Published in final edited form as:

J Biochem Mol Toxicol. 2014 December ; 28(12): 568–577. doi:10.1002/jbt.21599.

Minimal Detection of Nuclear Mutations in XP-V and Normal Cells Treated with Oxidative Stress Inducing Agents

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Abstract

Elevated levels of reactive oxygen species (ROS) can be induced by exposure to various chemicals and radiation. One type of damage in DNA produced by ROS is modification of guanine to 7,8-dihydro-8-oxoguanine (8-oxoG). This particular alteration to the chemistry of the base can inhibit the replication fork and has been linked to mutagenesis, cancer and aging. *In vitro* studies have shown that the translesion synthesis polymerase, DNA polymerase η (pol η), is able to efficiently bypass 8-oxoG in DNA. In this study we wanted to investigate the mutagenic effects of oxidative stress, and in particular 8-oxoG, in the presence and absence of pol η . We quantified levels of oxidative stress, 8-oxoG levels in DNA, and nuclear mutation rates. We found that most of the 8-oxoG detected were localized to the mitochondrial DNA, opposed to the nuclear DNA. We also saw a corresponding lack of mutations in a nuclear encoded gene. This suggests that oxidative stress' primary mutagenic effects are not predominantly on genomic DNA.

Keywords

DNA damage; DNA polymerase η ; mutagenesis; translesion synthesis; oxidative stress

INTRODUCTION

Oxidative stress is defined as an excess of reactive oxygen species (ROS) over antioxidants. ROS can occur as a consequence of normal respiration, as well as from external sources; it is a relevant challenge to normal homeostasis and human health. ROS takes many forms including the radical species superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($\cdot OH$), in addition to such non-radicals including H_2O_2 [1–3]. ROS can cause cellular damage by

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STATEMENT OF AUTHOR CONTRIBUTIONS

K.N. Herman performed and designed experiments, analyzed data, and prepared the manuscript. S.M. Toffton provided technical assistance with experiments. S.D. McCulloch designed experiments, provided intellectual input, and made final edits to the manuscript. All authors approved the final manuscript. K.N. Herman and S.D. McCulloch had complete access to the study data.

All authors declare no conflicts of interest.

attacking DNA directly, or indirectly, as well as damaging lipids and proteins. All of these processes can lead to various diseases, aging and cancer [1, 2].

One of the most abundant and mutagenic oxidative alterations is 7,8-dihydro-8-oxoguanine (8-oxoG), which can be formed by ROS directly attacking guanine bases within double stranded DNA. 8-oxoG can potentially Hoogsteen base pair with adenine, instead of forming a normal Watson-crick base pair with cytosine. This mispair causes a GC → TA transversion. Due to the structural similarity of 8-oxoG:A base pair to that of the T:A base pair, this mispair can be refractory to proof reading and possibly some repair mechanisms as well [4–6]. There are, however, multiple mechanisms to try and reduce the effects of 8-oxoG on the genome. One of these is 8-oxoguanine glycosylase (hOGG1) which is responsible for excising the damaged 8-oxoG base, thus initiating base excision repair. Additionally, there is human Mut T homolog (hMTH1) which hydrolyzes free 8-oxoG nucleotide triphosphate in the nucleotide pool and human Mut Y homolog (hMYH) which removes the mispaired A from the 8-oxoG:A pair [1]. The 8-oxoG remaining in the DNA during DNA replication is then able to inhibit progression of the replication fork and becomes a candidate for translesion synthesis (TLS) [7].

Studies have shown that the Y-family of DNA polymerases (pol) (DNA polymerase η , ι , κ and Rev 1) have some ability to bypass the damaged 8-oxoG base, usually inserting A [5, 8, 9]. *In vitro* lesion bypass assays and cell based plasmid replication assays have been used to evaluate pol η , showing it can bypass 8-oxoG, and that it does so with better efficiency than past normal guanines, but with very low fidelity [5, 10, 11]. For this reason, we decided to evaluate a cellular response of oxidative stress on nuclear DNA, in particular evaluating for effects of 8-oxoG in cells proficient in known DNA repair pathways (normal human fibroblast) and in cells deficient in functional pol η (Xeroderma pigmentosum variant (XP-V) cell). The goal was to evaluate the role of pol η in oxidative stress induced nuclear mutagenesis.

In order to evaluate for nuclear mutagenesis we chose to use the hypoxanthine-guanine phosphoribosyltransferase (HPRT) nuclear mutation assay with two chemicals which are known oxidative stress agents. Menadione (MD) is a general oxidative stress agent which affects complex I of the electron transport chain, causing the production of superoxide anion [12–14]. Methylene blue plus light (MBL), causes redox cycling and the production of superoxide. MBL has previously been shown to preferentially produce 8-oxoG in the treatment of plasmids [11, 15–17]. In addition to HPRT mutation rates, we also evaluated the level of oxidative stress and 8-oxoG generated during these treatments.

MATERIALS AND METHODS

Cell lines, growth conditions and treatments protocols

Cell lines used were GM02359-hTERT (XP-V strain XP115LO; referred throughout as XP-V), a pol η deficient line; and NHF1-hTERT (referred to as NHF), a normal fibroblast control line, both previously described [18–21]. Conditions for growth were as described previously [18]. Treatment conditions were determined by literature review in conjunction with preliminary cell viability studies. Menadione (USB Corporation, Cleveland, OH) was

used at 125 μM . Stock solutions of 1 mM were made in HBSS (Sigma-Aldrich, Saint Louis, MO) and filtered sterilized with a 0.2 μm filter (Genesee Scientific, RTP, NC) and diluted immediately prior to use in HBSS. Cells were treated for 20 minutes at room temperature [12, 13, 22]. Methylene blue plus light treatments were completed as follows: methylene blue (Calbiochem, EMD Biosciences, La Jolla, CA) stocks of 1 mM were prepared in pH2O and filter sterilized (0.2 μm). Cells were treated based on McBride *et al* and Lee and Pfeifer [11, 16]. Briefly, cell growth media was removed and replaced with 10 mM sodium phosphate buffer (pH 6.9) (Fischer-Scientific, Fair Lawn, NJ) containing deferoxaminemesylate (Calbiochem, EMD Biosciences, La Jolla, CA) at a concentration of 0.1 mM [11, 16]. Methylene blue was added to the phosphate buffer at a final concentration of 5 μM , then culture plates were exposed to an LED light (warm white 800 lumens, equivalence to 60W incandescent) at a distance of 18 inches for 15 minutes at room temperature [11, 15, 16]. For exposure to ultraviolet A radiation (UV-A), a dose of 400 mJ/cm^2 was used, from a 360 nm monochromatic lamp (EN-280L; Spectrolines). The setup is a dual 8-watt tube with a 2F082 filter. Fluence was determined by a SEL033 (#SEL0339663) detector from International Light technologies attached to a phototherapy UVA measurement system ILT1400SEL033. Ultraviolet B radiation (UV-B) was used at 10 mJ/cm^2 as previously described [18]. Addition of caffeine for specific treatments were as previously described [18]. The positive control for the flow cytometry was t-butylhydroperoxide (tBHP) (Sigma-Aldrich, Saint Louis, MO) at final concentration of 1 mM, treated for 1 hour at 37°C, as recommended by the manufacturer.

Total Cellular ROS detection

Cells were plated at 0.5×10^6 cells per P10 plate (Genesee Scientific, RTP, NC), incubated for 24 hours and then treated (as described above). Immediately after treatment, cells were washed with HBSS (Sigma-Aldrich, Saint Louis, MO) and then 3 mL of HBSS was added to the plates. CM-H₂DCFDA (DCF) (Gibco-Life Technologies, Grand Island, NY) dye was added to the HBSS to a final concentration of 1 μM (as pre-determined by preliminary background fluorescence studies). Plates were incubated for 30 minutes at 37°C in the presence of DCF. The cells were then washed again with HBSS and trypsinized, harvested in 5 mL polystyrene round-bottom tubes (BD Falcon, BD Biosciences: Franklin Lakes, NJ), washed again in HBSS, harvested and the cell pellet was brought up in 0.5 mL of HBSS and kept on ice in the dark until analysis by flow cytometry. Data was acquired on a Becton Dickinson LSRII cytometer (BD Biosciences, Franklin Lakes, NJ) using Diva 6.1 software. Data analysis was performed using FlowJo software.

Protein ROS detection

Detection of oxidized protein was performed as previously described with slight modifications [23–27]. Cells were plated at 80% confluency and grown for 24 hours. Cells were then treated and collected at 0, 4 or 24 hours after treatment. Treatments were as described above. Cells were trypsinized, washed with HBSS, and cell pellets collected. Cell pellets were lysed as described previously [28]. An equal volume of 20% trichloroacetic acid (TCA) was added to the lysate, followed by vortexing and centrifugation at 800 g for 5 minutes. The supernatant was discarded and 250 μL of 2N hydrochloric acid (HCL) was added to re-suspend the pellet. Then 250 μL of 2,4-Dinitrophenylhydrazine (DNPH) (Sigma-

Aldrich, Saint Louis, MO) in 2N HCL was added and the mixture was rotated for 1 hour at room temperature. 500 uL of 20% TCA was added and the tubes vortexed to mix. Protein pellets were harvested by centrifugation at 800 g for 5 min. Pellets were washed with ethanol:ethylacetate (1:1) three times. The pellet was then dried and resuspended in 8M urea. This mixture was centrifuged at 13,400 g for 5 minutes and the upper phase was collected. Protein concentrations were determined by Bio-Rad protein assay as described [28]. Samples (3 µg) were separated using 12% SDS-PAGE. Gels were wet transferred to nitrocellulose and Western blot analysis was performed. Antibodies used were used: Anti-DNP (D9656, Sigma-Aldrich, Saint Louis, MO); anti-β-actin (A5441). Imaging was on a Storm 865 (GE Life Science) using Cy5 labelled secondary antibodies: ECLPlex Goat anti rabbit Cy5 (PA45011V) and ECL Plex goat anti mouse Cy5 (PA45009V).

8-oxoG detection by alkaline gel

Replicative form plasmid DNA, M13mp18, from XL1 blue cells was purified by Qiagenminiprep kit. It was then treated with either: no treatment, methylene blue only, light only or methylene blue plus light. Samples were treated in 10 mM phosphate buffer, and underwent three ethanol precipitations to remove residual methylene blue. Two µg of treated plasmid was digested with FPG (New England Biolabs) under conditions recommended by the manufacturer. FPG was deactivated by heating for 10 minutes at 60°C, and samples were separated through a 1.5% alkaline gel as described [29].

8-oxo-dG detection by 2D Mass Spectrometry

5×10^6 cells were treated as described above and harvested for cell pellets at either 1 hour or 24 hours post treatment. Total cellular DNA was prepared from cells by the addition of lysis buffer (Qiagen 1045696) to which was added 20 mM 2,2,6,6-Tetramethylpiperidinoxy (Tempo) to prevent additional 8-oxoG production. Proteins were precipitated using neutralization solution (Qiagen 1045697). Samples were vortexed and centrifuged at 16,000 g for 15 minutes. Supernatant was collected and DNA/RNA precipitated with isopropanol. The pellet was resuspended in lysis/20 mM Tempo buffer, RNaseA (Sigma-Aldrich) was added and incubated for 30 minutes at 37°C. Protein precipitation solution was again added the sample was centrifuged at 16,000 g for 15 minutes, followed by a second isopropanol precipitation. DNA was resuspended in 30 µL of ppH₂O with 1 mM Tempo. 2D chromatography was performed as in Boysen *et al* [30]. See Supplemental Figure 3 for description of mitochondrial DNA preparation. 2D mass spectrometry was performed by Leonard Collins at the University of North Carolina Biomarker Mass Spectrometry Facility.

Mutation Frequency

The HPRT mutation assay was conducted as previously described [18–20].

Analysis of mutations at the *ura3-29* locus after MBL dosing

The wild-type (proficient in both OGG1 and RAD30 (pol η) base yeast strain used is the same as generated in a previous report (7B-YUNI300;*MATaCAN1his7-2leu2-::kanMX ura3-29:agp1 trp1-289 ade2-1 lys2- GG2899-2900*) [31]. OGG1 and/or RAD30 were deleted separately or in combination as described previously [31, 32]. They are referred to

here as OGG1 RAD30, *ogg1* RAD30, OGG1 *rad30*, and *ogg1 rad30*. Each strain contains a non-functional *ura3-29* locus (codon 86, TCT) which reverts to a functional state after GC → AT mutations. This change is suggestive of 8-oxoG dependent mutagenesis [33]. For one day treatments, cultures (10 ml) of each strain were grown from a single colony overnight at 30°C, 250 RPM in YPD. Cells were harvested by centrifugation, washed once in PBS, resuspended in 10 ml PBS, and methylene blue (Calbiochem, EMD Biosciences, La Jolla, CA) was added to final concentration of 5 µM. Cells were transferred to a P10 culture dish and exposed to an LED white light source as described above and previously [18]. Cells were then harvested and washed twice with 10 ml PBS. After the final wash, cells were re-suspended in 0.5 ml PBS and plated onto either complete synthetic media (CSM) or CSM-Uracil (CSM -Ura) to obtain total cell numbers and numbers of mutants at the URA3 locus, respectively. Mutation frequency was calculated as (Number of mutants/Total number of cells). Experiments were performed at least 3 times. For three day treatments, after the final wash a 100 µl aliquot of cells was added to 9.9 ml YPD and grown overnight at 30°C, 250 RPM. The dosing step was repeated on day 2 and again on day 3 before plating cells onto CSM/CSM -Ura as described above.

RESULTS

Production of Cellular Oxidative Stress

In order to verify that our treatments were creating oxidative stress, we performed flow cytometry using 2'-7' dichlorofluorescein (H2DCF) dye which becomes oxidized to the fluorescent dichlorodihydrofluorescein (DCF) in the presence of ROS [34]. Controls included cells which were both untreated and undyed, and cells that were untreated but dyed with H2DCF to give baseline values to compare against for increases in fluorescence caused by increased ROS. The positive control used was *tert*-butyl hydroperoxide (tBHP) at a dose of 1 mM for one hour at 37°C. Oxidative stress treatments evaluated included 125 µM MD, 5 µM MBL, 10 mJ/cm² UV-B, and 400 mJ/cm² UV-A. As expected, untreated, undyed cells displayed the lowest levels of fluorescence, and tBHP treated cells displayed the highest levels of fluorescence (Figure 1A and B; Table 1). The shift from left to right for untreated, dyed cells (Figure 1A and B, Clear curves; Supplementary Figures 1 and 2) indicates background fluorescence of the dye and/or basal levels of ROS. From this we see that there may be slightly different levels of ROS in the NHF compared to the XPV line under normal growth conditions, or that the cells have intrinsically different background fluorescence. The large increase in fluorescence caused by tBHP (Figure 1A and B, red curves) suggests a very high level of ROS production. This is consistent with the severe cytotoxicity observed by tBHP at this treatment level (data not shown). Surprisingly, despite using agents and doses that have been reported in the literature to cause oxidative stress, in our hands the treatments generated ROS at roughly the same levels as observed in untreated cells (Figure 1A and B, orange, green, purple curves). The exception to this is MBL, which in the NHF cells did cause a detectable increase in DCF fluorescence (Figure 1A). Although additional dyes to test for cell death were not done in conjunction with ROS staining, it seems likely that MBL treatment has affected cell survival, as suggested by the decrease in the percentage of the parent population for MBL compared to Untreated (Table 1). This holds true for both NHF and XPV cells, and is similar to the effect seen with tBHP exposure. This suggest that

despite the overall FITC-A level (i.e. level of detected ROS) for MBL being only slightly elevated in NHF cells and somewhat lower in XP-V cells compared to untreated samples, the treatment is in fact affecting the cell population in other ways.

Effects of oxidative stress - Protein Oxidation

In addition to direct detection of ROS we were interested in evaluating possible effects of oxidative stress within the cells. First, we chose to look at protein oxidation using a Western blot assay. After increased ROS, an antibody that recognizes a DNP conjugate to carbonylated proteins will show increased numbers of bands and a streakier appearance of lanes. Consistent with the flow cytometric detection of ROS results, Figure 1D–G shows moderate increases in oxidized proteins after most of the treatments and time points. The most prominent increase in oxidized protein is after MBL treatment, which correlates well with the above described flow cytometry based data.

8-oxoG detection by alkaline gel

Replicative form DNA of M13mp18 bacteriophage was used as a model to test the efficacy of MBL in generating 8-oxoG. DNA was exposed to MBL treatment, purified, and then treated with FPG, a protein that cleaves 8-oxoG from the DNA, leaving an abasic site. Separation of the DNA in an alkaline gel causes strand breaks at abasic sites, producing an increase in smaller fragments, or smear of DNA instead of discrete bands (Figure 2A). Figure 2B shows that only in the presence of both MB and white light, rather than each treatment alone, are detectable levels of 8-oxoG generated. The smear evident in the right most lane, confirms that the methylene blue plus light treatment creates 8-oxoG in greatly elevated levels compared to no treatment.

8-oxoG detection by 2D mass spectrometry

While the above assay shows that MBL can cause 8-oxoG in DNA in solution, we were also interested in whether our treatments were capable of generating 8-oxoG in DNA within cells. To test for this, we collected DNA from cells after treatments and analyzed it using mass spectrometry. We dosed cells with 125 μ M MD, 10 mJ/cm² UV-B, 400mJ/cm² UV-A or 5 μ M MBL and collected DNA 1 hour post treatment. We also collected DNA 24 hours post treatment for the MBL dose. As shown in Table II and Figure 2, we were unable to detect elevated 8-oxoG levels with MD, UV-B or UV-A treatments. However, we did observe a roughly 10 fold increase in 8-oxoG from the MBL treatment. This was observed in both cell lines, suggesting a similar mechanism of formation. Additionally, 8-oxoG levels 24 hours after treatment show that repair of the damage is occurring, and that the rate is similar in both cell lines (Figure 2C). Due to the fact that menadione uses Complex I of the mitochondrial respiratory chain for reduction and is known to interfere with mitochondrial respiration, and that methylene blue causes redox cycling of NADPH and NADH within the mitochondria [12, 35], we also looked at 8-oxoG levels specifically in DNA fractions highly enriched for mitochondrial DNA (Supplementary Figure 3). As seen in Table II and Figure 2D, once separated out from the total cellular DNA, we observed that most of the detected 8-oxoG is localized in the mitochondrial DNA and not in nuclear DNA fractions.

Evaluation of Nuclear Mutations using the HPRT locus

The fidelity of pol η has been assessed in numerous biochemical experiments and studies. Many of these studies have confirmed pol η performs low fidelity bypass of 8-oxoG *in vitro* [5, 10, 11, 36–38], while other studies have suggested that this bypass is “error free” (for lack of a better term) [33, 39, 40]. In addition to biochemical experiments, pol η also has been evaluated using damaged plasmids transfected into cells. This work suggests that the presence of pol η suppresses mutagenesis [11]. With this background of biochemical and plasmid based data, we wanted to transition into a cell based assay to investigate whether the presence of pol η would affect nuclear mutagenesis rates after oxidative stress.

Unexpectedly, we found that our oxidative treatments seemingly had no effect on nuclear DNA mutation frequencies, as measured by the HPRT assay (Table III). Untreated XP-V cells had a mutation frequency (MF) of 0.98×10^{-5} . Treatment with menadione (MF 2.33×10^{-5} ; 2.4 $\times$) and MBL (MF = 1.01×10^{-5} ; 1.0 $\times$) gave nearly identical frequencies. Similarly, untreated NHF cells gave a MF value of 0.55×10^{-5} , with menadione (1.65×10^{-5} ; 3 $\times$) and MBL (1.27×10^{-5} ; 2.4 $\times$) again causing very little difference. This is in comparison to our previously published work using environmentally relevant levels of UV-B (10 mJ/cm²) in which the MF of XPV cells was 25.8×10^{-5} (26 $\times$ higher than untreated) and for NHF cells the MF was 8.56×10^{-5} (15.6 $\times$ untreated) [18]. In addition we attempted alternative treatment protocols in an attempt to exacerbate the effects, in case a single, short oxidative stress inducing treatment was insufficient to generate damage/mutations at levels detectable in this assay. Repeated, low dose MD treatments caused cell death, regardless of the levels used. Repeated MBL treatments did not alter the mutation frequency, but did result in extremely low colony forming efficiencies (CFE). Treatments with H₂O₂ produced highly variable results (both within replicates and within different experiments) and were therefore unable to be used. We also attempted the addition of caffeine after MBL treatment (similar to as has been used to increase cytotoxicity effects of XP-V lines under UV-C and UV-B treatments) [18, 41, 42], but again this lowered the CFE (less than 1%) to a point that the mutation frequencies obtained were unreliable. These additional treatments are described in Supplementary Note I. In total, we were unable to find treatment levels/conditions that balanced survivability with mutations.

Oxidative stress dependent Ura3-29 mutations in yeast cells

In order to further explore the effects of MBL in cells, we used a set of yeast strains that were deficient in OGG1, RAD30, or both. OGG1 is the sole 8-oxoG repair enzyme in *S. cerevisiae* cells, and RAD30 (pol η) is the only Y-family member with significant lesion bypass ability. It has previously been shown that spontaneous mutations at the Ura3-29 locus are increased in an *ogg1* mutant strain, and that deletion of *rad30* further increases this mutation frequency [33]. Here, we performed similar experiments, but instead dosed cells either once or on 3 consecutive days with 5 μ M MBL (20 minute exposure). Consistent with our results described above, deletion of pol η alone did not affect the mutation frequency at a nuclear locus (Figure 3). We do see, however, that both deletion of either OGG1 alone or both OGG1 and RAD30 gives a large increase in the mutation frequency at the Ura3-29 locus. This is true after either a single exposure or 3 separate days of treatment. Determination of the sequence of functional Ura3 genes in mutants from the double

knockout strain showed that they were all GC → AT changes (data not shown), as expected if errors when bypassing 8-oxoG were the cause. These data suggest that when increased levels of 8-oxoG are present, the mutagenic propensity of it are partially mitigated by pol η, at least in yeast. They also suggest that the repair of 8-oxoG is more important in preventing mutagenesis than lesion bypass; whether or not a similar phenomenon is occurring in human cells remains to be seen. It is important to keep in mind that human pol η has a much greater *in vitro* error rate when bypassing 8-oxoG as compared to yeast pol η [5].

DISCUSSION

Numerous studies have evaluated the role of human polymerases encountering oxidative lesions; including plasmid replication assays and *in vitro* biochemical studies. In addition, studies assessing oxidative stress cytotoxicity have been performed, but none have combined both. Therefore, we treated cells with MD and MBL, in hopes of gaining a broader understanding of lesion bypass after oxidative stress. We chose MD for its properties as a general oxidative stress agent, and MBL for its defined ability to produce 8-oxoG in DNA [15]. Additionally, UV-B and UV-A were used for their well-known mutagenic effects in conjunction with their general consideration as oxidative stressors.

First, we tested for oxidative stress by flow cytometry. Our treatments, despite being published as oxidative stress agents, showed relatively low levels of detectible ROS (Figure 1A–C). However, these particular doses were chosen for their relatively minor cytotoxic effects to allow for evaluation with the long term mutagenicity assay; higher levels of the treatments could have produced larger quantities of ROS, but, it would enhance cytotoxicity, reducing the effectiveness of the HPRT assay. Our MD and MBL treatments show clear signs of oxidative stress, as evident through production of oxidized proteins (Figure 1D–G; Table I), as well as detectible 8-oxoG lesions in DNA (Figure 2; Table II). Despite seeing effects of oxidative stress throughout the cell, our nuclear mutation frequencies (assayed at the HPRT locus) of these treatments were disappointingly low and not significantly different from untreated cells. Attempts to exacerbate oxidative stress to increase mutation levels (Supplementary Note 1) were unsuccessful. Despite our additional efforts, we were unable to find conditions that balanced longer term survivability with detection of mutations, with many possible contributing factors. The first possibility is that the oxidative stress treatments used here were not enough on their own to cause detectable mutations. Under more ‘real world’ conditions exposure to multiple different agents simultaneously is more realistic, including combined exposures of sunlight, chemicals within food or water, and other sources of stress. Another possibility suggested by our yeast data and other previously published results [5, 11, 33] is, a pol η deficiency on its’ own is insufficient for 8-oxoG damage to cause mutations, and instead, there would need to be a concomitant deficiency in OGG1 or other repair factors to increase the 8-oxoG load. In addition, it is interesting to note that our MBL treatment caused 8-oxoG to be mainly localized in the mitochondria. MBL and MD both use the electron transport chain in order to create ROS, so it is perhaps not surprising to see effects in the mitochondria DNA; however it was unexpected to not see more general cellular effects caused by the increased ROS. This lack of effect could be due to the diffuse nature of ROS compared to a more direct damaging agent such as UV light. These results suggest that more research is needed to determine when the cells robust repair

mechanisms become overwhelmed by ROS, therefore permitting TLS to bypass the DNA damage caused by ROS and creating the potential for mutagenicity. Lastly, it is entirely possible that the HPRT assay has insufficient sensitivity to detect low levels of mutations, and therefore another method may be more suited to detecting these changes; including the potential for deep sequencing [43].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We would like to thank Dr. Yoshiaki Tsuji, Dr. Jun Ninomiya-Tsuji and Dr. Masaki Miyazawa from NC State University for technical advice. The University of North Carolina Biomarker Mass Spectrometry Facility is supported in part by P30-ES10126. This work was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health R01 ES016942 and T32 ES007046.

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Figure 1. ROS and Oxidative Stress Analysis
(A) Flow cytometric analysis of ROS levels, NHF cells. (B) Flow cytometric analysis of ROS levels, XP-V cells. For both A and B the peak the negative control is an untreated, undyed cell sample. (C) Color code key for Panels A and B. (D) Protein extracts from NHF cells were analyzed by Western blot with an α -DNPH antibody to detect oxidized protein, as a measure of oxidative stress. Treatments and times of analysis are as indicated. (E) Protein extracts from XP-V cells were analyzed by Western blot with an anti-DNPH antibody as described for Panel D. (F) Western blot for β -actin on gel from Panel D. (G) Western blot for β -actin on gel from E. (*) Indicates a sample that was not conjugated to verify the anti-DNPH antibody was specific.

Figure 2. 8-oxoG analysis

(A): Schematic of 8-oxoG detection assay in plasmid DNA. The presence of 8oxoG is revealed by cleavage with FPG, generating a smear of DNA during alkaline agarose gel electrophoresis. Replicative form M13mp18 DNA was used with: no treatment, methylene blue only, light only or methylene blue plus light. (B): Alkaline gel after FPG treatment indicating that the MBL treatments generate ample 8-oxoG lesions. (C): Analysis of 8-oxoG levels in total DNA isolated from NHF cells after the treatments listed. Whole cell DNA was isolated from NHF cells (black bars) and XP-V cells (white bars) and sent for mass

spectrometry. (D.): Enriched mitochondrial DNA was also isolated and analyzed. The results indicate that MBL preferentially damages mitochondrial DNA.

Figure 3. Oxidative stress induced mutation frequency at the URA3-29 locus is dependent on OGG1 and RAD30 activity

Yeast strains proficient or deficient in either OGG1, RAD30 (pol η), or both were treated with MBL (5 μM, 20 minutes) for 1 or 3 days. Reversion of the TCT Ura3-29 allele to TAT confers growth in the absence of uracil and suggests mispairing of A opposite 8-oxoG in the opposite strand [5, 33]. The statistics were run with Graphpad Prism 6 using a 2 way anova with a Tukeypost test. Significance is as follows: * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Table I
Oxidative Stress Flow Cytometry

Statistical analysis using FlowJo software for calculating ROS detection. The value of “FITC-A” indicates fluorescence which is correlated with ROS level. “Freq of Parent” indicates the percentage of cells gated and analyzed from the total population, a measure of cell viability.

	NHF		XP-V	
	Freq of Parent	FITC-A	Freq of Parent	FITC-A
No dye	93.7	470	61.2	304
tBHP	68.6	15922	25.6	6003
Untreated	93.8	1106	58.5	976
125 μ M Menadione	92.5	1075	53.8	571
10 mJ/cm ² UV-B	94.6	1014	67.0	869
400 mJ/cm ² UV-A	94.8	1091	68.3	738
5 μ M MBL	74.7	1388	27.1	786

Table II
Analysis of 8-oxoG damage in nuclear and mitochondrial DNA

Levels of 8-oxo-dG were measured in total DNA and fractions enriched for mtDNA using mass spectrometry. Values given are levels of 8-oxo-dG per 1×10^6 dG. Times given indicate when DNA was harvested after treatment. Untreated samples were similarly harvested 1 hour after mock treatment.

	8-oxo-dG/ 10^6 dG	
	NHF	XP-V
Untreated	1.2 (1)	2.1 (1)
125 μ M Menadione: 1 hr	1.5 (1.3 $\times$)	0.8 (0.7 $\times$)
10 mJ/cm ² UV-B: 1 hr	2.1 (1.8 $\times$)	1.3 (0.6 $\times$)
400 mJ/cm ² UV-A: 1 hr	1.8 (1.5)	0.5 (0.2 $\times$)
5 μ M MBL: 1 hr	11.8 (10 $\times$)	22.8 (11 $\times$)
5 μ M MBL: 24 hr	6.8 (5.7 $\times$)	14.5 (6.9 $\times$)
Untreated mtDNA	4.0 (3.3 $\times$)	6.1 (2.9 $\times$)
5 μ M MBL: 1 hr, mtDNA	76.4 (63.7)	170.9 (81.3 $\times$)

Table III
Evaluation of Mutations at the HPRT locus

The frequency of mutations at the genomic HPRT locus are shown. Treatments were as described in Materials and Methods. CFE, colony forming efficiency. MF, Mutation frequency. Data for untreated samples are the same as previously reported [18].

Cell Type		Untreated	125 μ M Menadione	5 μ M MBL
NHF	<i>CFE</i>	0.13	0.15	0.14
	<i>MF</i>	0.55×10^{-5}	1.65×10^{-5}	1.27×10^{-5}
XP-V	<i>CFE</i>	0.12	0.15	0.12
	<i>MF</i>	0.98×10^{-5}	2.33×10^{-5}	1.01×10^{-5}