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

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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 H_2O_2 and $FeSO_4$ induced significant numbers of AP sites in calf thymus DNA, which were predominantly cleaved 5′ to the AP sites (75% of total aldehydic AP sites). An increase in the number of 5′-AP sites was also detected in human cultured cells exposed to H_2O_2, 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 H_2O_2. These results suggest that 5′-oxidized AP sites induced by ROS are not efficiently repaired by the mammalian short patch base excision repair pathway.

Reactive oxygen species (ROS)[1] are generated continuously in cells during normal metabolic processes and by a number of exogenous agents, including ionizing radiation. ROS can react with cellular components such as proteins, lipids, and nucleic acids to induce DNA adducts such as 8-hydroxy-2′-deoxyguanosine (8-OH-dG) (1, 2). It is believed that these oxidized bases are predominantly repaired by a base excision repair pathway (3). In this process, a bifunctional 8-OH-dG-DNA glycosylase with apurinic/apyrimidinic (AP) lyase such as 8-hydroxy-2′-deoxyguanine-DNA glycosylase cleaves the *N*-glycosylic bond between 8-hydroxyguanine and deoxyribose and incises immediately 3′ to AP sites, leaving 3′-nicked AP sites (4, 5). The 3′-AP sites generated by the DNA glycosylase are subsequently excised by class II AP endonuclease (3), resulting in a 3′-hydroxyl group and a 5′-phosphate group. Repair is completed by polymerase and ligase activity. Recently, it has been reported that mammalian cell extracts repair 8-OH-dG preferentially via single nucleotide replacement reactions (6, 7). The contribution of nucleotide excision repair to the removal of 8-OH-dG was not significant in experiments using human cell extracts (6, 7). These results indicate that base excision repair plays a central role in counteracting oxidized base lesions.

In addition to base damage in DNA, ROS also induce lesions by hydrogen abstraction of the deoxyribose, frequently producing oxidized AP sites as well as DNA strand breaks (8). AP sites are also generated spontaneously by chemical depurination of labile oxidized bases and enzymatically by DNA glycosylases as mentioned above. Hydrogen abstraction has been examined extensively for model deoxyribose and polynucleotides (9). Although <10% of the hydroxyl radicals attack sugar residues in single-stranded polynucleotides, it has been proposed that oxidized AP sites induced by ROS may be one of the major oxidative lesions in double-stranded DNA (3, 9). These studies demonstrated that all hydrogen atoms of deoxyribose and ribose are potential targets for direct attack by oxygen radicals. In B-form duplex DNA, however, hydrogen atoms at the C-4′ and C-5′ positions of deoxyribose are the most accessible to ROS (10). ROS-induced sugar lesions and strand cleavage in genomic DNA are difficult to examine, mainly due to the large variety of products as well as their instability even at mild temperatures and neutral pH (11). Many oxidized sugars are very labile, as terminal sugar lesions tend to be modified spontaneously during experimental procedures.

We recently developed a sensitive aldehyde reactive probe slot-blot (ASB) assay to detect aldehydic AP sites in DNA, which can quantitate <1 AP site/10^6 nucleotides (12). Using this assay, we detected 50,000–200,000 AP sites in mammalian cells under normal physiological conditions (13). Large numbers of AP sites were detected in brain, heart, and colon DNAs, which appear to be continuously exposed to higher levels of oxidative stress. These endogenous AP sites were predominantly cleaved 5′ to the AP sites. Therefore, we hypothesized that oxidative stress directly induces 5′-nicked oxidized AP sites, which may contribute to a high steady-state level of AP sites in mammalian cells and tissues. To test this hypothesis, we have quantitated and characterized AP sites induced by ROS. We also have examined the repair efficiency of these AP sites in human cultured cells.

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-

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[1] 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.

eridinoxyl (TEMPO; Aldrich) on ice. DNA was then isolated by phenol/Sevag (chloroform:isoamyl alcohol, 24:1) extraction and purified as described (13).

Methoxyamine Treatment of Calf Thymus DNA

Calf thymus DNA (Sigma) was treated with 10 mM methoxyamine (MX) in 10 mM Tris-HCl/KOH (pH 7.4) and purified as described (12).

H_2O_2 and $FeSO_4$ 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 H_2O_2 and/or $FeSO_4$ in 10 mM Tris-HCl/KOH (pH 7.4) at 37 °C for 10 min with or without TEMPO. The AP site assay was performed immediately after the Fenton reaction. For measurement of oxidative base lesions, the Fenton reaction was quenched by addition of 15 mM TEMPO, and the DNA was recovered by precipitation with cold ethanol. 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 aldehyde reactive 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 with 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 Assay—Eight μg of DNA and 145 units of *Escherichia coli* exonuclease III (Exo III) (New England Biolabs Inc.) were incubated in 135 μl of 10 mM Tris-HCl/KOH (pH 7.5) containing 50 mM NaCl and 5 mM $MgCl_2$ at 37 °C for 1 min and immediately analyzed by the ASB assay.

3′-Cleavage 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 μ1 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 was 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 mV. The concentration of 8-OH-dG was normalized to the amount of DNA analyzed, as determined by UV absorbance.

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 (4×10^5 cells/ml). The cultured cells were exposed to H_2O_2 (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 extraction kit (Gentra 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 isopropyl alcohol. The DNA/RNA pellet was resuspended in lysis buffer with 10 mM TEMPO and incubated with RNases T1 (50 units/ml) and A (100 mg/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 $CaCl_2$ at 37 °C for 1 min to introduce 5′-nicked regular AP sites. In this experiment, $CaCl_2$ was used instead of $MgCl_2$ 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 $CaCl_2$ at 37 °C for 30 min. The aldehyde reactive probe 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 H_2O_2—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.

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 H_2O_2 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 $FeSO_4$ with or without H_2O_2 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 $FeSO_4$ and was further enhanced by H_2O_2 (Fig. 1*A*). TEMPO, a radical-trapping reagent containing a nitrone 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 H_2O_2 and $FeSO_4$ 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. 1*B*).

Base Lesions Induced by the Fenton Reaction—To test whether AP sites are major oxidative lesions induced by the Fenton reaction, we compared the number of AP sites, End

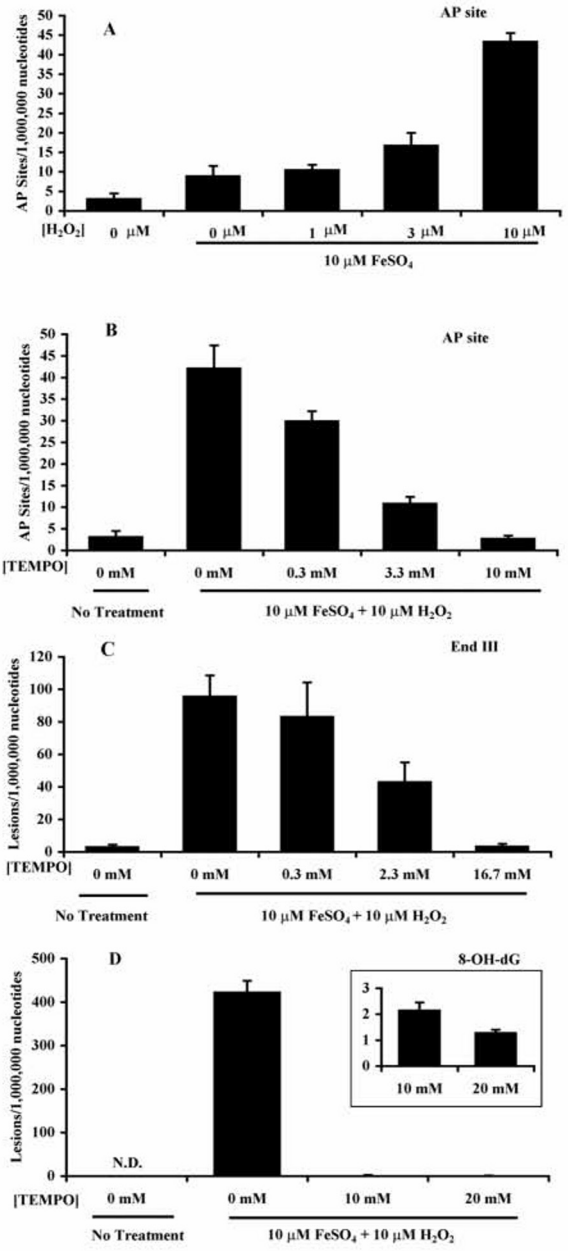

FIG. 1. **Detection of DNA lesions in calf thymus DNA following the Fenton reaction.** *A*, quantitation of AP sites in MX-pretreated calf thymus DNA; *B*, effect of TEMPO on AP site formation; *C*, quantitation of End III-sensitive sites in calf thymus DNA following the Fenton reaction with or without 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.

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

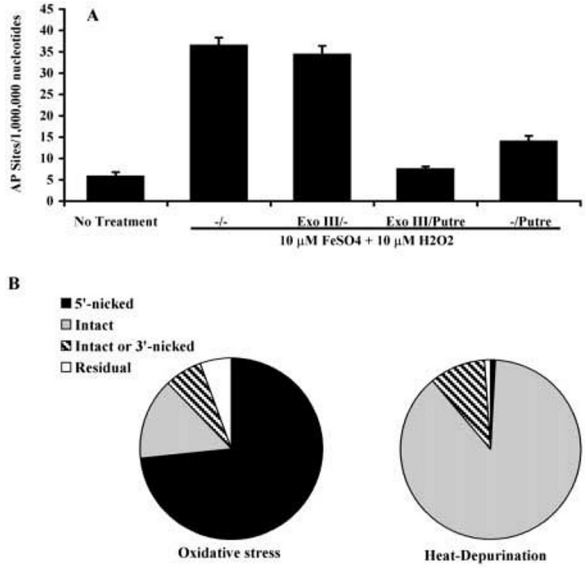

FIG. 2. **AP site cleavage assay of calf thymus DNA following the Fenton reaction.** *A*, quantitation of AP sites by the AP site cleavage assay. The original number of AP sites in calf thymus DNA was reduced by MX (*No Treatment*). DNA was then incubated with 10 μM H_2O_2 and 10 μM $FeSO_4$ (−/−). DNA 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).

(detection limit: 2 lesions/10^6 nucleotides) were around the detection limit. Using calf thymus DNA isolated in this laboratory, a combination of 10 μM H_2O_2 and 10 μM $FeSO_4$ 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 μM H_2O_2 and 10 μM $FeSO_4$ 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 sites as well as DNA strand breaks. AP sites are also generated spontaneously by chemical depurination of labile oxidized bases and unmodified bases and enzymatically by cleavage of the *N*-glycosylic bond between the sugar and modified bases. We recently developed an AP site cleavage assay to examine the site of cleavage at AP sites (13). To test whether the AP sites induced by ROS were 5′- or 3′-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 μM H_2O_2 and 10 μM $FeSO_4$ for 10 min. The number of AP sites increased to 37 AP sites/10^6 nucleotides (Fig. 2*A*). 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

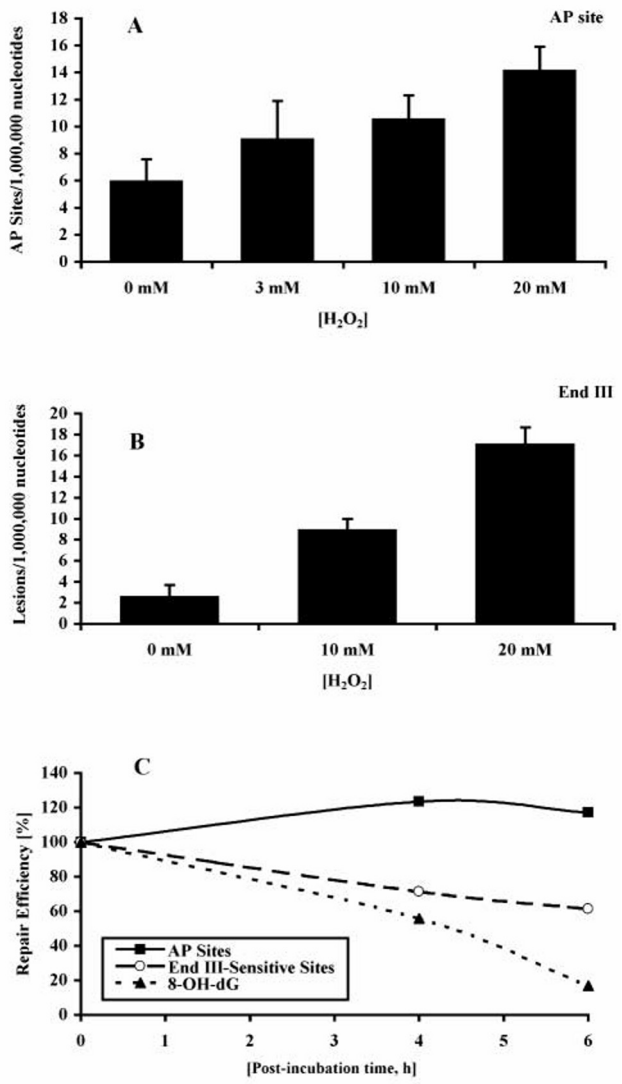

FIG. 3. **Induction and repair of oxidative DNA lesions in HeLa cells exposed to H_2O_2.** *A* and *B*, formation of AP sites and End III-sensitive sites, respectively, in cells exposed to H_2O_2 for 15 min; *C*, repair kinetics of AP sites, End III-sensitive sites, and 8-OH-dG at different time periods (0–6 h) after exposure to H_2O_2. The mean values were from four to five individual samples. *Bars* indicate S.D.

sites to 34 AP sites/10^6 nucleotides. This reduction was comparable to the data we published earlier (13) and may be due to the combination of enzymatic incision on the 5′-side by Exo III and nonspecific 3′-cleavage of AP sites during incubation with Exo III. In contrast, putrescine treatment resulted in significant reduction of the original number of AP sites. After incubation with Exo III followed by putrescine, the number of AP sites was reduced by 86% from the original number of AP sites in MX-pretreated calf thymus DNA exposed to the Fenton reaction. The summarized fractions of intact and cleaved AP sites and residual aldehydic lesions are shown in Fig. 2*B* (*left*). A major finding was that the AP site cleavage fractions induced by the Fenton reactions were different from those induced by heat/acid depurination (Fig. 2*B*, *right*) (13).

Oxidative DNA Lesions in Cells Exposed to H_2O_2—To evaluate AP site formation in cellular DNA by oxygen radicals, we exposed HeLa S3 cells to H_2O_2 at 3–20 mM without serum at 37 °C for 15 min. Toxicity of H_2O_2 to cells was determined by the trypan blue exclusion assay. The viability of cells was >95% when the cultured cells were harvested. HeLa cells showed a slight increase in the number of AP sites following

TABLE I
Number of 8-OH-dGs in HeLa cells exposed to 10 mM H_2O_2

H_2O_2 exposure	8-OH-dGs/10^6 nucleotides (±SD)[a]		
	0 h[b]	4 h	6 h
0 mM	0.05 ± 0.06[c]		
10 mM	2.72 ± 0.21	1.53 ± 0.44	0.47 ± 0.24

[a] 8-OH-dG was analyzed from four individual samples/group.
[b] Time after exposure to H_2O_2.
[c] The numbers of 8-OH-dGs were not detectable for two samples. The means ± S.D. were calculated for the sample as zero (individual values: 0.1, 0.1, <0.1, and <0.1).

exposure to H_2O_2 in a dose-dependent manner (Fig. 3*A*). The number of End III-sensitive sites and 8-OH-dGs was also increased by treatment with H_2O_2 (the ratio of induction of 8-OH-dGs, End III-sensitive sites, and AP sites at 10 mM H_2O_2 was ~0.6:1.4:1) (Fig. *3B* and Table I). If we assume that H_2O_2 exposure induces the Fenton reaction in cellular DNA, AP sites become among one of the major oxidative DNA lesions in cells. 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.

Repair Efficiency of Oxidative DNA Lesions in Cells Exposed to H_2O_2—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 H_2O_2. 8-OH-dG was repaired ~83% within 6 h, and oxidized pyrimidines were repaired ~40% (Fig. 3*C*). 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 H_2O_2 are more resistant to cellular excision repair pathways compared with oxidized bases.

Characterization of AP Sites in Cells Exposed to H_2O_2—The AP sites in cells exposed to H_2O_2 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 H_2O_2 (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 H_2O_2. To better understand the persistence of 5′-AP sites in cells after exposure to H_2O_2, 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′ to 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 H_2O_2 are repaired like 5′-regular AP sites or 5′-AP sites/ROS, the DNA from HeLa cells exposed to 20 mM H_2O_2 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

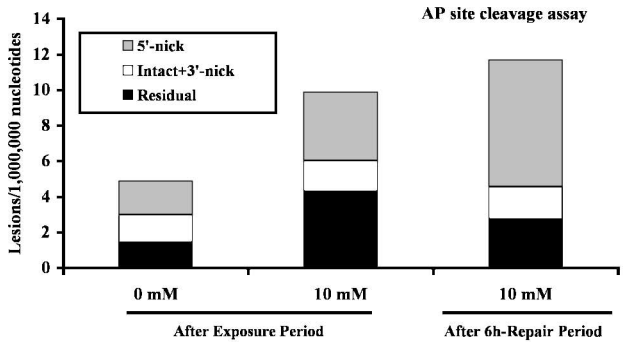

FIG. 4. **AP site cleavage assay of DNA isolated from HeLa cells exposed to H_2O_2.** AP site cleavage assays were performed for DNA isolated from HeLa cells immediately after exposure to 0 or 10 mM H_2O_2, and cells were post-incubated with fresh medium for 6 h after 10 mM H_2O_2 exposure. The mean values were from duplicate slots of four individual samples.

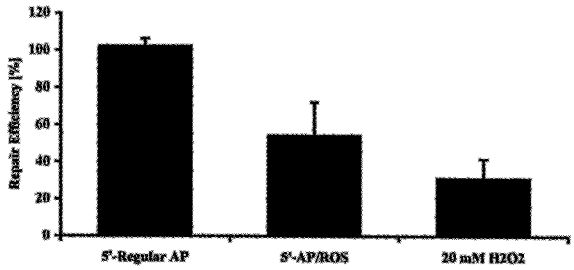

FIG. 5. **AP site repair assay by β-pol.** The efficiency of excision of 5′-nicked AP sites by the dRp lyase activity of β-pol was determined for 5′-regular AP sites and 5′-AP sites/ROS in calf thymus DNA and for 5′-AP sites in DNA from HeLa cells immediately after exposure to 20 mM H_2O_2. The efficiency of AP site repair was calculated by the reduction of AP sites by β-pol divided by the reduction of AP sites by putrescine. The mean values were from duplicate slots of three individual samples.

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 vivo* 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′-monoester phosphate terminus. In contrast, under anaerobic conditions, hydroxyl radicals induce C-4′-hydroxylated abasic sites with an equilibrium between C-4′-oxidized aldehydic AP sites. Hydrogen abstraction at C-5′ has also been proposed to produce 5′-cleaved aldehydic AP sites under aerobic conditions (8, 11). These AP sites with an aldehydic moiety should be a substrate for β-elimination and are detectable by the ASB assay. Therefore, we hypothesize that the 5′-AP sites induced by ROS represent oxidized AP sites such as 5′-cleaved C-4′- or C-5′-oxidized AP sites.

Whereas putrescine excised 5′-regular AP sites at 100 mM, β-pol efficiently cleaved 3′ to the 5′-AP sites at 80 nM. These results are in good agreement with the differences in the efficiency of cleavage of intact and 5′-nicked AP sites by putrescine (17) and β-pol (18). A 49-base pair oligonucleotide duplex DNA (20 nM) with a single intact or 5′-incised AP site (10,000 lesions/10^6 nucleotides) was cleaved ~50% by treatment with 200 and 5 nM β-pol, respectively, for 15 min at 37 °C (18). In contrast, we utilized long genomic DNA containing a much lower frequency of AP sites (20 lesions/10^6 nucleotides), which appears to be a more biologically relevant frequency based on the number of endogenous AP sites (13). Interestingly, β-pol excised the 5′-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 H_2O_2. 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 Lys^{72} 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 Lys^{72} 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, Lys^{72} 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 $MgCl_2$ for dRp lyase activity (21). However, Prasad *et al.* (18) proposed that β-elimination by β-pol is Mg^{2+}-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 Ca^{2+}, instead of Mg^{2+}, quite efficiently excised dRp moieties from the DNA backbone. These data indicate that Mg^{2+} 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 induce 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 H_2O_2. 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 H_2O_2. 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 aldehydic 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/10^7 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/10^7 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 artifactual DNA damage from oxidative stress. Therefore, the current DNA extraction method using a high concentration of TEMPO minimizes the artifactual induction of oxidative lesions during DNA extraction. In the present experiment, 10 mM H_2O_2 increased the number of 8-OH-dGs by a factor of >30 over the control. These data indicate that reduction of artifactual 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 H_2O_2 killed HeLa cells within 24 h at 20 mM, a limited number of cells survived after exposure to 10 mM H_2O_2 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.

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