequence (Bailey et al. 1998) and decreased membrane localization is associated with increased junctional permeability in endoscopic biopsies from patients with GORD (Jovov et al. 2011). The epithelium is an important barrier against reflux and impaired cell adhesion results in increased intracellular spaces and damage to the submucosa. A loss of E-cadherin has been observed in response to cheno-DCA (CDCA) and lithocholic acid and associated with acquisition of invasive potential in hepatobiliary carcinoma (Fukase et al. 2008). We have observed induction of Golgi fragmentation in response to both CDCA and lithocholic acid which may also contribute to repression of E-cadherin (unpublished observations).

Previous studies demonstrate a relationship between E-cadherin and EGFR pathways. EGFR activation reduced E-cadherin levels via the E-cadherin suppressor gene TWIST (Lo et al. 2007). A loss of E-cadherin has been shown to up-regulate EGFR expression (Wang et al. 2011). DCA has been demonstrated to activate EGFR in colon cancer cells (Raimondi et al. 2008). We investigated the effect of DCA on EGFR expression and activity in the QH cell line. We demonstrated that DCA impaired EGFR expression and glycosylation. Blocking EGFR activity with erlotinib did not attenuate this DCA reduction in E-cadherin expression and glycosylation; rather, it further potentiated this effect. This would suggest that DCA-mediated down-regulation of E-cadherin expression is not wholly dependent on EGFR activity. However, given that DCA modulated glycosylation and expression of both proteins, it is clear that multiple mechanisms are involved. N-Glycosylation of EGFR is required for correct folding and activation at the cell membrane. Defects in N-glycosylation of EGFR lead to spontaneous activation via ligand-independent receptor dimerization and phosphorylation (Matsumoto et al. 2008). Activation of EGFR causes phosphorylation of β-catenin and dissociation from E-cadherin, thus leading to a down-regulation of cell membrane associated E-cadherin and loss of tight junction formation.

We hypothesize that disruption to the Golgi structure by DCA impairs glycosylation of both EGFR and E-cadherin and consequently impairs their trafficking and retention at the cell membrane. In the context of BO and adenocarcinoma, DCA-mediated alterations of E-cadherin expression could impair cell–cell adhesion and lead to an invasive/metastatic phenotype.

In summary, we have demonstrated that a component of acid reflux, DCA, induced Golgi fragmentation, impaired protein secretion and altered glycoprotein expression. Impaired glycoprotein expression has multiple effects on cell processes such as adhesion, migration, inflammation and cell-to-cell communication. More recently, altered glycosylation has been associated with endoplasmic reticulum stress and decreased protein synthesis (Xu et al. 2010). Together with genetic and epigenetic alterations, post-translational modifications of proteins, such as glycosylation, warrant investigation as factors involved in the carcinogenic process. Establishing the mechanisms underlying glycosylation defects is crucial to understanding disease progression. This is the first study to demonstrate a link between bile acids and glycosylation defects in gastrointestinal disease and may be an important underlying mechanism of metaplasia and dysplasia of the esophagus.

Materials and methods

Cell culture and reagents

HET-1A squamous esophageal epithelial cells (ATCC, Rockville, MD) and QH Barrett’s metaplastic cells (also designated CP-A, kindly provided to us by Professor Peter Rabinovich, University of Washington) were cultured in bronchial epithelial cell basal medium (BEBM) culture medium + supplements (Lonza, Basel, Switzerland). For the QH cell line, medium was further supplemented with 5% fetal calf serum (Gibco-BRL, Grand Island, NY). DCA, dimethyl sulfoxide (DMSO) and brefeldin-A were obtained from Sigma-Aldrich Chemical Company (St Louis, MO). DCA was solubilized in DMSO. FITC-labeled lectins, WGA, ConA, PNA and UEA-1 were obtained from Vector Laboratories (Alexis Germany, Grunberg, Germany). Anti-GM130 antibody was obtained from Sigma-Aldrich Chemical Company. Anti-E-cadherin was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA).

Immunofluorescence staining

Cell lines were treated with DCA in serum and supplement-free medium, fixed with 4% paraformaldehyde, permeabilized with 0.1% (v/v) Triton X-100/phosphate buffered saline (PBS) followed by blocking with 5% bovine serum albumin (BSA)/PBS. Cells were incubated with anti-GM130 Golgi antibody (Sigma-Aldrich Chemical Company) or anti-E-cadherin antibody (Santa-Cruz Biotechnologies) followed by AlexaFluor-488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) and imaged using the Incell Analyser-1000 (GE Healthcare, Piscataway, NJ) and a Nikon T800 confocal fluorescent microscope (Carl Zeiss, Thornwood, NY).

Quantification of Golgi fragmentation using high-content analysis

The Incell Analyser-1000 is a microscope-based screening platform capable of large-scale objective analysis of fluorescently-labeled cells using automated image acquisition, data management and multi-parametric analysis. For analysis
of Golgi fragmentation, cells were treated in 96-well plates and Golgi was identified using a GM130 antibody as outlined in 2.4 above. Six fields of view per well were acquired using a ×20 objective in duplicate wells for n = 3 experiments. Fragmentation was measured using the Investigator software package (GE Healthcare) which uses an algorithm specific for detection of objects within the cell. The multi-target analysis algorithm was optimized to detect objects (Golgi fragments) within a cell using untreated cells with intact Golgi as a negative control and brefeldin-A (1 µg/mL)-treated cells as a positive control for Golgi fragmentation (Dinter and Berger 1998). The “organelle count” parameter was used to classify cells as having intact (organelle count of <2) or fragmented (organelle count of >2) Golgi with up to 2000 cells analyzed per treatment group.

**Gluc secretion assay**

To determine the effects of DCA on protein secretion, we used a GLuc assay (New England Biolabs, Ipswich, MA). Cells were transfected with a GLuc construct using Fugene HD (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s protocol. Twenty-four hours post-transfection, cells were treated with 1 µg/mL of brefeldin-A (a positive control), DMSO (a vehicle control) or DCA at indicated concentrations for either 1 or 6 h. The amount of luciferase protein secreted by the cells was quantified by measuring luminescence using a Wallac 1420 Victor2 luminometer (Perkin Elmer, Waltham, MA).

**Glycan profiling**

Cell surface glycan expression was assessed in HET-1A and QH cell lines following binding of the FITC-labeled lectins; WGA, ConA, PNA, UEA-1 and LCA (binding specificities as outlined in Supplementary data, Table S1). Cells were treated with DCA then fixed with 4% paraformaldehyde, blocked with 1% BSA for 30 min prior to staining with FITC-conjugated lectins for 30 min. Cells were washed with PBS and analyzed by flow cytometry. Fluorescence histograms with mean fluorescence intensity were generated using the Summit 4.3 software (Dako, Fort Collins, CO). Intracellular glycan expression and localization were assessed in response to DCA using the FITC-labeled lectins outlined above. Cells were treated with DCA, fixed with 4% paraformaldehyde, then permeabilized with 0.1% (v/v) Triton-X prior to blocking with 1% BSA and staining with FITC-conjugated lectins. Cells were imaged using the Incell Analyser-1000 (GE Healthcare).

**Western blotting for monitoring EGFR and E-cadherin glycosylation**

For analysis of EGFR and E-cadherin expression in response to DCA, cells were treated with DCA for short timepoint (300 µM for 6 h). Tunicamycin, a pharmacological inhibitor of N-glycan biosynthesis, was used at a final concentration of 1 µg/mL in a culture medium. Separately, a cell lysate was treated with 1 unit of PNGase-F, a recombinant endoglycosidase, which cleaves the GlcNAc-Asn linkage of high-mannose, hybrid and complex oligosaccharides from N-linked glycoproteins (Maley et al. 1989), according to the manufacturer’s instructions (New England Biolabs). Equal concentrations of protein from total cell lysates were fractionated on a 6% acrylamide gel, and EGFR and E-cadherin expression were analyzed by western blotting. Deglycosylated proteins exhibit an increase in mobility on a sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel due to a reduction in molecular weight.

**Statistical analysis**

Statistical comparison between groups was carried out using analysis of variance with Tukey honestly significant difference (HSD) post hoc correction to examine differences between groups. Data are graphically represented as the mean ± SEM. All P-values are two-sided and P-values of <0.05 were considered statistically significant in all analyses. All data were analyzed using the SPSS™ statistical software package (SPSS Inc., IL).

**Supplementary data**

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest**

None declared.

**Abbreviations**

AP-1, activator protein 1; BEBM, bronchial epithelial cell basal medium; BO, Barrett’s esophagus; CDCA, chenodeoxycholic acid; ConA, Concanavalin-A; DCA, deoxycholic acid; DMSO, dimethyl sulfoxide; EGFR, epithelial growth factor receptor; ERK, extracellular signal-related kinase; Fuc, fucose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylgalcosamine; GORD, Gastroesophageal reflux disease; HSD, honestly significant difference; JNK, cJun-N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa B; PBS, phosphate buffered saline; PKC, protein kinase C; PKD, protein kinase D; TRAIL, tumor necrosis factor–related apoptosis-inducing ligand; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; UEA-1, *Ulex europaeus* agglutinin-1; WGA, wheat germ agglutinate.
References


