Jenkins, G. Cronin, J. & Alhamdani, A. (2008). The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kB activation in oesophageal cells, with a mechanism of action involving ROS.. 23, 399-405.

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Mutagenesis vol. 23 no. 5 pp. 399–405, 2008
Advance Access Publication 30 May 2008
doi:10.1093/mutage/gen029

The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-κB activation in oesophageal cells, with a mechanism of action involving ROS

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Deoxycholic acid (DCA) is a secondary bile acid implicated in various cancers of the gastrointestinal (GI) tract. In oesophageal adenocarcinoma, DCA is believed to contribute to carcinogenesis during reflux where stomach contents enter the lower oesophagus. It is imperative that we understand the mechanisms whereby oesophageal carcinogens function in order that therapeutic options may be developed. We have previously shown that DCA can damage chromosomes and does so through its generation of reactive oxygen species (ROS). We show here, after detailed experiments, that DCA appears to have a non-linear dose response for DNA damage. DCA induces DNA damage (as measured by the micronucleus assay) at doses of 100 μM and higher in oesophageal OE33 cells, but fails to induce such DNA damage below this cut-off dose. We also show that in terms of NF-κB activation (as measured by up-regulation of two NF-κB target genes) by DCA, a similar dose response is observed. This dose–response data may be important clinically as DCA exposure to the oesophagus may be used as a way to identify the 10% of Barrett's oesophagus patients currently progressing to cancer from the 90% of patients who do not progress. Only quantitative studies measuring DCA concentrations in refluxates correlated with histological progression will answer this question. We further show here that ROS are behind DCAs ability to activate NF-κB as antioxidants (epigallocatechin gallate, resveratrol and vitamin C) abrogate DCAs ability to up-regulate NF-κB-controlled genes. In conclusion, low doses of DCA appear to be less biologically significant *in vitro*. If this were to be confirmed *in vivo*, it might suggest that reflux patients with low DCA concentrations may be at a lower risk of cancer progression compared to patients with high levels of DCA in their refluxate. Either way, antioxidant supplementation may possibly help prevent the deleterious effects of DCA in the whole GI tract.

Introduction

Oesophageal adenocarinoma (OA) is an extremely aggressive form of cancer which is increasing in incidence in the West. OA is caused by chronic gastro-oesophageal reflux disease (GORD) whereby stomach acids (and bile) are regularly regurgitated into the lower oesophagus. GORD affects up to 20–30% of the general population and ∼10% of these (i.e. 1–2% of the general population) acquire a metaplastic pre-malignant condition known as Barrett's oesophagus (BO) (1–3). Initially, it was thought that the acid component of the refluxate played the most significant role in the development of BO and its sequelae, adenocarcinoma. This led to the development of acid-suppressing drugs to minimize acid reflux with a view to eliminating symptoms and preventing carcinogenesis in the lower oesophagus of GORD patients. While acid suppression therapies are extremely effective at managing symptoms of GORD, there is little evidence that they have had an impact on trends in incidence of Barrett's oesophagus or OA (4,5). This has led to the theory that some non-acid constituent of reflux, such as bile, may be driving carcinogenesis.

Over the past 20 years, there have been many animal studies which have strengthened the link between bile acids and OA. This work has been invaluable in unpicking the relative contributions of acid and bile reflux to oesophageal damage and carcinogenesis. A lot of this work is summarized in a paper by Marshall *et al.* (6) and concludes that bile acid reflux generally worsens the oesophageal damage induced by acid reflux alone. Further studies have shown, through elegant surgical models, that bile acids promote OA development, perhaps even more so, than stomach acid alone (7,8)

As only ∼10% of patients with Barrett's oesophagus progress to OA (9), there has been considerable effort recently to try and identify biomarkers which correctly stratify the 'progressors' from the 'non-progressors'. It is possible that the type and concentration of bile acid in the refluxate of these patients may be a useful biomarker. The more damaging secondary bile acids (particularly in their unconjugated form) are generally thought to be more closely linked to OA development than their primary bile acid counterparts. The presence of these kinds of secondary bile acids in the oesophagus of patients with GORD has been known for some time (10–12). Table I shows the range of concentrations of deoxycholic acid (DCA), an injurious secondary bile acid linked to carcinogenesis, found in the upper gastrointestinal (GI) tract.

To support the labelling of bile acids like DCA, as carcinogens in their own right, there has been much evidence in recent years that such bile acids are capable of inducing molecular changes consistent with a pro-carcinogenic role. As well as the accumulating clinical evidence, there is good experimental data that they are capable of inducing DNA damage (13,14) and activating oncogenic signalling pathways (15–17). It is likely that bile acids cause these effects through their ability to induce intracellular reactive oxygen species (ROS) (14,18–20). Bile acid induction of oxidative stress is thought to be linked to their membrane-damaging role (as detergents). Mitochondrial membrane damage may well lead directly to increased ROS leakage into the cytoplasm (21). Therefore, the investigation of the role of ROS in the

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Table I. Levels of DCA in the oesophagus (and stomach) of patients with either GORD or Barrett's oesophagus

Study no.	Method	Average (DCA)	Maximum (DCA)	Reference
Ref. no. 23	HPLC on aspirated oesophageal fluid over a 15-h period. $N = 40$ patients	2[a] and 0 μM[a] (2.5 and 5.5 μM)	230[a] and 280 μM[a] (442 and 482 μM)	Nehra *et al.* (23)
Ref. no. 24	GC on gastric aspirates	3 μM (63 μM)	115 μM (1592 μM)	Darragh *et al.* (24)
Ref. no. 11	HPLC on oesophageal aspirates over a 17-h period. $N = 80$ patients	(35 μM)		Kauer *et al.* (11)

[a]Erosive oesophagitis and Barrett's oesophagus figures, respectively.
Data show both unconjugated DCA and DCA conjugated to either glycine or taurine (in brackets).

pathogenesis of Barrett's oesophagus (and OA) and the potential role of antioxidants in blocking these processes are fertile areas of research.

We have recently shown that the bile acid DCA is a clastogen, a mutagen (14) and can activate the pro-carcinogenic signalling molecule NF-κB (17). Indeed, of all the bile acids implicated in GI tract cancer, DCA appears to be a prime candidate carcinogen as it appears to be particularly bioactive. Our *in vitro* data support a role for DCA in particular as other bile acids (including the conjugated forms of DCA) do not induce similar molecular abnormalities. However, in terms of assessing individual candidate carcinogens for their ability to induce molecular defects, it is important to consider the effect of dose. We have shown recently that classic DNA-damaging agents and known carcinogens can display non-linear dose responses for DNA damage induction (22). Hence, at low doses, these carcinogens do not induce biologically relevant genetic changes. These non-linear effects are often described as 'thresholded' as they tend to consist of a 'no effect level' at low doses where no increase in DNA damage is seen above background. This is followed by a key point of inflexion whereafter subsequent dose-dependent increases in DNA damage are seen. The determination of the critical dose range where this transition takes place is essential if one wants to use this data to set safe human exposure levels. Finally, in order to complete the picture, as well as the dose response, the mechanism of action (MOA) responsible for the non-linear (thresholded) response is often required in order to convince the scientific community of the plausibility of the effect.

Given the role of bile acids (like DCA) in OA development, it is essential to understand the critical dose range whereby DCA is capable of inducing molecular abnormalities consistent with a pro-carcinogenic effect. Table I shows the known concentrations of DCA in the upper GI tract and displays the wide range of values seen in different patients (range 0 μM–282 μM) (11,23,24). At present, it is not known if variations in DCA concentration *in vivo* are involved in modulating the risks of OA development in individual patients. In terms of carcinogenic bile acids, we have strived here to assess if the carcinogenic bile acid DCA has a non-linear dose response and therefore, if there might be a critical dose of DCA exposure to the oesophagus associated with OA development. We have used both DNA damage induction and NF-κB activation as end points in these dose–response studies and have investigated the inherent capacity of oesophageal cells to scavenge ROS as the MOA behind any non-linearities.

This study may have an important clinical bearing on patients with GORD either

1. in stratifying patients into high and low cancer risk groups based on the level of DCA in their refluxate or
2. by identifying a MOA for DCA-induced molecular abnormalities that is amenable to chemoprevention.

Materials and methods

Cell lines

OE33 cells derived from a Barrett's related adenocarcinoma were newly purchased from the European Collection of Cell Cultures (Salisbury, UK) prior to the beginning of the study. Cells were grown in RPMI 1640 (Life Technologies, Paisley, UK), supplemented with 1% glutamine and 10% bovine calf serum (Life Technologies) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Micronucleus assay

For the micronucleus assay, 1–2 × 10^5 OE33 cells/ml were seeded into 25 cm^2 flasks, 24 h prior to treatment. The OE33 cells were then treated with neutral pH DCA (Sigma Aldrich, Poole, UK) overnight at various doses for 21 h, before harvesting. Concurrent with the exposure to DCA, cytochalasin B (4 μg/ml) was added to block cytokinesis and induce binucleated cells, and the cells were harvested by trypsin-mediated detachment 21 h later (i.e. 45 h after cultures were seeded).

Slides were prepared from 100 to 200 μl of the cell pellets using a Cytospin at 1200 r.p.m. for 8 min. The slides were checked using a light microscope and air dried before being fixed with 90% methanol at −20°C for 10 min (up to 10 slides prepared for each dose). The slides were subsequently stained with 10% Giemsa (BDH, Leicestershire, UK) for 8 min in phosphate buffer at pH 6.8 after being immersed in xylene and allowed to dry. After drying the slides and mounting the cover slips, the slides were visualized with an Olympus BH2 microscope at ×1000 magnification. All slides were scored blind and at least 1000 binucleated cells were counted per dose and assigned as micronucleus positive or negative. Duplicate cell cultures were established for each dose of the micronucleus study. The slides obtained from the duplicate cultures were scored together and the total number of micronuclei in each exposure group (as a % of the binucleate cell population) was noted. For the dose–response data, micronucleus data from up to five separate studies were pooled to enhance the number of events and unpick any subtle changes in dose response. Standard criteria for selecting micronuclei were used (25). To assess statistical significance between untreated cells and cells treated with varying doses of DCA, the Student's *t*-test was used to compare micronucleus frequency of the replicates between treated and untreated cultures.

For the antioxidant supplementation studies, the antioxidants were added to the OE33 cells 2 h prior to the addition of DCA [final concentrations; 50 μM for epigallocatechin gallate (EGCG) and 75 μM for resveratrol]. The media were changed prior to DCA addition to prevent direct chemical interaction in the media. DCA (200 μM) was added to the cells for 6 h along with cytochalasin B (4 μg/ml) and the media again changed (but including cytochalasin B) and further incubation continued for 16 h prior to the cells being harvested. The antioxidants tested (EGCG and resveratrol) were dissolved in dimethyl sulphoxide (DMSO) (see below) and DMSO alone was added to the cells in the negative controls. Fisher's exact test was used to assess if there was a significant difference between the numbers of micronuclei in the DCA-treated cells compared to the DCA+ antioxidant cells.

NF-κB induction

NF-κB activation was assayed by up-regulation of the known NF-κB target genes (*IL-8* and *I-κB*) in OE33 cells after exposure to varying doses of DCA for 6 h. These genes were chosen to represent DCA-induced NF-κB activity following previous studies, where they were shown to be common and reliable markers of NF-κB activity (17). Furthermore, the up-regulation of these genes

by DCA was shown to be blocked by several known NF-κB inhibitors, confirming their NF-κB dependence (data not shown). The DCA dose response for *IL-8* and *I-κB* expressions was performed in triplicate and representative expression changes plotted against dose.

Antioxidant supplementation and its effect on NF-κB-dependent gene expression

OE33 cells were treated with DCA (Sigma, Poole, UK) at a final concentration of 300 μM for 6 h duration, after they reached 60% confluency. This concentration of DCA is at the top end of the range of bile acid concentrations found in patients with Barrett's oesophagus (23), although the duration of exposure used was not physiological. For the antioxidant studies, each tested antioxidant (purchased from Sigma) was administered to the cells overnight prior to DCA exposure. This differed to the micronucleus study which involved 2-h pre-loading with the antioxidants. The doses of antioxidants were chosen based either on the literature or following empirical experiments in our laboratory. The media were subsequently changed and the cells treated with DCA in fresh medium. The solvent used for the antioxidants (DMSO, ethanol, water) was administered to the matched negative control flasks. EGCG was prepared as a 50-mM solution dissolved in DMSO and consequently added to the cells at final concentrations of 50 and 100 μM. Resveratrol was also prepared in DMSO as a 50-mM solution and was administered to the cells to achieve final concentrations of 50 and 75μM. Vitamin C was dissolved in distilled water to 50 mM and diluted into culture medium to give final concentrations of 50, 150 and 500 μM. Benzyl isothiocyanate was prepared in 100 mM solution in DMSO and was administered to the cells at final concentrations of 25 and 40 μM. Vitamin E (α-tocopherol) was prepared in 100 mM solution in ethanol (100%) and was administered to the cells at final concentrations of 50 and 100 μM.

Replicates of these cell culture experiments were performed to confirm the results. Real-time PCR was performed on both sets of RNA (twice) giving four data points per treatment.

RNA extraction and reverse transcription

After incubation with DCA, the OE33 cells were subjected to RNA extraction using the Midi RNeasy RNA extraction kit (Qiagen, Crawley, Surrey, UK). The extracted RNA was subsequently DNase treated using a DNA-free kit (Ambion, Huntingdon, UK). The RNA quantity was assessed by spectrophotometry at 260 nm and RNA quality was assessed by RT–PCR using primers for β-actin DNA.

Real-time PCR

RNA (500 ng) was first reverse transcribed into complementary DNA (cDNA) using a Retroscript RT kit (Ambion). Real-time PCR was then carried out using an iCycler PCR machine (BioRad, Hemel Hempstead, UK). cDNA was amplified and each cDNA batch was aliquoted in triplicate into 96-well plates using the appropriate PCR primers and the IQ Sybr-green supermix (BioRad). Primer sequences have previously been published (17). A reference standard curve was generated for each gene, on each plate, using a diluted cDNA reference; the relative gene expression levels were then read off this curve. As the same cDNA reference was used between plates, this allowed normalization between plates.

One-way analysis of the variance was utilized subsequent to standardization of the *IL-8* and *IκB* expressions by dividing by β-actin gene expression (a housekeeping gene). *Post hoc* multiple comparisons were carried out to determine if there was a significance difference between these ratios in the flasks exposed to DCA in isolation and those with different antioxidant concentrations plus the DCA.

Results

DCA displays a thresholded dose response for DNA damage

Table II shows the raw dose–response data for DCA with respect to its DNA-damaging ability as measured by the micronucleus assay. The micronucleus frequency (% binucleated cells with a micronucleus) was determined in several replicated studies and the data pooled for analysis. Over 37 000 binucleated cells were scored and over 700 micronuclei were identified. Up to five replicates were available for some doses; however, the 10- and the 25-μM doses were only performed once each. The error bars on Figure 1 display the inter-study variation (as standard deviation) and show good consistency between replicates. The background micronucleus frequency shown here is drawn from five different studies looking at OE33 cells and represents a pooled historical background figure from over 10 000 binucleate cells.

From the data in Table II, shown graphically in Figure 1, DCA clearly induced micronuclei (dashed line) in a non-linear manner *in vitro*. At low doses of DCA (<100 μM), there was no significant increase in micronucleus induction over the untreated controls. However, at doses of 100 μM and higher, there was a point of inflexion in the curve and significant increases in chromosome damage (micronucleus induction) were observed. Indeed, the 100-μM dose is the first dose to show a significant increase in micronucleus frequency ($P = 0.043$). Higher doses of DCA showed highly significant increases in micronucleus induction over the controls ($P < 0.001$ for 125, 150, 200 and 250 μM). It should be noted that significant toxicity (as measured by a fall in binucleate frequency—solid line in Figure 1) was associated with DCA doses >200 μM. We did not test the micronuclei here for kinetochore status as previously we have shown that DCA induces chromosome breakage events and not chromosome copy number changes (i.e. aneuploidy) (14).

DCA induces NF-κB-associated gene expression in a similar dose-dependent manner to DNA damage

DCA also induced NF-κB activity (as measured by the up-regulation of two NF-κB target genes, *IL-8* and *I-κB*) at

Table II. Clastogenicity data for DCA in OE33 cells

Dose of DCA (μM)	Mononucleates counted	Binucleates counted	Micronucleated binucleate cells	% Binucleate cells	% Micronucleus-containing cells
0	10638	10381	80	49.4	0.7
10	1750	1000	10	36.4	1
25	1512	1000	12	39.8	1.2
50	4010	3000	36	42.8	1.2
75	2162	2000	19	48.0	0.9
100	6702	5000	61	42.7	1.2
125	2799	2000	59	41.7	2.9
150	6240	4000	158	39.1	3.9
200	9975	3000	87	23.1	2.9
250	7438	2000	78	21.2	3.9
300	13704	3000	101	17.9	3.4

This table contains the raw data from the micronucleus studies displayed graphically in Figure 1. The total numbers of mononucleate cells, binucleate cells and micronucleated binucleate cells are shown. For some DCA doses, data have been pooled from up to five different studies (normally 1000 binucleates are scored per study). In the case of the negative control, data from more than five studies were available. For the 10- and 25-μM dose, only one set of data were available.

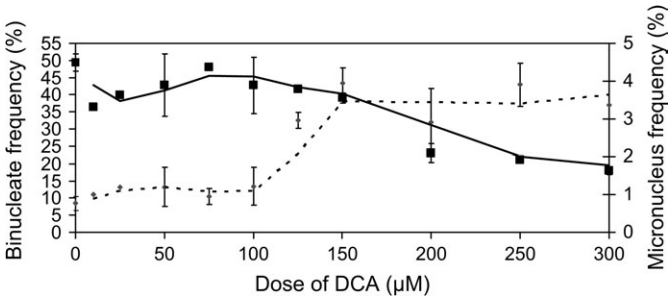

Fig. 1. The micronucleus frequency data (dashed line) and the binucleate frequency data (solid line) are plotted against dose of DCA (data from Table II). The graph shows a clear non-linear response with a no effect level up to 100 μM DCA and a sharp rise in micronuclei thereafter. Indeed, the 100-μM dose is the first dose showing a significant rise in micronucleus frequency above the background (using the *t*-test on the replicates). Doses of 200 μM or more show some toxicity as measured by the drop in binucleate frequency.

doses >100 μM (representative expression changes shown in Figure 2). This dose response is somewhat similar to the response for DNA damage seen in Figure 1. It was not possible to replicate all the doses of DCA used in the DNA damage study in the gene expression study. Furthermore, the data generated by real-time PCR in Figure 2 are not as amenable to generating dose responses as compared to the micronucleus assay, partly due to less power (fewer events) and due to inherent variation in gene expression between replicates. Hence, statistical significance was not achieved with any of the DCA doses here, but is seen at 300 μM in the studies shown in Figures 4–6. Nonetheless, the data generated in Figure 2 bear notable resemblances to the data in Figure 1, with respect to the doses of DCA capable of causing biological effects.

The MOA of DCAs dose response involves the generation of ROS

We have previously shown that DCA-induced chromosome damage as measured by the micronucleus assay can be abrogated by pre-exposure of the cells to equimolar amounts of the antioxidant vitamin C (14). In contrast, we show here that two other antioxidants (resveratrol and EGCG) do not block DCAs ability to damage chromosomes. In Figure 3, it is clear that pre-incubation of the OE33 cells, with resveratrol or EGCG prior to DCA exposure, has no effect on DCA-induced micronuclei at the dose (and exposure times) of antioxidant used here. In order to study the role of ROS in DCA-driven NF-κB-dependent gene expression, we similarly pre-exposed OE33 cells to several antioxidants prior to stimulating gene expression with DCA. Figures 4–6 show the results for ECGC, resveratrol and vitamin C. All three antioxidants abrogated DCA-driven gene expression, as measured by *IL-8* and *I-κB* gene expressions. Two other antioxidants, benzyl isothiocyanate and vitamin E (α tocopherol), failed to protect cells from DCA-induced gene expression changes. EGCG at its higher dose of 100 μM significantly abrogated both *IL-8* and *I-κB* expressions ($P = 0.048$ and $P = 0.041$, respectively). Resveratrol, at its higher dose of 75 μM, significantly abrogated the expressions of *IL-8* and *I-κB* ($P = 0.048$ and $P = 0.046$, respectively). For vitamin C, significant abrogation of gene expression occurred at the 150 and 500 μM levels for both genes ($P = 0.017$ and $P = 0.006$ for *IL-8* and $P = 0.04$ and $P = 0.026$ for *I-κB*).

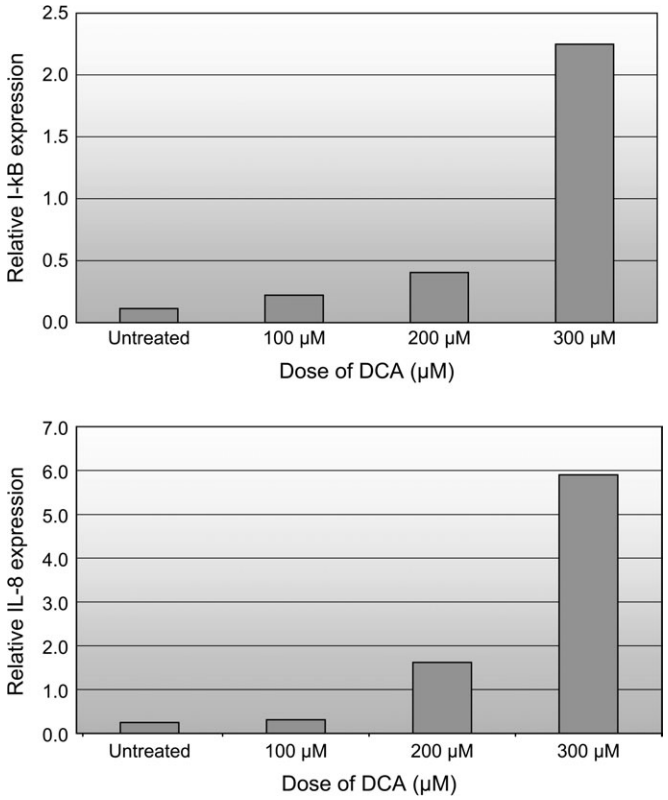

Fig. 2. Representative *IL-8* and *I-kB* gene expression levels are plotted against dose of DCA as a measure of NF-kB activity. Relative expression (normalized against the housekeeper β-actin) is shown. Both *IL-8* and *I-kB* are known transcriptional targets of NF-kB following DCA exposure (17) and are known to be NF-kB dependent (through the use of pharmacological inhibitors). Gene expression increases are evident at the 200- and 300-μM doses, less so at the 100-μM dose. This dose response is somewhat reminiscent of the DNA damage response in Figure 1.

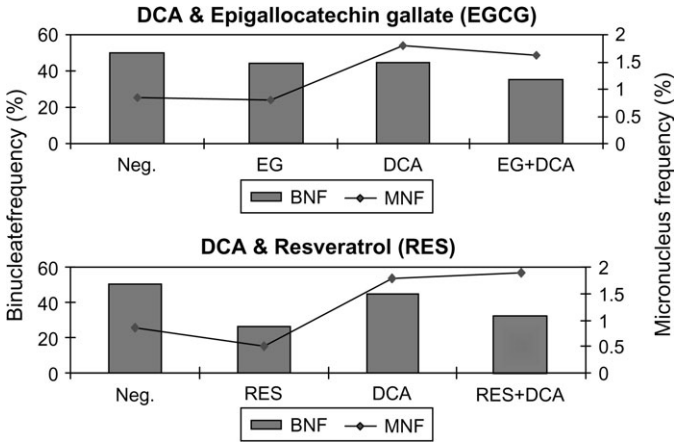

Fig. 3. The effect of resveratrol and EGCG supplementation on DCA-induced chromosome damage as measured by the micronucleus assay. Neither of these antioxidants (at these doses) prevented DCA-driven chromosome damage. This is in contrast to other studies by ourselves where we have shown that vitamin C (14) can block DCA-induced DNA damage.

Discussion

We have previously demonstrated that the bile acid DCA is a clastogen to oesophageal cells (14). We show here for the first time that DCA damages chromosomes in a non-linear

fashion. Furthermore, a broadly similar dose response was noted for DCAs ability to induce NF-κB activity (as measured by up-regulation of two well-known NF-κB-controlled genes).

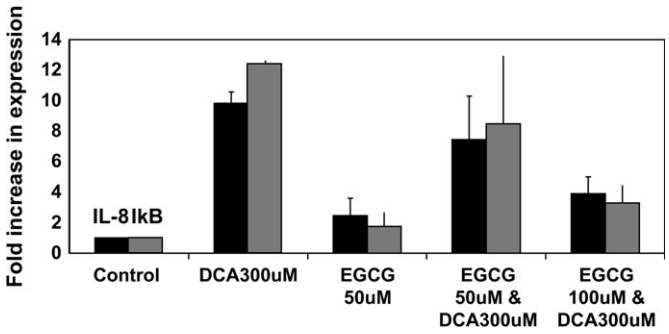

Fig. 4. The effect of pre-incubation with the tea polyphenol EGCG on DCA-induced *IL-8* and *I-kB* expressions in OE33 cells. Expression is normalized against the housekeeper β-actin and also normalized against the negative control which is given an expression value of 1. EGCG pre-incubation significantly abrogates DCA-driven NF-kB activity (as measured by *IL-8* and *I-kB* expressions) at the 100-μM dose.

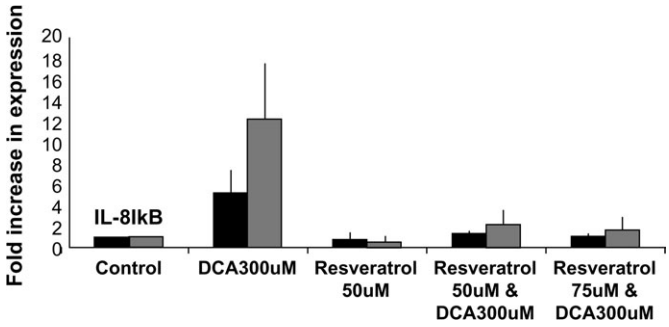

Fig. 5. The effect of pre-incubation with resveratrol on DCA-induced *IL-8* and *I-kB* expressions in OE33 cells. Expression is normalized against the housekeeper β-actin and also normalized against the negative control which is given an expression value of 1.Resveratrol pre-incubation significantly abrogates DCA-driven NF-kB activity (as measured by *IL-8* and *I-kB* expressions) at the 75-μM dose.

Interestingly, the data shown here suggest that low doses (<100 μM) of the bile acid DCA will neither cause DNA damage nor activate the procarcinogenic signalling molecule NF-κB in oesophageal cells. Hence, if these *in vitro* results were replicated *in vivo*, it would suggest that the concentration of damaging bile acids (like DCA) refluxing into the oesophagus might be useful in stratifying reflux patients into those at low or high risk of carcinogenesis. Moreover, there may well be a relationship between the dose of DCA in the refluxate and the individual risk of OA. This could be clinically important and may well lead to the development of a biomarker based on bile acid concentration, to assess individual risk of cancer development in refluxing patients. The quest for accurate biomarkers of cancer risk is seen as the 'holy grail' in the field of Barrett's oesophagus as it offers the possibility of screening the population for high-risk cases without over-burdening National Health Service endoscopy services.

The available *in vivo* data on bile acid composition in the refluxate of GORD patients demonstrate a wide range of concentrations of bile acids like DCA across patients. This can range from 0 to 280 μM (23). Our data show here that doses of 100 μM or more are capable of causing DNA damage and NF-κB-driven gene expression changes. Only a small number of refluxing patients will have DCA concentrations exceeding 100 μM. Hence, it may be possible that the 10% of Barrett's patients who progress to OA are also the patients with the highest DCA concentrations in their reflux. This possibility is definitely worthy of close examination in an effort to identify high-risk cancer patients early when treatment (often surgery) is still an option.

Other published studies looking at the effect of DCA *in vitro* support the conclusions here that 100 μM represents a critical cut-off concentration in terms of DCA activity. Glinghammar *et al*. (26) showed that 100 μM DCA was the lowest dose capable of inducing NF-κB activity as measured by a luciferase construct in colorectal cell lines. Song *et al*. (27) have also shown conclusively that 100 μM is a key concentration of DCA for inducing phospho-extracellular signal related kinase activity and cox-2 expression in SEG-1 cells. Furthermore, Qin *et al*. (28) showed that 100 μM DCA had a significant effect in terms of inducing ROS and *VCAM* and *ICAM* expressions in

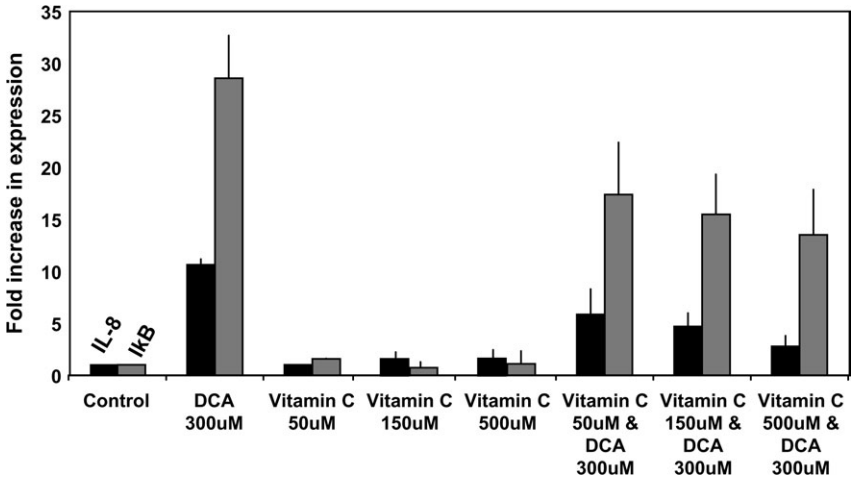

Fig. 6. The effect of pre-incubation with vitamin C on DCA-induced *IL-8* and *I-kB* expressions in OE33 cells. Expression is normalized against the housekeeper β-actin and also normalized against the negative control which is given an expression value of 1. Vitamin C pre-incubation significantly abrogates DCA-driven NF-kB activity (as measured by *IL-8* and *I-kB* expressions) at the 150- and 500-μM doses.

endothelial cells. Hence, our data fit well with the published literature and taken together build a strong case for DCA being biologically active (*in vitro* at least) at doses >100 μM.

In mechanistic terms, it is essential to understand how this dose dependence manifests itself and whether the mechanistic basis for this effect might be exploitable in therapeutic terms. We have shown here and elsewhere that the MOA attributable to DCA-induced biological effects involves the generation of ROS. Several antioxidants tested by us here and elsewhere (but interestingly not all) are capable of blocking DCA-driven DNA damage and NF-κB activity. Presumably, the non-linear dose response observed for DNA damage and possibly NF-κB activity is a consequence of a similar non-linear induction of ROS by DCA. Hence, at low levels of DCA exposure, there are limited amounts of ROS generated and/or that these levels of ROS are effectively dealt with by intrinsic antioxidant defences of the cell (antioxidant nutrients and enzymes such as superoxide dismutase, catalase and glutathione peroxidase). At doses of 100 μM or more, these antioxidant defences are saturated by the ROS generated and hence DCA activity, in terms of DNA damage and NF-κB activity, is observed. Therefore, because DCA-driven DNA damage induction and NF-κB activation involves ROS, and given the fact that some of the antioxidants were shown here to abrogate these effects *in vitro*, there is a strong case for antioxidant supplementation as a chemopreventative strategy in GORD patients. There is already excellent evidence that Barrett's patients intaking diets rich in antioxidants are protected against adenocarcinoma development (29,30). However, it is important to take note of the fact that not all antioxidants performed equally, hence some antioxidants may be more beneficial than others. Also, the doses used here vary (50 μM for EGCG, up to 500 μM for vitamin C) as does the time of incubation prior to DCA addition (2 h for DNA damage, overnight for NF-κB) and hence, any lack of effect seen here could be a consequence of the dosing regime. However, in both DNA damage induction and NF-κB activation, several antioxidants were protective and given that each separate study used the same antioxidants and used the same dosing regime, it was possible to rank each antioxidant's efficacy, with vitamin C apparently most effective, showing here suppression of gene expression changes and previously being shown by us to prevent DCA-driven micronuclei (14). Chemoprevention with antioxidants could be extremely important to GORD patients and patients with Barrett's oesophagus in terms of protecting them from OA, a deadly form of cancer. Coupling a chemoprevention strategy based on antioxidant supplementation to detailed measurement of bile acid concentration in gastric juice (with particular emphasis on DCA) would allow targeting the therapy to those who particularly need such intervention, on the basis of the levels of DCA in their refluxate.

Obviously, it is important to bear in mind that we have shown here data for only one bile acid at neutral pH. In reality, reflux involves exposure of the oesophagus to dozens of bile acids and the reflux background may be neutral in pH, weakly acidic or strongly acidic. Therefore, in order to fully convince the scientific community of the non-linear response to reflux in general in the oesophagus, more data are required from other reflux constituents. However, it is important to point out that we have previously shown that many of the conjugated bile acids (plus cholic acid) present in the refluxate do not cause DNA damage (14). In addition, we showed previously that acidification of DCA did not alter its DNA-damaging potential (14). Nonetheless, repeating the experiments presented here with other potentially active bile acids (like chenodeoxycholic acid) or with bile acid mixtures, representing those seen *in vivo*, would be beneficial. Furthermore, an important caveat to mention is that the shape of the dose response (Figure 1) may be influenced by the sensitivity of the assay used (the micronucleus assay). However, given the large numbers of cells studied here and the sharp increase in micronucleus frequency noted at the threshold doses and above, we feel that we are observing a true biological effect and are not being influenced by low sensitivity issues.

In conclusion, we have shown here that DCA, a secondary bile acid linked to several cancers of the GI tract, has a non-linear dose response for DNA damage as measured by the micronucleus assay. In addition, it shows a similar non-linear response for NF-κB activation. In both cases, 100 μM appears to be the cut-off dose. We further show that the MOA probably involves the generation of ROS by DCA and that these deleterious effects can be blocked by some antioxidants. This offers hope for firstly identifying refluxing patients at highest risk of cancer development (based on bile acid subtype and concentration) and further preventing oesophageal cancer development through antioxidant chemoprevention.

Funding

Swansea National Health Service Trust R&D Office; Tenovus the Cancer Charity (2002/5).

Acknowledgements

We wish to acknowledge the excellent technical support provided by Mrs Margaret Clatworthy. Conflicts of interest statement: None declared.

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Received on March 17, 2008; revised on April 21, 2008; accepted on April 25, 2008