Deoxycholic acid induces proinflammatory cytokine production by model oesophageal cells via lipid rafts

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ABSTRACT

The bile acid component of gastric refluxate has been implicated in inflammation of the oesophagus including conditions such as gastro-oesophageal reflux disease (GORD) and Barrett’s Oesophagus (BO). Here we demonstrate that the hydrophobic bile acid, deoxycholic acid (DCA), stimulated the production of IL-6 and IL-8 mRNA and protein in Het-1A, a model of normal oesophageal cells. DCA-induced production of IL-6 and IL-8 was attenuated by pharmacologic inhibition of the Protein Kinase C (PKC), MAP kinase, tyrosine kinase pathways, by the cholesterol sequestering agent, methyl-beta-cyclodextrin (MCD) and by the hydrophilic bile acid, ursodeoxycholic acid (UDCA). The cholesterol-interacting agent, nystatin, which binds cholesterol without removing it from the membrane, synergized with DCA to induce IL-6 and IL-8. This was inhibited by the tyrosine kinase inhibitor genistein. DCA stimulated the phosphorylation of lipid raft component Src tyrosine kinase (Src), while knockdown of caveolin-1 expression using siRNA resulted in a decreased level of IL-8 production in response to DCA. Taken together, these results demonstrate that DCA stimulates IL-6 and IL-8 production in oesophageal cells via lipid raft-associated signaling. Inhibition of this process using cyclodextrins represents a novel therapeutic approach to the treatment of inflammatory diseases of the oesophagus including GORD and BO.

1. Introduction

Low pH and bile acids in oesophageal refluxate have been associated with an increased risk of reflux oesophagitis and considered independent risk factors for the development of Barrett’s Oesophagus (BO). BO metaplasia, in turn, predisposes to oesophageal adenocarcinoma \cite{1,2}. A major therapeutic approach to the treatment of reflux oesophagitis is the use of proton pump inhibitors (PPIs). However, observations that PPIs have failed to halt the rising incidence of these diseases and that reflux oesophagitis in a rat model develops from cytokine-mediated inflammation rather than acid injury \cite{3}, suggest that other components of the refluxate, including bile acids are important factors in the development of reflux oesophagitis. In the model described by Souza et al., refluxate induces the epithelial cells to produce proinflammatory cytokines (including IL-8), attracting T lymphocytes that induce basal cell proliferation, followed by recruitment of neutrophils to the site of injury.

The bile acid, deoxycholic acid (DCA) has been shown to induce IL-8 expression via a number of signaling pathways in various cell types. MAPK signaling, in particular p38 and Erk1/2, has been shown to be involved in the induction of IL-8 by DCA in SKGT-4 oesophageal adenocarcinoma cells \cite{4}. The p38 MAPK inhibitor SB203580 and the Protein Kinase A (PKA) inhibitor H89 both partially blocked the DCA-induced increase in IL-8 in the 3D HEEC squamous oesophageal cell model but inhibition of Erk1/2 (PD98059), PKC (calphostin C) or Phosphatidylinositol-3-Kinase (PI3-K) (Wortmannin) had no effect in these cells \cite{5}. Jenkins et al. found that the DCA-induced increase in IL-8 mRNA in OE33 oesophageal adenocarcinoma cells could be attenuated by pretreating with the NF-kB inhibitor pyrrolidine dithiocarbamate \cite{6} while DCA (300 \textmu M) induced AP-1 transcription (Fra-1 and JunB).

Abbreviations: BO, Barrett’s Oesophagus; BEBM, bronchial epithelial cell basal medium; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; EGFR, epidermal growth factor receptor; GORD, gastro-oesophageal reflux disease; HRP, horseradish peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LCA, lithocholic acid; MCD, methyl-beta-cyclodextrin; PI3-K, phosphatidylinositol-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PPI, proton pump inhibitor; ROS, reactive oxygen species; Src, Src tyrosine kinase; TDCA, taurodeoxycholic acid; UDCA, ursodeoxycholic acid.

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complex) in SKGT-4 cells within one hour [4]. This is consistent with findings in colonic cells where DCA induction of IL-8 has been shown to be dependent on both NF-kB and AP-1 binding of the IL-8 5-prmoter [7]. DCA-induced reactive oxygen species (ROS) is also associated with an increase in IL-8 production in oesophageal epithelial cells [8]. Pretreatment with antioxidants such as epigallocatechin, reservatrol and vitamin C abrogate the ability of DCA to increase IL-8 mRNA expression in the oesophageal adenocarcinoma cell line OE33 [9].

There has been a lot of interest in therapeutically targeting the cytotoxic/pathological effects of bile acids including their proinflammatory activity. Much of this work has focused on bile acid receptors including FXR and TGR-5 [10,11]. In the context of the oesophagus, the anti-oxidant curcumin (50 μM), significantly abrogated the DCA (300 μM)-induced increase in IL-8 and I-kB mRNA expression in OE33 cells. This in vitro study was repeated in-vivo when curcumin supplementation in patients (500 mg tablet once daily) resulted in a significant decrease in IL-8 mRNA expression in Barrett’s tissue [12]. The hydrophilic bile acid, UDCA, has been shown to have beneficial effects in the treatment of liver disease and gallstones [13]. It generally has an antagonistic effect on the signaling pathways of the cytotoxic bile acids such as DCA, CDCA and LCA. Pretreatment of OE33 cells with UDCA (300 μM) followed by DCA resulted in a significant decrease in IL-8 mRNA expression compared to DCA treatment alone [14]. Peng et al. examined the protective effects of UDCA against DCA-induced injury in Barrett’s oesophagus patients and in Barrett’s cell lines [15]. They found that UDCA increased antioxidants and prevented DCA-induced DNA damage and NF-kB activation in both patient tissue and cell lines (UDCA pretreatment).

It is clear that bile acids, including DCA, are proinflammatory in multiple tissues including the oesophagus. DCA interacts with the cell in multiple different ways including via specific membrane transporters, receptors and, being a small hydrophobic molecule, directly with the lipid bilayer. In doing this, it has the capacity to activate a myriad cell signaling pathways. In this study, a panel of pharmacological inhibitors was screened for their ability to inhibit DCA-induced proinflammatory cytokine production in a model of normal oesophageal cells. In addition to using a range of broad-spectrum kinase inhibitors of DCA-activated pathways, we focused on membrane proximal targets to include receptors, ion channels and lipid and non-lipid components of lipid rafts. Mechanistic insight leading to the discovery and characterization of novel inhibitors of this inflammatory process would aid in the development of chemo-preventative agents for the prevention and management of GORD/inflammation of the oesophagus.

2. Materials and methods

2.1. Cell culture

HET-1A squamous oesophageal epithelial cells (ATCC, Rockville, MD), QH Barrett’s metastatic cells and GO Barrett’s dysplastic cells (also designated CP-A and CP-C respectively) kindly provided to us by Professor Peter Rabinovich, (University of Washington) were cultured in bronchial epithelial cell basal medium (BEBM) with supplements (Lonza, Basel, Switzerland). For the QH and GO cell lines the medium was further supplemented with 5% foetal calf serum (Gibco-BRL, Grand Island, NY). SKGT4 oesophageal adenocarcinoma cells (ATCC, Rockville, MD) were cultured in RPMI with 10% foetal calf serum. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The HET-1A cell line was derived in 1986 from human oesophageal autopsy tissue by transfection with simian-virus-40 large T-antigen (SV40T-antigen). While these cells do not develop a stratified squamous epithelium in organotypic culture [16], they are commonly used as a model of normal oesophageal squamous epithelium in cell culture.

For inhibitor experiments, when measuring cytokine mRNA Het-1A cells were seeded overnight at a density of 1 × 10⁵ cells per well and pre-treated the following day with inhibitor for 1 h before incubation with DCA (200 μM) for 6 h. When measuring secreted cytokine, Het-1A cells were seeded overnight at a density of 6 × 10⁵ cells per well and pretreated for 1 h with inhibitor before incubation with DCA (200 μM) for 24 h. In all cases, inhibitor was removed following the incubation with cells for one hour. Cells were washed with warm medium and medium containing DCA (200 μM) was then added for the time indicated. The panel of inhibitors used and their final concentrations is listed in Table 1.

2.2. Analysis of cytokine mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR)

2.2.1. RNA isolation

Cells were lysed with TRI Reagent (Sigma, St. Louis, MO, USA) and RNA was isolated by chloroform extraction and precipitated with isopropanol at room temperature for 10 min. Following centrifugation at 12,000 rpm, the RNA pellet was re-suspended in ethanol (75 %), centrifuged and then re-suspended in nuclease-free H₂O and quantified using a NanoDrop 8000 (Thermo Scientific, Wilmington, DE, USA).

2.2.2. cDNA synthesis using qRT-PCR

Reverse transcription was performed using 1 μg RNA and the GoScript Reverse Transcription System (Promega, Fitchburg, WI, USA). The reaction was performed in a cycle of 25 °C for 5 min, 42 °C for 1 h and 95 °C for 5 min to generate cDNA. The cDNA was added to a 384-well plate followed by the combination of TaqMan mastermix (Applied Biosystems, Carlsbad, CA, USA) and fluorescently labelled primer. Primers for IL-8, (Hs00174103_m1), and IL-6 (Hs00174131_m1) were used along with a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), (Applied Biosystems, Carlsbad, CA, USA). RT-PCR was performed using the Applied Biosystems 7900 H Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Fold inductions were calculated using the comparative Ct method as described in the ABI Prism manual using GAPDH as the internal control [17]. Gene expression levels of the cytokines IL-6 and IL-8 were expressed as the cycle threshold (Ct) value (IL-6 or IL-8)/ Ct value GAPDH. GAPDH levels (Ct values) were similar across all cell lines. Values are presented as fold change in gene expression relative to the control, which was normalized to 1.

2.3. Cytokine quantification

Secreted IL-6 and IL-8 protein was measured using a multiplex assay kit (MSD, Gaithersburg, MD, USA) according to manufacturer’s instructions. Cells were grown in 24-well plates until 70 % confluent and

Table 1

| Inhibitor                  | Inhibitor final concentration |
|----------------------------|------------------------------|
| Genistein                  | 100 μM                       |
| SB203580                   | 5 μM                         |
| PD98059                    | 20 μM                        |
| Erlotinib                  | 10 μM                        |
| PP2                         | 10 μM                        |
| Methyl-β-cyclodextrin       | 1 mM                         |
| Nystatin                   | 25 μg/mL                     |
| Ursodeoxycholic acid       | 200 μM                       |
| Bisindolylmaleimide        | 10 μM                        |
| EPA                        | 100 μM                       |
| Amlodipine                 | 10 μM                        |
| Aspirin                    | 50 μM                        |
| Wortmannin                 | 100 mM                       |
| ZJET-5MK                    | 10 μM                        |
| Ruthenium Red              | 5 μM                         |
| Chlorpromazine             | 10 μM                        |
pretreated with various inhibitors as indicated for 1 h prior to incubation with DCA (200 μM) for 24 h. The supernatant was removed from the wells and stored at −20 °C. The plate was read immediately following the assay on the Sector Imager (MSD, Gaithersburg, MD, USA) and concentrations of each cytokine calculated from the calibration curves.

2.4. Western blotting

Cells were lysed using RIPA buffer (20 mM Tris–HCl (pH 7.5) 150 mM NaCl, 1 mM Na₂EDTA 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) containing protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Total cell protein was quantified by using a BCA Protein Assay (Thermo Fischer Scientific, Waltham, MA, USA). Equal quantities of protein were separated by SDS-PAGE gel electrophoresis, transferred to PVDF membrane and probed with antibodies to phospho-Src, Src, cavolin (Cell Signaling Technology) or β-actin (Sigma Aldrich, St Louis, Missouri, USA) as loading control. Horseradish peroxidase (HRP) -conjugated secondary antibodies were diluted in blocking buffer (1:5000). HRP was detected by enhanced chemiluminescence and autoradiography.

2.5. Statistics

Statistical comparison between treatments was carried out using one sample t-tests or one-way ANOVA with Dunnett’s post-hoc correction with α < 0.05 considered significant. In all cases data are graphed as the mean ± standard error of the mean (SEM) with at least three separate replicates performed per experiment. All data were analysed using Graphpad Prism 5 (La Jolla, CA, USA).

3. Results

Basal IL-8 mRNA expression was quantified in four oesophageal cell lines representing the stages from normal to oesophageal adenocarcinoma before assessing the effect of DCA. The adenocarcinoma cell line, SKGT-4 was found to have an IL-8:GAPDH ratio of 0.039 ± 0.001 which was significantly higher (70 fold greater) than the normal Het-1A cell line, the metaplastic QH cell line and the dysplastic GO cell line (Fig. 1A). Fig. 1B shows the relative fold change of IL-8 mRNA in the four oesophageal cell lines after 6 h treatment with DCA (200 μM) compared to untreated cells. The fold change was 40.92 ± 6.01 for Het-1A cells, 22.38 ± 7.53 for QH cells, 10.54 ± 3.79 for GO cells and 6.23 ± 1.85 for SKGT-4 cells.

As the normal Het-1A cell line had the largest relative increase in IL-8 mRNA expression mediated by DCA, this was further investigated. Firstly, to assess if this large induction of IL-8 in Het-1A cells was specific to a high concentration of DCA, the experiment was repeated for a lower concentration of DCA, for taurodeoxycholic acid (TDCA) its common conjugated form and chenodeoxycholic acid (CDCA), another hydrophobic and relatively toxic bile acid. Incubation for 6 h with DCA (20 μM), TDCA (200 μM) or CDCA (200 μM) did not result in a significant increase on IL-8 mRNA expression in Het-1A cells as shown in Fig. 1C. DCA (200 μM) and CDCA (200 μM) significantly increased IL-8 protein in the supernatant (Fig. 1D). Conjugated DCA (TDCA (200 μM) or lower concentrations of the bile acid (DCA (20 μM)) did not significantly increase levels of secreted IL-8, which correlated with the corresponding mRNA levels in Fig. 1C. These findings are consistent with those of Jenkins et al. who found that DCA increased IL-8 mRNA expression only above a threshold concentration of approximately 100 μM [9].

To further investigate the signaling pathways involved in DCA-mediated induction of IL-8, a number of different inhibitors were selected based on pathways that are known to be activated by DCA. Inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA are shown in Fig. 2A in addition to results for inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA levels in Fig. 2B-D. Inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA are shown in Fig. 2A in addition to results for inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA levels in Fig. 2B-D. Inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA levels in Fig. 2B-D. Inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA levels in Fig. 2B-D. Inhibitors that significantly increased or decreased the DCA-induced increase in IL-8 mRNA levels in Fig. 2B-D.
incubation with DCA did not significantly affect the relative increase in IL-8 mRNA produced by DCA (200 μM). The increase in IL-8 mRNA in DCA-treated Het-1A cells was significantly attenuated by the tyrosine kinase inhibitor genistein, the p38 MAPK inhibitor SB203580, the MEK inhibitor PD98059, the cholesterol- binding agent MCD, the PKC inhibitor bisindolylmaleimide and by the hydrophilic bile acid, UDCA. Suppression of DCA-mediated IL-8 mRNA resulting from inhibition of p38 MAPK and Erk1/2 has been reported previously in other oesophageal cell lines [5]. Although erlotinib, the EGFR inhibitor did not significantly attenuate the increase in IL-8 mRNA caused by DCA, genistein, the broad spectrum tyrosine kinase inhibitor, almost completely eradicated the effect. UDCA pretreatment has been shown to decrease DCA induced apoptosis but not IL-8 expression in oesophageal cell lines [18]. In Het-1A cells, pretreatment with MCD before incubation with DCA (200 μM), caused a 5-fold decrease in IL-8 mRNA transcription. MCD has a high affinity for cholesterol resulting in lipid raft disruption and reduced likelihood of spontaneous receptor dimerisation [19] indicating that DCA may increase IL-8 mRNA via a lipid raft-mediated signaling mechanism.

Some of the inhibitors, shown in Fig. 2 were found to significantly enhance the fold change in IL-8 mRNA expression induced by DCA (200 μM) in Het-1A cells. This DCA-induced fold change in IL-8 mRNA was significantly increased in the presence of the caspase-8 inhibitor Z-IETD-FMK, the calcium chelator ruthenium red, the clathrin endocytosis inhibitor chlorpromazine and by the cholesterol-sequestering agent nystatin. The increase in IL-8 mRNA produced by treatment with DCA and nystatin underlines the importance of the membrane lipid raft component in DCA signaling, and how a disruption to its configuration and cholesterol composition can potentially lead to a pro- or anti-inflammatory response in these cells.

The Caspase-8 inhibitor Z-IETD-FMK almost trebled the IL-8 mRNA expression levels compared to DCA (200 μM) alone. The mechanism underlying this surprising effect is unclear. It is known that DCA can cause FAS activation in hepatocytes [20]. FAS signaling is classically associated with apoptosis through the death receptor complex and caspase-8. However FAS activation can also trigger inflammatory pathways via activation of p38, Erk and Jnk MAPK through the formation of a MyD88-FAS receptor complex [21]. Ruthenium red blocks TRPV channels and although it was thought that it may prevent Ca²⁺ induced PKC signaling, it also prevents Ca²⁺ influx and efflux causing cell swelling and in turn may further cause membrane perturbation leading to increased IL-8 signaling [22].

It is interesting to note that chlorpromazine and nystatin on their own increased the IL-8 mRNA expression by a factor of 95.11 ± 22.74 (p < 0.005) and 130.28 ± 105.30 (p = 0.2) respectively without DCA (Supplemental Fig. S1A). This indicates that they may work synergistically with DCA in increasing IL-8 mRNA expression. The caspase-8 inhibitor, Z-IETD-FMK and the calcium attenuator, ruthenium red, do not induce IL-8 mRNA in the absence of DCA. The panel of inhibitors that attenuated the DCA-induced increase in IL-8 mRNA did not significantly affect the IL-8 mRNA expression compared to untreated cells in the absence of DCA (Supplemental Fig. S1B).

Fig. 2B shows IL-8 protein secretion in the normal oesophageal Het-1A cell line in response to incubation with DCA in the presence and absence of the panel of inhibitors. Pretreatment with genistein or bisindolylmaleimide prior to incubation with DCA was found to decrease the levels of IL-8 to the greatest degree. SB203580, PD98059 and MCD pretreatment also significantly reduced the protein levels of IL-8 in the supernatant in response to DCA. UDCA pretreatment reduced the secreted IL-8 cytokine levels in the supernatant but not significantly (60 % decrease). Of the compounds that synergistically increased the IL-8 mRNA expression induced by DCA, only nystatin increased the levels of secreted IL-8 protein as seen in Fig. 2B.

Similarly to IL-8, IL-6 mRNA expression was increased by 200 μM DCA but not 20 μM DCA (Fig. 3A). DCA (200 μM) and CDCA (200 μM) significantly increased IL-6 protein levels in the supernatant. Lower concentrations of DCA (20 μM) or TDCA (200 μM) did not significantly increase levels of IL-6, which was in agreement with the corresponding effect on IL-8 protein levels.

Genistein, SB203580, PD98059, bisindolylmaleimide and MCD pretreatment significantly attenuated the increase in IL-6 protein secretion induced by DCA consistent with the pattern observed for IL-8. Ruthenium red pretreatment of Het-1A cells significantly decreased DCA-stimulated IL-6 protein production with no effect on secreted levels of IL-8. Het-1A cells pretreated with UDCA prior to incubation with DCA secreted lower levels of IL-6 protein compared to DCA alone but this was not statistically significant over three experiments. Therefore UDCA pretreatment decreases IL-8 and IL-6 protein secretion but not significantly despite significantly reducing IL-8 mRNA expression induced by DCA (200 μM).

Nystatin pretreatment increased the DCA induced IL-6 protein levels

Fig. 2. IL-8 mRNA and protein expression induced by DCA is affected by various pharmacological inhibitors. (A) Het-1A cells were seeded overnight at a density of 1 × 10⁵ cells per well and pretreated the following day for 1 h with erlotinib (10 μM), EIPA (100 μM), amlodipine (10 μM), aspirin (50 μM), wortmannin (100 nM), genistein (100 μM), SB203580 (5 μM), PD98059 (20 μM), UDCA (200 μM), MCD (1 mM), bisindolylmaleimide (Bis) (10 μM), Z-IETD-FMK (10 μM), ruthenium red (5 μM), chlorpromazine (10 μM), or nystatin (25 μg/mL) before incubation with DCA (200 μM) for 6 h. (B) Het-1A cells were seeded overnight at a density of 6 × 10⁶ cells per well and pretreated for 1 h with genistein (100 μM), SB203580 (5 μM), PD98059 (20 μM), UDCA (200 μM), MCD (1 mM), bisindolylmaleimide (Bis) (10 μM), CLI-095 (1 μg/mL), Z-IETD-FMK (10 μM), ruthenium red (5 μM), chlorpromazine (10 μM), or nystatin (25 μg/mL) before incubation with DCA (200 μM) for 24 h. Results are expressed as mean ± SEM of three experiments. Horizontal bars with indicators of significance (*) in red indicate inhibition of DCA-induced cytokine production following pre-treatment of cells with pharmacological inhibitor as indicated. Those in black indicate a statistical increase in cytokine production when cells are pre-treated with inhibitor followed by DCA.
in the supernatant with a similar fold change to the observed DCA-induced increase in IL-8 protein secretion. Pretreatment with Z-IETD-FMK or chlorpromazine prior to DCA did not result in any significant induced increase in IL-8 protein secretion. Pretreatment with Z-IETD-FMK or chlorpromazine prior to DCA did not result in any significant induced increase in IL-8 protein secretion. Pretreatment with Z-IETD-FMK (10 μM) before incubation with DCA (200 μM) for 6 h. All the treatments were compared to Het-1A cells treated with DCA (200 μM). (B) Het-1A cells were seeded overnight in 24-well plates at a density of 6 × 10^4 cells per well and treated the following day for 24 h with DMSO (0.1 %), DCA (20 μM), DCA (200 μM), TDCA (200 μM) or CDCA (200 μM). (C) Het-1A cells were seeded overnight in a 24-well plate at a density of 6 × 10^4 cells per well and pretreated for 1 h with SB203580 (5 μM), PD98059 (20 μM), genistein (100 μM), MCD (1 mM), UDCA (200 μM), bisindolylmaleimide (Bis) (10 μM), Z-IETD-FMK (10 μM), chlorpromazine (10 μM), ruthenium red (5 μM), or nystatin (25 μg/mL) before incubation with DCA (200 μM) for 24 h. Results are expressed as mean ± SEM of three experiments. Horizontal bars with indicators of significance (*) in red indicate inhibition of DCA-induced cytokine production following pre-treatment of cells with pharmacological inhibitor as indicated. Those in black indicate a statistical increase in cytokine production when cells are pre-treated with inhibitor followed by DCA.

Firstly, we validated the fact that DCA could induce Src phosphorylation in Het-1A cells. When these cells were incubated with DCA (200 μM) for 10 min there was a 1.76 fold increase in phospho-Src as depicted in Fig. 4A. Exposure of Het-1A cells to DCA (20 μM) or DCA (200 μM) for 30 min resulted in a 1.5 fold or a 2.0 fold increase in phospho-Src respectively.

Pre-treatment of Het-1A cells with the Src inhibitor PP2 caused a significant decrease (10 fold; 93 %) in the induction of IL-8 mRNA produced by DCA. However, pre-treatment of these cells with PP2 prior to incubation with DCA did not significantly decrease IL-8 protein secretion (12.7 ± 2.36 pg/mL, p = 0.45) (30 % decrease). (Fig. 4B). Het-1A cells pre-treated with PP2 prior to incubation with DCA secreted lower levels of IL-6 protein compared to DCA alone but this was not statistically significant over three experiments (data not shown). Therefore, PP2 pre-treatment decreases IL-8 and IL-6 protein secretion but not significantly despite significantly reducing IL-8 mRNA expression induced by DCA (200 μM). Our results demonstrate that DCA-induced IL-6 and IL-8 production is tyrosine kinase dependent; the broad spectrum tyrosine kinase inhibitor, genistein, decreased DCA-stimulated IL-8 mRNA by 97 % and protein by 100 % with the lipid raft-associated Src tyrosine kinase involved. We then investigated if the synergistic effect of DCA and nystatin on IL-8 production was tyrosine kinase dependent. Pre-treatment with genistein significantly inhibited
the synergistic response produced by DCA and nystatin \((p < 0.005)\). Cotreatment with nystatin and DCA (200 μM) caused a 680 ± 105 fold increase in IL-8 mRNA relative to untreated cells (Fig. 4C). Genistein pre-treatment prior to the addition of nystatin and DCA (200 μM) decreased the IL-8 mRNA expression to 8.32 ± 5.94 fold \((p < 0.005)\) relative to untreated cells (a 99% reduction in IL-8 mRNA expression levels). Neither PP2 or genistein alone increased IL-8 mRNA levels. Caveolins, and in particular caveolin-1, play an important role in the structural and signaling capacity of the sub-family of lipid rafts known as caveolae. Caveolin-1 binds multiple membrane receptors and enzymes including Src tyrosine kinase. Caveolin-1 expression was knocked down in Het-1A cells using siRNA and their ability to synthesise IL-8 mRNA in response to DCA was determined. Caveolin-1 knockdown reduced induction of IL-8 stimulated by DCA in Het-1A suggesting that DCA utilises these membrane microdomains to initiate proinflammatory signals in these cells (Fig. 4D). It is notable that knockdown of caveolin-1 was found to increase the basal levels of IL-8 mRNA in Het-1A cells 2.4 ± 0.6 fold \((p = 0.07)\). Taken together, these data indicate that lipid rafts and associated downstream tyrosine kinases are key to DCA stimulated proinflammatory cytokine (IL-6/IL-8) induction.

4. Discussion

As components of the refluxate, bile acids have been implicated in the pathogenesis of oesophageal diseases. Patients with GORD who have high concentrations of DCA in the refluxate are prone to oesophagitis and potentially progression to metaplasia and dysplasia. Here we demonstrate that there is an increase in both IL-8 and IL-6 mRNA and protein produced by normal oesophageal cells exposed to DCA. This is interesting because following organ culture (human biopsies), Fitzgerald et al. demonstrated increased levels of IL-8 (and IL-1beta) in Barret’s tissue when compared to adjacent normal squamous oesophagus [26]. Using a similar approach, Dvorakova et al. found increased levels of IL-6 to be secreted from Barretts’s tissue when compared to adjacent normal oesophageal or duodenal tissue [27]. We have uncovered a link between the ability of DCA to perturb the cell membrane and lipid raft (caveolin and Src) signaling, resulting in downstream IL-6 and IL-8 transcription and secretion.

In normal oesophageal cells DCA, but not conjugated DCA, stimulated an increase in IL-6 and IL-8 mRNA expression and protein secretion. Lower concentrations of DCA (20 μM) and the taurine-conjugated TDCA (200 μM) were not capable of significantly increasing IL-8 mRNA expression in Het-1A cells. DCA does not cause an increase in IL-8 below
a threshold concentration (approximately 100 μM) [9]. A higher concentration of TDCA may be required to initiate IL-8 signaling. It has previously been reported that treatment of another squamous oesophageal cell line, HEEC, with CA, TCA (1000 μM), CDCA and TCDCA (200 μM) caused a significant increase in IL-8 secretion at pH 7.5. Lowering the pH to 6.5 slightly lowered the IL-8 secretion for CA and CDCA but significantly increased the secretion for TCA and TCDCA [28]. Thus, bile acid concentration, conjugation status and pH are important factors in their proinflammatory activity.

It was found that pretreatment of Het-1A cells with the p38 inhibitor SB203580 and the MEK inhibitor PD98059 significantly decreased both the mRNA expression and secreted protein levels of IL-8 and IL-6 when incubated with DCA (200 μM). Inhibition of PKC activity using bis-dimethylamide significantly reduced IL-8 and IL-6 protein secretion in response to DCA. The novel PKC isoform, PKCγ, has previously been shown to be activated by Src [29]. PKCγ activation can stimulate NF-κB activation and IL-8 secretion in human bronchial epithelial cells [30]. In BHK-21 cells, DCA activates phospholipase C on the membrane and causes translocation of PKCα and PKCβ1 in a Ca2+ dependent manner [31]. Shah et al. also demonstrated cytochrome to membrane translocation of multiple PKC isoforms in response to DCA in colon cells [32]. Therefore, there may be a role for multiple PKC isoforms in DCA-stimulated IL-6/8 production in oesophageal cells.

The membrane proximal events mediated by DCA have long been the focus of investigation. Several studies have reported that DCA can indirectly transactivate the EGFR receptor (EGFR). This receptor is located in lipid rafts and is susceptible to mechanical dimerisation and activation, in a ligand independent manner [20,33]. EGFR and Src have been shown to exhibit co-operative signaling [34]. However, in the present study, the EGFR inhibitor erlotinib did not affect IL-8 mRNA production, whereas PP2, the Src inhibitor and genistein, the general tyrosine kinase inhibitor both significantly attenuate the DCA-induced increase in IL-8 mRNA. A study using pancreatic adenocarcinoma cells has shown a direct correlation between expression and activity of Src and expression of IL-8 [35]. Genistein significantly reduced the IL-8 response to DCA. This suggests that the IL-8/IL-6 production induced by DCA is independent of EGFR activity but is nevertheless dependent on associated signaling pathways.

Lipid rafts are islands of highly ordered saturated lipids and cholesterol that are laterally mobile in a more fluid disordered matrix of largely unsaturated lipids [36]. They are home to many signaling complexes including the Src family tyrosine kinases [37]. Remodelling of lipid rafts can cause receptor dimerisation and activation in a ligand-independent manner. Using synthetic vesicles, isolated cell membranes and live cells, Zhou et al. demonstrated that DCA was incorporated into non-raft (disordered) domains, further decreasing their order. This in turn altered plasma membrane proteolipid domains and modulated signal transduction [38]. In particular, they demonstrated that bile acid could increase segregation of Ras, which is embedded in nanoclusters in non-raft membrane domains, thus potentiating EGFR/MAPK signaling. These nanoclusters depend on lipids, including cholesterol, for their lateral segregation [39]. As the MAPK inhibitor, PD98059, significantly attenuated DCA-stimulated IL-6/8 production, modulation of non-raft membrane domains by DCA and subsequent downstream activation of MAPK may contribute to the mechanism leading to production of these cytokines. Using HCT116 colon cancer cells, Jean-Louis et al. also demonstrated perturbation of membrane organization by DCA-induced alteration of membrane microdomains and in particular, redistribution and clustering of cholesterol [19]. Nystatin alone or synergistically with DCA increased proinflammatory (IL-6/IL-8) cytokine production. Through its mechanism of binding cholesterol, sequestering it in the membrane and disrupting its intramembrane interactions, nystatin may activate similar signaling pathways to those stimulated by DCA. This may happen through stabilisation of membrane domains by nystatin and potentiation of DCA signaling in this model.

Caveolae membrane microdomains are rich in signaling molecules including Cav1. Cav1 is an important regulator of membrane organisation and cholesterol intracellular transport. Interestingly Jean Louis et al. demonstrated that DCA causes an increase in cholesterol levels in caveolae while simultaneously decreasing the level of Cav1 in these microdomains [19]. They further demonstrated that DCA causes internalisation of Cav1 protein from the membrane while not altering total cellular Cav1 expression. They postulate this as a mechanism of bile acid mediated activation of intracellular signaling. Fernandez-Rojo et al. demonstrated that Cav1 knockdown in AML12 hepatocytes and Cav1 -/- mice had altered bile acid signaling [40]. Here we demonstrate that knockdown of Cav1 decreased DCA-induced IL-8 further supporting evidence that bile acids modulate caveolae/Cav1 signaling.

Pretreatment with MCD significantly decreased IL-6/8 protein secretion in response to DCA. MCD is a member of a family of cyclic oligosaccharides with a cone-like structure that has a hydrophilic external surface and a lipophilic cavity. These compounds have been used as solubilizing agents in the food and pharmaceutical industry for many years. Cyclodextrins interact with lipids and cholesterol and it has been demonstrated that beta-cyclodextrins remove cholesterol from phospholipid bilayers [41]. As reviewed by Zidovetzki and Levitan (2007) and Leclercq (2016), MCD can affect membrane cholesterol in multiple ways, including its removal from both raft and non-raft domains, its redistribution between these domains and between different cellular membranes [24,42]. MCD can also affect membrane phospholipids and membrane proteins. In this study, depletion of caveolin-1 expression decreased the DCA-induced IL-8 mRNA expression indicating that these specialized raft domains are involved and that removal of cholesterol from these rafts could also disrupt IL-8 production. DCA can also mediate its effects via insertion/partition in non-raft domains [38] and as stated, this may be the mechanism of MAPK activation. Therefore, the effect of depletion of cholesterol in non-raft areas by MCD on downstream DCA signaling events cannot be ruled out.

Studies on the effect of MCD on lymphocytes and endothelial cells have demonstrated that MCD can cause the release of cell surface and intracellular proteins from membranes [43]. In lymphocytes, MCD released GPI-anchored and other cell surface proteins as well as membrane -associated Src family tyrosine kinases Lck and Fyn. Given that we have demonstrated a role for Src tyrosine kinase in DCA-stimulated IL-6/8 production, the observed effect of MCD on production of these cytokines may also be mediated, at least in part, by its inhibition of this kinase. Interestingly, in endothelial cells, MCD caused negligible release of caveolin-1, demonstrating a selective effect on protein release while also suggesting that MCD may not directly affect caveolin-1 in HET1A cells. It is unlikely that MCD had a significant effect on membrane phospholipids in HET1A cells at the concentration used in this study (1 mM), though the phospholipid composition of the membrane can affect the rate of MBD-induced cholesterol removal [44].

Further studies have uncovered molecular mechanisms of cyclodextrin interactions with cellular membranes, their lipid raft components and downstream effects on cell signaling and function. This in turn has led to investigations into the use of cyclodextrins as active pharmaceutical ingredients (reviewed by Pio di Cagno 2016 [45]). Hydroxypropyl-beta-cyclodextrin (HPβCD) complexed with lidocaine improved the antinociceptive effect of plain lidocaine in animal models of acute and persistent orofacial pain [46]. This is interesting in the context of the ‘Inflammaraft’ concept. These structures contain increased numbers of inflammatory mediators (e.g. pro-inflammatory cytokines, prostaglandins, pro-inflammatory and pain processing in inflammatory cells (e.g. glial cells) (reviewed in [47]). Studies in animal models of the cholesterol storage disorder, Niemann-Pick type C (NPC) disease demonstrate reduced neuronal cholesterol accumulation and prolonged lifespan in diseased mice (NPC -/- ) treated with HPβCD [48]. Clinical trials (including intrathecal infusion of HPβCD) in humans suffering from this disease are ongoing. Several studies have demonstrated beneficial effects of cyclodextrins on the cardiovascular system. Administration of MCD to
Here we demonstrate that DCA stimulates IL-6 and IL-8 production in oesophageal cells via lipid rafts and activation of associated downstream signaling pathways. The cholesterol-sequestering agent MCD attenuates the proinflammatory effects of this bile acid on the oesophagus, blocking IL-6 and IL-8 induction. These results suggest that MCD has potential to reduce inflammation of the oesophagus in the treatment of conditions of GORD and BO.

Author statement

Francis Quilty: Investigation, visualization, validation, formal analysis. Michael Freeley: Investigation, Siobhan Gargan: Investigation. John Gilmer: conceptualization, supervision Aideen Long: conceptualization, supervision, writing, review and editing.

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Appendix A. Supplementary data

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