4510–4516 *Nucleic Acids Research, 1999, Vol. 27, No. 22*

Nitric oxide-induced damage to mtDNA and its subsequent repair

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Received June 10, 1999; Revised August 27, 1999; Accepted September 27, 1999

ABSTRACT

Mutations in mitochondrial DNA (mtDNA) have recently been associated with a variety of human diseases. One potential DNA-damaging agent to which cells are continually exposed that could be responsible for some of these mutations is nitric oxide (NO). To date, little information has been forthcoming concerning the damage caused by this gas to mtDNA. Therefore, this study was designed to investigate damage to mtDNA induced by NO and to evaluate its subsequent repair. Normal human fibroblasts were exposed to NO produced by the rapid decomposition of 1-propanamine,3-(2-hydroxy-2-nitroso-1-propylhydrazino) (PAPA NONOate) and the resultant damage to mtDNA was determined by quantitative Southern blot analysis. This gas was found to cause damage to mtDNA that was alkali-sensitive. Treatment of the DNA with uracil-DNA glycosylase or 3-methyladenine DNA glycosylase failed to reveal additional damage, indicating that most of the lesions produced were caused by the deamination of guanine to xanthine. Studies using ligation-mediated PCR supported this finding. When a 200 bp sequence of mtDNA from cells exposed to NO was analyzed, guanine was found to be the predominantly damaged base. However, there also was damage to specific adenines. No lesions were observed at pyrimidine sites. The nucleotide pattern of damage induced by NO was different from that produced by either a reactive oxygen species generator or the methylating chemical, methylnitrosourea. Most of the lesions produced by NO were repaired rapidly. However, there appeared to be a subset of lesions which were repaired either slowly or not at all by the mitochondria.

INTRODUCTION

Nitric oxide (NO) is an inorganic gas that is a pleotrophic mediator of numerous physiological responses (1,2). It can be produced and released by a variety of cells in the body. While this gas plays an important role in regulating many biological functions, it can also, under certain conditions, be cytotoxic. The production of NO is a key component in cell killing caused by inflammatory processes (3). Additionally, overproduction of NO by non-immune cells through overactivation of specific isoforms of nitric oxide synthetase can have deleterious consequences. NO has been found to affect several different intracellular targets to exert its toxic effects. One of the prime sites for damage is the mitochondrion. NO has been shown to have a profound effect on mitochondria, causing impairment of mitochondrial oxidative metabolism by the inhibition of aconitase and possibly by inhibition of electron transport (4–6). While the impairment of aconitase may be at least partially responsible for reversible impairment of mitochondrial function, it is unlikely that the inhibition of this enzyme is the sole factor that ultimately leads to cell death (7). Since NO is a highly-reactive molecule that can interact with a variety of cellular components, it appears likely that damage to several key cellular constituents may lead to the final demise of the cell. One likely critical site for injury is DNA. In this regard, we have recently shown that NO damages mitochondrial DNA (mtDNA) to a greater extent than nuclear DNA (8). This is of importance for human disease because mutations in mtDNA have been associated with a variety of human illnesses, including some forms of diabetes mellitus (9,10), Alzheimer's disease (11,12), Parkinson's disease (13–15) and the normal process of aging (16–19). These mutations, which seem to accumulate with age, could be partially the result of either increased damage to mtDNA or decreased repair of this damage, or a combination of both factors.

DNA repair in mitochondria has been found to be selective for some types of damage. For instance, damage produced by agents such as UV-irradiation that cause bulky DNA adducts are not efficiently repaired (20). However, simple alkylation damage and lesions resulting from reactive oxygen species (ROS) are removed efficiently (21,22). Presently, to our knowledge, there is no information concerning the removal of damage to mtDNA caused by reactive nitrogen species (RNS). To overcome this dearth of information, we performed studies employing normal human fibroblasts, which were exposed to the NO generator 1-propanamine,3-(2-hydroxy-2-nitroso-1-propylhydrazino), otherwise known as PAPA NONOate (PAPA/NO). The results show that NO predominantly damages purines in mtDNA. Furthermore, most of these lesions in mtDNA, which are probably the result of deamination reactions, are repaired efficiently.

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MATERIALS AND METHODS

Cell culture

Karyotypically-normal human skin fibroblasts were obtained from the National Institute of Aging, Aging Cell Culture Repository, Coriell Institute for Medical Research, repository No.AG08498. Cells were maintained in Eagle's minimal essential media with Eagle's salts (Gibco BRL) supplemented with 15% fetal bovine serum (HyClone Laboratories), 50 μg/ml gentamicin (Sigma), 2× non-essential amino acids, 2× essential amino acids with L-glutamine (Gibco BRL) and 2× MEM vitamin solution (Gibco BRL). Cells were plated in 150 × 25 mm dishes and grown at 37°C, 5% CO_2 until near confluence (5–7 days).

Drug preparation and exposure

PAPA/NO purchased from Cayman Chemical was used to release nitric oxide (23,24). PAPA/NO dissociates to the free amine and nitric oxide in a pH-dependent manner. Stock solution (1 M) of PAPA/NO in 0.01 M NaOH (which is very stable and can be stored at 0°C for 24 h) was used to make various concentrations from 1 to 10 mM in Hanks' balanced salt solution (HBSS). Cells were rinsed with HBSS, then exposed to PAPA/NO for 10 min in a 5% CO_2, 37°C incubated environment. Control cultures were exposed to HBSS only, under the same conditions. After incubation, cells were either lysed immediately or rinsed in HBSS, then allowed to repair in cell culture medium.

Assay for nitric oxide-induced damage in mtDNA

Human skin fibroblast monolayer cultures were exposed to PAPA NONOate as described above. Cells were lysed at appropriate times in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% SDS and 0.3 mg/ml proteinase K. Lysates were incubated overnight at 37°C. High molecular weight DNA was extracted with an equal volume of phenol, phenol: chloroform (1:1) and chloroform. The DNA was treated with RNase (to a final concentration of 1 μg/ml) for 2 h and digested with *Kpn*I overnight at 37°C. Digested samples were precipitated, resuspended in TE buffer, and precisely quantified using a Hoefer TKO 100 minifluorometer and TKO standard kit. Samples containing 5 μg of DNA were heated for 20 min at 65°C and then cooled at room temperature. A sodium hydroxide solution was then added to a final concentration of 0.1 N, and samples were incubated for an additional 15 min at 37°C. After alkali treatment, samples mixed with loading dye were loaded onto a 0.6% alkaline gel and electrophoresed at 30 V (1.5 V/cm gel length) overnight in an alkaline buffer consisting of 23 mM NaOH and 1 mM EDTA. After standard gel washing, the DNA was transferred via vacu-blotting (American Bionetics, Vac-1000) onto a Zeta-Probe GT nylon membrane (Bio-Rad) and cross-linked to the membrane. The membrane was hybridized with a ^{32}P-labeled mtDNA specific probe. The probe was generated via PCR of a mouse mtDNA sequence as the template (22). Hybridization and washes were performed according to the manufacturer's directions. DNA damage and repair were determined as previously described (22).

Assay for mtDNA damage and repair at the nucleotide level by ligation-mediated PCR (LM-PCR)

We have previously published our modification of the LM-PCR procedure for the study of mtDNA (25). Briefly, following treatment of cells or a mtDNA sequence produced by extended length PCR (XLPCR) to give a 16.3 kb fragment (26) with PAPA/NO, DNA extraction, enzymatic digestion and quantitation, 10–15 μg of DNA was treated with NaOH to a final concentration 0.1 N for 15 min at 37°C. This treatment produces single-strand breaks at any abasic or sugar-modified site in the DNA. After alkali treatment, 1/10 vol of 3 M sodium acetate (pH 5.2) was added to samples and the DNA was precipitated with 2 vol of cold ethanol. Extractions, primer extentions and ligation were all performed as described previously (25), with the exceptions that 1 μg samples of genomic DNA and 10 ng samples of a PCR-generated mtDNA sequence were used (see Fig. 1 for sequences of primers 1 and 2). Following ligation, the reactions were precipitated with 0.3 vol of 10 M ammonium acetate, 6.6 mM EDTA and 20 μg glycogen. The precipitants were resuspended in 50 μl distilled water. PCR amplification, gel electrophoresis, blotting, probe preparation and hybridization were performed as previously described (25). Primer 2 was used to generate the hybridization probe.

1 5'- TAA AGA TTA AGA GAA CCA ACA CCT C - 3'

2 5'- AGT GAA ATG CCC CAA CTA AAT ACT ACC G -3'

Linker Primers (together- asymmetric linker)

LP25 5'-GCG GTG ACC CGG GAG ATC TGA ATT C-3'

LP11 5'- GAA TTC AGA TC -3'

Figure 1. Nucleotide sequence of primers used in LM-PCR.

RESULTS

To determine an appropriate concentration of the nitric oxide generator PAPA/NO to use for evaluating damage to and repair of mtDNA a dose response study was performed. Normal human fibroblasts were exposed to 5, 1 or 0.5 mM concentrations of PAPA/NO for 10 min. The cells were lysed immediately, total cellular DNA isolated, and Southern blotting performed to determine damage to mtDNA. Control cultures were incubated in drug diluent only. The results show that 5 mM PAPA/NO gave the appropriate number of strand breaks (~1 break per restriction fragment) to study repair (Fig. 2). Viability studies using trypan blue dye exclusion showed that ~80% of cells were viable 24 h after treatment with this concentration of PAPA/NO. In order to rule out the possibility that the PAPA/NO produced some other toxic product than NO which damaged mtDNA, HBSS containing 5 mM PAPA/NO was incubated for 1 h at 37°C to exhaust NO release. When cells were treated with this medium no damage was obtained (data not shown). To show that the damage was the result of exposure to NO, a study was performed using the NO scavenger carboxy-PTIO [2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, potassium salt]. Cells were treated with 5 mM PAPA/NO only, or 5 mM PAPA/NO plus 5, 7.5 or 10 mM carboxy-PTIO for 10 min. Control cultures received either the drug diluent HBSS alone or HBSS plus carboxy-PTIO. The results of these studies showed that the scavenger reduced the damage caused by NO in a progressive fashion (Table 1). Studies using isolated DNA have indicated

Table 1. A representative study in which normal human fibroblasts were exposed to 5 mM PAPA/NO only (5); or 5 mM PAPA/NO with 5, 7.5 or 10 mM carboxy-PTIO (5+Ct) for 10 min and lysed immediately

	NO	NO + 5 mM carboxy-PTIO	NO + 7.5 mM carboxy-PTIO	NO + 10 mM carboxy-PTIO
Breaks per restriction fragment	0.83	0.78	0.2	0.08
Percentage of damage reduction		6%	76%	91%

Control cultures were incubated in HBSS only (C) or HBSS containing 7.5 mM carboxy-PTIO (C + Ct). The methods for preparing the Southern blot are the same as described in Figure 2. Breaks per restriction fragment were determined as described in Materials and Methods. The percentage of damage reduction was calculated as the percentage of change in break frequency in the carboxy-PTIO treated cultures compared to the culture treated with PAPA/NO only. This study shows that the NO scavenger carboxy-PTIO was able to attenuate the mtDNA damage caused by PAPA/NO.

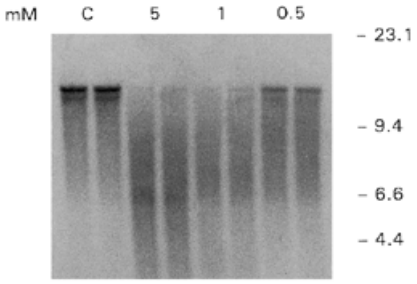

Figure 2. Normal human fibroblasts were exposed to 5, 1 or 0.5 mM PAPA/NO for 10 min and lysed immediately. Control cultures were incubated in drug diluent only (HBSS). High molecular weight DNA was isolated and digested to completion with *Kpn*I. Samples were exposed to 0.1 N NaOH prior to Southern blot analysis and hybridization with a mitochondrial probe. 5 mM PAPA/NO gave the appropriate amount of strand breaks (about one break per restriction fragment) to study repair.

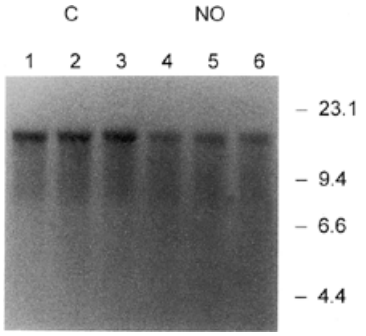

Figure 3. Normal human fibroblasts were exposed to 5 mM PAPA/NO for 10 min and lysed immediately. Control cultures were incubated in HBSS only. High molecular weight DNA was isolated and digested to completion with *Kpn*I. DNA from control and NO-exposed cells was either treated with 0.1 N NaOH (lanes 1 and 4), 3-MAG followed by 0.1 N NaOH (lanes 2 and 5), or UDG followed by 0.1 N NaOH (lanes 3 and 6). This figure is a representative autoradiograph from three separate experiments and shows that additional damage was not recognized by the glycosylases.

that NO predominantly damages DNA by deamination reactions with nitrous anhydride (N_2O_3) (27). Thus, it is likely that the damage to the mtDNA is the result of specific deamination reactions. The three possible reactions are (i) deamination of guanine to xanthine, (ii) deamination of adenine to hypoxanthine, and (iii) deamination of cytosine to uracil. To help determine whether these reactions occurred in mtDNA from cells which had been exposed to 5 mM PAPA/NO, total cellular DNA was isolated and treated in one of three ways. First, to identify xanthine, the DNA was exposed to alkali, which causes the unstable xanthine to fall out and breaks the strand at the resulting abasic site by a β-elimination reaction. Second, to recognize uracil, DNA was treated with uracil-DNA glycosylase (UDG) followed by alkali treatment to break the strand at the resulting abasic site. Finally, to identify hypoxanthine, the DNA was treated with 3-methyladenine DNA glycosylase (3-MAG), which removes hypoxanthine (28) followed by alkali treatment to break the strand at the resulting abasic site. The results of these studies show that no additional damage was detected using either glycosylase over that seen with alkali alone. These data suggest that the damage to mtDNA caused by RNS was predominantly due to the deamination of guanine to xanthine (Fig. 3). Next, LM-PCR was performed on a 200 bp sequence from the heavy strand of mtDNA from cells that were exposed to 1, 5 or 10 mM PAPA/NO to identify the nucleotide specificity for damage by RNS. Additionally, these studies were performed to evaluate the pattern of nucleotide damage caused by RNS in order to determine whether these species cause a signature pattern of damage. This sequence contained one of the break points for the 5 kb 'common deletion' that accumulates in mtDNA with aging (29,30). As can be seen in Figure 4, PAPA/NO produced a specific pattern of damage that was dose dependent. To compare the pattern of damage produced by NO with other DNA-damaging agents, cells were exposed to 2.5 mM of the methylating chemical methylnitrosourea (MNU). Since ~70% of the methylated bases formed by this chemical are alkali-labile N7 methylguanines, it was informative to compare the pattern of damage caused by this chemical with that produced by RNS. Also, for comparison, cells were exposed to ROS produced by the reaction of XO (10 mU/ml xanthine oxidase with 0.5 mM/ml hypoxanthine). Figure 5 is a representative autoradiograph of an LM-PCR study, and Figure 6 shows a comparison of the pattern of damage obtained from three separate experiments using each damaging agent. For these studies, 10 mM PAPA/NO was used. As can be seen

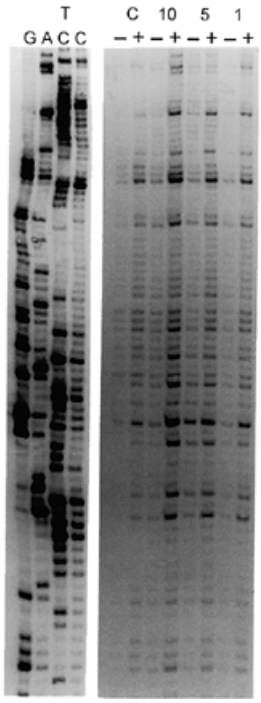

Figure 4. Dose response of NO-induced damage at the resolution of individual nucleotides in a sequence from the 'heavy strand' of mtDNA. Normal human fibroblasts were treated with either 10, 5 or 1 mM PAPA/NO for 10 min. Control cells (lane C) received HBSS only. After treatment the cells were lysed immediately, DNA was isolated, digested and subjected to LM-PCR. A + above a lane indicates treatment with 0.1 N NaOH.

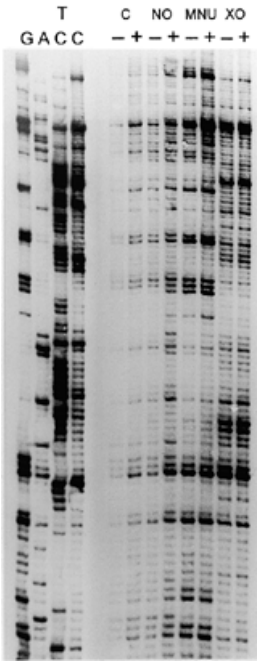

Figure 5. Comparison of 'heavy strand' mtDNA damage patterns using various genotoxins. Normal human fibroblasts were treated with 10 mM PAPA/NO for 10 min (NO), XO for 1 h or 2.5 mM MNU for 15 min. Control cultures (lane C) received only HBSS treatment. After treatment the cells were lysed immediately, DNA was isolated, digested and subjected to LM-PCR. A + above a lane indicates treatment with 0.1 N NaOH.

in these figures, all three agents gave distinctive patterns of damage. When compared to a Maxim–Gilbert sequencing ladder, it is apparent that most of the lesions produced by either PAPA/NO or MNU are on guanines; however, some adenines are also damaged. No damage to cytosines or thymines was observed. In agreement with our previous studies on oxidative damage in mtDNA (25), XO also damaged specific cytosines and thymines. To ascertain whether the pattern of damage obtained with NO was due to the chemical properties of this agent or was caused by association of the mtDNA with proteins, a 16.3 kb sequence of mtDNA was amplified by XLPCR. This DNA was exposed to 10 mM PAPA/NO and the pattern of damage compared with that obtained from cells exposed to this NO generator. The results displayed in Figures 7 and 8 show that the same pattern was obtained. Finally, to determine whether mitochondria can repair damage to its DNA caused by RNS, cells were treated with 5 mM PAPA/NO for 10 min and lysed immediately or rinsed and placed in culture media and allowed to repair for either 30, 120 or 240 min. Control cultures were incubated in drug diluent only. DNA samples were treated with alkali and quantitive Southern blots were performed. As can be seen in Figure 9, the damage produced by PAPA/NO was substantially repaired by 240 min. However, most of the damage was removed in the first 30 min. This finding reveals that there are efficient repair mechanisms in the mitochondria for most of the damage caused by RNS. However, there appears to be a subset of damage that is not removed efficiently.

DISCUSSION

There has been a considerable amount of work that has shown that NO is able to damage nuclear DNA (27,31–34). However, far less data have been forthcoming concerning mtDNA. To explore mtDNA damage and repair following exposure to NO we chose the NO generator PAPA/NO because it degrades rapidly to release NO (half life of 15 min) (24) and was tolerated well by the cells. This allowed us to evaluate repair in the absence of ongoing damage. Using quantitative Southern blotting, we were able to ascertain that this chemical caused damage to mtDNA in a dose-dependent fashion. That this damage was due to the generation of NO is supported by the observation that it was attenuated by treatment with the NO scavenger carboxy-PTIO. The observation that mtDNA is a vulnerable target for damage by NO corroborates previous findings from our laboratory using primary cultures of insulin-secreting β-cells (8). These experiments revealed that NO generated either exogenously with the NO generator (spermine/NO adduct) or endogenously by treatment with IL-1β, activates the inducible form of nitric oxide synthase and damages mtDNA. The results of the present investigation extend our previous findings by demonstrating that the predominant lesion that is found is alkali-labile. Data obtained using glycosylases that recognize either hypoxanthine (3-MAG) or uracil (UDG) demonstrate

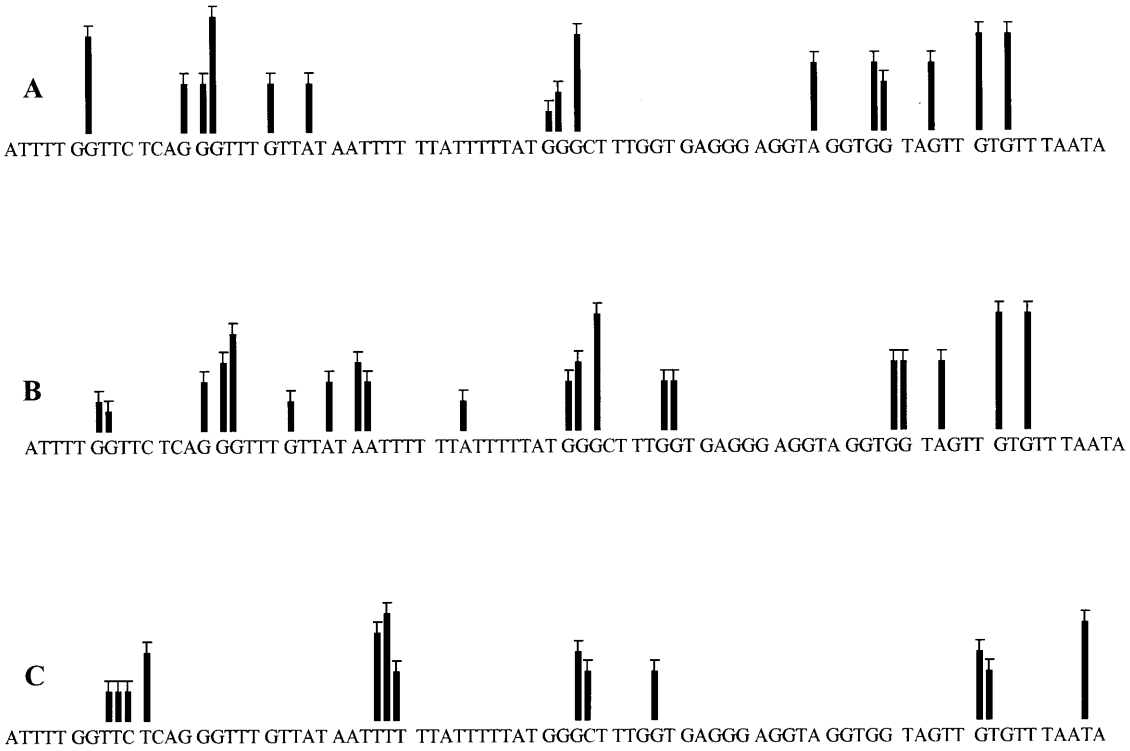

Figure 6. Nucleotide damage map. This map represents a 60 bp stretch within the 202 bp fragment of mtDNA analyzed. The bars above specific nucleotides indicate arbitrary values on a scale from 1 to 6 for intensity of damage observed for the particular nucleotides (mean ± SEM, n = 3). The damage represented was observed after exposure to MNU (**A**), PAPA/NO (**B**) or XO (**C**) as described in Figure 5.

that most of the damage in mtDNA is not uracil produced by the deamination of cytosine or hypoxanthine resulting from the deamination of adenine. However, in the case of hypoxanthine, it cannot be ruled out that this base adduct was not formed. Since hypoxanthine has been reported to be unstable (31,32), it is possible that it spontaneously falls out, forming an apurinic (AP) site before treatment with 3-MAG.

The studies using LM-PCR have shed new light on the damage caused by NO in mtDNA. They revealed that guanine is the most frequently damaged base, although some damaged adenines were also found when alkaline conditions were employed. No damage was detected at any pyrimidines in the sequence evaluated. For comparison, studies with the ROS generator XO showed that we are able to detect oxidative lesions to both purines and pyrimidines when they are present. The pattern and frequency of base damage was similar to that seen previously using the ROS generator alloxan (25). Therefore, it appears likely that the inability to detect pyrimidine lesions following exposure to NO was because few if any lesions are formed at these bases. These findings support the notion that the damage that occurred to mtDNA is through the formation of N_2O_3, which causes deamination of guanine to xanthine and adenine to hypoxanthine (31,32,34). In both cases, this lesion preceeded depurination to produce an AP-site which was converted to a strand break with an appropriate end for ligation by alkali treatment. That abasic sites predominantly are formed in DNA is in agreement with work by Tamir *et al.* (33) on plasmid DNA which was electroporated into Chinese hamster ovary cells. When these cells were treated with NO, a significant

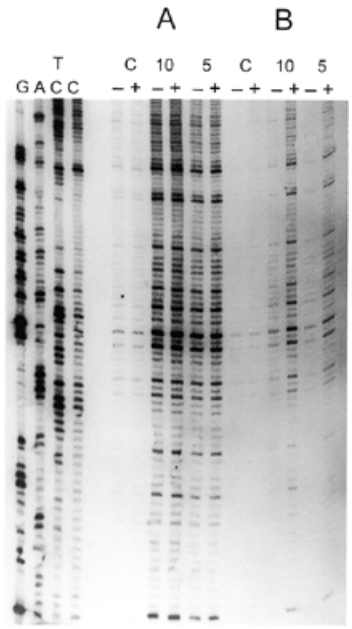

Figure 7. Comparison of the mtDNA damage profile obtained from normal human fibroblasts to a 16.3 kb XLPCR-amplified mtDNA sequence following exposure to 10 and 5 mM PAPA/NO for 10 min. After treatment, DNA was isolated, digested and subjected to LM-PCR. A + above a lane indicates treatment with 0.1 N NaOH. Controls received only HBSS. A lanes from the PCR-generated mtDNA sequence. B lanes for cellular mtDNA.

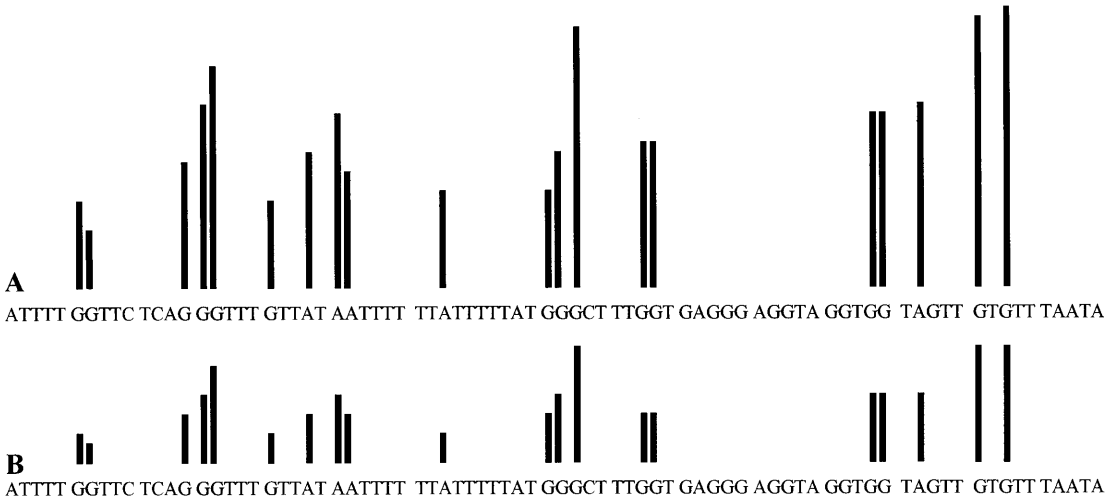

Figure 8. Nucleotide damage map. The map represents a 60 bp stretch within the 200 bp fragment of mtDNA analyzed. The bars above specific nucleotides indicate arbitrary values on a scale from 1 to 6 for intensity of damage observed for the particular nucleotides following exposure to 10 mM PAPA/NO for 10 min. (**A**) Cellular mtDNA, (**B**) PCR-generated mtDNA. The damage patterns are identical.

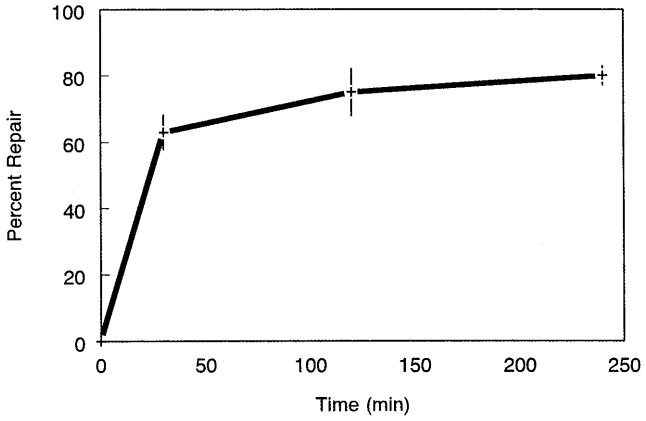

Figure 9. Repair of mtDNA from normal human fibroblasts exposed to 5 mM PAPA/NO for 10 min. Data are expressed as the mean ± SEM (n = 3) of the percent of alkali labile sites removed from the mtDNA at 30, 120 and 240 min after exposure to the NO generator. Note the rapid early repair followed by a more attenuated damage removal.

number of abasic sites were produced in the DNA. Additional work by these investigators using both DNA exposed to NO *in vivo* and *in vitro* has revealed that xanthine followed by hypoxanthine are the predominant base alterations (33,34).

By comparing the pattern of damage produced by NO with that generated by the alkylating agent MNU or the ROS generator XO it can be seen that, although all three of these agents damaged many of the same guanines, there were certain guanines that were only vulnerable to PAPA/NO. Therefore, it is possible to determine a signature damage pattern for RNS that is different from that produced by ROS or methylating toxins. This finding may prove useful for future studies of mtDNA in which the identity of the damaging agent is unknown. Additionally, it will be important to compare damage produced in mtDNA by different agents with patterns of known mtDNA mutations. The fact that NO produced the same pattern of damage when exposed to a PCR-generated mtDNA sequence establishes that the pattern of damage produced is due to the chemical properties of NO interacting within the sequence context of the DNA, rather than being influenced by the association of the DNA with the mitochondrial matrix proteins or other DNA-binding proteins.

NO has the ability to form *n*-nitroso compounds through the reaction with secondary and tertiary amines (35). These compounds could react with DNA to form 7-alkylguanine and 3-methyladenine. However, the LM-PCR studies showing that there is a different pattern of damage for guanines and adenines generated by NO and the alkylating toxin MNU indicates that the damage generated is not through the formation of alkylpurines.

These are, to our knowledge, the first studies to evaluate repair of NO damage in mtDNA. Initially, repair of NO-induced damage proceeded quickly. Approximately 70% of the damage was removed by 30 min. However, the rate of repair slowed so that only ~30% of the remaining damage was removed by 4 h. Although the exact reason for these apparent different rates in lesion removal is obscure, it probably relates to the type of lesion repaired. As this paper and others have previously suggested (33), the predominant lesion that is formed in DNA after exposure to NO is an abasic site. The present results indicate that this type of damage can be removed rapidly. The rapid repair of these lesions indicates that they are not alkylpurines because we have previously shown that the repair of these lesions is much slower. It requires more than 24 h to remove 70% of the alkylpurines formed in normal human fibroblasts exposed to an equivalent damaging dose of MNU (36). However, the slowly repaired component could at least in part be the repair of alkylpurines. It should be mentioned that NO can also produce other types of damage such as double strand breaks, DNA crosslinks and oxidative damage caused by NO-induced release of Fe (35,37). These types of damage are

repaired with far less efficiency in the nucleus, and it is likely that the same is true for the mitochondrion. It is interesting to note that when we studied repair of bleomycin-induced damage in mtDNA, which also forms a high proportion of abasic sites, we saw a similar pattern of repair (38). In this study, it was speculated that the slow phase of repair was the removal of double strand breaks. Clearly, more experimentation is warranted to determine the nature of the lesions produced in mtDNA and elucidate the mechanisms by which these lesions are repaired.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Wesley Driggers and Ms Nancy Thornley for their help with the preparation of this manuscript and Dr Steven Tannenbaum for his critical comments concerning it. This work was supported by United States Public Health Service Grants ES03456 and ES05865 from the National Institute of Environmental Health Sciences and AG12442 from the National Institute on Aging.

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