Oxidative Stress Induces DNA Damage and Inhibits the Repair of DNA Lesions Induced by N-Acetoxy-2-acetylaminofluorene in Human Peripheral Mononuclear Leukocytes


PMI-Strang Clinic, Department of Biochemical Epidemiology, New York, New York 10016 [R. W. P., G. A. D., M. M.J; Laboratory of Molecular Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [M. W. A., C. H. A., F. R.]; and Department of Molecular Ecogenetics, University of Lund, Wallenberg Laboratory, 220 07 Lund 7, Sweden [R. W. P., C. B.]

ABSTRACT

Human mononuclear leukocytes were exposed to prooxidants such as H₂O₂, phorbol-12-myristate-13-acetate, and 4-nitroquinoline-N-oxide, and the effects on induction of DNA damage and repair were evaluated. ADP ribosylation was activated by prooxidant exposure and the response was bimodal with peaks of activation occurring at about 30 min and 4–5 h. Other evidence for prooxidant-induced DNA damage was provided by nucleoid sedimentation assays. Unscheduled DNA synthesis (UDS) was only slightly induced by prooxidant exposure which suggested that either the DNA lesions were repaired by a short patch mechanism involving little UDS, or the repair process was inhibited by prooxidant exposures, or some combination of both. This point was clarified by the fact that the repair of DNA lesions induced by N-acetoxy-2-acetylaminofluorene, an inducer of large patch DNA repair, was inhibited in a dose-dependent manner by exposure to H₂O₂ and the inhibition was dependent on ADP ribosylation. In contrast, the repair of DNA strand breaks induced by prooxidant exposures as identified above were complete within about 8 h and the repair was independent of ADP ribosylation. Both ADP ribosylation and N-acetoxy-2-acetylaminofluorone-induced UDS were shown to be up- and down-regulated by the redox state of human mononuclear leukocytes indicating a unique mechanism of cellular control over DNA repair.

INTRODUCTION

The damage of DNA by physiological processes that generate oxidative stress are now well documented (1–3). Hydroxyl radicals (OH·), superoxide anion (O₂⁻), and hydrogen peroxide (H₂O₂) have all been shown to damage DNA in cells cultured in vitro (2, 4). Because of the strong association between DNA damage and mutation, it has often been hypothesized that metabolically induced oxidative stress is a major component of the processes of carcinogenesis and aging (1–3, 5, 6). Support for this hypothesis includes (a) strong correlations between metabolic rate and life span (1, 5), (b) tumor promoters are known to induce oxidative stress and DNA damage (1–4, 7–10), and (c) the fact that at least some DNA damage induced by oxygen radicals are physiologically involved in modulating DNA replication and cell differentiation (11–15) as well as DNA repair (4).

A role for defective DNA repair in the etiology of human cancers is recognized (16, 17). The rare autosomal recessive inherited chromosome-breaking syndromes (e.g., xeroderma pigmentosum, ataxia telangiectasia, Bloom’s syndrome and Fanconi’s anemia) have been associated with high incidences of cancer and defects in DNA repair (16, 17). However, recently the more widely inherited cancers of the colon (18) and breast (19) have also been linked to deficiencies in DNA repair synthesis capacity. These data have raised the question of whether or not a repair defect could be extended to include the more commonly occurring cancers. Colonic mucosa samples from individuals with genetic predisposition to colon cancer have an increased proliferative response to tumor promoters that can induce oxidative stress (20), an increased chromosome aberration frequency (Ref. 21 and as cited therein), and a decreased DNA repair synthesis (22). These observations suggest abnormalities in the oxidative stress responses among individuals destined to acquire colon cancer. Since our laboratory has also observed inhibited DNA repair responses in the mononuclear leukocytes from individuals at high risk for colon cancer (23–25), we have examined the possibility that cells exposed to oxidative stress can express an inhibition of DNA repair synthesis. Here we report that the repair of DNA strand breaks are unaffected by exposing cells to oxidative stress, but the repair of DNA lesions requiring UDS and ADPRT activity are markedly inhibited by prooxidants.

MATERIALS AND METHODS

Chemicals. NA-AAF and [³H]NA-AAF were supplied by Chemsyn Science Laboratories. 3AB, aphidicolin, BrdUr, BSO, cyclohexene-1-one, CdCl₂, CsSO₄, NAD⁺, GSH, and GSSG, H₂O₂, 4-NQO, and PMA came from Sigma Chemical Company. RPMI, fetal calf serum, and PHA came from Grand island Biological Company. [³H]Hadenine-labeled NAD⁺ was supplied by New England Nuclear Research Products. [³H]dThd and [³H]dCyd were products of Amersham.

Sampling. Peripheral blood samples from apparently healthy volunteers were obtained by venous puncture and collected into heparinized vacutainers (143 USP units/10-ml tube) or blood collection bags (7000 USP units/500 ml). All samples were processed within 4 h of collection. The HML fraction was isolated by conventional procedures involving density gradient centrifugation using an Isopaque-Ficoll cushion (1.077 g/ml) as already described (26). The HMLs were cultured either in RPMI 1640 medium (Gibco) or physiological saline and with or without serum supplements, as indicated.

ADPRT Assay. The procedure is adapted from the permeabilized cell technique of Berger (27) with modifications previously described (28). Duplicate samples of 5 x 10⁸ HMLs were cultured in 1% autologous plasma-supplemented physiological saline for 30 min at 37°C in the presence of a standardized 100 μM dose of H₂O₂. The cells were harvested by centrifugation at 500 x g for 10 min and then permeabilized.

Received 12/1/89; revised 2/26/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Environmental Medicine-PHL 875, NYU Medical Center, 550 First Avenue, New York, NY 10016. To whom requests for reprints should be addressed.

2 Present address: Chiropractic Basic Science Research Foundation in Boston, MA, and the Swedish Work Environment Fund and the Swedish Cancer Society in Sweden. (25, 132) indicates that this fact.

3 The abbreviations used are: UDS, unscheduled DNA synthesis; NA-AAF, N-acetoxy-2-acetylaminofluorene; 4-NQO, 4-nitroquinoline-N-oxide; 3AB, 3-aminobenzamide; BrdUr, 5-bromo-2'-deoxyuridine; BSO, buthionine sulfoximine; GSH, reduced glutathione; GSSG, oxidized glutathione; dThd, thymidine; dCyd, cytosine; PMA, phorbol-12-myristate-13-acetate; PHA, phytohemagglutinin; HML, human mononuclear leukocyte; ppt, precipitable; ADPRT, adenosine diphosphate ribosyl transferase; TCA, trichloroacetic acid.

4 Present address: Sandoz AG, 881/543/4002 Basel, Switzerland.
lized at 4°C by suspension for 15 min in 1.5 ml of ice cold buffer (10 mM Tris-HCl, pH 7.8; 1 mM EDTA; 30 mM 2-mercaptoethanol; 4 mM MgCl2). The cytoskeletons were recovered by centrifugation and then suspended in 50 µl permeabilization buffer, 25 µl reaction buffer (100 mM Tris-HCl, pH 7.8; 120 mM MgCl2), and 15 µl [3H]adenine-labeled NAD+ (27.1 Ci/mmol, diluted 1:418 with cold NAD+ to a final concentration of 240 µM). Incubation was continued at 30°C for 15 min. The reaction was terminated by addition of 0.5% sodium dodecyl sulfate and 0.5 ml 3 M NaCl at 60°C for 10 min. (ADP-ribose)2-protein complexes were precipitated with ice cold 7% TCA and then collected by centrifugation. Consequently, it does not distinguish nuclear poly-ADPR from membrane mono-ADPRT activities. Hence, we have referred to the ADP ribosylation in cytoskeletons as ADPRT. The data were recorded as cpm TCA ppt [3H]adenine-labeled NAD*/5 × 107 cells in the presence (activated levels) or absence (constitutive) of agents that induce oxidative stress. The induced level of ADPRT was calculated by subtracting the constitutive level from the activated level. The counting efficiency was 31.5%.

The NA-AAF-induced UDS Assay. The determination of UDS following a standardized exposure of HMLs to NA-AAF has been published elsewhere (29). Briefly, UDS was measured after incubation of 5 × 106 cells with and without a 10.0 µM dose of NA-AAF for 1 h at 37°C in 20% autologous plasma-supplemented RPMI medium. After exposure, HMLs were incubated in fresh culture medium for an additional 17 h in the presence of 10 mM hydroxyurea and [3H]dThd (10 µCi/µl/ml added of a 25 Ci/mmol solution). The cells were harvested by centrifugation at 400 × g, and the DNA was extracted and quantified by a modification of the Marmur method in which DNA is immobilized onto nitrocellulose filters (29). The data were recorded as cpm/ppt [3H]dThd/µg DNA by subtracting the cpm incorporated in the hydroxyurea-treated culture from the cpm incorporated in the parallel culture treated with both hydroxyurea and NA-AAF. Biochemical determinations were carried out in duplicate (i.e., usually involving 5–10 µg DNA/replicate). Repeat determinations on the cells from the same individual show a coefficient of variation of about ±15% (29, 30).

NA-AAF Binding to DNA and the Repair Efficiency Index. Binding studies were sometimes performed in parallel with NA-AAF-induced UDS determinations as follows: approximately 5 × 107 HMLs were suspended in 5 ml RPMI medium and supplemented with 1% autologous plasma, and then the cells were exposed to 10 µM [3H]NA-AAF (570 Ci/µmol) and incubated for 30 min at 37°C. The binding of NA-AAF to DNA is maximal after 30 min (i.e., between 30 min and 4 h incubation the values varied (+5%, and so the level of DNA damage induction is equivalent to the conditions used for UDS determinations). After incubation, the cells were pelleted at 400 × g for 10 min and washed once with saline. The DNA was extracted and purified, and radioactivity was counted and quantified, as we have discussed previously (29). The data were recorded as the average of duplicate biochemical determinations quantified as cpm [3H]NA-AAF incorporated/µg DNA. Repeat NA-AAF-binding determinations yield a coefficient of variation of about ±10% (30).

DNA repair proficiency indices could then be calculated by dividing the NA-AAF-induced UDS value by the [3H]NA-AAF binding to DNA value. Such ratios express the amount of DNA repair synthesis that occurred per unit of introduced DNA damage, and thus they correct the individual values for differences in the initial induction of DNA damage (i.e., as potentially influenced by NA-AAF metabolism). There is the possibility that NA-AAF-induced UDS could occur by a nick translation mechanism with the damaged strand containing the [3H]NA-AAF adduct being removed after UDS. However, the calculation of a DNA repair proficiency index still seems appropriate, because UDS induced by DNA damage from standardized NA-AAF exposure is estimated over 17 h, which is a period when the UDS response has become saturated in HMLs (31). Thus, the long quantification period for UDS would minimize any kinetic differences between removal of NA-AAF DNA lesions and the completion of UDS.

Nucleoid Sedimentation Assay. Isolated HMLs were exposed at 37°C to H2O2 (0–300 µM, 30 min), PMA (25 ng/ml, 1 h), or 4-NQO (2 µM, 30 min) in RPMI medium containing 10% fetal calf serum. After treatment, the cells were diluted with 10 volumes of medium, centrifuged, and resuspended in fresh medium. In some cases, HMLs were preincubated with 3AB (2 mM) for 30 min prior to chemical treatments and replaced in the medium after removal of chemicals. Cells were either immediately analyzed for DNA damage by the nucleoid sedimentation assay or incubated for up to 24 h and the DNA repair measured at various times. Cells were also incubated with or without 3% PHA for 24 h in the same complete medium prior to chemical exposure for analysis of DNA damage in stimulated HMLs.

Nucleoid bodies (= cell nuclei depleted of most proteins and lipids) were prepared from HMLs with the modified procedure of Cook and Brazell (32, 33). First, continuous sucrose gradients, 15–30% (w/w) containing 1.5 M NaCl, 10 mM Tris, 10 mM EDTA (pH 8) were formed using a gradient maker (Buchler Instruments, Fort Lee, MO). Lysis solution (150 µl) containing 1.5 M NaCl, 10 mM Tris, 10 mM EDTA, 0.5% Triton X-100 (pH 8) was layered on top of the preformed sucrose gradient followed by 50 µl cell suspension containing approximately 2.5 × 107 HMLs. Cells were allowed to lyse for 30 min at 4°C and spun in a swinging bucket rotor (SW 50.1; Beckman) for 30 min at 4°C at a speed of 25,000 rpm. To detect the nucleoid band the gradient also contained 1 µg/ml of the fluorescent dye Hoechst 33258. This concentration of the dye does not influence nucleoid sedimentation (33). The absolute sedimentation distance of the nucleoid band from the top of the tube was determined by detection of the visible fluorescence of the DNA Hoechst dye complex using a longwave UV lamp (Black ray, 366 nm; Fisher). For all figures, the sedimentation of nucleoids was plotted as a percentage of control nucleoid sedimentation.

Separation of Repaired DNA by Alkaline Isopycnic Centrifugation. DNA from freshly isolated HMLs was density labeled by incubating the cells (1 × 107/ml) with BrdUrd (40 µM) for 1 h. An additional 30-min incubation with 5 µM aphidicolin followed whenever the use of this agent was indicated. [3H]dCyd (5 µCi/ml) was then added to HMLs in the presence of BrdUrd (40 µM). After various incubation times, [3H]dCyd was removed by centrifugation of cells in a Sorvall RT 6000 table top centrifuge at 1400 rpm for 10 min. Cells were incubated with BrdUrd (40 µM) for an additional hour and then washed with RPMI medium and collected by centrifugation. After a second wash in phosphate-buffered saline (pH 7.4), HMLs were pelleted by centrifugation at 2500 rpm for 5 min and quickly frozen in liquid nitrogen and stored at –80°C for isolation of DNA.

DNA was isolated from samples containing approximately 5 × 107 HMLs and separated into nonreplicated and replicated BrdUrd-substituted fractions by isopycnic CsCl/Cs2SO4 gradient sedimentation (32–35). A purified DNA solution (3.5 ml) containing not more than 1 mg DNA was denatured with 0.35 ml 1 M NaOH. CsCl and Cs2SO4 were added to DNA to give a final concentration of 0.9091 and 0.2626 g/ml sample, respectively. Samples were centrifuged in a Beckman VTi 65 vertical rotor at 55,000 rpm at 20°C overnight. Gradients were fractionated into 0.3-ml aliquots for spectrophotometric determination of DNA concentration (1 mg DNA = 27.7 absorbance units at 260 nm). Aliquots of 0.1 ml/fraction were used for scintillation counting.

The Glutathione Assay. Total soluble cellular glutathione (GSH + GSSG) was measured by the enzymatic assay of Tietze (36). Briefly, cells were harvested in culture, washed (3 × 107), and then washed in physiological saline and acidified by resuspension in 500 µl 3% HClO4. The cells were sonicated and the acid-precipitated protein was removed by centrifugation at 0–10°C. Supernatants were neutralized with 0.5 M K2CO3, and the inorganic precipitate (KClO4) was removed by centrifugation before assaying the supernatant for glutathione by the enzymatic assay of Tietze (36). The glutathione determinations performed in this manner do not include glutathione-protein-mixed disulfides which are removed from the assay by HClO4 precipitation.

**RESULTS**

Activation of ADPRT by H2O2 and PMA. ADPRT requires DNA strand breaks for activation (for review see Ref. 37). Both H2O2 and PMA at the physiologically relevant doses of 30 µM and 20 ng/ml, respectively, gave time-dependent increases in
ADP ribosylation (Fig. 1). The ADPRT activation response was bimodal with one peak occurring after about 30 min and a second peak at around 4 h. The first peak of ADPRT activity was larger then the second peak with H$_2$O$_2$ treatment, whereas PMA induced a higher activity at 4 h.

Analysis of Prooxidant-induced DNA Damage and Repair by Nucleoid Sedimentation. Additional confirmation that oxidative stress induces DNA damage in HMLs was achieved using the method of nucleoid sedimentation in sucrose density gradients which has been shown previously to be highly sensitive to DNA strand breaks (32-35, 38-40). The data displayed in Fig. 2 clearly demonstrate that HMLs exposed to the prooxidants, H$_2$O$_2$ (30 min, 75 µM), PMA (1 h, 25 ng/ml), or the radiomimetic agent 4-NQO (30 min, 2 µM), all have reduced sedimentation at 0 time after exposure as compared to untreated controls. The differences in 0 time nucleoid sedimentation between the two subjects displayed in Fig. 3C also suggest interindividual variation in the in vitro DNA damage responses of HMLs to H$_2$O$_2$. Furthermore, there was no evidence that the repair of DNA strand breaks induced by prooxidants was inhibited, since nucleoid sedimentation levels comparable to untreated controls (i.e., 100% equals control level) could be achieved between 7 and 24 h of incubation following prooxidant exposure, which in turn indicates complete repair of the induced DNA breaks (Figs. 2 and 3). In the one study in which PMA was not removed from the medium (see lowest curve in Fig. 2), the DNA damage was more persistent, but repair of the single strand breaks did occur rapidly after 5 h.

Inhibitors of ADP ribosylation such as 3AB (41, 42) have been used to explore the role of ADPRT in the DNA repair process of various types of DNA lesions. One hypothesis for the role of ADPRT in DNA repair is that ADP ribosylation regulates the rejoining or the ligation steps of DNA repair (43). Repair of DNA damage induced by the prooxidants H$_2$O$_2$, PMA, or 4-NQO was also examined in the presence or absence of 2 mM 3AB in Fig. 3. For PMA- and 4-NQO-treated HMLs the presence of 3AB did not influence either the kinetics or the final restoration of nucleoid sedimentation to control levels. The presence of 3AB did increase the initial number of DNA strand breaks from the medium (see lowest curve in Fig. 2), the DNA damage was more persistent, but repair of the single strand breaks did occur rapidly after 5 h.

Fig. 1. Time course of the ADPRT response induced by H$_2$O$_2$ and PMA in HMLs. HMLs (5 x 10$^6$ cells/ml) cultured in physiological saline supplemented with 1% autologous platelet-poor plasma were exposed to either 30 µM H$_2$O$_2$ (left) or 20 ng/ml PMA (right) for the times indicated. Next the cells were pelleted by centrifugation at 600 x g for 10 min and permeabilized, and the induced ADPRT activity was estimated as described in "Materials and Methods." Points (bars), means (±SEM) of 1-5 experiments.

Fig. 2. Repair of DNA damage in HMLs exposed to H$_2$O$_2$, 4-NQO, and PMA. Freshly isolated HMLs were incubated for 30 min with H$_2$O$_2$ (75 µM, •) or 4-NQO (2 µM, ▲) or for 1 h with PMA (25 ng/ml, ▽). After treatment the chemicals were removed by washing the HMLs and resuspending in fresh media or additional PMA was added for a continuous PMA treatment condition (□). Sedimentation of nucleoids was measured immediately after removal of the chemical and after various incubations for up to 10 h.

Fig. 3. The induction and repair of DNA strand breaks in HMLs treated with various prooxidants. Duplicate HML aliquots were treated with 3AB (2 mM) or held at 37°C for 30 min incubation prior to treatment with prooxidants. HML samples were exposed at 37°C in RPMI medium containing 10% fetal calf serum to (A) PMA (25 ng/ml, 1 h), (B) 4-NQO (2 mM, 30 min), or (C) H$_2$O$_2$ (75 µM, 30 min). After treatment the prooxidants were removed from the medium, the HMLs resuspended in fresh medium, and the HML nucleoids measured. Sedimentation of nucleoids was also measured at various times after incubation for up to 24 h. All the curves plotted in have a significant positive slope when analyzed by linear regression (P < 0.05). A and B are paired comparisons of the same subject's HMLs. Data in C are from two different subjects; •, incubation time is 8 h; ○ or △, a paired comparison.
transportable through membranes, additional studies were carried out on viable HMLs. First, a dose-response relationship for H$_2$O$_2$-induced damage was established using the nucleoid sedimentation assay in which a rapid increase in DNA strand breaks was observed as the concentration of H$_2$O$_2$ was increased from 0–40 μM, which was followed by a slower rate in the strand breaks when the concentration of H$_2$O$_2$ was further increased from 40–300 μM. Second, H$_2$O$_2$-damaged DNA was examined by nucleoid sedimentation in HMLs stimulated to divide by mitogenic treatment (i.e., PHA). H$_2$O$_2$-induced DNA damage was assessed 24 h after treatment of HMLs with PHA and compared to HMLs without PHA stimulation. The DNA damage level was dramatically reduced in PHA-stimulated HMLs from 20–80% after exposure to H$_2$O$_2$ concentrations of 20–150 μM. Thus, HMLs programed to divide by PHA treatment had an increased capacity to inactivate H$_2$O$_2$, indicating additional antioxidant protection for proliferating lymphocytes.

Induction of DNA Repair Synthesis by Prooxidants. Induction of UDS from exposure to H$_2$O$_2$ or PMA was very small compared to UDS values induced by NA-AAF, an agent known to induce large patch DNA repair (44) (Table 1). In fact, of the 5 experiments reported in Table 1 involving either H$_2$O$_2$- or PMA-induced UDS, two contained data not significantly different from untreated controls, one was borderline significant, and two were significant at the 95% confidence level. The other model agent we have used to compare prooxidant-induced UDS is 4-NQO, which has been characterized as both a radiomimetic agent (i.e., short patch DNA repair inducer) and a stimulator of large patch DNA repair synthesis (44, 45). The level of 4-NQO-induced UDS was likewise on the average higher than the prooxidant-induced UDS levels but less than the UDS induced by NA-AAF (Table 1). These data suggest at least two possible interpretations: either (a) the DNA lesions introduced by prooxidants are not repaired by large patch excision repair (i.e., compare the difference between UDS induced by NA-AAF with that induced by H$_2$O$_2$ or PMA; Table 1) or (b) prooxidant exposures inhibit large patch DNA repair (i.e., compare the UDS induced by NA-AAF with that induced by 4-NQO, an inducer of both short and large patch excision repair; Table 1).

Inhibitory Effects of Oxidative Stress on UDS. NA-AAF is a well-characterized model carcinogen that induces a large patch type of DNA repair and therefore it is an efficient inducer of UDS (28, 44, 46). We have used NA-AAF-induced UDS as a model to assess whether H$_2$O$_2$ could inhibit the repair of DNA lesions induced by other genotoxins (i.e., NA-AAF) that are known to require endonuclease attack of DNA for large patch repair (28, 44, 46). NA-AAF-treated HMLs were pulsed with [3H]TdThd to radiolabel DNA for quantification of UDS after 30 min and 3 h treatment with H$_2$O$_2$. These periods were selected so as to correspond with the appearance of the ADPRT activation responses to oxidative stress already observed for HMLs in Fig. 1. The data presented in Fig. 4 show that HMLs first exposed to NA-AAF for 30 min to induce UDS and then to micromolar concentrations of H$_2$O$_2$ have suppressed levels of UDS compared to HMLs treated with NA-AAF but not treated with H$_2$O$_2$. The inhibition of NA-AAF-induced UDS was greater at 3 h after H$_2$O$_2$ treatment for each H$_2$O$_2$ dose examined.

The involvement of ADP ribosylation in the mechanism of H$_2$O$_2$ inhibition of UDS is also documented in Fig. 4. The degree of inhibition of NA-AAF-induced UDS by 30–100 μM

---

### Table 1

<table>
<thead>
<tr>
<th>Experiment and treatment</th>
<th>UDS $^a$ (dpm [3H]dCyd/μg DNA)</th>
<th>UDS $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>7.2, 6.3 (6.8)</td>
<td>9.7</td>
</tr>
<tr>
<td>+ 25 μM H$_2$O$_2$</td>
<td>12.8, 12.0 (12.4)</td>
<td>2.9</td>
</tr>
<tr>
<td>+100 μM H$_2$O$_2$</td>
<td>17.7, 19.1 (18.4)</td>
<td>5.6 (1,9, 9, 3)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>20.6, 17.5, 19.6 (19.3)</td>
<td>0.9 (−2, 4, 2)</td>
</tr>
<tr>
<td>+100 μM H$_2$O$_2$</td>
<td>22.6, 25.8 (24.2)</td>
<td>5.8 (2,1, 9, 5)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>20.8, 20.9 (20.3)</td>
<td>10.2 (6,5, 13, 9)</td>
</tr>
<tr>
<td>+300 μM H$_2$O$_2$</td>
<td>35.4, 38.2, 37 (36,8)</td>
<td>1.5 (−6, 1, 4)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>37.7, 38.8, 38.3 (38,3)</td>
<td>3.8 (0,1, 7, 5)</td>
</tr>
<tr>
<td>+300 μM H$_2$O$_2$</td>
<td>20.4, 20.5 (20.5)</td>
<td>12.2 (8,5, 15, 9)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>26.1, 22.5 (24.3)</td>
<td>10.0 (3,1, 6, 5)</td>
</tr>
<tr>
<td>+25 ng/ml PMA</td>
<td>35.4, 38.2, 37 (36,8)</td>
<td>1.5 (−6, 1, 4)</td>
</tr>
<tr>
<td>+200 μM H$_2$O$_2$</td>
<td>20.6, 17.5, 19.6 (19.3)</td>
<td>0.9 (−2, 4, 2)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>37.7, 38.8, 38.3 (38,3)</td>
<td>3.8 (0,1, 7, 5)</td>
</tr>
<tr>
<td>+300 μM H$_2$O$_2$</td>
<td>20.4, 20.5 (20.5)</td>
<td>12.2 (8,5, 15, 9)</td>
</tr>
<tr>
<td>+2.0 μM 4-NQO</td>
<td>19.0, 13.1 (16.1)</td>
<td>35.6 (31,9, 39, 3)</td>
</tr>
<tr>
<td>Untreated control</td>
<td>49.9, 53.5, 51.7 (51.4)</td>
<td>35.6 (31,9, 39, 3)</td>
</tr>
</tbody>
</table>

$^a$ HMLs were incubated either with H$_2$O$_2$, PMA, 4-NQO, or NA-AAF as indicated in 10% serum supplemented RPMI medium at 37°C for 60 min and then the cells were harvested by centrifugation. HMLs were resuspended in RPMI medium and cultured in the presence of 40 μM BrdUrd for 1 h. The incubation was then continued for an additional 30 min with 5 μM aphidicolin present. The HMLs were finally labeled with [3H]dThd for 1–4 h. The repaired DNA was extracted by the alkaline isopinic centrifugation, and the UDS was calculated as described in "Materials and Methods."

$^a$ UDS was calculated by subtracting untreated control values.

$^a$ 95% confidence intervals (in parentheses) were calculated for the differences between the experimental levels and their control levels using the t statistic and an overall pooled SE estimate determined from the mean variance of the 7 experiments. Confidence intervals involving 0 are not significant.

---

Fig. 4. Dependence of unscheduled DNA synthesis on ADP ribosylation in HMLs after induction of DNA damage with NA-AAF. HMLs were exposed to 10 μM NA-AAF in RPMI medium containing 20% autologous platelet-poor plasma for 30 min at 37°C after which they were treated for an additional 30 min with the indicated concentrations of H$_2$O$_2$. The cells were then harvested by centrifugation at 600 x g for 30 min and resuspended in fresh medium containing [3H]dThd and either the presence or absence of 2 mM 3AB after 30 min (top) or after 3 h (bottom) incubation at 37°C. Radiolabeling with [3H]dThd was for 2 h. This protocol allowed estimation of the effects of ADP ribosylation on UDS during the two peaks of prooxidant-induced ADPRT activities identified in Fig. 1. The values are % of control. Control values for NA-AAF-induced UDS after 30 min in the absence and presence of 3 mM 3AB were 234 and 216 cpm [3H]dThd/μg DNA, respectively, and after 3 h they were 121 and 112 cpm [3H]dThd/μg DNA, respectively.
H₂O₂ was significantly increased about 10–25% in the presence of 2 mM 3AB at both peaks I (30 min) and II (3 h) of the ADPRT activation responses of H₂O₂ (Figs. 2 and 4). Thus, H₂O₂ inhibits NA-AAF-induced UDS in a dose-dependent manner and the inhibitions can be further enhanced by inhibitors of ADPRT. The data suggest that ADP ribosylation of nuclear proteins prevent the inhibitory effects of H₂O₂ on large patch repair induced by NA-AAF DNA adducts.

There was no significant effect of 3AB on NA-AAF-induced UDS in the absence of H₂O₂ exposure (Fig. 4 legend). These data are consistent with our earlier report that UDS induced by NA-AAF alone is unaffected by ADPRT inhibitors (28). In general, ADPRT inhibitors do not block DNA repair synthesis but even appear to stimulate UDS under some conditions (42).

Effects of Redox State on DNA Repair. Oxidative stress has profound effects on the reduction/oxidation balance in cells (47), and glutathione, because it occurs in millimolar concentrations in cells (i.e., 90% of the cellular nonprotein thiol pool), is the primary cellular antioxidant (48). Therefore, we have examined the possibility that redox imbalances imposed on cells by oxidative stress might explain the inhibitions in NA-AAF-induced UDS. For the purpose of demonstrating the effects of redox imbalances on NA-AAF-induced UDS, we have grown cells in the presence of cyclohexene-1-one or BSO, which selectively deplete intracellular glutathione pools (49), or in the presence of GSH, which expands intracellular GSH pools (50). The data recorded in Table 2 strongly support the dependence of NA-AAF-induced UDS on the redox state of the HMLs. There was a dose-dependent increase in UDS in the presence of increasing levels of extracellular GSH and a corresponding dose-dependent decrease in NA-AAF-induced UDS with decreasing levels of glutathione pools. NA-AAF binding to DNA was also decreased by decreasing the glutathione pools, but the effect on DNA repair synthesis still remained after correction for this effect by calculating the DNA repair proficiency index (i.e., UDS/binding). The effect of redox state on NA-AAF binding to DNA no doubt relates to the fact that the esterase which deacetylates NA-AAF to a DNA-binding intermediate is also a NADPH-dependent enzymatic reaction (51).

Our data have also shown that ADP ribosylation was involved in the mechanism of inhibition of UDS by prooxidants (Fig. 4). Therefore, we have sought to demonstrate whether the redox state could directly modulate ADPRT activity. First, HMLs were treated with 100 µM H₂O₂ for 30 min to provide a higher level of ADPRT activity. Second, the cells were permeabilized as described in “Materials and Methods” but with the omission of mercaptoethanol. Finally, ADPRT was measured as TCA ppt cpm [³H]NAD⁺/5 × 10⁵ cells in the presence of increasing mm concentrations of either GSH or GSSG as described in “Materials and Methods.” Under these experimental conditions, there was a dose-dependent activation of ADPRT with increasing concentrations of GSH and a dose-dependent decrease in the presence of increasing concentrations of GSSG (Table 3). These data strongly support the fact that ADPRT is a sulfhydryl-containing enzyme with great dependence on the redox state for optimal activation.

The potential biological relevance of the redox state regulation of ADPRT activity is presented in Table 4. HMLs were given rather low doses of H₂O₂ (0–30 µM) to produce an in vitro induced oxidative stress after an 18-h incubation period at 37°C. The dependence of both constitutive and activated ADPRT (i.e., the response to an additional bolus of 100 µM H₂O₂ on glutathione pools is obvious from the data in Table 4. It is concluded that oxidative stress-induced redox imbalances can inhibit ADPRT function in viable HMLs.

### DISCUSSION

The data presented in this study suggest a possible mechanistic link between oxidative stress and the carcinogenic process which has not been previously postulated; namely, oxidative stress generated endogenously in cells or extracellularly initiates and promotes carcinogenesis by inhibiting the repair of certain types of DNA lesions through redox control of ADP ribosylation and UDS. There is additional support for our proposed mechanism already in the literature. First, the most common oxygen radical (.OH) damaging cellular DNA and generated by H₂O₂ (1–4, 52, 53) has been shown to be repaired efficiently and not to be dependent on ADP ribosylation (4). Second, extracellular generated O₂⁻ radical can damage cellular DNA and it was repaired very poorly (4). Third, human cells genetically deficient in glutathione could repair only about 70% of the DNA strand breaks induced by OH radicals generated by X-radiation compared to normal cells (54, 55). Likewise, cisplatinum-treated cells deficient in glutathione are also deficient in UDS (56). Fourth, tumor promoters have been reported to nonselectively inhibit both DNA repair and scheduled DNA synthesis (57, 58). Fifth, a promotable mouse cell line has 4–5 times more DNA strand breaks induced from prooxidant exposures than a nonpromotable mouse cell line (59), and this

### Table 2 Influence of glutathione on the regulation of NA-AAF-induced UDS in HMLs subjected to oxidative stress

<table>
<thead>
<tr>
<th>Experiment and treatment*</th>
<th>UDS (dpm/µg DNA)</th>
<th>% of control</th>
<th>NA-AAF binding to DNA (dpm/µg DNA)</th>
<th>% of control</th>
<th>Repair index (UDS/binding)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control (no GSH)</td>
<td>220, 213 (217)</td>
<td>118</td>
<td>338, 382 (360)</td>
<td>678</td>
<td>436, 340 (392)</td>
<td>1,054</td>
</tr>
<tr>
<td>+1 mM GSH</td>
<td>262, 248 (255)</td>
<td>136</td>
<td>325, 277 (301)</td>
<td>678</td>
<td>244</td>
<td>80</td>
</tr>
<tr>
<td>+5 mM GSH</td>
<td>270, 321 (295)</td>
<td>136</td>
<td>234, 272 (253)</td>
<td>678</td>
<td>184, 158 (171)</td>
<td>29</td>
</tr>
<tr>
<td>2. Control (no BSO)</td>
<td>273, 215 (244)</td>
<td>41</td>
<td>119, 80 (100)</td>
<td>678</td>
<td>32, 31 (32)</td>
<td>13</td>
</tr>
<tr>
<td>+0.5 mM BSO</td>
<td>32, 31 (32)</td>
<td>13</td>
<td>338, 382 (360)</td>
<td>678</td>
<td>244</td>
<td>80</td>
</tr>
<tr>
<td>+5 mM BSO</td>
<td>380, 446 (413)</td>
<td>50</td>
<td>436, 340 (392)</td>
<td>678</td>
<td>184, 158 (171)</td>
<td>29</td>
</tr>
<tr>
<td>3. Control (no cyclohexene-1-one)</td>
<td>207, 66, 38 (52)</td>
<td>50</td>
<td>244</td>
<td>62</td>
<td>184, 158 (171)</td>
<td>44</td>
</tr>
</tbody>
</table>

* Experiment 1 was carried out by treating 5 × 10⁵ HMLs cultured in 10% plasma-supplemented RPMI medium with 10 µM NA-AAF for 30 min at 37°C followed by another 30 min treatment in plasma-free RPMI medium with 100 µM H₂O₂. Next, 0, 1, and 5 mM glutathione was added and UDS was calculated after overnight incubation in the presence of 10 mM hydroxyurea and [³H]Tdr as described in “Materials and Methods.” Experiments 2 and 3 were carried out by first incubating HMLs in plasma-supplemented RPMI medium with 0.5 and 5 mM BSO or 1 and 4 mM cyclohexene-1-one for 18 h or 1 h, respectively, at 37°C. The induced UDS was calculated as previously described. Viability estimated by trypan blue exclusion after BSO or cyclohexene-1-one treatments were: experiment 2—untreated = 110 ± 1 ng/10⁶ cells, 0.5 and 5.0 mM BSO = 64 ± 1 and 65 ± 16 ng/10⁶ cells; experiment 3—untreated = 185 ± 3 ng/10⁶ cells, 1.0 and 4.0 mM cyclohexene-1-one = 46 ± 13 and 38 ± 2 ng/10⁶ cells.
greater sensitivity to strand breakage is consistent with a greater redox imbalance and an ultimate inhibition of DNA repair via glutathione pool depletion. Finally, DNA damage (14) and depletion of cellular GSH (60) are immunosuppressive, and a suppressed immune function is considered important to the development of cancers (61).

Moreover, in this study we have observed a bimodal response of ADPRT activity to oxidative stress (Fig. 1). The presence of peak II (i.e., 4 h peak) as a part of the time course activation response of ADPRT to PMA-induced oxidation has been previously observed in mouse and human fibroblasts (62). However, these authors did not observe the early peak of increased ADPRT activity to oxidative stress (Fig. 1). The presence of peak II (i.e., 4 h peak) as a part of the time course activation response of ADPRT to oxidative stress (Fig. 1).

Table 3 Modulation of ADPRT activity in HML cytoskeletons by the redox state of the reaction mixture

<table>
<thead>
<tr>
<th>Experiment and treatment</th>
<th>H$_2$O$_2$-activated ADPRT$^*$ (cpm TCA ppt $[^3H]NAD^+$/5 $\times 10^5$ cells)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Up-regulation of ADPRT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.5 mM GSH</td>
<td>893, 1099 (996)</td>
<td>100</td>
</tr>
<tr>
<td>+ 1.0 mM GSH</td>
<td>1268, 1644 (1457)</td>
<td>146</td>
</tr>
<tr>
<td>+ 2.5 mM GSH</td>
<td>1561, 1411 (1486)</td>
<td>149</td>
</tr>
<tr>
<td>+ 5.0 mM GSH</td>
<td>2982, 1666 (2324)</td>
<td>233</td>
</tr>
<tr>
<td>2. Down-regulation of ADPRT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.5 mM GSSG</td>
<td>729, 663 (696)</td>
<td>79</td>
</tr>
<tr>
<td>+ 1.0 mM GSSG</td>
<td>399, 551 (475)</td>
<td>68</td>
</tr>
<tr>
<td>+ 2.5 mM GSSG</td>
<td>360, 441 (401)</td>
<td>58</td>
</tr>
<tr>
<td>+ 10.0 mM GSSG</td>
<td>97, 177 (173)</td>
<td>20</td>
</tr>
</tbody>
</table>

$^*$HMLs in physiological saline were treated with 100 $\mu$M H$_2$O$_2$ for 30 min at 37°C in order to activate ADPRT and then pelleted by centrifugation at 600 x g. Centrifuged cell pellets were then permeabilized with hypotonic buffer minus mercaptoethanol, and ADPRT activity was estimated in the cytoskeletons in the presence or absence of the indicated concentrations of GSH and GSSG as described in “Materials and Methods.”

Table 4 Parallel dependence of the levels of glutathione and ADPRT activity in HMLs on chronic oxidative stress induced in culture over an 18-h period by in vitro dosed of H$_2$O$_2$

<table>
<thead>
<tr>
<th>H$_2$O$_2$ (pmol) pre-treatment</th>
<th>Total glutathione (ng/10$^6$ HMLs)</th>
<th>ADPRT activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>79</td>
<td>745</td>
</tr>
<tr>
<td>15</td>
<td>44 (44)$^*$</td>
<td>287 (25)</td>
</tr>
<tr>
<td>30</td>
<td>37 (53)</td>
<td>188 (51)</td>
</tr>
</tbody>
</table>

$^*$HML samples were cultured in 1% fetal calf serum-supplemented RPMI for 18 h at 37°C in the presence of 0, 15, or 30 pmol H$_2$O$_2$. Intra-cellular total glutathione (per 5 $\times 10^5$ HMLs) was then determined and parallel cultures (5 $\times 10^5$ HMLs/ml) were given an additional $\pm$100 pmol H$_2$O$_2$ dose for 30 min to estimate constitutive (–H$_2$O$_2$) and activated (+H$_2$O$_2$) ADPRT activity (see “Materials and Methods”).

% of control (0 pmol treatment).

The presence of these authors did not observe the early peak of increased ADPRT activity to oxidative stress (Fig. 1). These prooxidant states may lead to redox imbalances, and thereby they can explain the DNA repair deficiencies we have observed in HMLs coming from these types of human conditions that predispose to cancer. Furthermore, lymphocyte DNA damage and repair may also reflect immune function since both processes are up- and down-regulated by redox imbalances (Tables 2 and 3; 14, 49, 60).

REFERENCES

Oxidative Stress Induces DNA Damage and Inhibits the Repair of DNA Lesions Induced by N-Acetoxy-2-acetylaminofluorene in Human Peripheral Mononuclear Leukocytes


**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/15/4619

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/50/15/4619.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.