Induction of direct adducts, apurinic/apyrimidinic sites and oxidized bases in nuclear DNA of human HeLa S3 tumor cells by tetrachlorohydroquinone

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DNA damage induced by tetrachlorohydroquinone (Cl4HQ), the quinonoid metabolite of pentachlorophenol (PCP), was investigated in human HeLa S3 tumor cells. Formation of one major and two minor DNA adducts in cells treated with Cl4HQ (50–300 µM) was detected by 32P-post-labeling assay and the adducts accumulated over the course of the experiment (0.5–2 h), with total adduct levels estimated to be 3–6 per 10⁸ nucleotides. These adducts did not correspond to those derived from calf thymus DNA treated with tetrachloro-1,4-benzoquinone. Results from the apurinic/apyrimidinic (AP) sites assay indicated that the number of AP sites was 2-fold greater in cells exposed to Cl4HQ (300 µM) than the corresponding control. Further characterization of the AP sites confirmed that Cl4HQ induced predominantly (75%) putrescine-excisable AP sites in HeLa S3 cells. In parallel, the concentration of 8-hydroxy-2′-deoxyguanosine (8-HO-dG) in cells treated with Cl4HQ for 0.5 and 2 h was increased 2- and 5-fold, respectively, compared with the control. The extent of oxidative DNA damage induced by Cl4HQ was approximately two orders of magnitude greater than those of direct DNA adducts. Overall, it appears that reactive oxygen species mediate the parallel formation of AP sites and 8-HO-dG in HeLa S3 cells following treatment with Cl4HQ and that the contribution of depurination/depymidination of direct DNA adducts is relatively insignificant compared with the formation of oxidized AP sites. We conclude that putrescine-excisable AP sites represent a major type of ROS-mediated oxidative DNA damage in cellular DNA induced by Cl4HQ and may play a role in PCP-induced clastogenicity in mammalian cells.

Introduction

Tetrachlorohydroquinone (Cl4HQ), one of the reactive quinonoid metabolites of pentachlorophenol (PCP), is mutagenic and clastogenic in mammalian cells (1–8). Evidence indicates that Cl4HQ induces mutations at the hprt locus, micronuclei in Chinese hamster cells (1,2) and DNA single-strand breaks in Chinese hamster ovary cells and human fibroblasts (3–5).

Cl4HQ may also have contributed to the chromosomal damage induced by PCP in mammalian cells (8–10).

Tetrachlorohydroquinone and its semiquinone and quinone counterparts can undergo redox cycling to generate reactive oxygen species (ROS) as well as alkylate proteins and genomic DNA (8,11–13). Cl4HQ-derived ROS induces multiple types of DNA lesions in calf thymus DNA in the presence of Cu(II) (8,14), including the parallel formation of an oxidized base, DNA fragmentation and apurinic/apyrimidinic (AP) sites. The AP sites induced by Cl4HQ/Cu(II) in calf thymus DNA were characterized as predominantly putrescine-excisable AP sites, which is similar to those of the endogenous AP sites present in mammalian tissues and of calf thymus DNA treated with H2O2 (15). ROS are the main sources for the formation of AP sites in calf thymus DNA induced by PCP-derived quinonoids, whereas depurination/depymidination of direct DNA adducts of PCP quinonoids, such as tetrachloro-1,4-benzoquinone, is relatively insignificant (14). This raises the question as to whether similar types of DNA damage can be produced by Cl4HQ in mammalian cells and if such damage could contribute to Cl4HQ-induced clastogenicity in mammalian cells. A diagram of the chemical structures of PCP, Cl4HQ and the respective benzoquinone are depicted in Figure 1.

ROS-induced DNA damage in mammalian cells is subject to repair primarily by base-excision repair enzymes, such as 8-OH-dG glycosylase/lyase, which removes oxidized bases to generate AP sites (16). An additional repair enzyme, AP endonuclease, excises 3’-nicked AP sites to generate one nucleotide gaps. This process is followed by repair synthesis by DNA polymerase β and completed by ligase. If not repaired, AP sites can result in mutations, as well as chromosome aberrations (17).

To investigate the types of DNA damage induced by Cl4HQ in mammalian cells, we undertook the present investigation to study the formation of direct adducts and oxidative damage in nuclear DNA of human HeLa S3 tumor cells exposed to Cl4HQ. We examined the formation of AP sites and 8-HO-dG, as well as direct DNA adducts by the 32P-post-labeling method in Cl4HQ-treated and control HeLa S3 cells. We tested the hypothesis that putrescine-excisable AP sites are a major type of ROS-mediated oxidative DNA damage in cellular DNA induced by Cl4HQ.

Materials and methods

Chemicals

Cl4HQ (99%) and Cl4BQ (98%) were from Aldrich (Milwaukee, WI). Micrococcal nuclease, nuclease P1, potato apyrase (type VII) and spleen phosphodiesterase (type I) were purchased from Sigma (St Louis, MO). Whatman chromatography paper was from Fisher Scientific (Pittsburgh, PA). T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Polyethyleneimine (PEI)-cellulose thin layers were purchased from Alltech (Deerfield, IL). [γ-32P]ATP (sp. act. > 7000 Ci/mmol) was from ICN Pharmaceutical

Abbreviations: AP, apurinic/apyrimidinic; DMEM, Dulbecco’s modified Eagle’s medium; ESA, electrochemical array detector; BHT, butylated hydroxytoluene; HPLC-ECD, HPLC/electrochemical detection; 8-HO-dG, 8-hydroxy-2′-deoxyguanosine; PCP, pentachlorophenol; PEI, polyethyleneimine; ROS, reactive oxygen species; RAL, relative adduct levels; Cl4HQ, tetrachlorohydroquinone.
and washed with 70% ice-cold ethanol. The DNA pellets were resuspended in deionized water for 16 h by mixing at 4°C. DNA was quantified by spectrophotometry, assuming a $A_{260}$ of 1 when the DNA concentration was 50 µg/ml.

**DNA adduct analysis by $^{32}$P-post-labeling assay**

Covalent modification of DNA was analyzed by $^{32}$P-post-labeling after enrichment by nuclease P1 digestion, as described in Reddy and Randerath (19). DNA (5–10 µg) was digested with micrococcal nuclease and spleen phosphodiesterase, and the adducts were enriched by nuclease P1. The adducts were labeled with [γ-$^{32}$P]ATP (~100 µCi, sp. act. >7000 Ci/mmole) and T4 polynucleotide kinase as described by Gupta (20). Aliquots were spotted onto a polyethyleneimine cellulose sheet (12×20 cm) with a 15 cm wick (Whatman 1 chromatography paper). The plates were first developed in 1.0 M sodium phosphate (pH 5.8) (D1) overnight. The adducts were separated by development in the opposite direction as D1 with 3.6 M lithium formate and 8.5 M urea (pH 3.5). This was followed by development in 0.6 M lithium chloride and 0.5 M Tris–HCl, and 8.5 M urea (pH 8.0) perpendicular to the previous development onto a 2 cm wick (Whatman 3 mm chromatography paper). A final development with 1.7 M sodium phosphate (pH 6.0) onto a 2 cm wick (Whatman 1 mm chromatography paper) was performed in the same direction to reduce the background radioactivity. Adducts were visualized by autoradiography. Quantitation was performed by counting the radioactivity for each adduct and total nucleotides to determine the relative adduct levels (RAL) as described by Reddy and Randerath (19).

**Apurinic/apyrimidinic sites analyzed by ASB assay**

Apurinic/apyrimidinic sites were assayed based upon the reaction of the aldehyde group in an AP site with a probe bearing a biotin residue as described by Nakamura et al. (18). The AP site cleavage assay was performed as described by Nakamura and Swenberg (15).

**Analysis of 8-HO-dG by HPLC-ECD**

Quantitation of 8-HO-dG was based on a HPLC/electrochemical detection method (HPLC-ECD). DNA (100 µg) was hydrolyzed enzymatically to deoxyribonucleosides using deoxyribonuclease I, spleen phosphodiesterase, snake venom phosphodiesterase and alkaline phosphatase. The digest was separated by reversed phase HPLC, and 8-HO-dG quantitated using an electrochemical array detector (ESA, Chelmsford, MA), as described in Lin et al. (7).

**Statistical analysis**

All data are expressed as mean ± SD. The significance of differences in the results was evaluated with ANOVA, followed by Dunnett’s multiple comparison test.

**Results**

**DNA adduct analysis by $^{32}$P-post-labeling assay**

To determine the formation of direct DNA adducts, DNA isolated from HeLa S3 cells treated with Cl$_4$HQ was analyzed by $^{32}$P-post-labeling assay. One major and two minor adducts (adducts 1, 2 and 3) were detected in cells treated with 300 µM Cl$_4$HQ for 0.5 (Figure 2B) and 2 h (Figure 2C). The number of DNA adducts was estimated to be 3–6 adducts per $10^6$ total nucleotides (Table I). The relative percentages of the adducts were estimated as follows: 1, 47%; 2, 26% and 3, 27%. The adduct levels measured in cells treated with 300 µM Cl$_4$HQ
Induction of DNA damage by Cl\textsubscript{4}HQ

Fig. 3. Formation of AP sites in nuclear DNA of human HeLa S3 tumor cells treated with 0–300 \mu M Cl\textsubscript{4}HQ under physiological conditions at 37°C for 0.5–2 h. Data represent the mean ± SD of three to five determinations. Treatments marked with asterisks are statistically significantly different from control: * \(P < 0.05\); ** \(P < 0.01\).

Fig. 2. \(^{32}\)P-post-labeling maps of adducts of tetrachlorohydroquinone (Cl\textsubscript{4}HQ) with HeLa S3 cells treated with Cl\textsubscript{4}HQ: (A) control only; (B) 300 \mu M Cl\textsubscript{4}HQ for 0.5 h; (C) 300 \mu M Cl\textsubscript{4}HQ for 2 h; (D) DNA adducts in calf thymus DNA treated with tetrachlorohydroquinone Cl\textsubscript{4}BQ (1 mM) at 37°C for 2 h. The autoradiograph exposure time was 72 h at –80°C. Chromatography conditions are as described in Materials and methods.

Table I. Formation of DNA adducts (per 10\(^8\) total nucleotides) in nuclear DNA of HeLa S3 cells treated with Cl\textsubscript{4}HQ (300 \mu M) for 0.5–2 h at physiological condition

<table>
<thead>
<tr>
<th>Adducts</th>
<th>Duration of Cl\textsubscript{4}HQ treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>Adduct 1</td>
<td>1.3 (0.13)</td>
</tr>
<tr>
<td>Adduct 2</td>
<td>0.85 (0.09)</td>
</tr>
<tr>
<td>Adduct 3</td>
<td>0.88 (0.08)</td>
</tr>
<tr>
<td>Total adducts</td>
<td>3.0 (0.27)</td>
</tr>
</tbody>
</table>

for 2 h were 1.8-fold greater than those for the 0.5 h treatment, suggesting adduct accumulation. Relatively small amounts of direct adducts were detected in cells treated with 50 \mu M Cl\textsubscript{4}HQ for 2 h and the levels of individual adducts were estimated to be ~1–5 adducts per 10\(^9\) nucleotides (autoradiographs not shown) which was close to the limit of detection (1 adduct per 10\(^9\) nucleotides). Further investigation confirmed that these adducts did not correspond to those produced in calf thymus DNA treated with Cl\textsubscript{4}BQ (Figure 2D).

Detection and characterization of AP sites

To investigate whether Cl\textsubscript{4}HQ induces increases in AP sites in intact cells, human HeLa S3 tumor cells were incubated with Cl\textsubscript{4}HQ (0–300 \mu M) for 0.5–5 h under physiological conditions. Nuclear DNA was isolated and analyzed by ASB assay for AP sites. Results from the measurement of AP sites indicated that an increased number of AP sites was detected in cells treated with 300 \mu M Cl\textsubscript{4}HQ for 0.5 h (13.4 ± 1.34 versus 10.2 ± 1.49 AP sites per 10\(^8\) nucleotides) \((P < 0.05)\) and 2 h (15.7 ± 3.38 versus 7.86 ± 1.26 AP sites per 10\(^8\) nucleotides) \((P < 0.01)\) (Figure 3). Increases in the number of AP sites in cells treated with Cl\textsubscript{4}HQ at 50 \mu M for 0.5–2 h were not statistically significantly increased over the corresponding control. To test the hypothesis that AP sites induced by Cl\textsubscript{4}HQ in HeLa cells are excisable by putrescine, DNA was incubated with putrescine and immediately followed by the ASB assay. Results indicated that Cl\textsubscript{4}HQ induced predominantly putrescine-excisable AP sites (75%) in HeLa S3 cells (Figure 4).
time and that these DNA adducts did not co-elute with those tetrachlorohydroquinone on cell growth and the induction of DNA damage

Results of our analyses suggest that there is a trend for DNA and human DNA of mammalian cells following treatment with Cl$_4$HQ. In tetrachlorohydroquinone induced DNA strand break formation in PM2 DNA. In an effort to understand the genetic consequences of phenol. DNA damage can be derived from ROS that interact with hamster cells by tetrachlorohydroquinone, a metabolite of pentachloro-

was to extend our work in PCP quinonoid-induced DNA

Formation of 8-HO-dG in nuclear DNA of human HeLa S3 tumor (15). The predominant formation of putrescine-excisable AP cells treated with 0

Fig. 5. Formation of 8-HO-dG in nuclear DNA of human HeLa S3 tumor cells treated with 0–300 µM Cl$_4$HQ under physiological conditions at 37˚C for 0.5–2 h. Data represent the mean ± SD of three to five determinations. Treatments marked with asterisks are statistically significantly different from control: *P < 0.05; **P < 0.01.

Analysis of the formation of 8-HO-dG
To determine whether Cl$_4$HQ induces the formation of 8-HO-dG in HeLa S3 cells in parallel to the induction of AP sites, nuclear DNA derived from the control and treated HeLa S3 cells was assayed for the presence of 8-HO-dG by HPLC-ECD method as described above. Results indicated that the concentration of 8-OH-dG increased 2-fold in cells treated with 300 µM Cl$_4$HQ for 0.5 h over the corresponding control (0.43 ± 0.09 versus 0.21 ± 0.03 8-HO-dG per 10$^8$ dG) (Figure 5). The concentration of 8-OH-dG increased 5-fold in cells treated with 300 µM Cl$_4$HQ compared with the control 2 h after treatment (0.59 ± 0.13 versus 0.14 ± 0.03 8-HO-dG per 10$^8$ dG) (P < 0.001). No increase in 8-HO-dG was detected in cells treated with 50 µM Cl$_4$HQ.

Discussion
Our previous investigation provided clear evidence of parallel formation of 8-HO-dG, DNA fragmentation, and AP sites in calf thymus DNA treated with Cl$_4$HQ in the presence of the metal ion, Cu(II) (14). We demonstrated that, unlike estrogen and PAH-derived quinones, the AP sites induced by PCP quinonoids in calf thymus DNA were primarily mediated by ROS, whereas the contribution derived from depurination/depymidination of direct DNA adducts was relatively insignificant for the formation of AP sites (21,22). Our objective here was to extend our work in PCP quinonoid-induced DNA damage in calf thymus DNA to mammalian cells, where DNA damage is subject to the repair process.

It is increasingly evident that various types of oxidative DNA damage can be derived from ROS that interact with DNA. In an effort to understand the genetic consequences of oxidative damage in intact cells induced by PCP quinone and hydroquinone, we analyzed the relationship between the formation of direct adducts and oxidative damage in the nuclear DNA of mammalian cells following treatment with Cl$_4$HQ. Results of our analyses suggest that there is a trend for concentration- and time-dependent formation of direct adducts in nuclear DNA of HeLa S3 cells treated with Cl$_4$HQ. We observed that Cl$_4$HQ-derived DNA adducts accumulate over time and that these DNA adducts did not co-elute with those generated by Cl$_4$BQ in calf thymus DNA. Adduct levels were estimated to be ~10–20% of that observed in calf thymus DNA treated with Cl$_4$HQ (14).

In parallel, an increased number of AP sites and 8-HO-dG was detected in cells treated with Cl$_4$HQ over the corresponding control. Levels of AP sites and 8-HO-dG were estimated to be approximately two orders of magnitude greater than those of direct DNA adducts. However, we do not exclude the possibility that the relative levels of these distinct DNA lesions could be underestimated since the 32P-post-labeling method may not fully recover the direct adducts induced by Cl$_4$HQ. Further investigation indicated that the AP sites induced by Cl$_4$HQ in HeLa S3 cells were predominantly (75%) excised by putrescine. This result is comparable with those observed in calf thymus DNA treated with Cl$_4$HQ plus Cu(II), Cl$_4$BQ plus Cu(II) and NADPH (14), and is in good agreement with those observed in HeLa S3 cells treated with hydrogen peroxide (15). The predominant formation of putrescine-excisable AP sites along with the persistent presence of direct DNA adducts suggest that ROS are the main source for the induction of AP sites by Cl$_4$HQ in HeLa cells. Altogether, these results point to the formation of putrescine-excisable AP sites as one of the major types of oxidative DNA damage in intact cells induced by Cl$_4$HQ.

The ratio of the net increases of 8-HO-dG to AP sites (per nucleotide) in HeLa S3 cells induced by Cl$_4$HQ is estimated to be 1:6, which is 4-fold more than that estimated in calf thymus DNA treated with Cl$_4$HQ plus Cu(II) (ratio ~1:1.6) (14). We recently demonstrated that the Fenton reaction predominantly induced 8-HO-dG in calf thymus DNA compared with AP sites and oxidized pyrimidine bases (23). In contrast, 8-HO-dG was less than the number of AP sites in HeLa cells exposed to H$_2$O$_2$. In addition, while AP sites were persistent in HeLa cells during a 6 h repair period, 8-HO-dG was significantly repaired (~83%) within 6 h. Based on these results, we believe that repair enzymes, 8-HO-dG glycosylase/lyase (mOGG1), that cleave 8-HO-dG to form 3’ AP sites, is responsible for the difference observed between calf thymus DNA treated with Cl$_4$HQ plus Cu(II) and cells exposed to Cl$_4$HQ (24,25). Taken together, we theorize that quinonoid-derived ROS mediate the formation of putrescine-excisable AP sites, which may explain, in part, PCP-induced clastogenicity in mammalian cells.

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References


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