Nitric Oxide and Acid Induce Double-Strand DNA Breaks in Barrett’s Esophagus Carcinogenesis via Distinct Mechanisms

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Background & Aims: The luminal microenvironment including acid and nitric oxide (NO) has been implicated in Barrett’s esophagus carcinogenesis. We investigated the ability of acid and NO to induce DNA damage in esophageal cells. Methods: Transformed and primary Barrett’s esophagus and adenocarcinoma cells were exposed to either acid, (pH 3.5), ± antioxidant or NO from a donor or generated by acidification of nitrite in the presence of ascorbate ± NO scavenger. Phosphorylation of histone H2AX and the neutral comet assay were used to detect DNA double-strand breaks (DSBs). Intracellular levels of reactive oxygen species and NO were detected with fluorescent dyes. Mitochondrial viability was measured with a rhodamine dye. Long-term survival was assessed by clonogenic assay. Results: Exposure to acid (pH 3.5) for ≥15 minutes induced DSBs in all cell lines (P < .05). There was a concomitant increase in intracellular reactive oxygen species in the absence of mitochondrial damage, and pretreatment with antioxidants inhibited DNA damage. Exposure to physiologic concentrations of NO produced from the NO donor or acidification of salivary nitrite induced DSBs in a dose- (>25 μmol/L) and cell-dependent manner (adenocarcinoma > Barrett’s esophagus, P < .05). This occurred preferentially in S-phase cells consistent with stalled replication forks and was blocked with a NO scavenger. NO also induced DSBs in primary Barrett’s esophagus cells treated ex vivo. Cells were able to survive when exposed to acid and NO. Conclusions: Both acid and NO have the potential to generate DSBs in the esophagus and via distinct mechanisms.

The incidence of esophageal adenocarcinoma (EAC) has increased almost 6-fold over the past few decades in the United States and is a trend that is reflected in developed nations worldwide.1 The reason for this increase is unknown, and, because of the rapid rate of increase, it is likely that environmental factors play an important role. The metaplastic condition Barrett’s esophagus (BE) is a major risk factor for EAC.2 BE is widely accepted to be a consequence of chronic gastro-duodenal-esophageal reflux, and there is mounting evidence that reflux components play an important role in the progression to cancer. For example, cytoplasmic acid and bile can affect growth control and differentiation status. In in vitro and ex vivo models, acid stimulates either proliferation or differentiation, depending on the duration of acid exposure.3–5 Low pH can also inhibit apoptosis in vitro via activation of the mitogen-activated protein kinase pathways6 and down-regulation of the caspase cascade.6 Similarly, bile acids have been shown to stimulate proliferation in BE, although this effect was inhibited at low pH.7

Acid and bile may also promote progression via DNA damage-mediated genetic alterations. The level of DNA damage (alkali labile sites and single-strand breaks) is higher in BE mucosa compared with squamous mucosa from the same patient,8 and a number of recent studies have demonstrated that both acid and bile can induce single-strand DNA breaks, alkali labile sites, and oxidative damage in esophageal cell lines and tissue.9–11 Both acid and bile induce the intracellular production of reactive oxygen species (ROS) in esophageal cells, and the antioxidants can inhibit acid- and bile-induced DNA damage.10,11 Importantly, duodenoesophageal reflux has been shown to be potentially mutagenic in rat models of BE.12

Recently, high levels of nitric oxide (NO) have been found to be generated in the esophageal lumen during bouts of reflux because of the reduction of salivary nitrite by acid.13 Nitrite is converted to nitrous acid and a number of nitrosating species at acidic pH and can lead to the production of potentially carcinogenic N-nitroso compounds.14,15 However, in the presence of excess ascorbic acid (AA), nitrosating species are reduced to NO.16,17 Thus, the presence of AA in the gastric juice can protect...
against the potential effects of N-nitroso compounds yet, ironically, results in the production of NO. In the absence of reflux, this complex series of reactions occurs at the gastro-esophageal junction or gastric cardia, at which saliva first encounters gastric acid, and the highest concentrations of NO anywhere in the human body have been recorded at these locations. However, in the presence of reflux, production of NO moves proximally into the esophagus and at such high concentrations is able to cross rapidly the lipid membrane of epithelial cells lining the esophagus.

NO has been reported to be involved in a myriad of biologic processes including angiogenesis, apoptosis, gene expression, and DNA damage and has been implicated in carcinogenesis and tumour progression in a number of epithelial tissues including multiple organs of the gastrointestinal tract. It has also been found to be mutagenic in human cells and in mice. The ultimate effect of NO on cells is dependent on a number of factors including rate of production, rate of diffusion, and concentration of reagents such as the superoxide radical and oxygen with which NO forms peroxynitrite and N₂O₃, respectively. Inside the cell, NO and its reactive products may exert toxic effects by targeting lipids, proteins, or DNA. Because of its short half-life, the damaging effects of NO on DNA are thought to be mediated primarily through peroxynitrite or N₂O₃ and can result in deamination of purines and pyrimidines, base transitions, respectively.

This study aimed to investigate whether the production of NO from nitrite at acidic pH could induce DNA damage in esophageal cells in the form of double-strand breaks (DSBs). To differentiate between damage caused by NO and that caused by acid, we examined the ability of acid or NO alone and NO produced by acidification of nitrite to induce DNA damage. We used clonogenic assays to study cell survival following these insults. Finally, to determine the mechanism of DSB induction, we investigated whether antioxidant and NO scavenger treatment could inhibit acid and acidified nitrite-mediated damage, respectively.

Materials and Methods

Cell Lines

The EAC cell lines BIC, FLO, and SEG (kind gifts of Dr. D. Beer, University of Michigan, Ann Arbor, MI), were maintained in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS) (HyClone UK LTD., Northumberland, United Kingdom) and penicillin/streptomycin (Invitrogen). The BE nondysplastic cell line QhTERT and high-grade dysplasia cell lines ChTERT and GihTERT (also known as CP-A, CP-B, and CP-C, respectively, were kind gifts of Dr. P. Rabinovitch, University of Washington, Seattle, WA) were maintained in modified MCDB medium (Sigma-Aldrich, Dorset, United Kingdom).

**Induction of DNA Damage by Acid or NO Donor**

Cells were plated to give a cellular density of approximately 60%–70%. For acid treatments, cells were incubated for 15, 20, or 25 minutes with serum-free medium that was adjusted to pH 3.5 with 0.1 mol/L HCl, followed by incubation in serum containing medium at pH 7.4 for 20–30 minutes for a total incubation time of 45 minutes. This time point was chosen to allow for maximal phosphorylation of histone H2AX. An equivalent volume of phosphate-buffered saline (PBS) was added to normal pH, serum-free medium to control for differences in osmolarity. Where indicated, SEG cells were pre-incubated for 1 hour at 37°C with 5 mmol/L N-acetyl-l-cysteine (NAC; Sigma-Aldrich) diluted in medium, prior to incubation at low pH. For NO treatments, cells were incubated for 45 minutes in the presence of 25–200 μmol/L of NO donor MAHMA NONOate (NOC-9; Axxora, Nottingham, United Kingdom) dissolved in 0.1 mol/L NaOH (pH >10). Control cells were treated with an equivalent volume of 0.1 mol/L NaOH. Primary esophageal cells from endoscopic brushings of patients with confirmed BE were treated with 100 μmol/L MAHMA NONOate or an equivalent volume of 0.1 mol/L NaOH for 45 minutes in a similar fashion. All brushings were used with patient consent and ethics approval.

**Detection of Phosphorylated Histone H2AX by Fluorescence-Activated Cell Sorting and Immunocytochemistry**

Detection of phosphorylated histone H2AX (γH2AX) was performed by either fluorescence-activated cell sorting (FACS) or by immunocytochemistry using an antiphospho-histone H2AX (Ser139), mouse monoclonal (JWB301) antibody from Upstate (Hampshire, United Kingdom). For FACS, following treatment, cells were fixed in cold 70% ethanol and stored up to 2 weeks at −20°C. For analysis, cells were spun with an equal volume of PBS then resuspended in 1 mL cold permeabilization buffer (4% fetal bovine serum [FBS] and 0.1% Triton X-100 in PBS) for 10 minutes at room temperature. Cells were then centrifuged and incubated in 200 μL primary antibody (1:500 dilution) or an equivalent concentration of control mouse immunoglobulin (Ig)G (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at room temperature followed by 200 μL of Alexa Fluor 488 rabbit anti-mouse secondary antibody (Invitrogen; 1:200 dilution) for 1 hour at room temperature. Cells were rinsed and resuspended in PBS with 1 μg/mL DAPI.
(Sigma-Aldrich) prior to analysis of 10,000 cells/sample on a LSR II flow cytometer (BD Bioscience, Franklin Lakes, NJ). Analysis of FACS data was performed with FCS Express Version 3 software (DeNovo Software, Thornhill, Canada). Primary cells from endoscopic brushings were processed in the same way before spinning onto microscope slides, counter-stained with DAPI, and examined by confocal laser scanning microscopy.

For detection of γH2AX by immunocytochemistry, cells were fixed with ice-cold 5% acetic acid/95% ethanol for 5 minutes then incubated for 30 minutes at room temperature in blocking buffer (3% bovine serum albumin [BSA] in PBS) then incubated in 200 μL primary antibody (1:2000 dilution) or an equivalent concentration of control mouse IgG for 1 hour at room temperature, followed by 200 μL of Alexa Fluor 488 anti-mouse secondary antibody (1:500 dilution) for 1 hour at room temperature. Cells were then washed 5 times with PBS mounted in VECTASHIELD Mounting Medium with DAPI, followed by 200 μL of Alexa Fluor 488 anti-mouse secondary antibody (1:500 dilution) for 1 hour at room temperature, and viewed by confocal laser scanning microscopy (Carl Zeiss, Ltd, Welwyn Garden City, United Kingdom).

Cell Viability and Clonogenic Survival

Cell viability 24 hours after incubation at pH 3.5 for 10, 20, 30 minutes was assessed by trypan blue exclusion assay using the automated Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Fullerton, CA). Clonogenic survival assays were performed immediately after incubation in acid for 10, 15, or 20 minutes or 45 minutes following the addition of the indicated doses of MAHMA NONOate. Cells were plated into 96-well plates at a density of 1, 10, or 100 cells per well with a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark) and incubated at 37°C for 10–12 days. Wells were scored as positive or negative for colony growth, and the fraction of surviving cells was calculated by comparison with control treated cells plated at a density of 1 cell/well.30 Clonogenic survival was not assessed in QhTERT cells because they did not readily form colonies from single cells.

Apoptosis

Apoptosis was examined with the Annexin V-FITC Fluorescence microscopy kit (BD Biosciences). SEG cells grown on coverslips were treated with serum-free medium at pH 3.5 or pH7.4 (control) for 25 minutes followed by 20-minute or 6-hour incubation in normal pH medium then labelled with Annexin V-FITC according to manufacturer’s instructions. Cells treated with 100 μmol/L staurosporine for 6 hours were used as a positive control. The percentage of Annexin V-FITC positive cells was calculated in 3 randomly placed fields (>200 cells scored per sample) and the experiment repeated twice.

Detection of Intracellular ROS or NO

Cells were incubated with 5 μmol/L 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA; Invitrogen) or 2μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen) in PBS at room temperature for 45 minutes for the detection of ROS or NO, respectively. Cells were washed and incubated at 37°C in phenol red free DMEM (Sigma-Aldrich) containing serum for 30 minutes to allow deesterification of the dye. Cells were then incubated with phenol red and serum-free DMEM adjusted to pH 3.5 with 0.1 mol/L HCl or serum-free medium at pH7.4 with added PBS (control) for 10, 15, 20, or 25 minutes. For NO experiments, cells were treated with 25, 50, or 100 μmol/L MAHMA NONOate or an equivalent volume of 0.1 N NaOH in serum containing medium for the indicated periods. Immediately following treatment, 10,000 cells per sample were analysed on a LSR II flow cytometer. For some experiments, cells were spun onto slides, and confocal laser-scanning microscopy was used to take pictures of ROS or NO-induced fluorescence.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was assessed by uptake of the selective dye, tetramethylrhodamine ethyl ester (Invitrogen). Briefly, cells were harvested and incubated with tetramethylrhodamine ethyl ester (final concentration 0.1 μmol/L) for 5 minutes at room temperature and analyzed by FACS on a LSR II flow cytometer. Cells treated with 50 μmol/L carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma-Aldrich) were used as a positive control.

In Vitro Measurement of NO Production From Nitrite

The production of NO from the reaction between salivary nitrite and acidic reflux was reproduced in vitro by the addition of nitrite (Merck Biosciences, Nottingham, United Kingdom) to acidified medium containing AA. Serum- and sodium bicarbonate-free DMEM (Sigma-Aldrich) was adjusted to pH 3.5 using a 50-mmol/L citrate buffer (citic acid and sodium citrate dihydrate). Ten milliliters of acidified medium was stirred continuously in a 37°C cabinet to which freshly prepared AA solution was added to a final concentration of 0.5, 1, or 2 mmol/L. Nitrite in the form of sodium nitrite solution (Merck Biosciences) was added to the medium, and the production of NO was measured using an ISO-NOP probe (World Precision Instruments, Stevenage, United Kingdom).

Phosphorylation of histone H2AX was measured by FACS as above in SEG cells treated with 600 μmol/L sodium nitrite or 600 μmol/L sodium nitrate in medium at pH 3.5 or 7.4 with 2 mmol/L AA for 5 minutes followed by fresh, normal medium for 40 minutes. In some experiments, cells were preincubated for 1 hour with 80 μmol/L
of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO; Axxora) in normal medium and then treated with sodium nitrite or sodium nitrate in the presence of a further 80 μmol/L PTIO.

**Immunoblotting**

Samples containing equal amounts of protein (150 μg or 50 μg for β-actin blot) were separated by 15% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Primary and secondary antibodies were added in 1% milk powder in PBS and incubated for 2 to 6 hours at room temperature or overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were detected by ECL Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, United Kingdom). The antibodies and the dilutions used are as follows: antiphospho-histone H2AX (Ser139), mouse monoclonal (JWB301) antibody was from Upstate (1:1000), and anti-β-actin rabbit monoclonal (13E5) antibody (1:1000) was from Cell Signaling Technology (Danvers, MA); ImmunoPure goat anti-mouse and goat anti-rabbit antibodies (both 1:4000) were from Pierce (Rockford, IL).

**Statistical Analysis**

All experiments were performed in duplicate or triplicate and repeated on at least 2 separate occasions. Analysis of variance (ANOVA) was used to test for significant differences between means within a group and the Student t test was used to test for statistical differences between 2 mean values. P < .05 was required for statistical differences.

**Results**

**Ability of Acid to Induce DNA Double-Strand Breaks**

The ability of acid (pH 3.5) to induce DSBs was investigated using phosphorylation of histone H2AX (γH2AX), which is an early and specific marker. In preliminary experiments, incubation at pH 3.5 for <10 min did not induce DSBs (data not shown). However, incubations ≥15 minutes led to an increase in fluorescence of the entire cell population as shown by the representative FACs histograms (Figure 1A). This was confirmed by the confocal images (Figure 1B). Quantitative FACs data from all of the cell lines demonstrated that incubation at pH 3.5 resulted in a significant increase in γH2AX compared with control in all cell lines (P < .05), which was not dependent on dose (as defined by duration of incubation at pH 3.5) except in FLO cells, although this increase was not statistically significant (P = .1 by ANOVA; Figure 1C). Thus, acid can induce DNA DSBs in BE nondysplastic, high-grade dysplasia, and adenocarcinoma cell lines as demonstrated by the phosphorylation of H2AX.

**Effect of Acid on Cell Viability and Survival**

If left unrepaired, DNA damage, particularly DSBs, can lead to cytostatic and cytotoxic effects; therefore, we next assessed the effect of acid on cell viability and survival. In addition, we wanted to be sure that any DSBs we detected were mediated by acid rather than occurring as a by-product of acid-induced apoptotic cell death.

A trypan blue exclusion assay demonstrated >80% viability 24 hours after a 10-minute incubation for all cell lines, and all except nondysplastic QhTERT showed >80% viability after 20-minute incubation (Figure 1D). Incubations longer than 30 minutes or at pH below 3.0 significantly decreased viability (data not shown) and justified our decision to restrict our DNA damage experiments to 25 minutes or less at pH 3.5. In contrast, the clonogenic assay revealed cell-line specific differences in long-term survival following incubation at pH 3.5 (Figure 1E). Adenocarcinoma cells (BIC, FLO, and SEG) that had been incubated for 10 minutes at pH 3.5 showed 75%–90% survival compared with 53% and 34% for the high-grade dysplasia BE cell lines GiHTERT and ChTERT, respectively (P = .0001 adenocarcinoma vs high-grade dysplasia cells). GiHTERT cells showed similar survival to BIC and SEG cells after 15-minute incubation but did not form any colonies after 20-minute incubations even when 100 cells were plated per well. BIC and SEG cells showed significantly reduced survival of 22%–25% (P = .00001 by ANOVA) and <1% (P = 2 × 10^-8 by ANOVA) after 15 minutes or 20 minutes at pH 3.5, respectively, compared with FLO cells.

We also examined acid-induced apoptosis in SEG cells by Annexin V-FITC labeling. Control treated cells had 1.8% ± 0.9% Annexin V positive cells compared with 14.0% ± 3.2% and 10.8% ± 2.9% at 20 minutes (the same time point at which γH2AX was measured; Figure 1C) and 6 hours after treatment at pH 3.5 for 25 minutes, respectively (P < .05 vs control, P = .3, 20 minutes vs 6 hours, Figure 1F). Staurosporine-treated cells (positive control) showed 93.4% ± 4.8% positive cells. Therefore, for the pH and doses we had examined, we were confident that at least in SEG cells the acid-induced DNA DSBs were primarily a direct effect of the acid and were not a consequence of apoptotic cell death.

**Acid Induces Intracellular Production of ROS**

Acid-induced production of ROS has been suggested to be the mediator of oxidative damage in esophageal cell lines. Therefore, we investigated whether incubation at pH 3.5 was inducing intracellular production of ROS in our cell lines and whether this might be responsible for the production of DSBs. Cells were preincubated with the cell permeable ROS detection dye DM-H2DCFDA, which fluoresces upon reaction with H2O2, HO·, or ROO· radicals, or ONOO⁻. Incubation of SEG cells at pH 3.5 led to a dose-dependent increase in
ROS-mediated fluorescence (Figure 2A). Confocal images of SEG cells incubated for 20 minutes at pH 3.5 showed that the production of ROS was diffuse and predominantly throughout the cytoplasm (Figure 2B). Similar dose-dependent increases in ROS production were observed in all cell lines (Figure 2C; *P* < .05 compared with corresponding control treatment). There was no particular trend in the level of ROS production when nondysplastic and high-grade dysplasia BE cells were compared with adenocarcinoma cells. Hence, the level of ROS production does not correlate with long-term survival (Figure 1C).

Pretreatment with the antioxidant NAC reduced acid-induced γH2AX fluorescence in SEG cells treated with acid for 15 minutes (Figure 2D). The mean increase in γH2AX fluorescence following 15 minutes at pH 3.5 was reduced from approximately 2.5-fold to 1.5-fold by pretreatment with NAC (Figure 2E; *P* = .01), indicating that acid-induced DSBs are largely mediated by ROS.

Because mitochondria are a rich intracellular source of ROS, we next determined whether disruption of the mitochondria might be the source of acid-induced ROS (Figure 2F). CCCP-treated positive control cells had depolarized mitochondria as indicated by the low fluorescence compared with the bright fluorescence of untreated control cells (Figure 2F, pink outline vs black histogram). In contrast, there did not appear to be any substantial loss of mitochondrial membrane polarization following incubation at pH 3.5 (Figure 2F, red and blue outlines). Thus, disruption of the mitochondria does not appear to be the source of the acid-induced intracellular ROS production. Next, we examined the ability of NO to induce DNA damage.

**In Vitro Production of NO From Nitrite**

To investigate the ability of NO to cause DNA damage to BE nondysplastic, high-grade dysplasia, and adenocarcinoma cells in vitro, we first determined the concentration range of NO produced from physiologi-
cally relevant concentrations of nitrite and AA at physiologic pH. Gastric juice AA concentrations as high as 2 mmol/L and salivary nitrite concentrations as high as 2 mmol/L have been reported. The addition of 100, 300, or 600 μmol/L sodium nitrite to acidified medium containing 2 mmol/L AA led to a dose-dependent production of NO (Figure 3A; P = 1 x 10^-6, P = 4 x 10^-8, and P = 6 x 10^-7 for pH 3.5, 3.0, and 2.5, respectively). Likewise, the concentration of NO produced from 600 μmol/L sodium nitrite added to acidified medium was dependent on the concentration of AA (Figure 3B; P = 7 x 10^-6, P = 4.0013, and P = 3 x 10^-6 for pH 3.5, 3.0, and 2.5, respectively). In contrast, addition of 600 μmol/L sodium nitrite to acidified medium without AA produced 0.6–1.9 μmol/L NO and undetectable levels when added to pH 7.4 medium containing 2 mmol/L AA (data not shown). Overall, the concentration of NO produced from physiologic levels of these 3 variables ranged from 11 to 279 μmol/L, which compares well with those measured in vivo at either the distal esophagus or at the gastric cardia.

We next examined whether concentrations of NO in the range from 25 to 200 μmol/L were sufficient to cause DNA damage to BE nondysplastic, high-grade dysplasia, and adenocarcinoma cell lines in vitro. Because exposure to acid alone can cause DNA damage, we used the chemical NO donor MAHMA NONOate, rather than nitrite at low pH, to investigate the ability of NO alone to induce DNA damage. This donor has a half-life of approximately 3 minutes at pH 7.4, and, thus, addition of the donor to cells in culture will replicate a burst of NO exposure as might be expected during reflux episodes. To confirm that NO was entering the cells in vitro, the NO-reactive fluorescent dye DAF-FM diacetate was used. The fluorescence of FLO cells preincubated with the dye and treated with 100 μmol/L MAHMA NONOate peaked at 20 minutes and decreased over time so that by 100 minutes it matched that of control cells (Figure 3C). Significant increases in fluorescence were seen across all cell lines but were best seen in QhTERT and FLO cells (Figure 3D). Both QhTERT and FLO cells showed a dose-dependent increase in NO-induced fluorescence when examined after 30-minute incubation with the indicated dose of MAHMA NONOate (Figure 3D; P = .04 and P = .01 for QhTERT and FLO, respectively). Confocal images of FLO cells treated with or without 100 μmol/L MAHMA NONOate for 20 minutes showed that the fluorescence was evenly distributed throughout the cell (Figure 3E).
NO Induces DNA DSBs

We next examined whether these concentrations of NO could induce DNA DSBs in BE nondysplastic, high-grade dysplasia, and adenocarcinoma cell lines in vitro. FACS histograms of SEG cells incubated with MAHMA NONOate for 45 minutes revealed a corresponding increase in the proportion of cells with higher fluorescence compared with isotype control antibody labelled cells (Figure 4A, red line vs black histogram), indicative of cells containing NO-mediated DSBs. Similarly, confocal images of SEG cells incubated with MAHMA NONOate revealed an increase in fluorescence in a proportion of cells exposed to NO (Figure 4B). Isotype control labelled cells did not show any γH2AX fluorescence (data not shown). Quantitative analysis of FACS data revealed a dose-dependent increase in γH2AX fluorescence up to 100 μmol/L MAHMA NONOate in all cell lines (Figure 4C and 4D). There was no further increase in the proportion of SEG cells with γH2AX when cells were treated with 500 μmol/L MAHMA NONOate compared with 200 μmol/L MAHMA NONOate (data not shown). For nondysplastic and high-grade dysplasia BE cell lines treated with 200 μmol/L NO, the maximum percentage of cells with DSBs was 30%–37% (Figure 4C) compared with 55%–60% in cancer cell lines (Figure 4D; P = .002).

To confirm the presence of DSBs in these cells, a neutral comet assay was performed with SEG cells after incubation with MAHMA NONOate for 45 minutes. Unlike the alkaline comet assay, which detects single- and double-strand breaks as well as alkali labile sites, the neutral comet assay only shows DSBs. Incubation with the NO donor resulted in the formation of comet tails in SEG cells, confirming that NO was indeed causing DSBs (Figure 4E).

The ability of NO to induce DSBs in primary BE cells was also tested. Confocal microscopy images of primary cells obtained from endoscopic brushings of BE segments showed NO-induced γH2AX in 2 of 10 patients tested (Figure 4F). Both undamaged and damaged nuclei were regular and oval in appearance, and the presence of condensed and irregularly shaped apoptotic nuclei were not observed in either control or treated samples.

NO Induces Transient DSBs in S-Phase Cells

Given that the concentrations of NO used caused DSBs in only a proportion of cells, we next costained anti-γH2AX-labelled cells with DAPI to investigate whether there was any cell cycle dependence of NO-induced damage. Two-dimensional plots of FACS analyses of SEG cells revealed that cells in the late G0/G1 and S-phase predominantly showed an increase in γH2AX.
following exposure to NO for 45 minutes (Figure 5A). In contrast, anti-\(\gamma\)H2AX labelling of cells in early G0/G1 and G2/M was mostly unchanged. In control samples, cells in G2/M show higher baseline levels of \(\gamma\)H2AX labelling than cells in G0/G1, a phenomenon that has been reported to reflect the higher DNA content of these cells.29

Incubation with up to 200 \(\mu\)mol/L MAHMA NONOate for 45 minutes did not affect long-term survival in either high-grade dysplasia BE or adenocarcinoma cells (Figure 5B), indicating that these concentrations of NO are not cytotoxic and suggest that the DSBs are repaired. Although QhTERT (nondysplastic) cells did not readily form colonies in the clonogenic assay, qualitative assessment of QhTERT cell cultures following exposure to NO revealed that they continued to grow normally (data not shown). The kinetics of NO-mediated \(\gamma\)H2AX in SEG cells is in keeping with this data (Figure 5C) because the loss of \(\gamma\)H2AX has been correlated with repair in the absence of cell death.34,35 Cells in S-phase show extensive \(\gamma\)H2AX 45 minutes after the addition of 100 \(\mu\)mol/L MAHMA NONOate, but, by 90 minutes, there is no increase compared with control cells (Figure 5C).

Western blotting confirmed that NO caused \(\gamma\)H2AX (Figure 5D). Control lysates displayed a basal level of \(\gamma\)H2AX at the predicted molecular size of approximately 15 kilodaltons, which was increased after just 15 minutes incubation with 100 \(\mu\)mol/L MAHMA NONOate and more noticeably in positive control cells that had been exposed to ionizing radiation or ultraviolet radiation (Figure 5D). This increased expression was maintained for a further 30 minutes but had begun to decrease 60 minutes after the addition of the NO donor. This is consistent with the kinetics of \(\gamma\)H2AX assessed by FACS (Figure 5C). A band of approximately twice the molecular weight of \(\gamma\)H2AX was also up-regulated by ionizing radiation, ultraviolet radiation, or NO and possibly represents \(\gamma\)H2AX doublets.

**Nitrite-Produced NO Induces Double-Strand DNA Breaks**

We next investigated whether replication of nitrite chemistry in vitro could induce DSBs. SEG cells were incubated at pH 3.5 or 7.4 with 2 mmol/L AA and 600 \(\mu\)mol/L nitrate or nitrite with or without PTIO (NO scavenger) for 5 minutes followed by 40 minutes in normal medium without AA, nitrate/nitrite, or PTIO. Cells treated with nitrite at pH 3.5 showed significantly increased \(\gamma\)H2AX compared with nitrate-treated cells at pH 3.5 (Figure 6A, \(P = 7 \times 10^{-6}\)) or nitrite-treated cells at pH 7.4 (\(P = 3 \times 10^{-4}\)). The NO scavenger PTIO reduced the number of cells with DSBs from 35% to 21% (\(P = .003\)). Similar to cells treated with the NO donor at pH 7.4, nitrite-induced damage occurred preferentially in S-phase cells (Figure 6B), suggesting that NO not acid-
induced ROS were responsible for DSB induction. The absence of any effect in the experiments performed at pH 3.5 with nitrate compared with pH 7.4 with nitrate is further evidence that this was mediated by NO not acid. This is consistent with the earlier studies in which acid-induced damage only became apparent at 15 minutes.

Discussion

In this study, we have shown for the first time that acid and NO induce double-strand DNA breaks in BE nondysplastic, high-grade dysplasia, and adenocarcinoma cells and thus may contribute to the genetic rearrangements in the progression from BE to EAC. DNA DSBs are critical lesions that can promote genomic instability and are the primary event in the creation of gross genetic abnormalities such as chromosomal translocations.36 There are several well-documented cases in which translocations are known to play an important role in cancer, including the Philadelphia chromosome in chronic myelogenous leukaemia.37 The progression from BE to EAC is accompanied by a number of genetic and chromosomal abnormalities including gene mutations, chromosomal translocations, and aneuploidy. However, it is unclear whether they are caused by a mutagen or are a consequence of abnormal growth regulation.

DSBs are formed through the action of exogenous agents, such as ionizing radiation, or endogenous agents, such as metabolically generated ROS. NO induced DSBs preferentially in S-phase cells in the esophageal adenocarcinoma cell line SEG, which is reminiscent of replication fork collapse that occurs as a consequence of a variety of DNA lesions in replicating cells.40–42 Similar findings were recently reported in human B cells treated with NO-releasing aspirin.43 Impaired processing of stalled replication forks to restart replication or failure to protect stalled forks can lead to accumulation of mutations and genomic aberrations. Significantly, it has been suggested that genome rearrangements in cancer are largely due to faulty chromosome replication.44 Although not examined here, it is likely that both acid and NO are also inducing other types of DNA damage including single-strand breaks, abasic sites, and oxidative and nitrosative base lesions such as 8-nitroguanine.27,45

In contrast to NO-induced damage, we found that acid-induced ROS production mediated a shift in fluorescence of the entire cell population (Figure 1A), suggesting that DSBs occurred in all cells regardless of cell cycle status. This was confirmed by examining γH2AX in relation to cell cycle status by costaining with DAPI (data not shown) and indicates that different mechanisms are responsible for NO- and acid-induced DNA damage in BE nondysplastic, high-grade dysplasia, and adenocarcinoma cells. With the exception of QhTERT cells, acid treatment did not dramatically reduce cell viability in the short-term, which is in keeping with the findings of others.9 The sensitivity of the QhTERT cell line possibly reflects its origin from nondysplastic BE,46 whereas the remaining cell lines are derived from high-grade dysplasia (ChTERT and GihTERT) or adenocarcinomas and thus have possibly already acquired greater resistance to acid-induced cytotoxicity. However, long-term survival data demonstrate that the lasting effects of acid are more
duced DSBs, indicating that ROS are responsible for acid-induced production of ROS. Furthermore, we demonstrated that antioxidant treatment inhibited acid-induced DSBs, indicating either insufficient inhibition of ROS or the presence of non-ROS mechanisms of DNA damage. The source of acid-induced intracellular ROS is unclear, although we and others have shown that damage to the mitochondria is unlikely to be responsible. Thus, our data would support the use of antioxidant therapy in preventing the progression of BE to EAC. However, because of the involvement of AA in the production of NO in the esophageal lumen, we would favor the use of antioxidants other than vitamin C. In fact, oral coadministration of nitrite plus AA in rats has been shown to induce tumors of the proximal stomach, and, in mice, intragastric administration of nitrite and AA induced DNA damage within gastric epithelial cells.

We used physiologic levels of nitrite, AA, and pH to measure NO levels that might arise in the esophageal lumen during reflux episodes. In agreement with other studies, the production of NO was dependent on both the concentration of AA and nitrite and also the pH. We recognize that our in vitro experiments did not take into account the dilution of these components, particularly nitrite, that would occur in vivo when saliva mixes with gastric juice; however, the concentration of nitrite used (600 μmol/L) was at least 2- to 3-fold lower than the highest levels reported for human saliva following a meal. Furthermore, in a study of 10 patients, esophageal luminal concentrations of NO up to 60 μmol/L have been detected within BE segments during episodes of reflux. We initially chose to use a NO donor to examine the ability of NO to induce DNA damage because acid was also found to induce DSBs, and we wanted to separate this from the damage caused by NO. To do so, we used a NO donor with a short half-life of several minutes, meaning that, within 15 minutes, >95% of the donor had degraded and released NO. We believe that such a pulsatile exposure would most closely mimic the production of NO in vivo during bouts of reflux. For the same reason, when replicating the luminal nitrite chemistry in vitro, we used a 5-minute pulse of acid plus nitrite before replacing with normal medium. As with acid treatments, phosphorylation of H2AX was assessed 45 minutes after the addition of NO because it has been previously reported that it is maximal 30–60 minutes after the generation of a DSB. Approximately 70 μmol/L NO was produced from 600 μmol/L nitrite under these conditions in earlier experiments (Figure 3A), and the percentage of cells with DSBs (35%) are similar to when an equivalent dose of the NO donor was used (Figure 4D). This approach more closely represents how epithelial cells at the luminal surface would be exposed to NO. The damage caused appeared to be a consequence of nitrite reduction rather than acid-induced ROS production because incubation with acid and nitrate did not induce DSBs. However, the NO scavenger PTIO only partially

![Figure 6. Nitrite-induced DSBs in Barrett’s esophagus nondysplastic, high-grade dysplasia, and adenocarcinoma cells. SEG cells were treated with 600 μmol/L nitrate or nitrite in the presence of 2 mmol/L ascorbate at pH 3.5 or 7.4 with or without PTIO, and γH2AX was detected by FACS (A). Data show mean percentage of positive cells ± SEM. Statistical analysis by Student t test, *P = 7 × 10^-6, #P = 3 × 10^-5, **P = .003. (B) 2-D FACS plots of γH2AX and DAPI-labelled SEG cells treated with 600 μmol/L nitrate or nitrite at pH 3.5.](image-url)
inhibited acidified nitrite-induced DSBs, suggesting that products other than NO could be involved, such as nitrous acid.

Significantly, exposure to short bursts of physiologic concentrations of NO induced DNA DSBs but did not affect long-term survival (Figures 4 and 5B). Similar concentrations of NO could be produced from exposure to acidified nitrite for 5 minutes (Figures 3A, 3B, and 6). In contrast, acid-mediated DSBs require exposure to pH 3.5 for >15 minutes (Figure 1), which also results in significantly reduced cell survival. Thus, luminal NO more so than acid may be important in the accumulation of DNA DSBs in the esophageal epithelium. Importantly, our data indicate that, at physiologic concentrations, NO can freely diffuse across the plasma membrane and accumulate throughout the cytoplasm and nucleus in epithelial cells grown in monolayers and that exposure to a single physiologic dose is sufficient to induce DNA damage without inducing cell death.

In summary, we have demonstrated that physiologic, luminal concentrations of NO can cause DNA damage in the form of DSBs in BE nondysplastic, high-grade dysplasia, and adenocarcinoma cells without loss of cell survival. In addition, we showed for the first time that acid can induce double-strand DNA breaks in these same esophageal cell lines and that this is mediated by intracellular production of ROS. Our data provide a mechanistic rationale for previously proposed hypotheses linking increased nitrate intake because of use of nitrogen-based fertilizers or economic status with trends in the incidence of EAC. This study also lends support to the idea that antioxidant therapy, other than vitamin C, may be an appropriate strategy in preventing the progression of BE to EAC.

References
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