Hydrogen Peroxide Induces Topoisomerase I-mediated DNA Damage and Cell Death*

Received for publication, October 16, 2003, and in revised form, December 11, 2003
Published, JBC Papers in Press, December 19, 2003, DOI 10.1074/jbc.M311370200

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Reactive oxygen species modify DNA, generating various DNA lesions including modified bases such as 8-oxo- guanine (8-oxoG). These base-modified DNA lesions have been shown to trap DNA topoisomerase I (TOP1) into covalent cleavage complexes. In this study, we have investigated the role of TOP1 in hydrogen peroxide toxicity. We showed that ectopic expression of TOP1 in Saccharomyces cerevisiae conferred sensitivity to hydrogen peroxide, and this sensitivity was dependent on RAD9 checkpoint function. Moreover, in the mammalian cell culture system, hydrogen peroxide-induced growth inhibition and apoptosis were shown to be partly TOP1-dependent as evidenced by a specific increase in resistance to hydrogen peroxide in TOP1-deficient P388/CPT45 murine leukemia cells as compared with their TOP1-proficient parental cell line P388. In addition, hydrogen peroxide was shown to induce TOP1-DNA cross-links. These results support a model in which hydrogen peroxide promotes the trapping of TOP1 on oxidative DNA lesions to form TOP1-DNA cleavage complexes that contribute to hydrogen peroxide toxicity.

Reactive oxygen species (ROS)† (i.e. hydrogen peroxide, superoxide anion, and hydroxyl radical) generated during oxidative stress are known to damage proteins, nucleic acids, and cell membranes and have been implicated in cancer, aging, and several chronic neurodegenerative diseases (1–4). There are a spectra of DNA lesions generated by ROS, with the most abundant being a base modification to 7,8-dihydro-8-oxoguanine (8-oxoG) (5, 6). In vivo measurements estimate a high number (10⁵) of such oxidatively modified lesions formed in a cell each day (7). Conversion of guanine to 8-oxoG causes a selective mis-incorporation of adenine opposite to 8-oxoG that can lead to G-T transversions in the DNA (8, 9). In the absence of repair, mis-incorporation of adenine opposite to 8-oxoG can lead to apoptosis and the carcinogenic benzo[a]pyrene-DNA adducts, and aberrant DNA structures (e.g. nicks, mismatches, abasic sites, and AraC-substituted DNA), are now known to trap TOP1-DNA cleavage complexes (20–23). It has been recently demonstrated that oxidative base lesions such as 8-oxoG can increase TOP1 binding to DNA and also induce a 3–7-fold increase in TOP1 cleavage complexes (24, 25).

In this study, we have investigated the role of TOP1 in oxidative DNA damage both in yeast and cultured mammalian cells using hydrogen peroxide, an ROS known to cause oxidative DNA damage primarily through the hydroxyl radical that results from the Fenton reaction (26–28). We found that in Saccharomyces cerevisiae, ectopic expression of TOP1 sensitized cells to hydrogen peroxide. Moreover, in the mammalian system, TOP1-deficient P388/CPT45 murine leukemia cells exhibited an increased resistance to hydrogen peroxide in both growth inhibition and apoptosis assays as compared with their TOP1-proficient parental P388 cells. These results, coupled with the demonstration of TOP1-DNA cross-links in HeLa cells treated with hydrogen peroxide, suggest a potential role for TOP1 in hydrogen peroxide cytotoxicity.

**MATERIALS AND METHODS**

**Drugs, Yeast Strains, Plasmids, and Cell Lines—Camptothecin (CPT), etoposide (VP-16), and vinblastine were purchased from Sigma. VM-26 (teniposide) was obtained as a gift from Bristol-Myers-Squibb Co. ARC-111 (8,9-dimethoxy-2,3-methyleneedioxy-5-[2-(N,N-dimethylamino)ethyl]-5H-dibenzo[a,j]1,6-naphthyridin-6-one) (topo) was a gift from Dr. Edmund J. LaVoie (Rutgers University) (29). All yeast strains used in this study are listed in Table I (30–34). In experiments requiring ectopic expression of TOP1, strains were transfected with pGALyTOP1 or pGALyTOP1 (30, 35). Strains transformed with yCP50 served as vector controls, whereas strains transformed with pGALyTOP1 expressed yeast TOP1 under the control of the yeast GAL1 promoter. Strains carrying pGALyTOP1 expressed mutant yeast TOP1 with its active site tyrosine 727 mutated to phenylalanine (36). The tdp1 mutant HNY66, the rad9 mutant HNY243, the rad18rad9 double mutant HNY244, the rad6tdp1 double mutant HNY361, and the rad6tdp1top1 triple mutant HNY328 were all derived from the parental strain HNY102 (Table I (37). The mouse leukemia parental cell line P388 and its CPT-resistant subline P388/CPT45 (38–41) were cultured in suspension at 37 ºC in a 5% CO₂ incubator and maintained by regular passage in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum, 2-glutamine (2 mM), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 50 μM 2-mercaptoethanol.

**Yeast Clonogenic Survival Assay—Sensitivity of yeast cells to hydro-
gen peroxide was performed by exposing log phase cells to synthetic complete medium without uracil but with galactose as the carbon source (SC-ura/GAL). After galactose induction, cells were diluted and treated with hydrogen peroxide for 1 h at 30 °C. Subsequently, diluted samples were plated in duplicate and clonogenic survival was assayed by counting the number of viable colonies.

MTT Assay—Growth inhibition was determined using the MTT assay as described previously (42). The MTT assay was performed using 96-well microtiter plates. For determination of IC_{50} values, cells were exposed continuously to varying drug concentrations, followed by MTT assays at the end of the fourth day.

DNA Fragmentation Assay—For detection of apoptosis, chromosomal DNA fragments released from nuclei during apoptosis were measured as described previously (43). Briefly, 2.5 × 10^{6} cells were treated with the appropriate drug for 4 h at 37 °C. Cells were then pelleted and lysed in 0.4 ml lysis buffer (10 mM Tris- HCl, pH 8.0, 20 mM EDTA, and 0.2% Triton X-100) and incubated on ice for 20 min. After centrifugation to remove nuclei, chromosomal DNA in the supernatant (including both high molecular weight DNA and nucleosomal DNA fragments) were extracted with phenol/chloroform, and precipitated with 1:10 volume of ethanol.

CTD assay—The volume of the lysate was adjusted with 1% Sarkosyl to 2 ml and layered gently on top of the gradient. The samples were centrifuged at 31,000 rpm for 24 h. Fractions were obtained using a different Beckman SW41 rotor at 31,000 rpm for 24 h. Fractions were collected from the bottom of the tube, and 100 μl of each fraction was diluted with 100 μl of 25 mM sodium phosphate buffer (pH 6.5) and then spotted onto a nitrocellulose membrane in a slot-blot apparatus. The membrane was washed with sodium phosphate buffer and immunoblotted with anti-TOPO1 antibodies.

Immunoblotting Analysis—Treated cells were lysed directly with 2X SDS sample buffer. Proteins were separated in a 7% SDS-PAGE gel and subsequently transferred onto a nitrocellulose membrane. The membrane was stained with Ponceau to confirm protein loading. Immunoblotting of the membrane was performed using human anti-TOPO1 scleroderma antibodies (45).

FACS Analysis for Activated Caspase in Apoptotic Cells—Caspase activity was measured using the CaspACE FITC-VAD-FMK in situ marker from Promega according to the manufacturer’s instructions. Briefly, 10^{6} cells were stained with 25 μM FITC-VAD-FMK at 37 °C in the dark for 40 min. Cells were then washed and resuspended with PBS (phosphate-buffered saline). FACS analysis of cells was performed with excitation of 488 nm and emission of 525–550 nm.

RESULTS

Hydrogen Peroxide Sensitivity of Yeast top1 Mutant Strains—To examine the role of TOPO1 on hydrogen peroxide sensitivity, we determined the effect of TOPO1 underexpression on hydrogen peroxide sensitivity. Four top1 mutant strains together with their isogenic wild-type strains (see Table I) were used in a clonogenic survival assay. As shown in Fig. 1, top1 mutants TG107 and RS190 were more resistant to hydrogen peroxide as compared with their corresponding isogenic wild-type strains, CH335 and W3031-a, respectively. These results suggest that TOPO1 contributes to hydrogen peroxide sensitivity in these strains. However, the other two top1 mutant yeast strains, JCW27 and EKY3, exhibited about the same sensitivity to hydrogen peroxide as the corresponding wild-type strains, JCW25 and FY250, respectively. These results indicate that the strain background is important in conferring TOPO1-dependent sensitivity to hydrogen peroxide.

Ectopic Expression of TOPO1 Confers Sensitivity to Hydrogen Peroxide—To further examine the role of TOPO1 in hydrogen peroxide sensitivity, we determined the effect of TOP1 overexpression on hydrogen peroxide sensitivity. The yeast TOP1 deletion strain JN2–134 was transformed with a plasmid carrying the yTOP1 gene under a GAL-inducible promoter. As a control, JN2–134 cells were transformed with either the vector alone or the vector carrying the mutated yTOP1 gene (the active site tyrosine mutant, yTOP1_{Y727F}). The yeast cells were grown in selective media and subsequently induced with galactose overnight. After hydrogen peroxide treatment, cells were plated and assayed for colony formation. The results are shown in Fig. 1A. The ectopic expression of yTOP1 conferred sensitivity to hydrogen peroxide in a dose-dependent manner as compared with cells expressing the vector alone or cells expressing mutant yTOP1. As shown in Fig. 2B, the same result was obtained using a different top1 mutant strain, RS190 (see Fig. 1), which was shown to be resistant to hydrogen peroxide. Introduction of the yTOP1 gene to RS190 conferred sensitivity to hydrogen peroxide as compared with the cells bearing plasmids with the active site yTOP1_{Y727F} mutant or vector alone (Fig. 2B). Similar results were also obtained for another top1 mutant strain, TG107 (data not shown). These experiments demonstrate that overexpression of TOP1 confers hydrogen peroxide sensitivity to yeast with different genetic backgrounds.

Hydrogen Peroxide Sensitivity in tdp1 and rad9 Mutant Yeast—Tdp1p is a yeast tyrosine-DNA phosphodiesterase that has been implicated in the repair of TOP1 cleavable complexes (37, 46). Mutations in TDP1 sensitize cells to TOP1 poisons such as camptothecin (CPT) (37). This sensitivity to TOP1 poisons is also significantly increased in a rad9 mutant background defective in the G2 checkpoint (37, 47). In order to examine the potential role of TOPO1 in hydrogen peroxide sensitivity, we determined the hydrogen peroxide sensitivity in
The tdp1 and rad9 mutants. As shown in Fig. 3A, the tdp1 mutant was more sensitive to hydrogen peroxide and CPT (see the inset) than its corresponding wild-type strain. Similarly, the rad9 mutant (Fig. 3B) showed considerably more sensitivity to hydrogen peroxide as compared with its wild-type strain. The hydrogen peroxide sensitivity in the rad9tdp1 and rad9top1 double mutants, and the rad9top1tdp1 triple mutant was also investigated. As shown in Fig. 3C, the rad9 and rad9tdp1 mutants showed increased sensitivity to hydrogen peroxide as compared with their top1 mutant strains, again suggesting that hydrogen peroxide sensitivity depends on TOP1. In addition, hydrogen peroxide sensitivity (Fig. 3D), like CPT sensitivity (Fig. 3C), was affected by the RAD9 status and to a lesser extent by the TDP1 status. These results demonstrate that hydrogen peroxide sensitivity mirrors CPT sensitivity in its TDP1 and RAD9 dependence.

Selective Resistance of TOP1-deficient P388/CPT45 Murine Leukemia Cells to Hydrogen Peroxide—We have also investigated the role of TOP1 in hydrogen peroxide sensitivity in mammalian cells. The mouse leukemia P388 cells and the subline P388/CPT45 selected for CPT resistance were used in the current study. The P388/CPT45 cells are highly resistant to CPT (over 45-fold). By contrast, these cells were not significantly more resistant to VM-26 or vinblastine as compared with their TOP1-proficient parental cell line P388 (Fig. 4). The IC50 (μM) values for the various drugs in these cells are listed in Table II.

Apoptosis Induced by Hydrogen Peroxide Is Reduced in TOP1-deficient Cells—Hydrogen peroxide is known to induce both necrosis and apoptosis (48, 49). We have investigated the role of TOP1 in hydrogen peroxide-induced apoptosis by assaying two apoptotic endpoints, nucleosomal DNA fragmentation, and caspase activation. As shown in Fig. 5, A and B, increasing concentrations of hydrogen peroxide were shown to induce nucleosomal DNA fragments indicative of apoptotic cell death in the P388 cells, but not in the P388/CPT45 cells. By contrast, VP-16 was shown to induce nucleosomal DNA fragments in both P388 and P388/CPT45 cells, confirming the specificity of TOP1-dependent resistance in P388/CPT45 cells to hydrogen peroxide-mediated apoptosis. In addition to nucleosomal DNA fragmentation, apoptotic caspase activation was also monitored in the cell line pair upon treatment with hydrogen per-
Following treatment with hydrogen peroxide, cells were exposed to FITC-conjugated VAD-FMK (a caspase inhibitor that specifically binds to activated caspase), and then analyzed for FITC fluorescence by flow cytometry. As shown in Fig. 5, C and D, increasing concentrations of hydrogen peroxide induced a significant increase in activated caspase in the parental P388 cells as compared with the TOP1-deficient P388/CPT45 cells, as evidenced by a shift in FITC peak fluorescence. CPT (a TOP1 drug) and VM-26 (a TOP2 drug), which were used as controls, were also shown to induce apoptosis in the P388 cells, as demonstrated by the corresponding increase in activated caspase in these drug-treated cells. However, CPT- but not VM-26-induced caspase activation was reduced in the TOP1-deficient P388/CPT45 cells. This result suggests that the apoptotic pathway in TOP1-deficient P388/CPT45 cells is not altered. Consequently, the specific resistance to hydrogen peroxide in P388/CPT45 cells is likely due to TOP1 alteration (i.e. TOP1 deficiency) rather than other genetic changes.

Hydrogen Peroxide Traps TOP1-DNA Covalent Complexes in Cells—The ICE bioassay was used to demonstrate the trapping of TOP1-DNA covalent complexes by hydrogen peroxide in HeLa cells. Following treatment with hydrogen peroxide, cells were lysed in 1% Sarksyl and subjected to CsCl gradient fractionation. Collected fractions were subsequently immunoblotted for TOP1 in order to detect TOP1-DNA covalent complexes. The results are shown in Fig. 6. Hydrogen peroxide (25 mM, 30 min) was able to trap a small amount of TOP1-DNA complexes (see fraction 2). As a positive control,
the TOP1-specific poison CPT (25 μM, 30 min) was shown to trap TOP1-DNA covalent complexes as expected (see fractions 1 and 2).

**DISCUSSION**

In the current work, we have demonstrated that hydrogen peroxide toxicity is increased in yeast expressing TOP1. In our experiments, ectopic expression of yTOP1 in *S. cerevisiae* sensitized cells to hydrogen peroxide while the catalytic inactive yTOP1V727F mutant did not confer increased sensitivity, suggesting that the catalytic function of TOP1 is important in conferring hydrogen peroxide sensitivity. However, the phenotype resulting from the overexpression of a gene product could be due to a gain of function and may not reflect the physiological role of the gene product. Consequently, we investigated the effect of hydrogen peroxide on cells that underexpress TOP1. Four isogenic pairs of top1 mutant strains were examined for their sensitivity to hydrogen peroxide. Only two (i.e. TG107 and RS190) of the four top1 mutant strains exhibited increased resistance to hydrogen peroxide, suggesting that the TOP1-dependent lethality depends on the genetic background. In other words, TOP1-independent lethality can be the major pathway for hydrogen peroxide cytotoxicity in certain genetic backgrounds. It should be pointed out that ectopic expression of wild-type yTOP1 was able to restore sensitivity to hydrogen peroxide in the two top1 mutant strains (i.e. TG107 and RS190) that exhibited resistance to hydrogen peroxide, further supporting a role for yTOP1 in oxidative damage response.

**TDP1** has been suggested to be involved in the repair of TOP1 cleavage complexes (37, 46). However, our studies with tdp1 mutants have failed to demonstrate a significant role for TDP1 in hydrogen peroxide sensitivity/resistance. This result is probably not surprising since several pathways are known to be involved in the repair of TOP1-DNA covalent complexes (46, 50). Among them, the RAD9 pathway has been implicated in TOP1-DNA covalent complex repair since rad9 mutants exhibit increased sensitivity to the TOP1-specific drug CPT (37). RAD9 is a *S. cerevisiae* DNA damage checkpoint gene responsible for damage-induced cell cycle arrest at G1 and G2 (47). Several studies have demonstrated that hydrogen peroxide selectively causes RAD9-dependent cell cycle arrest in G2 (51). In addition, increased sensitivity to oxidatively damaging agents such as hydrogen peroxide is S-phase-specific and depends on RAD9 function (52). Our experiments with the rad9 top1 and rad9tdp1 double mutants have indeed demonstrated that hydrogen peroxide cytotoxicity, like CPT cytotoxicity, is both TOP1- and RAD9-dependent, suggesting the potential involvement of TOP1 cleavage complexes. The strong dependence of hydrogen peroxide cytotoxicity on TOP1 in rad9 mutant background further supports the notion that hydrogen peroxide, like CPT, kills cells through TOP1 cleavage complexes whose repair is defective in rad9 background.

It should be noted that our results regarding hydrogen peroxide parallel those obtained by Nitiss et al. (53) using methyl methanesulfonate and other DNA-damaging agents (e.g. UV).
FIG. 5. **Hydrogen peroxide-induced apoptosis is partly TOP1-dependent.** For experiments shown in panels A and B, P388 (A) and P388/CPT45 (B) cells were treated with hydrogen peroxide (125, 250, and 500 μM), VP-16 (50 μM) or a TOP1-targeting drug ARC-111 (topoval) (5 μM) for 4 h. Treated cells were analyzed for nucleosomal DNA fragmentation by 1.8% agarose gel electrophoresis as described under “Materials and Methods.” For experiments shown in panels C and D, P388 (C) and P388/CPT45 (D) cells were treated with H$_2$O$_2$ (125, 250, and 500 μM), CPT (2 μM), or VM-26 (2 μM) for 14 h followed by detection of activated apoptotic caspase using the FITC-conjugated caspase inhibitor FITC-VAD-FMK as described under “Materials and Methods.” For H$_2$O$_2$ treatment, the wider end of the triangle in the figure represents the highest concentration.
H$_2$O$_2$ Induces TOPI-mediated DNA Damage and Cell Death

They have shown that overexpression of yTOP1 confers sensitivity of yeast cells to these DNA-damaging agents. It is plausible that the underlying mechanism of TOPI-mediated lethality in both studies is the same (i.e. trapping of TOPI-DNA covalent complexes).

We have also investigated the potential role of TOPI in hydrogen peroxide cytotoxicity in cultured mammalian cells. P388/CPT45 cells, which are derived from P388 murine leukemia cells by selecting for CPT resistance (over 1000-fold more resistant to CPT), are deficient in TOPI (40, 41). We have shown that P388/CPT45 cells were up to 6-fold more resistant to hydrogen peroxide than P388 cells as demonstrated by the MTT assay. This result suggests that hydrogen peroxide cytotoxicity is partly TOPI-dependent. However, P388/CPT45 cells may contain mutations in other genes unrelated to the TOPI defect, which may affect the DNA repair and apoptosis pathways. In order to rule out this possibility, other cytotoxic agents (i.e. VM-26 and vinblastine) were used in the current study. P388/CPT45 cells were shown to be specifically resistant to hydrogen peroxide and CPT, but not VM-26 (a TOPI-specific drug) or vinblastine (a tubulin-specific drug). Similar results in these cells were obtained using apoptosis assays. Hydrogen peroxide was shown to induce apoptosis in P388 cells as evidenced by both nucleosomal DNA fragmentation and caspase activation assays. Hydrogen peroxide-induced apoptosis was partially reduced in TOPI-deficient P388/CPT45 cells, suggesting that hydrogen peroxide-induced apoptosis is partly TOPI-dependent.

The precise role of TOPI in hydrogen peroxide cytotoxicity is still unclear. It is possible that TOPI may reduce the expression of certain genes involved in the repair of oxidatively damaged DNA. Consequently, top1 mutants or TOPI-deficient cells would become more resistant to hydrogen peroxide. The other possibility is that hydrogen peroxide may trap TOPI into potentially lethal covalent complexes in mammalian cells as in yeast. Indeed, we have demonstrated that hydrogen peroxide induces TOPI-DNA cross-links in HeLa cells using the ICE bioassay, suggesting the formation of TOPI-DNA covalent complexes. We have also demonstrated that the TOPI-DNA cross-links are reversible upon removal of hydrogen peroxide, suggesting that they may reflect reversible TOPI cleavage complexes.

It is unclear how hydrogen peroxide may trap TOPI into cleavage complexes. Previous studies using purified TOPI have demonstrated that hydrogen peroxide does not directly trap TOPI into covalent complexes (54). However, hydrogen peroxide may indirectly trap TOPI into covalent complexes by generating oxidative DNA lesions (i.e. 8-oxoG) mediated by the hydroxyl radical through the Fenton reaction. Indeed, TOPI has been shown to bind to sites of oxidatively damaged DNA to form TOPI-DNA cleavage complexes (24). These TOPI-DNA cleavage complexes are expected to cause S-phase-specific cytotoxicity (17, 18).

Like in yeast, TOPI-independent pathways are certainly important for hydrogen peroxide cytotoxicity. P388/CPT45 cells are over 1000-fold more resistant to CPT (40, 41), but only 6-fold more resistant to hydrogen peroxide, suggesting the presence of TOPI-independent pathways for hydrogen peroxide cytotoxicity. There are likely many TOPI-independent pathways contributing to hydrogen peroxide cytotoxicity due to the nonspecific chemical reactivity associated with hydrogen peroxide. One of these pathways could involve TOPI. Previous studies have demonstrated that TOPI can be directly trapped into cleavage complexes by high concentrations of hydrogen peroxide and may thus contribute to hydrogen peroxide cytotoxicity (54). Another possible pathway could involve telomeres since extensive telomere shortening has been demonstrated in cells treated with hydrogen peroxide (55, 56). The importance of these various pathways in hydrogen peroxide cytotoxicity may depend on a variety of factors. For example, the role of TOPI in hydrogen peroxide cytotoxicity may be particularly important in proliferating cells since TOPI-mediated lethality is substantially S-phase-specific (17, 18). In non-proliferating cells such as postmitotic neurons, TOPI-independent pathways may become more important. Clearly, further studies are necessary to elucidate the roles of various cytotoxic pathways in the cellular response to hydrogen peroxide.

Acknowledgments—We thank Drs. James C. Wang, John Nitiss, and Howard Nash for providing us with various yeast strains, Dr. Mary-Ann Bjornsti for the TOPI plasmids, and Drs. Michael R. Mattern and Randal K. Johnson for the P388 and P388/CPT45 murine leukemia cells.

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L. F. Liu and S. Desai, unpublished results.
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J. Biol. Chem. 2004, 279:14587-14594.
doi: 10.1074/jbc.M311370200 originally published online December 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M311370200

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