BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 1–5 (1997)
ARTICLE NO. RC977065

Formation of Novel C^1-Oxidised Abasic Sites in Alkylperoxyl Radical-Damaged Plasmid DNA

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Received June 26, 1997

We have recently shown that peroxyl radicals react with DNA to form alkali-labile sites. To further characterise these lesions, we studied their susceptibility to digestion by repair endonucleases that recognise different types of abasic sites. We found that peroxyl radical-damaged pSP189 plasmids were resistant to cleavage by T4 endonuclease V, an enzyme that incises DNA at "regular" and C^4-oxidised abasic residues. In contrast, the DNA was digested by exonuclease III, an enzyme that recognises "regular" and C^1-oxidised abasic sites. The presence of Trolox during exposure to peroxyl radicals reduced subsequent DNA cleavage by exonuclease III, while prior incubation of damaged plasmids with methoxyamine potentiated digestion by this enzyme. These findings suggest that peroxyl radical-induced DNA damage involves the generation of novel C^1-oxidised deoxyribose residues.

Endogenously-produced genotoxicants are receiving increasing attention as likely contributors to spontaneous mutagenesis (1–3). One key source of such species is lipid peroxidation, an autocatalytic cascade affecting polyunsaturated fatty acids that generates various DNA-damaging products such as malondialdehyde and a number of enals (4). The recent detection of adducts from these aldehydes in mammalian DNA has confirmed that lipid peroxidation is a major contributor to spontaneous DNA damage *in vivo* (5, 6). To gain a fuller appreciation of the genotoxic properties of other lipid peroxidation products, we recently commenced study of the DNA-damaging properties of peroxyl radicals (7). These oxygenated lipid radicals catalyse the propagation phase of lipid peroxidation, and their likely formation in nuclear membranes adjacent to chromatin attachment sites suggests a possible role in spontaneous DNA damage (8–10). We recently showed that a range of base substitutions are produced upon bacterial processing of peroxyl radical-damaged DNA, although G → T transversions were by far the most common (7). Since the premutagenic damage was alkaline-sensitive and SOS-dependent, we hypothesised that abasic sites were probably responsible for the G → T transversions (7). However, since other genetic lesions can also generate this mutation, the present work was intended to directly assess the contribution of depurination events.

Due to the toxic properties of abasic sites, most cells are equipped with apurinic/apyrimidinic (AP) endonucleases which repair these lesions (11). The majority of AP endonucleases, which includes such *E. coli* enzymes as endonuclease IV and exonuclease III, cleave double-stranded DNA immediately 5′ to an abasic site (12, 13). In contrast, a smaller group of AP endonucleases (such as *E. coli* endonuclease III and T4 endonuclease V) incise DNA 3′ to an abasic site via a β-elimination reaction mechanism (14, 15). Recent substrate-specificity studies have revealed that these two classes of AP endonucleases also show differences in their activity against certain types of abasic sites (16–18). Whilst "regular" or nonoxidised abasic sites are readily digested by all AP endonucleases, those enzymes which cleave 5′ to an abasic site show a higher reactivity against DNA that contains C^1-oxidised sugars, while digesting C^4-oxidised deoxyribose residues less readily. In contrast, endonucleases which incise the DNA at the 3′ side cleave both types of oxidised AP sites to a similar degree with the exception of T4 endonuclease V which shows minimal recognition of 1′-oxidised sites (16).

In this study we have exploited the differences in substrate specificity of various AP endonucleases in an effort to characterise the DNA damage caused by peroxyl radicals. Double-stranded pSP189 plasmids were treated with the peroxyl radical generator 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) and then digested with either exonuclease III or T4 endonucle-

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Abbreviations: AP site(s), apurinic/apyrimidinic site(s); AP endonuclease(s), apurinic/apyrimidinic endonuclease(s); AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride.

0006-291X/97 $25.00

ase V (19). We predicted that if a premutagenic lesion other than an abasic site was present in the modified DNA, it would not be cleaved by either AP endonuclease. Conversely, if "regular" abasic sites were the major lesion, the damaged DNA should be digested by both enzymes. In contrast, if the damage involves C^1-oxidised sugars the DNA would be cleaved by exonuclease III only, while the presence of C^4-oxidised sugars would enhance digestion by both enzymes although to a lesser extent than "regular" AP sites. Collectively, our results indicate that while abasic sites are major contributors to peroxyl radical-induced mutagenesis, the DNA damage is more complex than the simple depurination events initially envisaged.

MATERIALS AND METHODS

Materials. 2,2′-azobis (2-amidinopropane)-HCl was obtained from Polysciences, Inc. (Warrington, PA). Double-stranded pSP189 plasmid DNA, amplified in the bacterial strain Ab2463, was a generous gift from Prof. L. J. Marnett, Vanderbilt University, Nashville, TN. Methoxyamine and Trolox were purchased from Sigma-Aldrich Pty., Ltd. (Australia). Exonuclease III and T4 endonuclease V were obtained from Boehringer Mannheim Pty., Ltd. (Australia) and Astral Scientific (Australia) respectively. All other reagents were purchased from standard commercial suppliers.

Peroxyl radical modification of pSP189 DNA. To prepare peroxyl radical-treated plasmids, double-stranded pSP189 DNA (0.1 mg/mL) was incubated for 2 hr at 37 °C in open reaction vessels containing 100 μM 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) in sodium phosphate buffer (50 mM, pH 7.0). Control DNA samples were treated similarly in the absence of AAPH. Following incubation, the DNA was recovered using a BIORAD DNA purification kit and then quantitated via absorbance measurements at 260 nm (20). In some experiments, the same DNA modification conditions outlined above were used except that various concentrations of Trolox (0 to 200 μM) were included in the reaction mixtures.

Endonuclease relaxation assay. AAPH-modified pSP189 DNA (0.5 μg) was incubated for 1 hour at 37 °C with either exonuclease III (0–10 units) or T4 endonuclease V (0–10 units) in a final volume of 10 μL. The reaction media used during exonuclease reactions comprised 50 mM Hepes/KOH (pH 7.6), 1 mM dithiothreitol, 10 % glycerol (w/v) and 5 mM $MgCl_2$ (21), while the buffer used in T4 endonuclease V reactions contained 20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 1 mM EDTA (16). In experiments where the effects of methoxyamine were investigated, modified DNA was incubated with methoxyamine (0 to 250 mM) for 30 mins at 37 °C prior to the addition of exonuclease III (7.5 units) (22). All endonuclease reactions were stopped by adding 2 μL of 2 % sodium dodecyl sulfate (16), and the DNA was loaded onto a 0.8 % agarose gel containing 0.5 μg/mL ethidium bromide. The DNA was then resolved for 2 hours at 65 V after which it was visualised under UV light (20). The supercoiled (form I) and relaxed (form II) DNA was then quantitated via densitometry using a Kodak DC40 digital camera and *Digital Science* electrophoresis analysis software.

RESULTS

Effects of increasing AP endonuclease concentration. Other workers have shown that high concentrations of the peroxyl radical generator AAPH cause nicking of DNA, converting supercoiled plasmids (form I) to their relaxed counterpart (form II) (23). To ensure that this type of damage was minimised during the preparation of our substrate DNA, we used the lowest concentration of AAPH which was mutagenic in our previous experiments (i.e. 100 μM) (7). Nonetheless, some nicking (approx. 20%) was associated with exposure to even this low concentration of AAPH (*data not shown*). To determine whether abasic site formation accompanied this damage, peroxyl radical-modified pSP189 plasmids were then digested with two dissimilar AP endonucleases, T4 endonuclease V and *E. coli* exonuclease III (Figure 1). Intriguingly, we found that the modified DNA was readily digested by exonuclease III, with a concentration-related loss of form I (supercoiled) DNA (*solid squares*, Figure 1) and a corresponding increase in form II (relaxed) DNA (*solid triangles*, Figure 1) accompanying treatment with the enzyme. Note that despite the loss of form I DNA, form II DNA formation declined in the presence of 10 units of enzyme. A similar finding has been reported by others following the use of high concentrations of exonuclease III, and most likely reflects degradation by the 3′–5′ exonuclease activity associated with this protein (24).

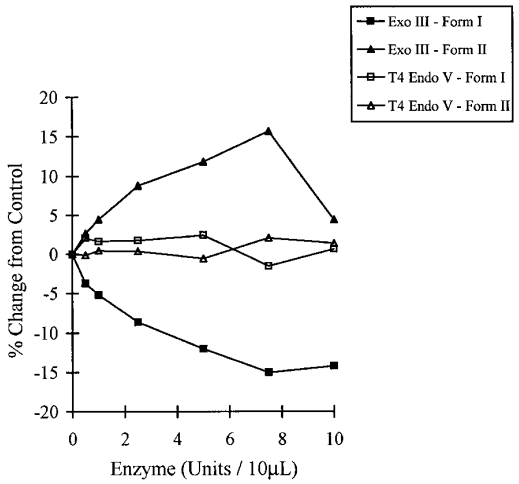

FIG. 1. Effect of increasing enzyme concentration on the digestion of AAPH-modified pSP189 plasmids by two AP endonucleases. AAPH-treated double-stranded pSP189 DNA (0.5 μg) was incubated with either exonuclease III (0–10 units) or T4 endonuclease V (0–10 units) for 1 hour at 37°C in a final reaction volume of 10 μL. The reaction products were then resolved on an ethidium bromide-containing 0.8% agarose gel prior to densitometric quantitation of form I (*supercoiled*) and form II (*relaxed*) DNA. The results are expressed as a percentage change over the amount of forms I and II DNA present in non-digested, AAPH-modified plasmids. Each data point represents the mean of two independent observations.

The exonuclease III experiments provided clear evidence for the induction of abasic sites by peroxyl radicals. However, the finding that the damaged DNA was completely resistant to degradation by T4 endonuclease V suggested that it did not contain "regular" abasic sites which are formed by simple hydrolysis of the *N*-glycosyl bond of deoxynucleotides (Figure 1). We found

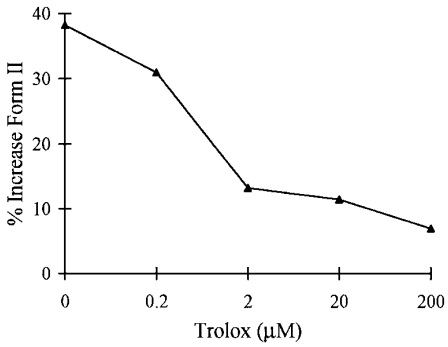

FIG. 2. Effect of increasing concentrations of the radical scavenger Trolox on the generation of exonuclease III-sensitive lesions in pSP189 plasmids during exposure to peroxyl radicals. Double-stranded pSP189 DNA was treated with AAPH (100 μM) in the presence of Trolox (0 to 200 μM) and then digested with 7.5 units of exonuclease III. The reaction products were then resolved on an ethidium bromide-containing 0.8% agarose gel prior to densitometric quantitation of form II (*relaxed*) DNA. The percentage of DNA in the form II (relaxed) state is expressed as the percentage change over the amount present in non-digested, unmodified DNA. Each data point represents the mean of two independent observations.

that even in the presence of up to 10 units of T4 endonuclease V, the proportion of DNA molecules in the supercoiled (*open squares*, Figure 1) and relaxed (*open triangles*, Figure 1) forms remained essentially the same as in undigested substrate DNA. In a separate control experiment, we established that this lack of activity was not due to a poor batch of enzyme or inappropriate assay conditions, since T4 endonuclease V readily digested depurinated pSP189 DNA that contained "regular" abasic sites generated by heating the plasmids under mildly acidic conditions (*data not shown*). On the basis of substrate specificities for exonuclease III and T4 endonuclease V reported by Haring *et al.* (1994) (16), the most consistent interpretation of our data is that peroxyl radicals almost exclusively generate a subgroup of "oxidised" abasic sites that contain a C^1-oxidised sugar residue, rather than "regular" depurinated sites.

Effects of a radical scavenger on exonuclease III digestion of AAPH-modified DNA. We have previously reported that AAPH-induced mutagenesis in the M13 forward mutational assay is abolished by the water-soluble vitamin E analogue, Trolox (7). To assess whether this peroxyl radical scavenger blocked the generation of exonuclease III-sensitive lesions, plasmids were exposed to 100 μM AAPH in the presence of a range of Trolox concentrations prior to digestion with exonuclease III (Figure 2). The presence of Trolox resulted in a concentration-dependent decrease in DNA cleavage by exonuclease III, confirming that peroxyl radicals mediate the formation of the exonuclease III-sensitive abasic sites in the DNA.

Effects of methoxyamine on exonuclease III digestion of AAPH-modified plasmids. The reaction of methoxyamine with abasic sites has previously aided clarification of the mechanism of action of various AP endonucleases (12, 22, 25–27). Whilst the derivatisation of abasic sites by methoxyamine inhibits the action of most mammalian 5′-acting AP endonucleases (22, 27), such modified sites are readily cleaved by the bacterial counterparts to these enzymes (12, 22). Indeed, it has been suggested that the presence of *o*-methylhydroxylamine adducts at abasic sites actually enhances cleavage by bacterial AP endonucleases, since the ring-opened abasic sugar may enhance enzyme access during 5′ phosphodiester bond hydrolysis (12). Our present results support this proposal, since pretreatment with methoxyamine produced a concentration-related enhancement of exonuclease III-catalysed digestion of AAPH-damaged DNA (Figure 3). By showing that reaction with methoxyamine facilitates cleavage by exonuclease III, this finding strengthens the conclusion that abasic sites are key DNA damage products of peroxyl radicals.

DISCUSSION

Our results provide the first conclusive demonstration that peroxyl radicals, ubiquitous products of lipid peroxidation, generate abasic sites during their interactions with DNA. Since our previous experiments showed that the most common mutations in peroxyl radical-damaged DNA occur at guanine residues, it seems likely that the AP endonuclease-sensitive sites result from the loss of guanine bases (7). These findings are consistent with previous studies that have detected alkaline-sensitive lesions at, or immediately 5′ to, guanine nucleotides in DNA following exposure to peroxi-

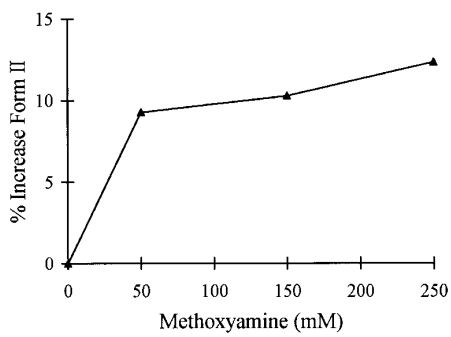

FIG. 3. Effect of methoxyamine on the susceptibility of peroxyl radical-damaged DNA to digestion by exonuclease III. Following exposure to 100 μM AAPH for 2 hr at 37°C, double-stranded pSP189 plasmids were incubated with increasing concentrations of methoxyamine (0 to 250 mM) for 30 minutes prior to digestion with 7.5 units of exonuclease III. The reaction products were then resolved via agarose gel electrophoresis prior to densitometric quantitation of form II (*relaxed*) DNA. The extent of digestion (form II formation) is expressed as the percentage increase over the amount of digestion occurring in AAPH-modified plasmids. Each data point represents the mean of two independent observations.

dised lipids and transition metal catalysts as a source of peroxyl radicals (28–31). Unfortunately, the genotoxicity observed in these types of experiments is difficult to interpret since a plethora of other lipid decomposition products are also formed, hence a specific form of DNA damage cannot be attributed to any one particular species. This problem was avoided in the present study by using the azo initiator compound AAPH which generates peroxyl radicals in a metal-free environment (32). Such species are a valid surrogate for lipid-derived peroxyl radicals, since study of reaction rate constants have shown that the nature of the organic substituent attached to the oxygen has little effect on the reactivity of peroxyl radicals (33).

The inability of T4 endonuclease V to cleave peroxyl radical-treated DNA indicates that the depurinated lesions are not "regular" abasic sites such as are formed upon hydrolysis of the *N*-glycosyl bond of deoxynucleotides, since the latter are cleaved by all AP endonucleases (16). Instead, the damage is more likely to involve C^1-oxidised abasic sites which are more readily recognised by AP endonucleases that cleave 5′ to an abasic site (16). The sugar derivative formed upon C^1-oxidation of deoxyribose, 5-methylene-2-furanone, is the primary product of reactions between DNA and "chemical nucleases" such as copper-phenanthroline and cationic manganese-porphyrin complexes (16, 34, 35). Copper/phenanthroline-damaged plasmid DNA has been found to be a substrate for *E. coli* exonuclease III but not for T4 endonuclease V—the latter preferring DNA that contains C^4-oxidised abasic sites such as are generated by iron/bleomycin complexes (16, 36, 37). While our present findings raise the possibility that the chemistry underlying peroxyl radical-induced depurination resembles that produced by copper/phenanthroline, confirmation of this proposal awaits more detailed chemical analysis.

Confirmation that C^1-oxidised deoxyribose is the major product of reactions between DNA and peroxyl radicals would be significant, as this form of sugar damage is only minor during DNA damage by hydroxyl radicals or ionising radiation, which preferentially form C^4- and to a lesser extent, C^2-oxidised residues (16). Indeed, we have already obtained preliminary evidence that the chemistry of sugar damage by peroxyl radicals is different to that produced by Fenton products such as hydroxyl radicals. The main product of the C^4-oxidation of deoxyribose is malondialdehyde, which forms a pink chromogen upon reaction with thiobarbituric acid (38, 39). Interestingly, we have observed a strong yield of thiobarbituric-acid reactive substances following treatment of deoxyribose solutions with iron/H_2O_2, but not following exposure to high concentrations of AAPH (*unpublished observation*). Whether the extent of C^1-oxidation produced by these two radicals is also different remains to be confirmed using more definitive means of chemical analysis. If it could be shown that distinct classes of endogenous oxygen-centred radicals (eg. peroxyl radicals versus hydroxyl radicals) produce different types of sugar damage, then quantitation of such damage in DNA from cells and tissues might provide an important insight into specific free radical fluxes in living systems.

ACKNOWLEDGMENTS

This study was supported by a Priming Grant from the National Health and Medical Research Council of Australia. We are grateful to L. J. Niedernhofer for advice concerning the preparation of pSP189 plasmid DNA.

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