5'-Nicked Apurinic/Apyrimidinic Sites Are Resistant to β-Elimination by β-Polymerase and Are Persistent in Human Cultured Cells after Oxidative Stress*

(Received for publication, October 13, 1999, and in revised form, December 7, 1999)

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Genomic DNA is continuously exposed to oxidative stress. Whereas reactive oxygen species (ROS) preferentially react with bases in DNA, free radicals also abstract hydrogen atoms from deoxyribose, resulting in the formation of apurinic/apyrimidinic (AP) sites and strand breaks. We recently reported high steady-state levels of AP sites in rat tissues and human liver DNA (Nakamura, J., and Swenberg, J. A. (1999) Cancer Res. 59, 2522–2526). These AP sites were predominantly cleaved 5' to the lesion. We hypothesized that these endogenous AP sites were derived from oxidative stress. In this investigation, AP sites induced by ROS were quantitated and characterized. A combination of H2O2 and FeSO4 induced significant numbers of AP sites in calf thymus DNA, which were predominantly cleaved 5' to the AP sites (75% of total aldehydeic AP sites). An increase in the number of 5'-AP sites was also detected in human cultured cells exposed to H2O2, and these 5'-AP sites were persistent during the post-exposure period. β-Elimination by DNA β-polymerase efficiently excised 5'-regular AP sites, but not 5'-AP sites, in DNA from cells exposed to H2O2. These results suggest that 5'-oxidized AP sites induced by ROS are not sufficiently repaired by the mammalian short patch base excision repair pathway.

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* This work was supported in part by NIEHS Superfund Basic Research Program Grant P42-ES05948 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The abbreviations used are: ROS, reactive oxygen species; 8-OH-dG, 8-hydroxy-2'-deoxyguanine; AP, apurinic/apyrimidinic; ASB, aldehyde reactive probe slot-blot; TEMPO, 2,2,6,6-tetramethylpiperidinoxyl; MX, methoxyamine; Exo III, E. coli exonuclease III; End III, E. coli endonuclease III; HPLC, high pressure liquid chromatography; β-pol, DNA β-polymerase; dRp, deoxyribose phosphate.

Experimental Procedures

DNA Isolation from Calf Thymus

Thymus was harvested from a newborn Holstein calf and quickly frozen on dry ice. After thawing, the calf thymus was homogenized in lysis buffer (Gentra Systems, Inc.) with 10 mm 2,2,6,6-tetramethylpip-
eridinoxyl (TEMPO; Aldrich) on ice. DNA was then isolated by phenol/Sevag (chloroform/isoamyl alcohol, 24:1) extraction and purified as described (13).

**Methoxamine Treatment of Calf Thymus DNA**

Calf thymus DNA (Sigma) was treated with 10 mM methoxamine (MX) in 10 mM Tris- HCl/ KOH (pH 7.4) and purified as described (12). H2O2 and FeSO4, Treatment of Calf Thymus DNA

Calf thymus DNA isolated in our laboratory or commercially obtained calf thymus DNA (Sigma) pretreated with MX was incubated with H2O2 and FeSO4 in 10 mM Tris- HCl, pH 7.4, containing 1 mM EDTA. The DNA concentration was measured by a UV spectrophotometer, and the DNA solution was then prepared at 0.1 or 1 µg/100 µl of TE buffer. After washing the DNA pellet with 70% ethanol, DNA was resuspended in distilled water containing 1 mM TEMPO.

**ASB Assay**

The AP site assay was performed following a procedure slightly modified from that reported by Nakamura and Swenberg (13). Briefly, 8 µg of DNA in 150 µl of phosphate-buffered saline was incubated with 1 mM adenylic acid probe at 37 °C for 10 min. After precipitation using cold ethanol, DNA was resuspended in TE buffer (10 mM Tris- HCl, pH 7.4, containing 1 mM EDTA). The DNA concentration was measured by a UV spectrophotometer, and the DNA solution was then prepared at 0.5 or 1 µg/100 µl of TE buffer. Heat-denatured DNA was immobilized on a nitrocellulose membrane (Hybond-C Super, Amersham Pharmacia Biotech). The nitrocellulose membrane was soaked in 5× SSC and then baked in a vacuum oven for 30 min. The membrane was preincubated with 10 ml of Tris- HCl containing bovine serum albumin for 15 min and then incubated in the same solution containing streptavidin-conjugated horseradish peroxidase at room temperature for 45 min. After rinsing the nitrocellulose membrane, the enzymatic activity on the membrane was visualized by enhanced chemiluminescence reagents. The nitrocellulose filter was then exposed to x-ray film, and the developed film was analyzed using an Ultrascan XL scanning densitometer.

**AP Site Cleavage Assay**

The AP site cleavage assay was performed as described (13) with a slight modification.

**Regular AP Site Assay**—The number of total AP sites was measured by the ASB assay as described above.

**5'-Cleavage AP Site Assay**—Eight µg of DNA and 145 units of Exo III (New England Biolabs Inc.) were incubated in 155 µl of 10 mM Tris- HCl/ KOH (pH 7.5) containing 50 mM NaCl and 5 mM MgCl2 at 37 °C for 1 min and immediately analyzed by the ASB assay.

**3'-Cleavage AP Site Assay**—Eight µg of DNA, 10 mM EDTA, and 100 mM putrescine were incubated in 135 µl of 10 mM Tris- HCl/ KOH at 37 °C for 30 min and immediately analyzed by the ASB assay.

**Detection of Residual AP Sites**—Eight µg of DNA and 145 units of exonuclease III in 110 µl of 10 mM Tris- HCl/ KOH were incubated at 37 °C for 1 min, immediately followed by addition of 0.1 volume of 100 mM EDTA. The sample was incubated with 100 mM putrescine in the reaction buffer at 37 °C for 30 min, immediately followed by the ASB assay.

**E. coli Endonuclease III-sensitive Site Assay**

Oxidative pyrimidine bases are repaired by E. coli endonuclease III (End III), leaving AP sites on the DNA backbone (3). End III is kindly provided by Dr. Y. W. Kow (Emory University). The End III-sensitive site assay was performed as described (13).

**8-OH-dG Assay**

Quantitation of 8-OH-dG was based on an HPLC/electrochemical detection approach that was modified from a method previously described by Richter et al. (14). DNA was hydrolyzed enzymatically to deoxyribonucleosides using deoxyribonuclease I, spleen phosphodiesterase, snake venom phosphodiesterase, and alkaline phosphatase. The digest was separated by reversed-phase HPLC, and 8-OH-dG was quantitated using an electrochemical array detector (ESA). Electrochemical oxidation was monitored at 200, 300, 375, 450, 525, 600, 700, and 800 nV. The concentration of 8-OH-dG was normalized to the amount of DNA analyzed, as determined by UV absorbance.

**RESULTS**

**AP Sites Induced by the Fenton Reaction**—One of the most significant oxygen radicals is the hydroxyl radical, which is generated by the reaction of reduced transition metals with H2O2 via the Fenton reaction (15). To address whether oxygen radicals induced by the Fenton reaction directly generate AP sites in DNA, calf thymus DNA pretreated with MX was incubated with 10 µM FeSO4 with or without H2O2 at 37 °C for 10 min under neutral pH conditions. The number of AP sites in MX-pretreated calf thymus DNA increased following treatment with 10 µM FeSO4 and was further enhanced by H2O2 (Fig. 1A). TEMPO, a radical-trapping reagent containing a nitroxide group (9), is known to reduce the number of 8-OH-dGs in mammalian tissues at 1 mM (16). To investigate whether TEMPO inhibits AP site formation by the Fenton reaction, MX-pretreated calf thymus DNA was reacted with 10 µM H2O2 and FeSO4 with or without TEMPO. TEMPO prevented AP site formation in a dose-dependent manner and completely protected DNA from AP site formation at concentrations of 10 mM (Fig. 1B).

**Cell Culture**

HeLa S3 cells were obtained as suspension cells from the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill. After centrifugation, cells were resuspended in 25 ml of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Life Technologies, Inc.) without serum (4 × 106 cells/ml). The cultured cells were exposed to 25% H2O2 (Sigma) at 37 °C for 15 min, immediately followed by centrifugation. After washing twice with cold phosphate-buffered saline, cell pellets were frozen and stored at −80 °C until use. To test the repair efficiency of oxidative DNA lesions, cells washed in phosphate-buffered saline were further resuspended in 20 ml of Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 with 10% bovine serum (Hyclone Laboratories) and cultured at 37 °C for up to 6 h.

**DNA Isolation from Cultured Cells**

DNA isolation from cultured cells was performed using the PureGene DNA isolation kit (Genta Systems, Inc.). Briefly, cell pellets were thawed and lysed in lysis buffer supplemented with 20 mM TEMPO. After protein precipitation with a protein precipitation solution, the DNA/RNA mixture in the supernatant was precipitated with isopropanol. The DNA/RNA pellet was resuspended in lysis buffer with 10 mM TEMPO and incubated with RNAses T1 (50 units/ml) and A (100 µg/ml) at 37 °C for 30 min, followed by protein and DNA precipitation. The DNA pellet was resuspended in sterilized distilled water with 1 mM TEMPO. The DNA solution was stored at −80 °C for assays.

**AP Site Repair Assay with Human β-pol**

The AP site repair assay was performed by a procedure slightly modified from the AP site cleavage assay.

**5'-Regular AP Sites**—Eight µg of DNA pretreated with heat/acid buffer (12) and 90 units of Exo III were incubated in 45 µl of 50 mM Hepes/KOH (pH 7.4) containing 50 mM NaCl and 8 mM CaCl2 at 37 °C for 1 min to introduce 5'-nick regular AP sites. In this experiment, CaCl2 was used instead of MgCl2 to avoid further DNA degradation by the exonuclease activity of Exo III. The DNA solution was subsequently incubated with human β-pol (a gift from Dr. S. H. Wilson, NIEHS, National Institutes of Health) or putrescine at different concentrations in 67.5 µl of 50 mM Hepes/KOH (pH 7.4) containing 50 mM NaCl and 5.4 mM CaCl2 at 37 °C for 30 min. The alkaline-processed reaction was performed in the mixture supplemented with 3.4 µl of 100 mM EDTA, 64.1 µl of 50 mM Hepes/KOH, and 15 µl of 10 mM aldehyde reactive probe at 37 °C for 10 min and immediately analyzed by the ASB assay.

**Calf Thymus DNA Pre-exposed to the Fenton Reaction and DNA Isolated from Cells Exposed to H2O2—Eight µg of DNA pre-exposed to the Fenton reaction was incubated with human β-pol or putrescine at different concentrations as described above and analyzed by the AP site assay.

**Repair Efficiency**—The efficiency of AP site repair was calculated by the reduction of AP sites by β-pol divided by the reduction of AP sites by putrescine.

**5'-Nicked AP Sites Induced by Oxidative Stress**

DNA isolated from cultured cells was performed using the PureGene DNA isolation kit (Genta Systems, Inc.). Briefly, cell pellets were thawed and lysed in lysis buffer supplemented with 20 mM TEMPO. After protein precipitation with a protein precipitation solution, the DNA/RNA mixture in the supernatant was precipitated with isopropanol. The DNA/RNA pellet was resuspended in lysis buffer with 10 mM TEMPO and incubated with RNAses T1 (50 units/ml) and A (100 µg/ml) at 37 °C for 30 min, followed by protein and DNA precipitation. The DNA pellet was resuspended in sterilized distilled water with 1 mM TEMPO. The DNA solution was stored at −80 °C for assays.
III-sensitive sites, and 8-OH-dGs in calf thymus DNA following the Fenton reaction. End III cleaves the N-glycosylic bond between deoxyribose and most oxidized pyrimidines, leaving 3'-cleaved AP sites (3). The number of End III-sensitive sites was calculated from the number of AP sites with End III treatment minus the number of AP sites with putrescine treatment. Since commercially available calf thymus DNA contains relatively large amounts of oxidative base lesions even without any treatment, we isolated DNA from fresh calf thymus with 10 mM TEMPO in this experiment. Whereas the steady-state level of AP sites was detected at 8 lesions/10^6 nucleotides in isolated calf thymus DNA, endogenous 8-OH-dG (detection limit: 1 lesion/10^7 dGs) was not detectable, and End III-sensitive sites (detection limit: 2 lesions/10^6 nucleotides) were around the detection limit. Using calf thymus DNA isolated in this laboratory, a combination of 10 mM H_2O_2 and 10 mM FeSO_4 generated End III-sensitive sites and 8-OH-dG at 96 and 424 lesions/10^6 nucleotides, respectively (Fig. 1, C and D). These results indicated that the Fenton reaction induced by 10 mM H_2O_2 and 10 mM FeSO_4 produced predominantly 8-OH-dG, followed by pyrimidine base lesions and AP sites (the ratio of 8-OH-dGs, End III-sensitive sites, and AP sites was 9.7:2.2:1). In addition to AP sites, the formation of these oxidative base lesions by the Fenton reaction was almost completely protected by TEMPO at concentrations ranging from 10 to 20 mM (Fig. 1, C and D). In the subsequent experiments, we isolated DNA from cultured cells with lysis buffer supplemented with 20 mM TEMPO to avoid artifactual formation of oxidative base lesions as well as AP sites.

AP Site Cleavage Assay for AP Sites Induced by the Fenton Reaction—ROS can induce sugar lesions directly by hydrogen abstraction of deoxyribose, resulting in AP site formation; 3'- or 5'-nicked or intact, the AP site cleavage assay was performed for MX-pretreated calf thymus DNA exposed to the Fenton reaction. MX-pretreated calf thymus DNA containing 5.9 ± 0.9 (mean ± S.D.) AP sites/10^6 nucleotides was incubated with 10 mM H_2O_2 and 10 mM FeSO_4 for 10 min. The number of AP sites increased to 37 AP sites/10^6 nucleotides (Fig. 2A). In this assay, we used Exo III as the class II AP endonuclease to identify 3'-cleavage of AP sites and putrescine to detect 5'-nicks. Immediately after the Fenton reaction, DNA was incubated with Exo III and/or putrescine, followed by the ASB assay. A single treatment of Exo III reduced the number of AP

**Fig. 1.** Detection of DNA lesions in calf thymus DNA following the Fenton reaction. A, quantitation of AP sites in MX-pretreated calf thymus DNA; B, effect of TEMPO on AP site formation; C, quantitation of End III-sensitive sites in calf thymus DNA following the Fenton reaction with or without TEMPO; D, quantitation of 8-OH-dG in calf thymus DNA following the Fenton reaction with or without TEMPO. The mean values were from duplicate slots of four individual samples. Bars indicate S.D. N.D. indicates that the number of lesions was under the detection limit.

**Fig. 2.** AP site cleavage assay of calf thymus DNA following the Fenton reaction. A, quantitation of AP sites by the AP site cleavage assay. The original number of AP sites in calf thymus DNA was reduced by MX (No Treatment). DNA was then incubated with 10 μM H_2O_2 and 10 μM FeSO_4 (−/−). DNA was then incubated with Exo III and/or putrescine (Exo III/−, Exo III only; Exo III/Putre, Exo III plus putrescine; −/Putre, putrescine only), and the number of remaining AP sites in calf thymus DNA was measured by the ASB assay. The mean values were from duplicate slots of four individual samples. Bars indicate S.D. B, summary of AP site cleavage assay of DNA following the Fenton reaction or heat/acid buffer treatment (13).
sites and residual aldehydic lesions are shown in Fig. 2. Reaction. The summarized fractions of intact and cleaved AP sites was reduced by 86% from the original number of AP sites. After incubation with Exo III followed by putrescine, the number of AP sites-cleavage of AP sites during incubation with these oxidized DNA lesions. Furthermore, these data suggest that the repair of 8-OH-dG may be more efficient compared with the repair of AP sites and oxidized pyrimidine base lesions.

Repar Efficiency of Oxidative DNA Lesions in Cells Exposed to H2O2—To further investigate the repair efficiency of these oxidative DNA lesions, the cultured cells were post-incubated in fresh medium with 10% serum for up to 6 h after the exposure to 10 mM H2O2. 8-OH-dG was repaired ~83% within 6 h, and oxidized pyrimidines were repaired ~40% (Fig. 3C). In contrast, we detected no reduction in the number of AP sites after the 6-h repair period. The data further confirmed that AP sites induced by H2O2 are more resistant to cellular excision repair pathways compared with oxidized bases.

Characterization of AP Sites in Cells Exposed to H2O2—The AP sites in cells exposed to H2O2 were characterized using the AP site cleavage assay. The number of 5'-AP sites and residual aldehydic lesions increased 2–3 times compared with controls after exposure to 10 mM H2O2 (Fig. 4). These lesions tended to accumulate during the repair period. In contrast, the combined fraction of 3'-nicked and intact AP sites did not increase in cells exposed to H2O2. To better understand the persistence of 5'-AP sites in cells after exposure to H2O2, we tested whether β-pol could excise 5'-AP sites introduced by oxidative stress. MX-pretreated calf thymus DNA exposed either to the Fenton reaction or to heat/acid buffer followed by the incision 5'-AP sites by Exo III was incubated with β-pol or putrescine. The efficiency of AP site repair was calculated by the reduction of AP sites through β-elimination by β-pol divided by the reduction of AP sites through β-elimination by putrescine. β-pol efficiently excised 5'-regular AP sites at a concentration of 60 ng/67.2 μl (Fig. 5). In contrast, 5'-AP sites directly introduced by ROS were less efficiently excised from the DNA backbone by β-pol. To address whether 5'-AP sites in cells exposed to H2O2 are repaired like 5'-regular AP sites or 5'-AP sites/ROS, the DNA from HeLa cells exposed to 20 mM H2O2 was incubated with β-pol at 60 ng/67.2 μl, followed by the ASB assay. These 5'-AP sites were also excised less efficiently by β-pol compared with 5'-regular AP sites. We also detected a β-pol-resistant AP site fraction after a combined treatment of Exo III and β-pol (data not shown).

**DISCUSSION**

A large number of AP sites are produced continuously by spontaneous depurination in mammalian cells (12), leaving intact AP sites. Oxidative stress also induces labile ring-saturated pyrimidine adducts that result in intact AP sites by chemical depyrimidination. These intact AP sites are subsequently incised 5' to AP sites by class II AP endonuclease. Most
oxidative base lesions are also excised by bifunctional DNA glycosylases with AP lyase activity, which introduce 3'-AP sites. In addition, hydrogen abstraction directly induces both 5'- and 3'-nicked AP sites (8–10). Therefore, a significant number of intact, 5'- and 3'-cleaved AP sites may be induced in cells under oxidative stress conditions. The present study demonstrated that oxidative stress predominantly induced 5'-cleaved AP sites in DNA in vitro and in vivo. Furthermore, 5'-nicked AP sites directly induced by ROS were efficiently released from the DNA backbone through β-elimination by putrescine, but not by β-pol (Fig. 5). In contrast, 5'-cleaved regular AP sites induced by heat/acid treatment followed by Exo III were efficiently excised by either putrescine or β-pol. These results indicate that the 5'-AP sites induced in vitro and in vitro by ROS are repaired differently than 5'-regular AP sites. In B-form duplex DNA, ROS most likely induce sugar lesions directly by abstraction of hydrogen atoms at the C-4' or C-5' position of deoxyribose (9–11). Under aerobic conditions, hydrogen abstraction at C-4' results in DNA cleavage to produce the 3'-phosphoglycolate terminus, the base propenal, and the 5'-monooester phosphate terminus. In contrast, under anaerobic conditions, hydroxyl radicals induce C-4'-monoester phosphate terminus. In contrast, under anaerobic conditions, the 3'-phosphoglycolate terminus, the base propenal, and the substrate for 8, 11). These AP sites with an aldehydic moiety should be a 9-oxidized aldehydic AP 9-dRP (deoxyribose phosphate) moiety in long genomic DNA as efficiently as those in oligonucleotides at similar concentrations. These data suggest that β-pol efficiently recognizes and excises 5'-cleaved regular AP sites under physiologically relevant conditions.

Aldehydic AP sites were relatively minor oxidative DNA lesions generated by the Fenton reaction in in vitro experiments, whereas these AP sites became one of the major oxidative lesions in genomic DNA from cells exposed to H₂O₂. Furthermore, 5'-cleaved AP sites were more persistent compared with oxidative base lesions in cultured cells after exposure to oxidative stress. As described above, putrescine, but not β-pol, efficiently excised 5'-AP sites induced by ROS. These data indicate that 5'-AP sites induced by oxidative stress are not repaired efficiently by cellular excision repair pathways. However, the results regarding the efficiency of repair by putrescine indicate that the lesions are potentially repairable through β-elimination by an amine moiety. It has been proposed that the amine residue Lys⁷² in β-pol forms a Schiff base intermediate with the AP site and cleaves 3' to the AP site (19). The difference in dRP lyase activity between putrescine and β-pol for 5'-AP sites induced by ROS suggests that the amine moiety of Lys⁷² in β-pol may not efficiently reach the aldehydic moiety of 5'-nicked oxidized AP sites. This inefficiency might be explained as follows: 1) β-pol inefficiently recognizes these 5'-aldehydic AP sites induced by ROS; or 2) after β-pol recognizes 5'-oxidized AP sites, Lys⁷² in β-pol does not reach the aldehydic moiety of these AP sites due to structural difference of oxidized AP sites. Although 5'-nicked C-4'-oxidized AP sites induced by bleomycin followed by human AP endonuclease are excised by β-pol (20), the excision efficiencies of β-pol for 5'-nicked C-4' or C-5'-oxidized AP sites directly induced by oxidative stress are still unknown. We hypothesize that 5'-oxidized AP sites directly induced by ROS may be repaired by the Flap endonuclease-1-dependent long patch base excision pathway. In our previous study, we found large numbers of endogenous 5'-nicked AP sites in rat tissues and human liver (13). Interestingly, the cleavage fractions of AP sites induced by the Fenton reaction are similar to those of endogenous AP sites in rat and human tissues. Although it has been believed that AP sites are efficiently repaired, oxidized AP sites are not excised as efficiently as regular AP sites in cells. Therefore, we believe that endogenous AP sites arise primarily from oxidized AP sites rather than from regular AP sites. We suggest that the high steady-state level of AP sites might be due to an inefficient short patch base excision repair pathway by β-pol.

It was originally demonstrated that β-pol required MgCl₂ for dRP lyase activity (21). However, Prasad et al. (18) proposed that β-elimination by β-pol is Mg²⁺-independent based on inhibition of dRP lyase activity by EDTA and restoration of dRP lyase function by supplementing with NaCl. Our results also showed that Ca²⁺, instead of Mg²⁺, quite efficiently excised dRP moieties from the DNA backbone. These data indicate that Mg²⁺ in not an essential cofactor for the dRP lyase activity of β-pol.
The human enzymes counteracting most oxidative base lesions are bifunctional DNA glycosylases such as human 8-hydroxy-2'-deoxyguanine-DNA glycosylase and human endonuclease III (22), leaving 3'-AP sites after releasing modified bases. Subsequently, class II AP endonuclease removes the 3'-blocked termini by 3'-phosphoesterase activity to create a 3'-OH group for DNA repair synthesis (3). Although ROS induces significant numbers of oxidized base adducts, there was no accumulation of the combined fraction of intact and 3'-nicked AP sites in cellular DNA after exposure to H2O2. These data suggest that class II AP endonuclease efficiently excises a large number of 3'-cleaved AP sites. In in vitro repair assays, 8-OH-dG and oxidized pyrimidines were repaired mainly by a short patch base excision repair pathway (6, 7, 23). However, the DNA repair synthesis at 8-OH-dG was less efficient than that at regular AP sites (7). Therefore, it has been proposed that the first three processes from base release to excision of 3'-AP sites may be rate-limiting steps. Our data suggest that 3'-phosphoesterase activity to repair 3'-AP sites is not rate-limiting in base excision repair. Based on these results, the excision of modified bases may be one of the rate-determining processes in the 8-OH-dG base excision repair pathway. Furthermore, in human cultured cells, oxidative stress induced AP endonuclease and rendered cells resistant to oxidative stress (26). These results also raised the possibility that 3'-AP sites generated by bifunctional DNA glycosylases may induce AP endonuclease. In addition to 3'-AP sites, ROS also induced other 3'-phosphate lesions, including 3'-phosphoglycolate. These 3'-blocked termini might be one of the reasons for AP endonuclease induction in cells under oxidative stress conditions. Although the Fenton reaction directly induced a significant number of intact AP sites in the in vitro system, the number of intact AP sites was not increased in cells exposed to H2O2. Both regular AP sites and C-4'-oxidized AP sites without strand breaks directly induced by bleomycin are repaired by an interaction of AP endonuclease and β-pol in vitro using oligonucleotides (20, 27). Based on these data and our experiments, the regular and oxidized aldehydeic AP sites with no cleavage on either side induced by ROS appear to be efficiently repaired in cells through a base excision repair pathway.

A high concentration of TEMPO almost completely protected the formation of AP sites, End III-sensitive sites, and 8-OH-dG induced by a high level of oxidative stress. Furthermore, DNA extracted from fresh calf thymus also showed very low amounts of 8-OH-dG (<1 lesion/10^9 nucleotides). In contrast, the range of steady-state levels of 8-OH-dG measured by HPLC/electrochemical detection has varied from 4 to 800 lesions/10^9 nucleotides in mammalian cells and tissues (28). There are many factors that artifactually induce oxidative DNA lesions during DNA extraction (24). The trapping of free radicals by TEMPO appears to be quite efficient for preventing artificial DNA damage from oxidative stress. Therefore, the current DNA extraction method using a high concentration of TEMPO minimizes the artificial induction of oxidative lesions during DNA extraction. In the present experiment, 10 mM H2O2 increased the number of 8-OH-dGs by a factor of >30 over the control. These data indicate that reduction of artificial oxidative DNA lesions will also allow us to more accurately determine dose-response relationships as well as the repair kinetics of these lesions after oxidative stress.

Further studies are needed to understand the biological consequences of 5'-AP sites persisting in cells under normal physiological conditions as well as after oxidative stress. Although H2O2 killed HeLa cells within 24 h at 20 mM, a limited number of cells survived after exposure to 10 mM H2O2 and started growing within 1–2 days (data not shown). These results suggest that 5'-oxidized AP sites are repairable by cellular DNA repair pathways. Recently, Jackson et al. (25) demonstrated that oxidative stress, but not UV radiation or methylating agent, induces frameshift mutations in microsatellite DNA. They proposed that a common lesion such as a strand break is more likely to contribute to genomic instability than the alteration of a specific nucleotide. It is possible that 5'-oxidized AP sites might be involved in the frameshift mutation in microsatellite DNA. To date, it has generally been believed that AP sites are repaired very efficiently in genomic DNA; however, the high steady-state level of 5'-nicked AP sites as well as persistent 5'-cleaved AP sites after oxidative stress suggest that some fraction of AP sites may not be efficiently repaired by the mammalian excision repair pathway.

Acknowledgments—We thank Drs. S. H. Wilson, Y. W. Kow, and K. McDorman for providing human β-pol, E. coli End III, and fresh calf thymus, respectively.

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