Mutagenesis induced by the nitric oxide donor sodium nitroprusside in mouse cells

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Nitric oxide (NO) is an important bioactive molecule derived from endogenous or exogenous sources. NO can exhibit genotoxicity through the formation of reactive nitrogen species. Nitric oxide releasing compounds, such as sodium nitroprusside, are widely used for the therapy of hypertension and other disorders. Here we have characterized the mutagenicity of sodium nitroprusside in mouse embryo fibroblasts carrying the cII mutation reporter gene. Sodium nitroprusside dose-dependently increased the cII mutant frequency to levels ~5-fold above background. The mutational spectrum induced by sodium nitroprusside was characterized by an increase in the fraction of G→T transition mutations (P < 0.003) but the proportion of transition mutations was not increased. We discuss the potential origin of the G→T mutations induced by this compound in mammalian cells.

Introduction

Nitric oxide (NO) has become one of the most important molecules in the area of biological and medical investigation in recent years (1–3). NO is a ubiquitous, physiologically active molecule involved in many biological functions and disease states including vasodilation, endotoxic shock, sexual function, neurotransmission, cerebral ischemia and inflammation (1–3). During an inflammatory response, inducible nitric oxide synthase (iNOS) is upregulated and NO is produced in large quantities in macrophages, Kupffer cells, hepatocytes and other cells (3,4). Reactive oxygen and nitrogen species produced during inflammation participate in the elimination of infectious agents. Beckman (5) and Schmidt et al. (6) have suggested that NO has a double-edged role in specialized tissues and cells. This means that NO is not only an important bioregulatory agent but may also be an endogenous cytotoxin, mutagen and/or carcinogen. The reactive agents released during an inflammatory response can damage tissues and cells and cause the formation of protein and DNA adducts in the inflamed areas. There are clear associations between chronic inflammation and cancer risk, for example for Helicobacter pylori infection and gastric cancer (7) and ulcerative colitis and colon cancer (8–10).

In the presence of oxygen, NO forms reactive nitrogen species (N₂O₃ and peroxynitrite, ONOO⁻) that can lead to deamination and oxidation of DNA bases, respectively (11,12). Several reports have indicated that NO is mutagenic in vitro. Exposure to low concentrations of NO results in significantly enhanced mutation frequencies in Salmonella typhimurium strain TA1535 (13). NO-releasing compounds, including spermine-NO complex, Na₂O₂N₂ (NET2) and glyceryl trinitrate, are mutagenic in S. typhimurium strain TA1535 and almost all of the analyzed mutants contained C→T transitions in the hisG46 (CCC) target codon consistent with a cytosine deamination mechanism (12,14). Mutagenicity of NO has also been shown in mammalian cells. When TK₆ human lymphoblastoid cells were treated by directly introducing NO gas into the medium, cells were mutated at both the HPRT and TK loci, DNA strand breaks were induced and purine bases were deaminated in a concentration dependent manner (15).

Sodium nitroprusside (SNP) is an NO-releasing compound that has been used as an anti-hypertensive agent since the 1920s (6). Although the data are very limited, it has been shown that long-term use of nitrates as donors of nitric oxide has the potential to induce genotoxicity as assessed by measurement of micronucleated lymphocytes (16). In previous laboratory studies, SNP has been shown to cause DNA strand breaks (17), and to cause a dose-dependent increase of the mutant frequency at the gpt locus in g12 cells (18) and at the HPRT locus in MN-11 cells (19). However, the exact nature of the mutations induced by SNP in mammalian cells has not yet been analyzed. In this report, we have determined the mutagenic capacity and mutational specificity of sodium nitroprusside using the BigBlue® transgenic mouse mutation assay system with the cII gene as the mutational target.

Materials and methods

Cell culture and SNP treatment

Early-passage embryonic fibroblasts of BigBlue® mice were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SNP was obtained from Aldrich Chemicals (St Louis, MO). After the cells reached 70% confluence, they were treated with various doses of SNP for 24 h. After the treatments, the medium was removed and replaced with complete growth medium. The cells were grown for an additional 8 days for use in mutation analysis. Cells were passaged once at Day 4 after completion of the treatment.

Measurement of nitrite concentration

Nitrite (NO₂⁻) is a relatively stable product of more reactive nitrogen intermediates that can be readily measured chemically. To quantify the amount of NO released by SNP, NO₂⁻ was spectrophotometrically determined at 550 nm according to the Griess reaction (20) with a calibration curve using known concentrations of sodium nitrite (Figure 1). The measurements were performed at different incubation times (1, 4, 12 and 24 h) with different concentrations (0, 100, 300 and 500 µM) of SNP. Results were expressed as concentration of NO₂⁻.

cII mutant frequency analysis

The cII mutant frequency was quantified by using the λ Select-cII Mutation Detection System for BigBlue® Rodents (Stratagene, La Jolla, CA). The assay system is based on the ability of the λ phage to multiply either lytically or lysogenically in Escherichia coli host cells (21). The commitment of the λ phage to lysis or lysogeny upon infection of the host is regulated by a chain of events, of which cII transcription is a determining event (22). The cII protein activates the transcription of cI repressor and λ integrase, both of which obligate the phage to undergo lysogenization. Only λ LIZ shuttle vectors...
amplified by PCR with two primers (5′ cII under- goes lysogenic growth, but phage with mutant positions 50 and 5′ of the G1250 strain were mixed with 1:1000 dilutions of DNA disgust cII genes undergo lytic growth and give rise to plaques. When incubated at 37°C, non-cII mutants also undergo a lytic cycle. The cII mutant frequency was calculated by dividing the number of mutant plaques by the calculated total number of plaques.

**cII mutational spectrum analysis**

For sequencing analysis, mutant plaques were selected and replated at low density to verify the mutant phenotype and to isolate plaques. Single well-isolated plaques were picked, placed into 25 μl of Tris–EDTA buffer and boiled for 5 min. A 433-bp segment containing the cII gene and flanking regions was amplified by PCR with two primers (5′-CCACACCTATGTTGATTG-3′; positions +345 to +365). The PCR products (100 ng) were sequenced with a Big Dye terminator cycle sequencing kit (ABI Prism, Applied BioSystems, Foster City, CA) on an ABI DNA sequencer. Each mutation was confirmed by sequencing terminator cycle sequencing kit (ABI Prism, Applied BioSystems, Foster City, CA) on an ABI DNA sequencer. Each mutation was confirmed by sequencing.

In this experiment it was shown that the NO₂ concentration increased in an SNP concentration- and time-dependent manner.

**SNP-induced mutagenesis**

We determined whether SNP induced specific mutations in the cII gene of mouse embryo fibroblasts carrying a λ transgene array (BigBlue® mice). The cells were treated with incremental concentrations of SNP (100, 300 and 500 μM) for 24 h and then were allowed to grow for mutation fixation for an additional 8 days. Cell survival as determined by trypan blue staining was 50% at the highest concentration of SNP used. After 8 days, the cells were analyzed to determine the mutant frequency and the mutational spectrum of the cII gene.

Induction of mutations in the cII gene by SNP increased dose- dependently, although the increase was not linear. The induced cII mutant frequency was 25.30 × 10⁻⁵ at the highest dose of SNP (500 μM), showing an ~5-fold increase in the number of mutants relative to control (5.28 × 10⁻⁵) (Table I).

To analyze the mutational spectra, we sequenced the DNA isolated from the verified mutant cII plaques induced by SNP at 500 μM (number of sequenced plaques = 158) and derived spontaneously in the control group (number of sequenced plaques = 151) for the entire length of the cII gene. In both spectra, we observed three 'jackpot' mutations at nucleotide positions 179–184 (G insertion/deletion), 211 (G→C transversion), and 221 (T→G transversion). These jackpot mutations, already found in the cII gene in previous studies by us and by others (25–29), are assumed to occur in the early development of the transgenic rodent by unknown mechanisms and to undergo clonal expansion such that many cells from a single tissue harbor the same type of mutation. Therefore, it is appropriate to exclude these jackpot mutations from the comparative mutation spectra analysis. The jackpot mutations accounted for 27% of the spontaneous and 13% of the SNP-induced cII mutations. The remaining non-jackpot mutations were predominantly single-base substitutions, which constituted 74 and 84% of the spontaneous and SNP-induced cII mutations, respectively. The λ-based mutation detection system does not allow the recovery of large deletions. In the case of spontaneous mutations, the most frequent mutations were G→A transitions (35%) and A to G transitions (15%). For SNP-induced mutations, G to A transitions (29%), A→G transitions (17%), and G→T transversions (27%) occurred predominantly (Figure 2). However, only the percentage of G→T transversions was statistically significantly different from the control (P < 0.003; χ² test). When one considers the absolute mutant frequencies between the control cells and the SNP-treated cells, other types of mutations were also increased. Figure 2A shows that the absolute mutant frequencies are increased, in order of the highest-fold increase, for G→T transversions, A→G transitions, A→C transversions, G→A transitions, and the relatively rare A→T mutations and

**Results**

**NO₂ formation**

Nitrite (NO₂) concentrations were determined in order to reflect the production of NO after exposure to SNP. NO₂ is the product of oxidation of NO (23), so the increase of NO₂ concentration can be regarded as an indicator of the production of NO (18,24). An increase in NO₂ concentration in the medium was observed after exposure to a single concentration of SNP with increase of time (1, 4, 12 and 24 h). A dose-dependent increase in NO₂ concentration was also observed within the dose range of 100, 300 and 500 μM of SNP. In this experiment it was shown that the NO₂ concentration increased in an SNP concentration- and time-dependent manner.

![Fig. 1. Production of nitrite after exposure of cells to SNP. Mouse embryo fibroblasts were treated for different periods of time with the indicated concentrations of SNP. Production of nitrite is indicative of the release of nitric oxide from SNP.](image-url)
Fig. 2. Types of mutations induced by the NO donor SNP at the cII locus in transgenic mouse embryo fibroblasts. Mouse embryo fibroblasts were exposed to 500 μM SNP for 24 h. Mutations induced at the cII gene after a growing period of 8 days were analyzed by DNA sequencing. White columns, mutations in untreated (control) cells; black columns, mutations in SNP-treated cells. In this analysis, recurrent jackpot mutations at positions 179–184, 211 and 221 were excluded. (A) absolute mutant frequencies; (B) relative frequencies (percentage) of each type of mutation.

deletions were also increased. The absolute frequency of G → C transversions remained unchanged.

Discussion

The mutations induced by nitric oxide delivered directly as a gas (predominantly A → G) differ from those induced by NO-releasing chemicals (predominantly G → A and G → T) in supF mutation assays (30,31). Nitric oxide is known to cause DNA damage by two major pathways, deamination and base oxidation. The deamination of bases (12,15) is promoted by the powerful nitrosating agent N2O3 formed from NO and molecular oxygen. Base deamination will yield hypoxanthine from adenine, xanthine and oxanosine from guanine, uracil from cytosine, and thymine from 5-methylcytosine. All of these deamination reactions are mutagenic. For example, adenine deamination can lead to A → G transition mutations, a type of mutation that was increased in SNP-treated cells. There is some in vitro evidence that xanthine, a deamination product of guanine, can base-pair with thymine, guanine, or adenine, as well as, cytosine (32,33). Mis-pairing of xanthine with adenine, or insertion of adenine opposite an apurinic site resulting from loss of xanthine, could account for the G → T mutations observed. However, recently it has been shown that 2′-deoxyxanthosine is rather stable in DNA at physiological conditions (34,35). The guanine moiety of DNA reacts with NO to yield another deamination product, oxamine (Oxa), although the extent of its formation is not clear (36,37). Oxa can react further with polyamines to form cross-linked adducts, for example spermine-Oxa (Oxa-Sp). Oxa-Sp is a strong block to DNA synthesis and can elicit G → T transversions (38).

In a recent study, exposure of cells to NO led to relatively small increases in deaminated bases and Oxa was not detected (39). However, since SNP may lead to DNA lesions other than base deamination, the mutations observed could also be the result of processing and polymerase bypass of these other lesions.

The combination of NO and superoxide, O2 −, produces the reactive molecule ONOO−, peroxynitrite. Peroxynitrite decomposition and DNA damage chemistry is highly complex due to dependence on pH and carbon dioxide concentrations. This chemistry has been reviewed comprehensively (40,41). The peroxynitrite pathway will predominantly lead to oxidation of guanine. The reaction of ONOO− with dG produces several primary products including 8-nitro-2′-deoxyguanosine (8-nitro-dG), which can depurinate to yield an abasic site, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), 5-guanidino-4-nitroimidazole, and 2,2-diamino-4-[(2-deoxy-β-D-erythro-pentofuranosyl)aminol]-5(2H)-oxazolone (oxazolone). 8-oxo-dG efficiently undergoes further oxidation to give a range of products including, for example spiropyrinimidohydrantoin (Sp) and dehydroguanidino-hydrantoin (DGH) (41). Several of these dG-derived products are known or have the potential to cause G → T transversions, including 8-oxo-dG, 8-nitro-dG (after depurination), and oxazolone (40).

SNP generates reactive oxygen species (ROS) during the redox cycling of nitroprusside (42), and it is converted to a number of products, such as nitrite, NO, iron, cyanide, and oxygen-free radicals including superoxide radical and hydroxyl radical (42). Nitrite is not likely to be mutagenic at the concentrations and pH used here (31,43). It is plausible that the observed G → T transversions predominantly induced by SNP may be derived from oxidation of guanine residues in DNA. Peroxynitrite (ONOO−) is a potent oxidizing agent formed from reaction of NO and superoxide radicals and reacts almost exclusively with dG residues (44). In mutagenesis experiments using the supF system, ONOO− induced mainly G → T transversions (45,46). The latter pathway would be most consistent with the mutations induced by SNP in our mammalian cell system. It has also been postulated that a product of the reaction between nitric oxide and intracellular glutathione, such as GSNO or some species derived from it, may underlie the promutagenic activity of SNP (24). Finally, oxygen-free radicals may be produced from SNP by low molecular weight reducing agents (42) and, without the involvement of NO, could be directly responsible for oxidation of dG and dT leading to an increase of mutations at G/C and A/T base pairs. Clearly, further studies are required to
identify the reactive intermediates and the type of DNA damage involved in SNP mutagenesis.

Acknowledgement

This work was supported by NIH grant CA084469 to G.P.P.

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Received on August 23, 2006; revised on September 22, 2006; accepted on October 3, 2006