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Sodium deoxycholate causes nitric oxide mediated DNA damage in oesophageal cells

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Abstract
Patients with chronic gastro-oesophageal reflux disease experience the reflux of acid and bile into the distal oesophagus. The secondary bile salt sodium deoxycholate (NDC) is implicated in the induction of mucosal injury during reflux episodes. This study hypothesized that NDC damages DNA in oesophageal cells by an oxidative mechanism. In the oesophageal cell line HET1-A, increased production of nitric oxide (NO) was measured in NDC-treated cells. Protection from DNA strand breaks induced by NDC (10 μM) was observed in cells coincubated with the nitric oxide scavenger C-PTIO ($p < 0.012$) or pre-incubated with the NO synthase inhibitor L-NAME ($p < 0.009$) or the NFκB inhibitor, TPCK ($p < 0.036$). Collectively these data implicate the involvement of NFκB and nitric oxide synthase in the DNA damage induced by NDC in oesophageal cells. In conclusion, NDC-driven NO production may play an important role in inducing DNA damage during episodes of gastro-oesophageal reflux and thereby contribute to reflux-related carcinogenesis.

Keywords: *Sodium deoxycholate, bile, comet assay, nitric oxide, reflux, Barrett's oesophagus*

Abbreviations: *BO, Barrett's oesophagus; ECM, epithelial cell medium; FCS, foetal calf serum; GORD, gastro-oesophageal reflux disease; LDH, lactate dehydrogenase; LOS, lower oesophageal sphincter; LMP, low melting point; NDC, sodium deoxycholate; NO, nitric oxide; NOS, nitric oxide synthase; OA, oesophageal adenocarcinoma; $ONOO^-$, peroxynitrite ROS/RNS radical oxygen species and radical oxygen species*

Introduction

A recent global consensus has defined gastroesophageal reflux disease (GORD) as a 'condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications' [1]. The most common manifestation is reflux oesophagitis. The exact prevalence of GORD is difficult to estimate due to regional variation, self-medication and lack of referral [2]. However, recent estimates suggest the prevalence is high in Western populations, affecting 10–20% of adults [3].

The aetiology of GORD is multifactorial, involving lower oesophageal sphincter dysfunction and neural abnormalities that often coexist with hiatal hernia [4]. Several lifestyle factors may be involved, including physical activity and diet [5] and twin studies have also revealed a genetic contribution to this disease [6,7].

GORD is associated with complications that include oesophagitis, Barrett's oesophagus (BO) and oesophageal adenocarcinoma (OA) [8]. Factors within refluxate, including gastric acid, pepsin, bile salts and pancreatic enzymes, can cause mucosal injury and inflammation. Patients with erosive oesophagitis or BO experience significantly more acid and bile reflux than individuals with non-erosive reflux disease [9] and exposure to increased concentrations of the secondary bile acid deoxycholic acid is observed in the disease process [10].

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Reactive nitrogen species (RNS) are increasingly implicated in reflux-related diseases. Peroxynitrite ($ONOO^-$) has been found at higher concentrations in oesophagitis and Barrett's epithelium relative to normal oesophageal epithelium [11,12]. Vaninetti et al. [13] reported a step-wise increase in the expression of iNOS mRNA in oesophageal tissue obtained from patients with GORD, BO or OA, respectively. Using an integrated genomic and proteomic approach, Ostroski et al. [14] observed increased expression of neuronal NOS and inducible NOS genes in BO tissue.

Within the oesophagus, nitric oxide (NO) is involved with the timing of peristalsis and triggering of transient lower oesophageal sphincter muscle relaxation [15]. NO could therefore contribute to the development of GORD via an effect on oesophageal motor function. NO may also promote the development of Barrett's oesophagus and oesophageal adenocarcinoma via established effects on necrotic or apoptotic cell death, enhancing proto-oncogene expression and stimulating Cox-2 activity [16,17]. Reactive RNS have also been shown to be genotoxic and mutagenic in prokaryotic and eukaryotic cells [18] and in this regard are noteworthy, because accumulation of nitrotyrosine residues has recently been associated with p53 mutations in OA tissue [13].

We have previously demonstrated that deoxycholate stimulates DNA damage in the form of strand breaks in oesophageal cells [19]. In this study, we test the hypothesis that the DNA damage induced by sodium deoxycholate occurs via the formation of NO. We also assess the role of NF-κB in deoxycholate-stimulated NO production and DNA damage.

Materials and methods

Epithelial cell medium (ECM) was obtained from Promocell GmbH (Heidelberg, Germany). DMEM, McCoys medium, foetal calf serum (FCS) and DAF-FM diacetate were obtained from Invitrogen (UK). FNC coating mix® was obtained from Stratech (UK). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (C-PTIO) was obtained from Axorra (UK). Cytotox 96® LDH assay kit was obtained from Promega (UK). Nitrate reductase from *Aspergillus niger* was obtained from Roche Diagostics (UK). All other reagents including the NFκB inhibitor N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) were supplied from Sigma (UK).

Cell treatments

HET1-A cells were grown in ECM. HT29 cells were grown in McCoys medium supplemented with 10% (FCS). Each cell line was cultured without antibiotics in 75 cm^2 flasks at 37°C in 5% CO_2. Prior to experimental treatments, cells were detached from culture vessels with trypsin and 2×10^5 cells placed into wells of a 24-well tissue culture plate with medium, pre-coated with FNC coating mix for at least 16 h to allow adhesion and recovery.

Cells were treated with the secondary bile salt sodium deoxycholate (NDC) for 3 h in media without FCS. Cells were pre-incubated with the nitric oxide (NO) scavenger C-PTIO (100 μM) or the NO synthase inhibitor (NOS) L-NAME (100 μM) for 1 h prior to the addition of NDC. Alternatively, cells were pre-incubated with the NFκB inhibitor (TPCK) (5 μM) for 30 min prior to the addition of NDC.

Cell viability

Lactate dehydrogenase (LDH) release into the supernatant was assayed under each experimental treatment using the Cytotox 96® LDH assay kit.

Nitrite/nitrate assay (Griess)

The total production of NO was determined by enzymatic conversion of nitrate (NO_3^-) to nitrite (NO_2^-) with nitrate reductase followed by assay of total NO_2^- by the Griess assay [20]. Briefly, 1×10^6 cells/well were seeded into a 24-well plate and allowed to adhere. After treatment with NDC with or without the NOS inhibitor L-NAME the cells were washed three times and incubated in fresh culture medium at 37°C for 20 h. Supernatant was collected, centrifuged at 13 000 rpm for 10 min in an MSE microfuge to pellet any debris or proteinaceous material. Supernatants (50 μl) were collected and added to triplicate wells of a 96-well microtitre plate with 0.05 U of nitrate reductase, FAD 200 μM and NADPH 6 mM in 50 mM HBSS (pH 7.4). This mixture was incubated for 30 min in the dark at 37°C. Lactate dehydrogenase (7.5 U) in sodium pyruvate (30 mM) was added to each well for 10 min in the dark at 37°C to oxidize any unreacted NADPH. For the Griess assay, 50 μl of acidified sulphanilamide was added to the wells and incubated at 4°C for 10 min, followed by 50 μl of N-naphthylethylenediamine hydrochloride, incubated at 4°C for 30 min. This minor modification to the Griess assay has been reported to improve sensitivity [21]. The absorbance of the samples was read at 540 nM in an IEMS spectrophotometer (Lab systems).

Detection of NO by DAF-FM diacetate

Nanomolar concentrations of NO can be successfully detected by the cell permeant fluorescent probe DAF-FM diacetate. This is deacetylated by intracellular esterases to form DAF-FM, which becomes trapped within the cell and highly fluorescent in a reaction with NO [22]. Following the method of

Leikert et al. [23], cells (2×10^5/well) were seeded into a black fluorescent microplate (Thermo Electron UK) and pre-incubated with DAF-FM (0.1 μM) for 1 h in HBSS/2 mM L-arginine at 37°C. NDC in HBSS/2 mM L-arginine was then added and the fluorescence monitored after 3 h with excitation at 495 nM and emission at 515 nM on a Thermo Varioskan plate reader.

Comet assay

The comet assay (or single cell gel electrophoresis assay) permits detection of alkali-labile sites and single-strand breaks in DNA within single cells under alkaline conditions [24]. Following treatment with NDC, cells (2×10^5) were resuspended in 1 ml of 0.8% low melting point (LMP) agarose in PBS at 37°C. The cell suspension (100 μl) was applied onto slides pre-coated with 1% normal melting point agarose, a coverslip applied and briefly incubated on ice to allow the cell suspension to solidify. A further 80 μl of LMP agarose was subsequently applied as above. Slides were placed in cold lysis buffer (2.5 M NaCl, 1 mM EDTA, 10 mM Tris, adjusted to pH 10, made to 10% DMSO and with 1% Triton X-100 added immediately prior to use) at 4°C in the dark for at least 1 h. Slides were then incubated in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) at room temperature for 40 min to facilitate DNA unwinding. This was immediately followed by electrophoresis at 23 V, 300 mA for 20 min. Slides were washed in neutralization buffer (0.4 M Tris, pH 7.5), then stained with 20 μg/ml ethidium bromide. Comets were visualized by epifluorescent microscopy at 250× magnification. For each treatment, the percentage of DNA present in the comet tail of 75 cells per slide counted in duplicate was quantitated using an image analysis system (Komet 4; Kinetic Imaging Ltd, UK).

Statistical analysis

Comet assay data did not fit a normal distribution. Therefore, the median value from each treatment was calculated. Experiments were carried out on at least three separate occasions and the data analysed by ANOVA. The statistical significance of individual treatment groups in comparison with controls was subsequently determined with Dunnett's multiple comparison post-test. ANOVA with post-hoc testing using Tukeys HSD was used to assess statistical significance of DAF-FM fluorescence. Independent *t*-tests without assumption of equal variance were applied for experiments where ANOVA was not suitable. In all analyses, values of $p < 0.05$ were considered significant (SPSS version 13; Chicago, IL).

Results

Viability

The release of LDH, regarded as a sensitive indicator of membrane integrity, was employed to provide a measure of cell viability. LDH leakage was always observed to be $< 20\%$, suggesting limited toxicity under the experimental conditions employed.

Nitrite (Griess assay)

In cells or aqueous solutions NO is converted to NO_2^- or NO_3^-. Although nitrite was detectable in the supernatant of untreated HET1-A cells at relatively low (micromolar) amounts, NDC treatment resulted in a dose-dependent increase in NO_2^-, with more than double the level being measured following 500 μM NDC compared to untreated control cultures (Figure 1). However, NO_2^- production in unstimulated cells was only detectable if the cell number was increased to 1×10^6 cells/0.5 ml, the cells left to recover from treatment for 20 h and the accumulated total NO_2^- measured after enzymatic conversion of nitrate in the supernatant to NO_2^-. HT29 cells, previously reported to produce NO upon stimulation, served as a positive control (Figure 1). Pre-incubating the cells in the NOS inhibitor L-NAME (100 μM) significantly suppressed ($p < 0.001$) NO_2^- production in HET1-A cells, below the background level of untreated control cultures and below the level of NO_2^- produced by cells treated only with NDC (Figure 2).

Nitric oxide (DAF-FM)

As NO_2^- levels were too low to detect by the Griess assay during a 3 h treatment with NDC (data not

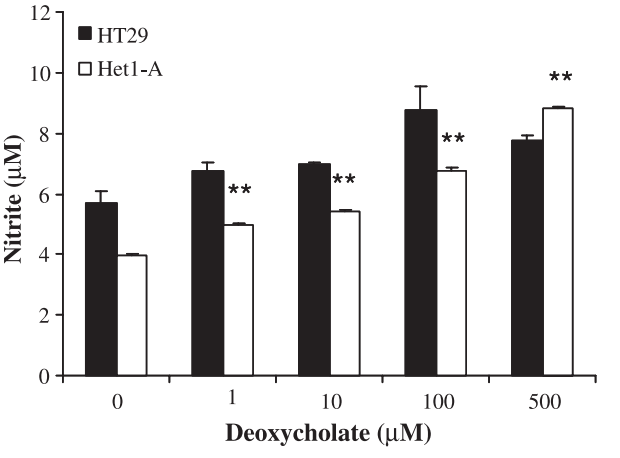

Figure 1. Nitrite production from NDC-treated cells. Results represent total NO_2^- from supernatant with prior enzymatic conversion of NO_3^- to NO_2^-. HT29 cells (positive control (■)); HET1-A cells (□). Cells were allowed to recover for 20 h prior to assay of NO_2^-. Results are expressed as the mean value of NO_2^- (μM) of triplicate cultures plus SEM from three separate experiments. **Indicates significant at $p < 0.01$ relative to control cells.

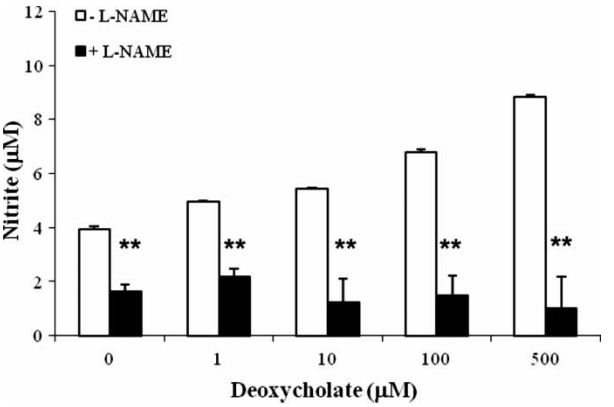

Figure 2. Nitrite production from NDC-treated HET1-A cells, (□) or pre-incubated with L-NAME (100 μM) for 1 h prior to treatment with NDC for 3 h (■). Cells were allowed to recover for 20 h prior to assay of NO_2^- in the presence or absence of L-NAME (100 μM). Results are expressed as the mean value of NO_2^- (μM) of triplicate cultures plus SEM from three separate experiments. **Indicates significant at $p < 0.01$ relative to control cells.

shown), we used a more sensitive probe for NO, namely DAF-FM to permit comparison of NO production with DNA damage at these early time points. In this assay NDC resulted in significant fluorescence of DAF-FM after 3 h at doses as low as 100 μM ($p < 0.01$; Figure 3). NO_2^- production was reduced to below the level of untreated control cells by pre-incubation with L-NAME.

DNA damage

NDC caused a dose-dependent increase in DNA damage in HET1-A cells as measured by the comet assay (Figure 4). Cells pre-incubated with L-NAME (100 μM) for 1 h prior to treatment were protected against DNA damage (Figure 4) stimulated by NDC, at both 10 μM ($p < 0.009$) and 100 μM ($p < 0.001$).

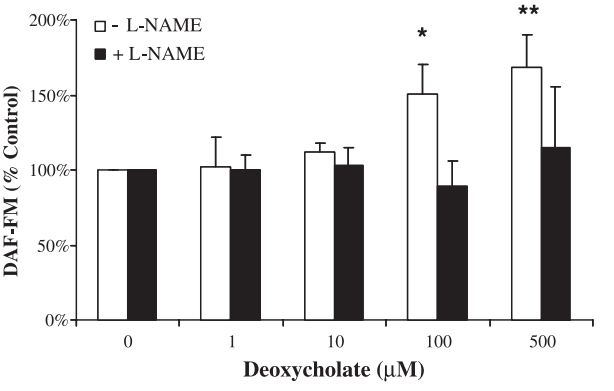

Figure 3. Fluorescence of DAF-FM in HET1-A cells treated with NDC, (□); Pre-incubated in L-NAME 100 μM (■). Data are expressed as the mean plus SEM percentage fluorescence of triplicate determinations compared to control cultures (C) from three separate experiments. *Indicates significant at $p < 0.05$, **indicates significant at $p < 0.01$ relative to control cells without L-NAME.

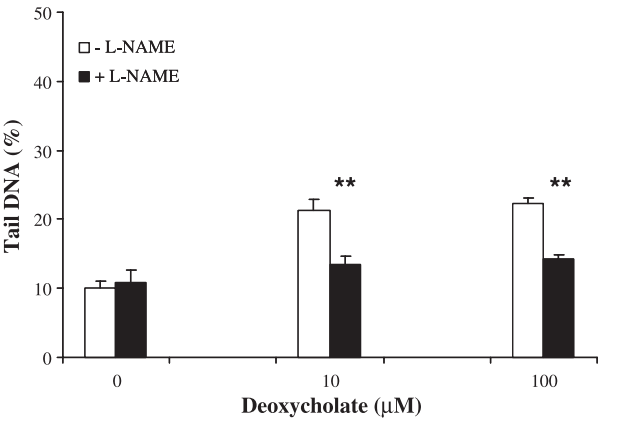

Figure 4. DNA damage in HET1-A cells treated with NDC (□) or pre-incubated in L-NAME 100 μM prior to treatment with NDC (■). Data are presented as mean plus SEM for three separate experiments. **Indicates significant at $p < 0.01$ relative to control cells without L-NAME.

Heterogeneity across experiments masked the full effect of the addition of C-PTIO. Coincubation of cells with the NO scavenger C-PTIO (100 μM) caused a small but non-statistical increase in levels of DNA damage, but this was not sufficiently high enough to alter the protective effect of C-PTIO against NDC-mediated DNA damage (Figure 5). This was significant ($p < 0.012$) at the lower dose of NDC (10 μM). These data with a NO scavenger (C-PTIO) or NOS inhibitor (L-NAME) indicate that reducing NO decreases damage to DNA in the cells.

We next sought to inhibit the activity of NFκB using the inhibitor TPCK, in order to test the hypothesis that NDC-induced stimulation of NOS was down stream of NFκB activation. Used at a concentration of 5 μM and for a pre-incubation period of 30 min, cell viability was high (> 89%). TPCK reduced DNA damage significantly in cells treated with NDC (10 μM and 100 μM) ($p < 0.036$ and $p < 0.034$, respectively) (Figure 6).

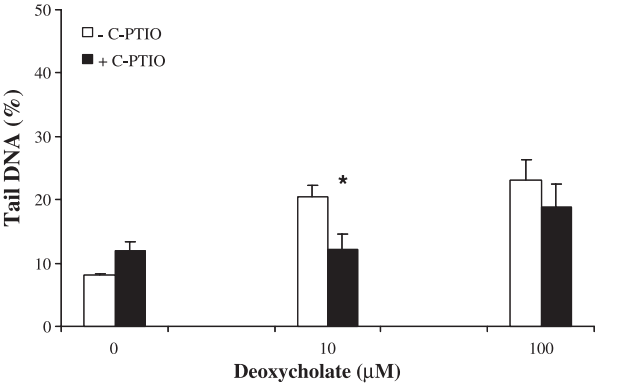

Figure 5. DNA damage in HET1-A cells treated with NDC (□) or with NDC and NO scavenger/C-PTIO 100 μM (■). Data are presented as mean plus SEM for three separate experiments *Indicates significant at $p < 0.05$ relative to control cells without C-PTIO.

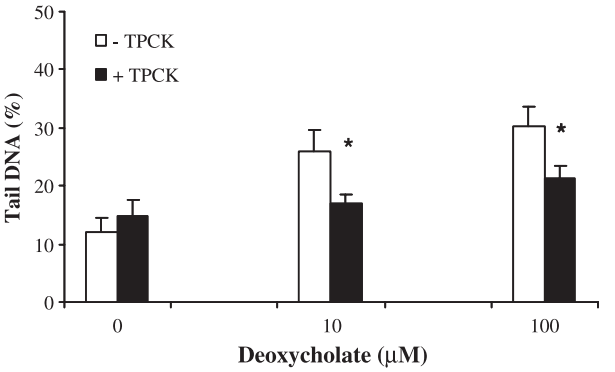

Figure 6. DNA damage in HET1-A cells treated with NDC (□) or pre-incubated in TPCK 5 μM prior to treatment with NDC (■). Data are presented as mean plus SEM for three separate experiments. *Indicates significant at $p < 0.05$ relative to control cells without TPCK.

Discussion

This study in an SV40 antigen transformed cell line (HET1-A) derived from normal oesophageal squamous epithelium [25] tested the hypothesis that NDC-induced DNA damage was mediated by NO-derived radicals. Production of NO was detected after a short (3 h) exposure to NDC and the accumulation of its metabolite NO_2^- was quantified in the culture medium after NDC was removed. Production of NO was associated with DNA strand break damage. Scavenging the NO radical with C-PTIO or inhibition of NOS using L-NAME reduced DNA damage stimulated by NDC. A similar effect was achieved by incubating cells with the NFκB inhibitor TPCK, suggesting the production of NO was NFκB-dependent. These results are consistent with DNA damage being formed by NO subsequent to an induction of NOS, stimulated by activation of the nuclear transcription factor NFκB. Bile salts, in particular the hydrophobic secondary salts such as NDC, have been reported to alter cellular redox balance [26]. Dvorak et al. [27] reported increased oxidative stress in BO tissue and oesophageal cell lines exposed *in vitro* to a bile acid cocktail at low pH. Jenkins et al. [28,29] recently demonstrated that deoxycholate causes ROS release, followed by activation of NFκB and increased expression of downstream targets such as IL-8 and IKK in oesophageal cell lines. Increased levels of NFκB have been observed in a rat model of oesophagitis [30] and during the step wise progression of BO towards OA in oesophageal tissue [31]. Normally sequestered with IκB in the cytoplasm, NFκB is held in an inactive state. Phosphorylation of IκB and its subsequent degradation allows nuclear translocation of NFκB where it can act as a transcription factor for a wide variety of genes including the constitutive and inducible isoforms of NOS. This process is rapid, redox-sensitive and induced by a variety of signals that converge upon NFκB to modulate responses, in particular to extracellular signals. In this study, inhibition of NFκB with the serine protease inhibitor TPCK reduced NDC mediated DNA damage. A recent study by Zhen et al. [32] reported upregulation of eNOS and iNOS expression by reactive oxygen species in human coronary artery endothelial cells, an effect which was dependent on NFκB activation. It therefore seems possible that the NFκB mediated NO production and associated DNA damage observed in this study may have occurred as a downstream consequence of ROS release by deoxycholate treated cells.

NO can react with DNA directly via a variety of mechanisms inducing strand breaks, deamination of DNA bases or the generation of oxidative lesions, but also indirectly by several mechanisms such as inhibition of DNA repair and nitrosylation of proteins [33,34].

The level of NO produced from HET1-A cells was relatively low when compared to other stimulated epithelial cells such as HT29 and extremely low when compared to primed inflammatory cells reported in other studies [35]. Nevertheless, use of the NO scavenger C-PTIO demonstrated that NO generated during NDC exposure was responsible for almost doubling DNA damage levels, as measured by strand breaks in this cell line. Bile acids have been reported to stimulate NO production in other cell and tissue types including colonic mucosa, vascular endothelial cells and hepatocytes [36–38], but this is the first report in oesophageal cells. The inclusion of C-PTIO with NDC at the higher concentration of 100 μM failed to provide cells significant protection from NDC-induced DNA damage. We speculate that it is likely other radical species may become involved as the concentrations of NDC increases. For example, increasing mitochondrial oxidative stress would facilitate the production of a variety of ROS such as superoxide and this could interact with NO to produce the energetic radical peroxynitrite.

The production of NO over a sustained period of time (20 h) in cells exposed to NDC suggests induction of NOS, although in this study we have not investigated the specific NOS isoform involved, nor have we investigated transcriptional regulation of iNOS.

Sustained NO production (20 h) in response to a relatively short (3 h) exposure to physiological concentrations of NDC supports the hypothesis that brief episodes of gastroesophageal reflux in patients with GORD promote long-term adaptive responses that predispose towards the development of BO. This adaptive response has been reported by Crowley-Weber et al. [39] and latterly by Dall'Agnoll et al. [40] in colon adenocarcinoma cell lines subjected to chronic exposure to NDC. In these cell lines the development of apoptosis resistance, activation of NFκB and the increased formation of S-nitrosylated proteins were observed.

Our study with a squamous oesophageal derived cell line suggests that NDC in refluxate has the potential to stimulate the production of RNS, via an NFκB-dependent mechanism, and result in DNA damage. Caution must be exercised in the extrapolation of our *in vitro* results to normal cells *in vivo*, because relatively higher oxygen tensions encountered *in vitro* might facilitate the formation of RNS. However, both NFκB and NOS activity have been found to be elevated in oesophagitis, BO and OA, as have elevated levels of DNA damage and impaired defence against oxidative stress [31,41]. In susceptible individuals, ineffective mucosal defences together with the anatomical and physiological abnormalities associated with GORD could allow exposure of oesophageal stem cells to bile acids, resulting in NO-stimulated DNA damage. The accumulation of cytogenetic changes or mutations associated with DNA strand breaks could confer a survival advantage through clonal selection of bile resistant phenotypes in the oesophageal epithelium and promote the development of BO. Further accumulation of DNA lesions in these clones would be expected to increase the risk of neoplastic progression. This pathway may constitute an important target for chemoprevention strategies. In this regard, Jenkins et al. [29] have recently reported protection from deoxycholate-mediated DNA damage (micronucleus assay) by pre-treatment with vitamin C in an OA cell line. In rats, inhibition of iNOS by a selective pharmacological inhibitor or by dietary supplementation with freeze-dried berries [42] has been reported to reduce oesophageal tumour progression induced by treatment with N-nitrosomethylbenzylamine. Furthermore, high concentrations of NO can be generated within the gastroesophageal junction and the acid stomach via the reduction of salivary nitrite [43] and Gago et al. [44] recently observed NO within exhaled air following the consumption of red wine. Thus, the distal oesophagus is exposed to multiple potential sources of NO.

In conclusion, we have demonstrated that treating oesophageal cells *in vitro* with NDC at levels physiologically relevant to patients with BO or GORD causes NO-mediated DNA damage in a dose-dependent manner. This process requires functional NFκB and downstream induction of NOS. Further studies are now required to define how deoxycholate modulates NFκB signalling and transcriptional regulation of NOS resulting in DNA damage and to establish whether these events occur during episodes of reflux *in vivo*.

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