Evidence for Production of Hydroxyl Radicals by Pentachlorophenol Metabolites and Hydrogen Peroxide: A Metal-Independent Organic Fenton Reaction

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Received March 18, 2000

The production of hydroxyl radicals by tetrachlorohydroquinone (TCHQ, a major metabolite of the widely used biocide pentachlorophenol) in the presence of H2O2 was studied by salicylate hydroxylation method. HPLC with electrochemical detection was used to measure the levels of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) formed when the hydroxyl radicals react with salicylate. We found that TCHQ and H2O2 could produce both 2,3- and 2,5-DHBA when incubated with salicylate. Their production was markedly inhibited by hydroxyl radical scavenging agents dimethyl sulfoxide and ethanol, as well as by tetrachlorosemiquinone radical scavengers desferrioxamine and other hydroxamic acids. In contrast, their production was not affected by the nonhydroxamate iron chelators diethylenetriaminepentaacetic acid (DTPA), bathophenanthroline disulfonic acid, and phytic acid, as well as the copper-specific chelator bathocuprione disulfonic acid. A comparison of product formation and distribution from the reaction of ferrous iron with hydrogen peroxide (the classic Fenton system) strongly suggests that the same hydroxyl radical adducts are formed as in the TCHQ/H2O2 experiments. Taken together, we propose that hydroxyl radicals were produced by TCHQ in the presence of H2O2, probably through a metal-independent organic Fenton reaction.

Key Words: tetrachlorohydroquinone; tetrachlorosemiquinone radical; hydrogen peroxide; desferrioxamine (Desferal; deferoxamine); organic Fenton reaction; hydroxyl radical; salicylate hydroxylation.

Tetrachlorohydroquinone (TCHQ) has been identified as a major toxic metabolite of the widely used wood preservative pentachlorophenol, and has also been implicated in its genotoxicity (1–7). TCHQ could bind DNA and cause single strand breaks (SSB) in isolated DNA (6), in human fibroblasts (6, 7), in V79 cells (8), in Chinese hamster cells (CHO) (9), and in liver tissues of mice (10, 11). TCHQ could also induce micronuclei and mutations at the HPRT locus of V79 cells (12, 13), and the formation of 8-hydroxy-2-deoxyguanosine in V79 cells (14) and in B6C3F1 mice (10).

The ability of TCHQ to induce DNA SSB has been previously attributed to its ability to form hydroxyl radicals through a radical-driven Fenton reaction (6). This proposal was based on the fact that TCHQ-induced DNA SSB was completely protected by the iron chelator desferrioxamine (DFO). In a recent study, we found that the protection by DFO against TCHQ-induced DNA SSB was not due to its binding of iron but rather to its efficient scavenging of the reactive tetrachlorosemiquinone radicals, and the formation of the less reactive DFO nitric oxide radical (15). Based on these new findings, we proposed that the DNA damage induced by TCHQ might be through an organic Fenton reaction. In this study, we will present evidence to support this hypothesis. The production of hydroxyl radicals by TCHQ and H2O2 was studied by salicylate hydroxylation method. HPLC with electrochemical detection was used to measure the levels of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) formed when the hydroxyl radicals react with salicylic acid (SA) (16–21).

MATERIALS AND METHODS

Chemicals. Tetrachlorohydroquinone (TCHQ), 4-(2-hydroxy-ethyl)-1-piperazinethanesulfonic acid (HEPES), diethylenetriaminepentaacetic acid (DTPA), bathophenanthroline disulfonic acid (BPS), and phytic acid (PA) were purchased from Sigma. Desferrioxamine B (DFO) was obtained as the mesylate salt (Desferal) from Ciba-Geigy (Basle, Switzerland). Acetohydroxamic acid (AHA), benzohydroxamic acid (BHA), and bathocuprione disulfonic acid.
Salicylic acid (SA) was incubated for 30 min at 37°C in HEPES buffer (100 mM, pH 7.4) with either TCHQ, or H2O2, or their combination. A LiChrospher 100 RP-18, 5 microns, 25 cm × 4 mm (E. Merck) column was used to separate the two major SA hydroxylation products, 2,3- and 2,5-dihydroxybenzoic acid (DHBA). The mobile phase contained citric acid 0.03 M, acetic acid 0.03 M, methanol 1% (vol.), and sodium azide 0.28 g/ml. The mobile phase was titrated with solid NaOH to pH 3.0 and then with solid CH3COONa to a final pH of 3.6. The samples were run in parallel with standards of 2,3-DHBA and 2,5-DHBA. Electrochemical detector with potential of 1.0 V against Ag/AgCl was used for this assay. Each point represents the mean of two separate experiments with the SD less than 5%.

ESR studies. Samples (0.05–0.1 ml) for ESR measurements were drawn by a syringe into a gas-permeable Teflon capillary (Zeus Industries, Raritan, NJ) of 0.032 inch inner diameter, 0.0015 inch wall thickness and 8 cm length. The filled capillaries were inserted into a quartz ESR tube (open at both ends) which was then placed vertically in the cavity dewar. ESR spectra were recorded by using a JES-Fe-3XG (JEOL, Tokyo, Japan) spectrometer with 100-kHz field modulation. Spectra were recorded with a microwave power of 4 mW and a modulation amplitude of 0.1 mT or less.

RESULTS

Tetrachlorohydroquinone (TCHQ) and hydrogen peroxide (H2O2), when incubated with salicylic acid (SA), could synergistically produce both 2,3- and 2,5-dihydroxybenzoic acid (DHBA), while either of them alone was much less efficient (Fig. 1). The production of DHBAs was dose-dependent on TCHQ, H2O2, and SA, respectively (Figs. 2A, 2B, and 2C). In order to further confirm that the hydroxylated products detected in TCHQ/H2O2 system were from the reaction of SA with free hydroxyl radicals (‘OH), we investigated the generation of these products in a model system. Hydroxyl radical generation was performed using a classic Fenton system (Fe(II) plus H2O2) in the same reaction mixture (Fig. 3). The same hydroxylated products were detected in Fe(II)/H2O2 system as evidenced by the same retention time (data not shown). The production of DHBAs was markedly inhibited by hydroxyl radical scavenging agents dimethyl sulfoxide (DMSO) and ethanol in both Fe(II)/H2O2 and TCHQ/H2O2 systems (Fig. 4). In addition, the product distribution from the reaction of ferrous iron with hydrogen peroxide (the ratio of 2,3-DHBA to 2,5-DHBA is 1.21) is also similar, but different than that from the TCHQ/H2O2 experiments (the ratio is between 0.85 and 0.99). Together, these results strongly indicate that ‘OH was probably produced by TCHQ/H2O2 system.

In order to get further evidence, we also employed a more direct method, the ESR spin trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 100 mM). A typical DMPO-OH radical signal was observed with TCHQ/H2O2 system (data not shown). Similar effects were also observed in both SA hydroxylation and ESR spin-trapping system for tetrachloro-1,4-benzoquinone (TCBQ, the oxidation product of TCHQ) in the presence of H2O2 (data not shown).
The catalytic role of transition metals possibly contaminating the reaction system was carefully examined and ruled out. The nonhydroxamate iron chelators, diethylenetriaminepentaacetic acid (DTPA), phytic acid (PA), and bathophenanthroline disulfonic acid (BPS), as well as the copper-specific chelator bathocuprione disulfonic acid (BCS) had no effect on DHBAs production by TCHQ/H2O2, while the trihydroxamate iron chelator desferrioxamine (DFO), which is not only a strong iron chelators, but also a known efficient tetrachlorosemiquinone radical scavenger (15), markedly inhibit their production (Fig. 5). Similar marked inhibition was also observed for other mono-hydroxamates such as aceto- and benzohydroxamate (Fig. 5). In contrast, the DHBAs production by Fe(II)/H2O2 was markedly inhibited both by the nonhydroxamate iron chelators and by the hydroxamate iron chelator DFO (Fig. 4).

**DISCUSSION**

Desferrioxamine (DFO; Desferal; deferoxamine) is a linear trihydroxamic acid siderophore that forms a kinetically and thermodynamically stable complex with ferric iron (22). Its high binding constant (log $\beta = 31$) and its redox properties (E = $-0.45$ V), render the bound iron unreactive for the catalysis of oxygen radicals production (23). Although DFO has been repeatedly used to probe iron-catalyzed hydroxyl radical formation in biological systems (22–24), several recent studies demonstrated that this trihydroxamate compound has a mechanism of action other than as an iron chelator (summarized in Ref. 15). In our previous report, we showed that the protective effect of desferrioxamine (DFO) on tetrachlorohydroquinone (TCHQ)-induced DNA damage was not due to the chelation of iron, but rather to scavenging of the reactive tetrachloro-
2,5-DHBA in TCHQ/H₂O₂ system is around 1 (the oxidation of 2,3-DHBA. In contrast, the ratio of 2,3-DHBA to Fenton reaction, which should be in favor of the formation of hydroxyl radicals in classic was confirmed by salicylate hydroxylation study. TCHQ radical is crucial in the results and theoretical considerations, we propose that the production of ·OH by TCHQ/H₂O₂ is through a metal-independent organic Fenton reaction

TCSQ• + H₂O₂ → TCBQ + ·OH + OH⁻,

where TCSQ• (tetrachlorosemiquinone radical) substituted for ferrous iron in the classic Fenton reaction, TCBQ (tetrachloro-1,4-benzoquinone) is the oxidation product of TCHQ. We think this is a novel mechanism for hydroxyl radical production, and this might explain, at least partly, the genotoxicity of pentachlorophenol and its metabolites.

REFERENCES


