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Role of Hydrogen Peroxide in the Formation of DNA Adducts in HL-60 Cells Treated with Benzene Metabolites

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We have investigated the influence of peroxides on DNA adduct formation in HL-60 cells treated with polyphenolic metabolites of benzene. Treatment of HL-60 cells with 50 μM hydroquinone (HQ), 500 μM catechol (CAT) or 200 μM 1,2,4-benzenetriol (BT) resulted in adduct levels of 0.27, 0.21 and 0.21 $\times$ 10^{-7}, respectively. Addition of 50–250 μM H_2O_2 or 250 μM cumene hydroperoxide to HL-60 cells increased DNA adduct formation 2.7–10-fold following treatment with HQ or CAT but had no effect on adduct formation by BT. Treatment of HL-60 cells with the combinations of HQ plus either BT or phorbol myristate acetate (PMA) potentiated DNA adduct formation by 2.5–4-fold. Significant elevations of cellular H_2O_2 levels occurred after treatment of HL-60 cells with either PMA, CAT or BT. These results indicate that cellular levels of H_2O_2 regulate the peroxidase dependent activation of benzene metabolites to form DNA adducts.

Human exposure to benzene occurs from both industrial and environmental sources (1,2). Benzene has to be metabolized (3) to exert its hematotoxic (4,5) leukemogenic (6) and carcinogenic effects (7). The principal metabolites of benzene are phenol, hydroquinone (HQ), catechol (CAT) and to a lesser extent 1,2,4-benzenetriol (BT) (8,9). After exposure to benzene these metabolites are known to accumulate in the bone marrow where they can be further oxidized to exert their toxic effects (10). The further oxidation of these compounds in the bone marrow is probably due to peroxidase enzymes such as myeloperoxidase and macrophage peroxidase (11,12). Although the mechanism by which benzene exerts its toxic and leukemogenic effects in the bone marrow is not yet understood, the formation of DNA adducts by benzene metabolites may play a important role in this process. Previously we have reported that the polyphenolic metabolites of benzene form DNA adducts in peroxidase containing cells such as promyelocytic HL-60 cells, mouse bone marrow macrophages and human bone marrow cells treated *in vitro* (13–15). However we were not able to detect DNA adduct formation in cells lacking detectable peroxidase activity (15). These results suggest a important role for peroxidase enzymes in the activation of benzene metabolites to form DNA adducts.

Cellular hydrogen peroxide (H_2O_2) production is the endproduct of many naturally occurring processes in cells (16). In addition stimulation of cells with PMA and other protein kinase C activators can generate large amounts of H_2O_2 (16–18). Recent studies have also demonstrated that treatment of cells with carcinogenic agents results in increased levels of H_2O_2 (19,20). In cells containing peroxidase enzymes, cellular hydroperoxides are reduced by peroxidase enzymes such as myeloperoxidase. As a result of this process the peroxidase enzyme is converted to a intermediate termed Compound I. A wide variety of phenolic compounds including the benzene metabo-

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Abbreviations: hydroquinone, HQ; catechol, CAT; 1,2,4-benzenetriol, BT; hydrogen peroxide, H_2O_2; cummene hydroperoxide, CuOOH; phorbol myristate acetate, PMA; relative adduct level, RAL.

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lites can serve as reducing agents for this intermediate and in the process become oxidized to semiquinone radicals (21). This oxidation step is believed to play an important role in the formation of DNA adducts by the polyphenolic benzene metabolites (13,15). Work from our laboratory has demonstrated that combinations of benzene metabolites potentate the levels of DNA adduct formation by 3–6 fold (14). Based on the studies reported above, we hypothesize that the potentiation of DNA adducts observed may be due to alterations in cellular hydroperoxide levels.

The purpose of these studies was to examine the effects of hydrogen peroxide and organic hydroperoxides on the formation of DNA adducts in HL-60 cells treated with polyphenolic benzene metabolites and to determine if alterations in levels of cellular hydroperoxides may be responsible for the observed potentiation of DNA adduct formation by combinations of benzene metabolites.

MATERIALS AND METHODS

Culture conditions and cell treatments. HL-60 cells were grown as previously described (13,15). HQ, CAT, and BT (Aldrich) were dissolved in distilled water and added directly to the culture medium at final concentrations of 0–250 μM for 4 or 24 h. Hydrogen peroxide (30%, Sigma) was diluted to a 1.5% solution with distilled water and added to the cells at the same time as the benzene metabolites at final concentrations of 50–250 μM. CuOOH (Sigma) was diluted in ethanol and added simultaneous with the benzene metabolites. PMA (Sigma) was dissolved in dimethylsulfoxide, further diluted with distilled water and added to the culture 15 minutes prior to the addition of HQ at a final concentration of 25 nM. Following treatment the cells were collected and stored at −70°C until the DNA was isolated.

Hydrogen peroxide assay. Hydrogen peroxide release by HL-60 cells was determined using the phenol red assay as previously described (17,22). Briefly, 5×10^6 cells were washed and resuspended in 5 mL buffered phenol red solution containing 140 mM NaCl, 10 mM potassium phosphate, 0.1 mg/mL phenol red, 0.05 mg/mL horseradish peroxidase Type II (Sigma) and 5 mM glucose. The cells were treated with either 250 μM HQ, CAT or BT; the combination of 50 μM HQ plus 250 μM CAT or 250 μM BT or with PMA (25 nM) and incubated for 1 h at 37°C. The samples were centrifuged and 10 μl of 1M NaOH was added to each mL of the supernatant. Hydrogen peroxide production was measured as the increase in A_{610} due to the oxidation of phenol red. The assay was calibrated with known concentrations of hydrogen peroxide, and the results were expressed as nmole H_2O_2 produced in 10^6 cells.

[^{32}*P*]-*postlabeling.* The DNA isolation and the P_1 nuclease enhanced [^{32}P]-postlabeling was performed as previously described (13–15).

RESULTS

The effect of hydrogen peroxide and cumene hydroperoxide on DNA adduct formation by benzene metabolites in HL-60 cells. HL-60 cells were treated for 24 h with either HQ, CAT or BT alone or in combination with H_2O_2. Control samples were treated with either distilled water or different concentrations of H_2O_2. DNA adducts produced in HL-60 cells by these treatments were measured by [^{32}P]-postlabeling. No DNA adducts were detected in the control samples (data not shown). Treatment of HL-60 cells with 50 μM HQ for 24 h resulted in the formation of two DNA adducts 1 and 3 with a RAL of 0.27×10^{-7} (Fig. 1A). The combination treatment of 50 μM HQ plus 50 μM H_2O_2 significantly increased the DNA adduct levels in HL-60 cells compared to treatment with 50 μM HQ alone (Table 1). The addition of H_2O_2 to the treatment medium containing HQ also resulted in the formation of a new adduct #2 which was not observed after treatment with 50 μM HQ by itself (Fig. 1B). The RALs produced by combination treatment of HQ with H_2O_2 were linearly related to the H_2O_2 concentrations, however higher concentrations of H_2O_2 did not change the adduct map observed in Fig. 1B.

The effect of the organic hydroperoxide CuOOH on the formation of DNA adducts by treatment of HL-60 cells with HQ was studied. Treatment with CuOOH by itself produced no detectable DNA adducts (results not shown). However, the combination treatment of 50 μM HQ plus 250 μM CuOOH resulted in a 8-fold increase in the DNA adduct level (Table 1) and a adduct profile (Fig. 1C) similar to that observed after combination treatment with H_2O_2 (Fig. 1B).

Twenty-four h administration of 500 μM CAT resulted in the formation of 3 DNA adducts 2, 4 and 5 (Fig. 1D) with a RAL of 0.21×10^{-7}. The addition of 50 μM H_2O_2 to the treatment potentiated the overall level of these DNA adducts by 3.9-fold (Fig. 1E, Table 1). Combination

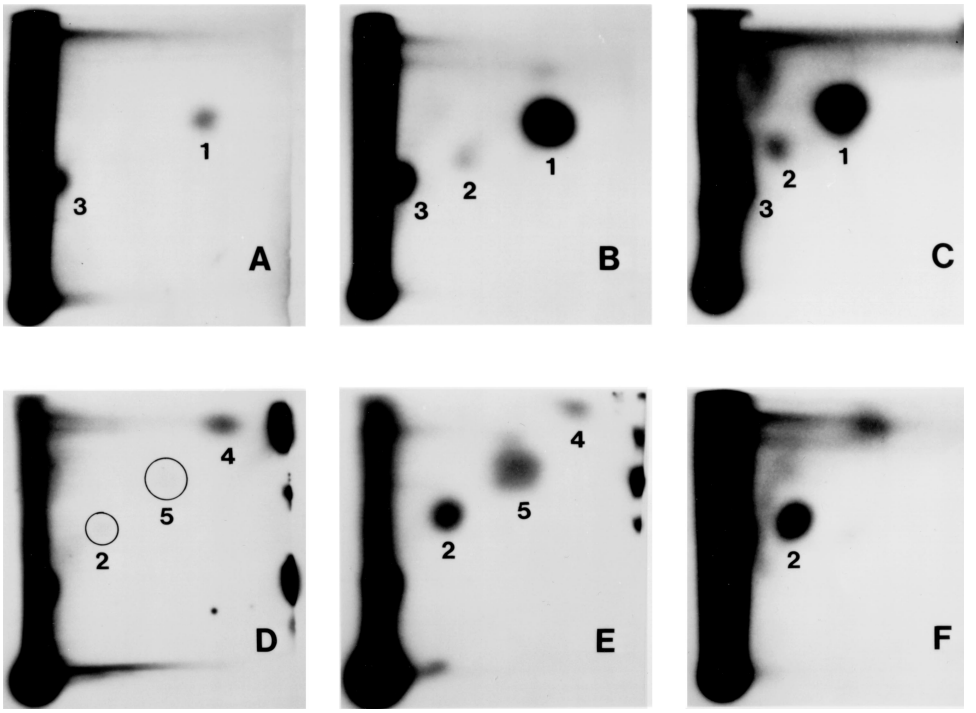

FIG. 1. [^{32}P]-postlabeling detection of DNA adducts in HL-60 cells treated for 24 h with 50 μM HQ (A), 50 μM HQ plus 250 μM H_2O_2 (B), 50 μM HQ plus 250 μM CuOOH (C), 500 μM CAT (D), 500 μM CAT plus 250 μM H_2O_2 (E) and 200 μM BT (F). The autoradiograms were exposed for 8 to 24 h at −70°C.

TABLE 1The
Effect of Hydrogen Peroxide, Cummene Hydroperoxide and Phorbol Myristate Acetate on DNA Adduct Formation in HL-60 Cells Treated with Benzene Metabolites

Concentrations (μM)	Treatment time (h)	Relative adduct level ($\times 10^{-7}$)	Potentiation
HQ (50)	24	0.27 ± 0.05	—
HQ (50) + H_2O_2 (50)	24	0.73 ± 0.16*	2.7
HQ (50) + H_2O_2 (100)	24	1.30 ± 0.22**	4.8
HQ (50) + H_2O_2 (250)	24	2.00 ± 0.21**	7.4
HQ (50) + CuOOH (250)	24	2.16 ± 0.17**	8
CAT (500)	24	0.21 ± 0.044	—
CAT (500) + H_2O_2 (50)	24	0.83 ± 0.081**	3.9
CAT (500) + H_2O_2 (100)	24	1.15 ± 0.14**	5.4
CAT (500) + H_2O_2 (250)	24	2.10 ± 0.35**	10.0
BT (200)	24	0.21	—
BT (200) + H_2O_2 (100)	24	0.16	—
BT (200) + H_2O_2 (250)	24	0.17	—
BT (250)	24	0.64 ± 0.01	—
HQ (50) + BT (250)	24	3.8 ± 0.28**	4.2
HQ (100)	4	0.14 ± 0.008	—
HQ (100) + PMA (0.025)	4	0.35 ± 0.038*	2.5

* $p < 0.01$.
** $p < 0.001$.

TABLE 2H
nf2O_2 Production by HL-60 Cells Treated with Various Benzene Metabolites

Treatment (μM)	N	H_2O_2 (nmole/10^6 cells)
Control	4	0.30 ± 0.035
HQ (250)	6	0.38 ± 0.083
CAT (250)	6	1.09 ± 0.095**
BT (250)	6	1.78 ± 0.095**
HQ (50) + CAT (250)	5	1.46 ± 0.140**
HQ (50) + BT (250)	5	1.78 ± 0.096**
PMA (0.025)	2	6.34

** $p < 0.001$ (vs. control).

treatments of CAT with 100 or 250 μM H_2O_2 potentiated adduct formation 5.4 and 10.0 fold, respectively (Table 1).

Treatment of HL-60 cells with 200 μM BT resulted in the formation of one DNA adduct (#2) (Fig. 1F) with a RAL of 0.21×10^{-7}. Addition of 250 μM H_2O_2 had no effect on DNA adduct formation (Table 1).

Hydrogen peroxide production by HL-60 cells treated with benzene metabolites. Recent studies have suggested that treatment of cells with various carcinogenic agents can modify the cellular levels of hydroperoxides (19,20). In order to determine if HL-60 cells were undergoing similar processes, we have measured the levels of H_2O_2 under our treatment conditions. HL-60 cells produced low levels of H_2O_2 (Table 2). The cellular level of H_2O_2 was not significantly influenced by treatment with HQ. In contrast a 1h treatment with 250 μM of either CAT or BT significantly potentiated the levels of H_2O_2. Also the combination of 50 μM HQ plus either 250 μM CAT or 250 μM BT produced a significant increase in the levels of H_2O_2 compared to the control cells (Table 2).

DNA adduct formation by combination treatment with polyphenolic metabolites. HL-60 cells were treated with the combination of 50 μM HQ and 250 μM BT for 24 h. This treatment significantly potentiated the levels of DNA adducts compared to the individual treatments (Table 1) and produced a adduct map (Fig. 2A) similar to that observed in the combination treatments of HQ with H_2O_2 and CuOOH (Figs. 1B and 1C).

DNA adduct formation bh HQ in PMA-induced HL-60 cells. The above results suggest that the levels of DNA adducts produced by treatment of HL-60 cells with either HQ or CAT are related to the cellular levels of H_2O_2. In order to test this hypothesis we have treated HL-60 cells with

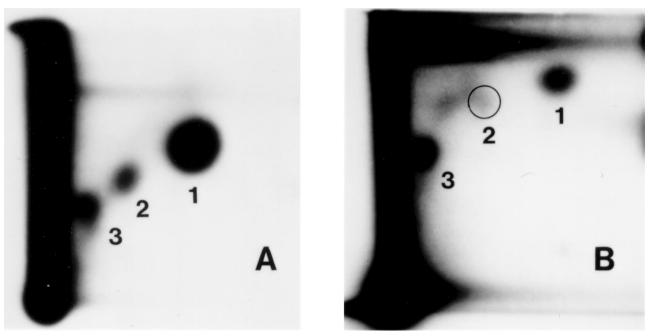

FIG. 2. [^{32}P]-postlabeling detection of DNA adducts formed in HL-60 cells either treated with the combination of 50 μM HQ plus 250 μM BT for 24 h (A) or pretreated for 15 min with 25 nM PMA followed by 100 μM HQ for 4 h (B). The autoradiograms were exposed for 24 h at −70°C.

PMA which is known to significantly increase the levels of H_2O_2 (16, 19). Incubation of HL-60 cells with 25 nM PMA resulted in the release of 6.34 nmole H_2O_2 per 10^6 cells (Table 1). In parallel studies, treatment of HL-60 cells with 100 μM HQ for 4 h resulted in a DNA adduct pattern similar to that shown in Fig. 1A with a RAL of 0.14×10^{-7}. Pretreatment of these cells with 25 nM PMA prior to the administration of HQ produced a 2.5 fold elevation in the total adduct level (Table 1) and a 5.4 fold increase in the level of adduct 1 (Fig 2B).

DISCUSSION

The addition of H_2O_2 to the cell culture medium potentiated DNA adduct formation 2.7-10 fold in HL-60 cells following treatment with either HQ or CAT. The increase in the levels of DNA adducts were linearly related to the amount of H_2O_2 added. A similar potentiation in DNA adducts levels was also obtained by the addition of CuOOH. Previous studies in our laboratory have demonstrated that the formation of DNA adducts by these metabolites in treated cells is dependent upon peroxidase activation (15). Taken together our results suggest a model whereby the formation of DNA adducts by HQ and CAT is regulated by the cellular levels of H_2O_2 and other peroxides. Cellular peroxidase serve to reduce these peroxides to either H_2O or the corresponding alcohol. During this process the peroxidase enzyme is oxidized. HQ and CAT serve as reducing substrates for the oxidized enzyme. However as a consequence, these phenolic compounds are oxidized to reactive intermediates which lead to DNA adduct formation. Consistent with this model is the result that addition of H_2O_2 to the medium did not increase DNA adduct formation in HL-60 cell treated with the fully oxidized para-benzoquinone (G. Lévay, unpublished observation).

In contrast to the results with HQ and CAT, the role of H_2O_2 in the activation of BT to form DNA adducts is unclear. BT is known to be a reducing co-substrate for peroxidase enzymes (22) and the formation of DNA adducts in cells treated with BT was dependent on peroxidase activity (G. Lévay, unpublished observation). However the addition of H_2O_2 to HL-60 cells did not influence the DNA adduct levels produced by BT treatment.

As described above, our studies suggested that the levels of cellular peroxides limit the formation of DNA adducts by HQ and CAT. PMA is a protein kinase C activator, capable of initiating the production of large amounts of H_2O_2 (16,19). Treatment of HL-60 cells with 25 nM of PMA increased the cellular release of H_2O_2 by 20-fold. Pretreatment of HL-60 cells with PMA prior to the addition of HQ potentiated the level of DNA adduct 1 by 5.4-fold. Therefore increasing cellular peroxide levels either by exogenous addition or endogenous generation significantly increased DNA adduct formation in HL-60 cells treated with either HQ or CAT.

Previous studies have reported that treatment of polymorphonuclear lymphocytes and mouse skin with carcinogenic agents results in the generation of H_2O_2 (19,20). Our studies demonstrated that treatment of HL-60 cells with CAT and BT resulted in a significant increase in the cellular levels of H_2O_2. Furthermore treatment with the combination of HQ plus either CAT or BT also significantly increased H_2O_2 levels compared to untreated cells. The increased levels of H_2O_2 may be due to the generation of superoxide which either enzymatically or non-enzymatically dismutates to H_2O_2.

In addition to increasing the levels of H_2O_2, the combination of HQ plus BT elevated by 4-fold the levels of DNA adducts, and produced a DNA adduct profile similar to that observed with the combination of HQ and either H_2O_2 or CuOOH. Earlier reports from our laboratory, demonstrated that combination treatment of HL-60 cells with HQ and either CAT or BT resulted in a 3–6-fold elevation in DNA adduct formation (14). At that time, the mechanisms responsible for the observed potentiation of adduct levels was not understood. However based on our current studies, we interpret these results to suggest that treatment of HL-60 cells with combinations of benzene metabolites results in increased generation of H_2O_2 and subsequent peroxidase-dependent increases in DNA adduct formation by these metabolites.

We believe that these results have significance to understanding the mechanisms by which benzene and related compounds are activated to form DNA adducts *in vivo*. In addition these results may also have significance to smoking related DNA adduct formation in alveolar cells and lung tissue (23). The polyphenolic benzene derivatives are present in high concentrations in cigarette smoke (24). These compounds in the presence of peroxidase enzymes such as the alveolar macrophage peroxidase might result in enhanced DNA adduct formation in respiratory tissues.

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