Transition mutation in codon 248 of the p53 tumor suppressor gene induced by reactive oxygen species and a nitric oxide-releasing compound

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Exposing the human bronchial epithelial cell line BEAS-2B to the nitric oxide (NO) donor sodium 1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) at an initial concentration of 0.6 mM while generating superoxide ion at the rate of 1 µM/min with the hypoxanthine/xanthine oxidase (HX/XO) system induced C:G→T:A transition mutations in codon 248 of the p53 gene. This pattern of mutagenicity was not seen by ‘fish-restriction fragment length polymorphism/polymerase chain reaction’ (fish-RFLP/PCR) on exposure to DEA/NO alone, however, exposure to HX/XO led to various mutations, suggesting that co-generation of NO and superoxide was responsible for inducing the observed point mutation. DEA/NO potentiated the ability of HX/XO to induce lipid peroxidation as well as DNA single- and double-strand breaks under these conditions, while 0.6 mM DEA/NO in the absence of HX/XO had no significant effect on these parameters. The results show that a point mutation seen at high frequency in certain common human tumors can be induced by simultaneous exposure to reactive oxygen species and a NO source.

Introduction

Nitric oxide (NO) is produced by nitric oxide synthases (NOS), which catalyze the conversion of arginine to citrulline (1). The physiological effects of NO are characterized by their apparent dichotomy: in some cases it is a regulatory molecule, whereas in others it has toxic effects (2,3). During inflammation, simultaneous production of reactive oxygen species (ROS) and NO has been proposed to play a part in cytotoxicity, given that NO and superoxide (O2−) can rapidly interact to form the strong oxidant peroxynitrite (ONOO−) (k = 6.7×109/M/s) (4,5). Such species seem to be implicated in bactericidal and tumoricidal properties of activated macrophages (6–9).

In addition, clinical studies have shown that inducible nitric oxide synthase (iNOS) activity is increased in certain human diseases, such as colon cancer (10), ulcerative colitis and Crohn’s disease (11). However, it is not yet known which role NO plays in the development of these pathologies. Nevertheless, chronic inflammation, such as due to Helicobacter pylori infection, liver fluke infection and ulcerative colitis, has been implicated in carcinogenesis (12–14).

In chronic inflammation, epithelial cells are potential targets of the reactive species produced by the inflammatory cells. Therefore, we studied the effects of ROS and NO on human epithelial cells, using the NO donor sodium 1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO) and the superoxide/hydrogen peroxide-producing system hypoxanthine (HX)/xanthine oxidase (XO) (15). The interaction between ROS and NO was characterized by determination of superoxide and urate production, soluble guanylyl cyclase stimulation and dihydrorhodamine oxidation. The biological effects of our system were described by measuring lipid peroxidation, DNA fragmentation and mutagenesis, which are ROS-induced cellular effects (16–20).

Here we provide evidence that, in contrast to our observations with DEA/NO alone (21), co-generation of NO and superoxide can induce mutations (specifically C:G→T:A transitions at position 1 of codon 248) in the p53 gene of human cells in culture.

Materials and methods

Cell line

The biological experiments were performed using BEAS-2B cells, a human bronchial epithelial cell line transformed by SV40 large T antigen (22). They were cultured in LHC-8 serum-free medium (23) (Biofluids, Rockville, MD) in an atmosphere containing 3.5% CO2 at 37°C in dishes coated with 10 µg/ml human plasma fibronectin (Upstate Biotechnology, Lake Placid, NY), 20 µg/ml bovine serum albumin (Sigma, St Louis, MO) and 20 µg/ml collagen (Vitrogen 100; Celtrix, Santa Clara, CA).

Cell treatment

The effects of ROS were studied using the HX/XO system at the following concentrations: 300 µM HX (Sigma) and 0.006 U/ml bovine XO (Roche Molecular Biochemicals, Rotkreuz, Switzerland). The reversion of HX/XO-induced cytotoxicity by addition of 78 U/ml bovine catalase (Roche Molecular Biochemicals) and 30 U/ml human superoxide dismutase (SOD) proved that the observed effects were due to ROS.

The effects of NO were investigated using the NO donor DEA/NO (Chemical Abstracts Registry no. 92382-74-6), which releases 1.5 molecules of NO per DEA/NO anion when dissolved in aqueous medium. It is a member of the diaziniumdioxide (or NONOate) family, stable compounds in alkaline solution which have the property to release NO at pH 7.4 without any additional activation (24).

DEA/NO decomposition measurement

The decomposition of the NO donor DEA/NO in LHC-8 medium was followed spectrophotometrically at 250 nm (ε250 nm = 6.5/M/cm) (25), using a Beckman DU-64 spectrophotometer.

Xanthine oxidase activity estimation

The XO activity, and thus the efficiency of the ROS-producing system, was estimated using two products of the HX/XO system, superoxide and urate (26). Standard conditions in all of the experiments reported here employed initial concentrations of 300 µM HX and 6 mL/ml XO. Superoxide production was followed spectrophotometrically measuring horse ferricytochrome c (Sigma) reduction to ferrocyanochrome c at 550 nm (ε550 nm = 21/M/cm) (27,28) in the absence or presence of bovine SOD (Roche Molecular

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Abbreviations: DEA/NO, sodium 1-(N,N-diethylamino)diazen-1-ium-1,2-diolate; DHR 123, dihydrorhodamine 123; ENU, ethylnitrosourea; fish-RFLP/PCR, ‘fish-restriction fragment length polymorphism/polymerase chain reaction’; HPO, hydroperoxides; HX, hypoxanthine; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; NO, nitric oxide; NOS, nitric oxide synthases; RH 123, rhodamine 123; ROS, reactive oxygen species; SOD, superoxide dismutase; XO, xanthine oxidase.

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Biochemicals, Mannheim, Germany) in order to determine the superoxide-specific reduction of cytochrome c. This assay was carried out in the presence of 20 μM ferricytochrome c. Urate formation was monitored by UV absorbance at 305 nm, in the absence or presence of porcine uricase (Roche Molecular Biochemicals) to determine the uric acid-specific absorbance. These assays were performed in cell-free culture medium at 37°C, using a Beckman DU-64 spectrophotometer.

**Soluble guanyl cyclase stimulation assessment**

In order to determine the fate of NO in the presence of oxygen radicals, we studied the stimulation of soluble guanylyl cyclase. cGMP accumulation was measured after cells were exposed to DEA/NO, in the absence or presence of HX/XO, using a radioimmunoassay (29). BEAS-2B cells were plated in 12-well dishes (100,000 cells/well) and exposed for 10 min to 5 μM DEA/NO ± HX/XO, in the presence of 0.5 mM isobutylmethylxanthine (Calbiochem, La Jolla, CA). The treatment was stopped by addition of cold ethanol (−20°C, final concentration 66%). The cells were then collected, frozen in liquid nitrogen and evaporated to dryness under vacuum. The pellet was resuspended in 250 μl of 50 mM sodium acetate buffer (pH 4), acetylated for 10 min with acetic anhydride/triethylamine (2.1 μl per 100 μl buffer) and tested for cGMP using anti-cGMP antibodies (Meloy Laboratories, Springfield, VA).

**Dihydrorhodamine 123 oxidation**

The oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine 123 (RH 123) was followed spectrophotometrically at 501 nm (ε<sub>501</sub> nm = 78.78 mM/cm) (30, 31). DEA/NO and HX/XO ± DEA/NO were assayed in 1 ml of LHC-9 buffer containing 50 μM DHR 123 and 100 μM diethylenetriaminepentaacetic acid at 37°C using a Beckman DU-64 spectrophotometer.

**Mutation detection**

The mutagenicity of our system was investigated using the previously described "fish-restraction fragment length polymorphism/polymerase chain reaction" (fish-RFLP/PCR) method (21) to detect mutations within codon 248 (CGG) of the p53 tumor suppressor gene, which has been described as a preferential target in many human tumors (32). BEAS-2B cells were grown to 60% confluence and treated with HX/XO ± DEA/NO for 24 h; as a positive control, they were exposed for 20 min to 4 mM ethylendiaminetetraacetic acid (EDTA), which has been shown to induce G→A transitions within codon 248 of the p52 gene (21). The cells were harvested 72 h later. The genomic DNA was extracted, purified and submitted to the fish-RFLP/PCR method as previously described (21). Briefly, a DNA aliquot of 200 μg was denatured in 0.2 M NaOH (5 min at 65°C), then the solution was neutralized by simultaneous addition of 0.2 M HCl and 100 mM HEPES (pH 7.4). Then SP6-biotinylated exon VII antisense RNA was added at a final concentration of 0.08 mg/ml. SP6 exon VII antisense RNA was synthesized from plasmid pSP72-X7, in which the entire exon VII of p53 (110 bp) has been inserted into pSP72 (Promega, Madison, WI). Hybridizations were carried out for 16 h (65°C) then 5 μl of streptavidin-agarose (Sigma) was added. The mixture was incubated for 45 min at room temperature. Agarose beads were spun in a microfuge and washed with 10 mM HEPES (pH 7.4) containing 1 mM EDTA. Purified DNA was eluted in 0.2 M NaOH to hydrolyse the RNA. After neutralization with 100 mM Tris (pH 7.4) plus 0.2 M HCl and addition of yeast tRNA (0.5 mg/ml), the samples were phenol extracted and precipitated in silanized tubes with 3 vol ethanol. The purified DNA was resuspended in 10 mM Tris (pH 9), 3 mM MgCl<sub>2</sub> and 0.3 mM deoxynucleotide triphosphate (dNTP) containing CTTGCCACAGGTCTCCCCAA and AGGGTGTCAGCGCCACACAGA (0.4 μM) as 5' and 3' primers, respectively, and amplified for five cycles (94°C for 30 s, 60°C for 1 min and 78°C for 30 s) using 0.5 μl of T7 polymerase (Stratagene, La Jolla, CA). The samples were then digested with MspI (30 min at 37°C). This PCR/restriction step was repeated once again and the samples were amplified for 20 cycles under the same conditions as described above. Following a third digestion with MspI the samples were amplified for 25 cycles using AGGGTGTCAGCGCCACACAGA and TGTGCCAGGTGTCGAAGTC as 5' and 3' primers, respectively. The 146 bp fragment obtained was purified after electrophoresis on agarose gel and sequenced using exon-Pro (Stratagene) following the manufacturer’s instructions.

Cell survival after HX/XO + DEA/NO exposure was 60%. Since incubation of the cells with spin-coated HX/XO + DEA/NO resulted in no cytotoxic effect, mutagenesis was not investigated under this condition.

**DNA fragmentation analysis**

Cells treated at 60% confluence for 24 h were tested for DNA single-strand fragmentation using the alkaline unwinding method (33, 34), which is based on the partial denaturation of DNA containing single-strand breaks.

**Lipid peroxidation evaluation**

The level of lipid peroxidation was determined using a colorimetric assay (Lipid Peroxidation Assay Kit; Calbiochem) to detect malondialdehyde (MDA) and hydroperoxides (HPO), which are degradation products of lipid peroxides. Cells were treated for 24 h when 60% confluent. Under such conditions, <10% cytotoxicity was found. After treatment, the cells were pelleted by centrifugation, washed with phosphate-buffered saline and resuspended in 20 mM Tris–HCl (pH 7.5). Following lysis by freezing/thawing, cellular extracts were tested for lipid peroxidation and protein content (BCA Protein Assay; Pierce, Rockford, IL). The peroxidation assay was performed following the manufacturer's instructions, in the presence of 1% Lubrol (ICN, Costa Mesa, CA).

**Results**

**NO/ROS interaction**

In order to verify the efficiency of the ROS-producing system, XO activity was estimated using two products of the HX/XO system, superoxide and urate (26). As expected, an increased reduction of cytochrome c was observed in the presence of HX/XO (Figure 1A), proving that this system produced superoxide. The initial production of O<sub>2</sub>− was estimated to be 1.076 ± 0.006 μM/min (Table I). Urate production followed similar kinetics (data not shown). The addition of DEA/NO to the HX/XO system decreased superoxide-induced cytochrome c reduction >10-fold (Figure 1A and Table I); this inhibition was nearly totally during the first 15 min, which corresponds to the period when >90% of the DEA/NO decomposed as measured spectrophotometrically (24). Since these data suggest that superoxide had interacted with NO, we confirmed the availability of NO in the presence of ROS by determining the stimulation of soluble guanylyl cyclase (29), a well-known enzymatic target of NO. Although 5 μM DEA/NO was able to increase cGMP production in BEAS-2B cells 5-fold, the addition of HX/XO reduced this DEA/NO-induced stimulation to a low value (Figure 1B), meaning that NO had been scavenged before reaching its enzymatic target. The inhibition of DEA/NO-induced guanylyl cyclase stimulation by the HX/XO system supported the hypothesis that NO interacted with superoxide. To determine the generation of reactive nitrogen species, the oxidation of DHR 123 in the presence of diethylenetriaminepentaacetic acid was used. DHR 123 was found to be oxidized by HX/XO at an initial rate of 0.013 ± 0.001 μM/min. The addition of DEA/NO to HX/XO increased DHR 123 oxidation (Figure 1C), which then occurred with an initial rate of 0.131 ± 0.017 μM/min (Table I). The 12-fold decrease in analytically detectable superoxide production observed by incubation with HX/XO and DEA/NO (relative to the rate in the absence of DEA/NO) corresponded to a 10-fold increase in DHR 123 oxidation, indicating the formation of a new species.

**Mutagenesis**

The fish-RFLP/PCR technique developed to detect mutations in codon 248 of the p53 gene is based on two-step enrichment of a mutant target. In the first step the target gene is extracted from total genomic DNA and in the second the mutated sequence is amplified by coupled RFLP/PCR. This allows the detection of mutants from a large amount of DNA that would not fit in a standard PCR assay. The sensitivity of the assay is 1 base in 10<sup>6</sup> (21). The short period of cell growth before collection (72 h) does not allow clonal expansion of cells expressing mutated p53 since one cell division occurs.

To test whether ROS and NO were able to induce mutations in codon 248 of the p53 gene, we applied the fish-RFLP/PCR...
Lipid peroxidation is a well-known effect of ROS. Accordingly, the level of lipid peroxide metabolites (MDA and HPO) was increased ~1.25-fold when the cells were treated with HX/XO (Figure 4). The addition of DEA/NO resulted in a dose-dependent increase in the HX/XO effect, while DEA/NO alone had no effect per se (Figure 4). The basal level of MDA + HPO found in our cells was 0.99 ± 0.67 nmol/mg protein (mean ± SD, n = 30), which is consistent with values obtained in other studies (35–37).

**Discussion**

Nitric oxide is a key regulatory molecule which mediates many physiological processes, such as vasodilation, bronchodilation, neurotransmission and platelet aggregation (2). It is also a well-known toxic agent as well as a constituent of air pollution and cigarette smoke. Endogenous NO seems to be implicated in some human diseases, such as atherosclerosis (38), neuro-degenerative diseases and cancer (39). In particular, NO takes part in chronic inflammation. Indeed, NO is produced by the
Fig. 3. Influence of DEA/NO on HX/XO-induced DNA strand breakage. 
(A) DNA single-strand breaks were measured in cells treated for 24 h with HX/XO ± DEA/NO. Data are expressed as means ± SEM (n = 4–12) of the difference between treated and untreated cells in the percentage of damaged DNA found. Student’s t-test was used to compare the effects of the different treatments (*P < 0.05, ††P < 0.01 relative to untreated cells). 
(B) ELISA detection of HX/XO-induced double-strand breaks in the absence or presence of DEA/NO. The DNA double-strand breakage was evaluated in BEAS-2B cells exposed for 24 h to HX/XO ± 600 μM DEA/NO in LHC-8 medium. Results are expressed as a ratio of double-strand fragmentation observed in treated cells versus control and represent means ± SEM (n = 12–16). Student’s t-test was used to compare the effects of the treatments (*P < 0.05, **P < 0.01).

Fig. 4. Modulation of HX/XO-induced lipid peroxidation by DEA/NO. Lipid peroxidation was determined in BEAS-2B cells exposed for 24 h to HX/XO ± DEA/NO. Results are expressed as the ratio of lipid peroxidation [nmol (MDA + HPO)/mg protein] observed in treated cells versus control and represent means ± SEM (n = 9–30). Statistical analysis was performed using Student’s t-test to compare results obtained after different treatments (*P < 0.05).
increase in 5-hydroxy-2'-deoxycytidine (55), which has also been shown to induce C→T transition mutations (56). The G→C transversion observed in the second position of codon 248 has already been described in human fibroblasts exposed to hydrogen peroxide and FeCl$_3$ (57). The addition of DEA/NO to HX/XO leads preferentially to C→T mutation and it is possible that NO increases the damage induced by oxidative attack on 5-methylcytosine (52), for example by limiting its repair.

To test the hypothesis that NO might serve as an endogenous mutagen, we developed a human cell system where NO was either endogenously produced or generated by DEA/NO (21). Using two different approaches, the fish-RFLP/PCR genotypic assay and a phenotypic mutation assay, we have shown that NO per se is not detectably a mutagen in cultured human bronchial cells (21). Similar results have been observed in another study where human iNOS cDNA was constitutively expressed in V79 Chinese hamster cells. Cells expressing iNOS had no increase in the frequency of hprt mutations compared with parental cells (58). In the present study, we have evidence that the interaction of NO and superoxide is able to induce mostly G:C→A:T mutations, while superoxide induces three different mutations. The mutant fraction can only be estimated based on the sensitivity of the system, whose detection limit is 1 base in 10$^6$ (21). We concluded that NO significantly increases C→T transitions in the first base of codon 248 of the p53 gene.

Several studies have attempted to define the 'in vivo' role of NO in the mutagenesis process resulting from inflammation. To mimic inflammation conditions under which tissues are exposed to different reactive oxygen species released by inflammatory cells, stimulated macrophages have been co-cultured with cells expressing a β-galactosidase reporter plasmid. These experiments demonstrated that inhibition of NOS decreases DNA damage (59). In addition, in a lacZ transgenic mouse model, developed to assess the mutagenic effect of inflammation, the lacZ gene mutation frequency due to inflammation was twice the mutation frequency observed in controls and such an increase was abolished by administration of the NOS inhibitor N-methyl-L-arginine (60), suggesting a role for NO in the mutagenesis process. In another recent study, an increased hprt mutation frequency dependent on iNOS activation was detected in macrophages stimulated to produce NO (61). Furthermore, NO exposure is mutagenic to *Salmonella typhimurium* TA1535 (46,62,63), where the major mutations found are C:G→T:A transitions.

On exposure of the cells to HX/XO and DEA/NO, C:G→T:A mutations could form by decreasing the endogenous defense provided by endogenous sulfhydryls, which are scavengers of nitrosating species. Indeed, NO alone has been shown to be mutagenic in TK6 cells (64); this may be due to the fact that TK6 cells have a low glutathione level (65) compared, for example, with NO-resistant V79 cells (66). On the other hand, in the presence of NO and O$_2^-$ there may be formation of new reactive species. In stimulated macrophages, O$_2^-$ precedes (67) or accompanies (68) NO formation and induced DNA damage can be totally suppressed by a NOS inhibitor, suggesting that only a NO metabolite is involved in genotoxicity. It had already been demonstrated that phagocytes from control patients, but not from granulomatous disease patients deficient in superoxide production, are mutagenic in *Salmonella* (69), indicating a role for superoxide. NO and O$_2^-$ can rapidly interact to form the strong oxidant peroxynitrite (4,5). The latter was previously shown to induce lipid peroxidation (70), DNA damage (49,71–77) and cell death (7,77,78). Nitric oxide, or rather its derivatives such as ONOO$^-$, has been implicated in many forms of DNA damage, such as base modifications and DNA strand breakage (79). Peroxynitrite or simultaneous production of superoxide and NO, either by SIN-1 or by activated macrophages, have been shown to induce DNA single-strand breaks (71,80). In our system, DEA/NO potentiated HX/XO-induced single-strand breakage. Different mechanisms may be responsible for this effect, including direct modification of DNA or inhibition of DNA repair enzymes (59,81). In addition to single-strand breaks, we observed DNA double-strand breakage in BEAS-2B cells treated with HX/XO + DEA/NO. This double-stranded fragmentation could result from single-strand breaks (59) or from activation of endonucleases during apoptosis. The latter phenomenon was observed in cells surrounding activated macrophages (82). It was reported that acute/intense exposure to ONOO$^-$ induced cell necrosis, whereas mild/less severe exposure to this species induced apoptosis (77). Under our conditions, DNA fragments were found in both the cellular fraction and the supernatant (data not shown), suggesting that a necrotic phenomenon occurred, perhaps as a consequence of absence of phagocytosis of apoptotic cells, as proposed by others (78).

Finally, in our system, NO potentiated ROS-induced lipid peroxidation. We do not yet know the mechanism involved in this pro-oxidant activity of NO, although DEA/NO alone did not induce lipid peroxidation, suggesting that reaction with O$_2^-$ was necessary. A possible explanation is the formation of H$_2$O$_2$ from ONOO$^-$ resulting from the presence of HEPES in LHC-8 medium. Indeed, LHC-8 contains ~23 mM HEPES and this tertiary amine has been shown to interact with peroxynitrite, leading to formation of H$_2$O$_2$ (83). However, in the presence of 20 mM HEPES only 5–13% of the total peroxynitrite is converted into H$_2$O$_2$ (83,84), the other part remaining as reactive nitrogen/oxygen species. On the other hand, ONOO$^-$ has been demonstrated to cause lipid peroxidation (70). DEA/NO-induced potentiation of lipid peroxidation could enhance DNA strand breakage since lipid peroxide derivatives were found to cause DNA single-strand breaks (85,86). In addition, peroxidation could lead to etheno-adducts which may lead to mutations (48) such as C:G→T:A, as observed in this study.

Altogether, our results suggest that NO may be a component of inflammation-induced carcinogenesis.

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References


p53 mutations from co-generation of NO and O2−


