A Novel Mechanism for Metal-independent Hydroxyl Radical Production by Hydrogen Peroxide and Halogenated Quinones

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Abstract: The hydroxyl radical (•OH) has been considered to be one of the most reactive oxygen species that are produced in biological systems. Frequently, •OH formation is ascribed to the transition metal-catalyzed Fenton reaction. During the study of the molecular mechanism for the genotoxicity of the wood preservative pentachlorophenol (PCP), we found that •OH could be produced by H_2O_2 and PCP metabolite tetrachloro-1,4-benzoquinone (TCBQ) independent of transition metal ions. Further studies showed that TCBQ, but not its corresponding semiquinone radical, is essential for •OH production. Metal-independent production of •OH could also be observed with other halogenated quinones and H_2O_2. Based on these data, we propose that •OH production by TCBQ and H_2O_2 is not through a semiquinone-dependent organic Fenton reaction, but rather through a novel nucleophilic substitution and homolytical decomposition mechanism. This represents a novel mechanism for •OH production not requiring the catalysis of redox-active transition metal ions, and may partly explain the potential carcinogenicity of the widely used biocides such as PCP and other polyhalogenated aromatic compounds.

Keywords: Hydroxyl radical, Tetrachloro-1,4-benzoquinone, Electron Spin Resonance (ESR) spin-trapping, Hydrogen peroxide, Carcinogenicity.

1. INTRODUCTION

1.1. Pentachlorophenol (PCP) and its Major Quinoid Metabolites

PCP is a one of the most widely used biocides and has been used primarily as a wood preservative [1]. Because of its efficiency, broad spectrum and low cost, PCP has also been used as algaecide, bactericide, fungicide, herbicide, insecticide and molluscicide [1-3]. In the US, approximately 97% of PCP usage was as a wood preservative [1]. In China and other developing countries, PCP has also been used to kill snails to prevent snail fever. Its worldwide usage and relative stability make PCP a ubiquitous environmental pollutant [1-3]. In fact, PCP has been detected in body fluids and tissues of people who are not occupationally exposed to it [1-3].

Following chronic exposure of mice to PCP, various tumors were observed [4, 5]. In humans, malignant lymphoma and leukemia have been associated with occupational exposure to PCP [1-3]. PCP has been listed as a priority pollutant by the US Environmental Protection Agency (EPA), and classified as a group 2B environmental carcinogen by the International Association for Research on Cancer (IARC) (1). While the precise mechanism of PCP's genotoxicity remains to be elucidated, it has been suggested that its quinone and semiquinone metabolites play an important role (Scheme **1**). Tetrachlorohydroquinone (TCHQ) could be produced through PCP dechlorination by liver microsomal cytochrome P450s [2, 3, 6]. TCHQ can be further oxidized to tetrachloro-1,4-benzoquinone (TCBQ) via its corresponding semiquinone, the tetrachlorosemiquinone radical (TCSQ•) [7].

Redox-cycling of quinoid compounds is a well-known phenomenon [8-10]. The cyclic (auto)oxidation and reduction reactions with the formation of semiquinone radicals can produce large amounts of reactive oxygen species (ROS) by reducing molecular oxygen to superoxide ($O_2^{\bullet-}$), which in turn can induce oxidative stress [8-10]. However, it is generally accepted that $O_2^{\bullet-}$ itself is not directly attacking DNA, but only after its dismutation to hydrogen peroxide (H_2O_2) and subsequent metal-mediated cleavage to hydroxyl radical (•OH). This reaction sequence is called the Haber-Weiss reaction, or superoxide-driven Fenton reaction (M represents transition metals, especially iron and copper) [10]:

$$2\,O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$O_2^{\bullet-} + M^{n+} \rightarrow O_2 + M^{(n-1)+}$$

$$H_2O_2 + M^{(n-1)+} \rightarrow {}^{\bullet}OH + OH^- + M^{n+}$$

While PCP itself does not show any reactivity towards DNA, TCHQ was found to induce single strand breaks in various biological systems [7, 11]. TCHQ also induced the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in cells [12] and mice [11]. Recently, TCHQ was found to induce formation of direct adducts, AP sites and oxidized bases in human HeLa S3 cells [13].

1.2. Molecular Mechanisms for PCP Quinoid Metabolites-Induced DNA Damage

Both oxidative DNA damage and direct DNA adducts have been implicated in PCP-induced mouse liver carcinogenesis [11, 13]. The most commonly analyzed biomarker of oxidative DNA damage, 8-OH-dG, was detected in the livers of mice treated with PCP and TCHQ, and the levels were increased significantly over the corresponding controls [11]. Recent findings in rats that had been administered PCP also revealed a statistically significant increase in 8-OH-dG in hepatic DNA over control [14]. Redox cycling of PCP quinoid metabolites to generate ROS is believed to play an important role in PCP genotoxicity.

Among ROS, •OH is regarded as the most reactive one produced in biological systems. DNA damage resulting from attack by •OH includes base oxidation, deoxyribose damage, strand breaks and apurinic/apyrimidinic (AP) sites. •OH can induce AP sites by direct hydrogen abstraction from the sugar moiety of DNA.

2. AN UNEXPECTED MECHANISM OF PROTECTION BY DESFERRIOXAMINE AGAINST TCHQ-INDUCED DNA DAMAGE

As mentioned above, TCHQ has been identified as one of the main toxic metabolite of PCP. TCHQ can induce DNA single strand breaks, and has also been implicated in PCP-associated genotoxicity. The ability of TCHQ to induce DNA damage has been previously attributed to its ability to form •OH through the classic metal-dependent Fenton reaction (see above). This notion was based on the fact that TCHQ-induced DNA damage was com-

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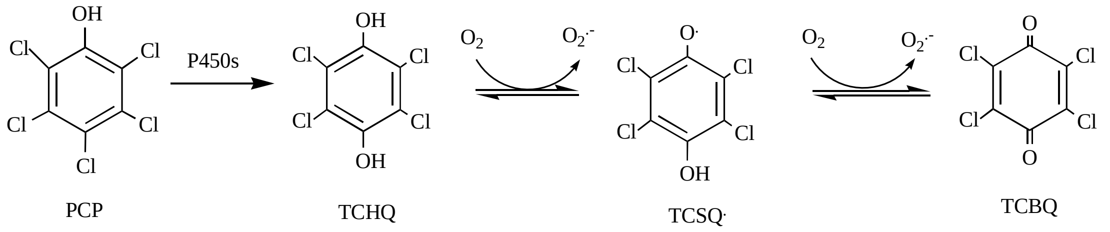


Scheme 1. Pentachlorophenol (PCP) and its major, potentially carcinogenic metabolites.

pletely prevented by desferrioxamine (DFO, also called Desferal® and deferoxamine), which has been used as an iron chelating agent for the treatment of iron overload. DFO is a naturally-occurring trihydroxamic acid siderophore that forms a kinetically and thermodynamically stable complex with ferric iron. Its high binding constant ($\log \beta = 31$) and its redox properties ($E^o = -0.45$ V), render the bound iron un-reactive for the catalysis of •OH production. Diethylenetriaminepentaacetic acid (DTPA) is an analog of the widely used chelating agent ethylenediaminetetraacetic acid (EDTA). DTPA could also form a kinetically and thermodynamically stable complex with ferric iron ($\log \beta = 28$; $E^o = +0.03$ V). Both DFO and DTPA have been widely used to study the role of iron in various chemical and biological systems, and therefore we employed these two structurally different but relatively specific iron chelating agents to probe whether iron played any role in TCHQ-induced DNA damage. We found [15] that DFO provided remarkable protection against TCHQ-induced DNA single strand breaks in isolated DNA, while other iron chelators such as DTPA did not. To better understand its underlying molecular mechanism, the autooxidation process of TCHQ yielding TCSQ• intermediate was studied. We found that DFO led to a marked reduction in both the concentration and half-life of TCSQ•. Interestingly, the decay of TCSQ• was accompanied by concurrent formation of DFO-nitroxide radicals. In contrast, DTPA had no detectable effect on TCHQ autooxidation. These results suggest that the protection by DFO against TCHQ-induced DNA damage was not due to its binding of iron, but rather due to its scavenging of the reactive TCSQ• [15].

Then we extended our study from isolated DNA to human fibroblasts, and from DFO to other hydroxamic acids. We found [16] that co-incubation of DFO provided marked protection against both the cyto- and genotoxicity induced by TCHQ. Similar patterns of protection were also observed for three other hydroxamic acids. Spectral studies showed that the three hydroxamic acids also effectively scavenged the reactive TCSQ• and enhanced the formation of the less reactive and less toxic chloranilic acid. The results of this study demonstrated that the protection by DFO and other hydroxamic acids against TCHQ-induced cyto- and genotoxicity in human fibroblasts is mainly through scavenging of the reactive TCSQ• and not through prevention of the Fenton reaction [16].

3. MOLECULAR MECHANISM OF GENOTOXICITY INDUCED BY PCP QUINOID METABOLITES

3.1. Metal-independent •OH Production by PCP Quinoid Metabolites and H_2O_2

The above results suggest that iron was not involved in TCHQ-induced DNA damage. In another word, TCHQ-induced DNA damage may not be due to the iron-mediated •OH production through the classic Fenton reaction! Then the question became what was the underlying molecular mechanism for PCP quinoid metabolites-induced •OH production? To test whether •OH can be produced by PCP quinoid metabolites, we first employed the well-known salicylate hydroxylation method. HPLC with electrochemical detection was used to measure the levels of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) formed when •OH reacts with salicylate. We found [17] that TCHQ and H_2O_2 could produce both 2,3- and 2,5-DHBA when incubated with salicylate. Their production was markedly inhibited by the •OH scavenging agents, but not affected by the non-hydroxamate iron chelators and the copper-specific chelator. Similar effects were also observed with TCBQ and H_2O_2. Based on these results, we suggested that •OH could be produced by TCHQ and H_2O_2, possibly through a metal-independent Fenton-like reaction [17].

Since the salicylate hydroxylation method cannot provide direct evidence for •OH formation, a more specific method, such as secondary radical ESR spin-trapping with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), was employed to further substantiate and extend our previous observations. A typical DMPO/•OH signal, and DMPO/•CH_3 signal derived from •OH attack on DMSO, will be more conclusive evidence for •OH production from H_2O_2 and TCHQ or TCBQ. We found [18] that when incubated with DMPO, the combination of TCBQ and H_2O_2 produced the DMPO/•OH adduct, while either compound alone did not. The formation of DMPO/•OH was markedly inhibited by the •OH scavenging agents DMSO and formate, with the concomitant formation of the characteristic DMPO adducts with •CH_3 and •COO^-, respectively. These secondary radical ESR spin-trapping results provided definitive evidence that •OH could indeed be produced by TCBQ and H_2O_2.

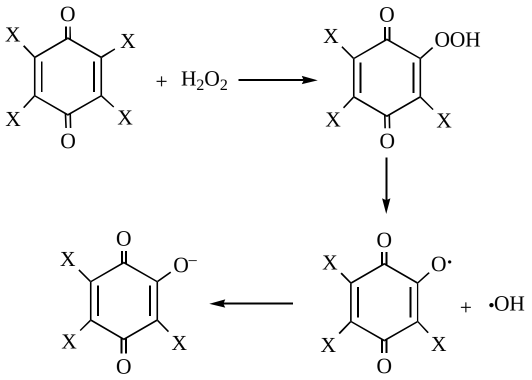
Then a critical question arose: was the production of •OH by TCBQ and H_2O_2 metal-dependent or –independent? To answer this question, the potential role of catalytic transition metals contaminating the reaction system was carefully examined by using several structurally different and relatively specific metal chelating agents for iron and copper. Neither the DMPO/•OH signal, nor the DMPO/•CH_3 signal produced by the DMPO/TCBQ/H_2O_2 system in the absence and presence, respectively, of DMSO was affected by the addition of various nonhydroxamate iron and copper chelating agents. In contrast, the formation of DMPO/•OH by the DMPO/Fe(II)/H_2O_2 system was almost completely inhibited by the nonhydroxamate iron chelating agents. These results clearly demonstrated that the production of •OH by TCBQ and H_2O_2 is *independent* of transition metal ions. It should be noted that the metal-independent production of •OH was not limited to TCBQ and H_2O_2, but was also observed with other halogenated quinones. In contrast, no •OH production was detected from H_2O_2 and the nonhalogenated quinone, 1,4-benzoquinone [18].

3.2. Mechanism of Metal-independent •OH Production by TCBQ and H_2O_2

Based on the above experimental results, we first proposed (17, 18) that the production of •OH by TCBQ and H_2O_2 might be through a metal-independent semiquinone-mediated organic Fenton reaction:

$$\text{TCSQ}^{\bullet} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + \text{TCBQ},$$

where TCSQ• substitutes for ferrous iron in the classic, metal-dependent Fenton reaction. This type of reaction between semiquinone radicals and H_2O_2 has been previously proposed by Koppenol and Butler [19], who suggested that such reactions are thermodynamically feasible and do not require metal ions for catalysis.



Scheme 2. Proposed mechanism for •OH production by tetrahalo-1,4-benzoquinone (TXBQ, X = F, Cl, Br) and H_2O_2: a nucleophilic reaction may take place between TXBQ and H_2O_2, forming a trihalo-hydroperoxyl-1,4-benzoquinone (TrXBQ-OOH) intermediate, which can decompose homolytically to produce •OH and trihalo-hydroxy-1,4-benzoquinone radical (TrXBQ-O•). TrXBQ-O• may disproportionate, or get an electron, to form the ionic form of trihalo-hydroxy-1,4-benzoquinone (TrXBQ-O^-).

If the above mechanism were correct, then the production of $^{\bullet}$OH from H_2O_2 and TCBQ should be dependent on the concentration of TCSQ$^{\bullet}$, i.e., the higher the concentration of TCSQ$^{\bullet}$, the more $^{\bullet}$OH should be produced. Furthermore, the main product of this reaction should be TCBQ. Using secondary radical ESR spin trapping method, we found that DMPO/$^{\bullet}CH_3$ and DMPO/$^{\bullet}$OH adducts can be produced by H_2O_2 and TCBQ in the presence of DMPO and DMSO. However, no DMPO/$^{\bullet}CH_3$ and DMPO/$^{\bullet}$OH adducts were detected from H_2O_2 and TCHQ, the reduced form of TCBQ, although high concentrations of TCSQ$^{\bullet}$ could be produced during the autooxidation of TCHQ. Interestingly, if TCHQ was promptly oxidized to TCBQ with myeloperoxidase, DMPO/$^{\bullet}CH_3$ and DMPO/$^{\bullet}$OH adducts could be detected again, similar to that produced by TCBQ. Furthermore, the formation of DMPO/$^{\bullet}CH_3$ and DMPO/$^{\bullet}$OH was found to be directly dependent on the concentrations of TCBQ and H_2O_2. These results strongly suggest that TCBQ, but not its corresponding semiquinone radical TCSQ$^{\bullet}$, is essential for $^{\bullet}$OH production. Therefore, the production of $^{\bullet}$OH by TCBQ and H_2O_2 appears not to occur through a semiquinone-dependent organic Fenton reaction.

To get more information on the mechanism of $^{\bullet}$OH production by TCBQ/H_2O_2, the time and concentration dependent production of DMPO/$^{\bullet}$OH by TCBQ/H_2O_2 was studied. Two distinct phases were observed: the first fast phase (about 30 seconds) and the second slower phase. This indicates that $^{\bullet}$OH may be produced by two-step reactions between TCBQ and H_2O_2. Furthermore, UV-visible spectral studies showed that there was a direct interaction between TCBQ and H_2O_2, with the reaction mixture changing quickly from the original yellow color to a characteristic purple color. The final reaction products between TCBQ and H_2O_2 were then identified by mass spectrometry, and found to be the ionic form of trichloro-hydroxy-1,4-benzoquinone (TrCBQ-OH). By using oxygen-18-enriched H_2O_2, we confirmed that H_2O_2 is the source and origin of the oxygen atom inserted into the reaction product TrCBQ-OH.

It has also been shown [20] that both TCBQ and H_2O_2 were consumed with a stoichiometric ratio of about 1:1, and H_2O_2 accelerated the rate of TCBQ decomposition by two orders of magnitude with the loss of chloride. Thus, the metal-independent production of $^{\bullet}$OH by TCBQ and H_2O_2 may be not through a previously proposed semiquinone-mediated organic Fenton reaction. Based on the above experimental results and the fact that H_2O_2 is a better nucleophile than H_2O [21], we proposed a novel mechanism for $^{\bullet}$OH production by H_2O_2 and TCBQ [22] (Scheme **2**): a nucleophilic reaction may take place between TCBQ and H_2O_2, forming an unstable trichloro-hydroperoxyl-1,4-benzoquinone (TrCBQ-OOH) intermediate, which can decompose homolytically to produce $^{\bullet}$OH and trichloro-hydroxy-1,4-benzoquinone radical (TrCBQ-O$^{\bullet}$). TrCBQ-O$^{\bullet}$ then may disproportionate to form the ionic form of trichloro-hydroxy-1,4-benzoquinone (TrCBQ-O^-). In the presence of excess of H_2O_2, TrCBQ-O^- may further react with H_2O_2 via similar pathway to produce another $^{\bullet}$OH. The production of $^{\bullet}$OH by other halogenated quinones and H_2O_2 should be through a similar mechanism (Scheme **2**). We also found that TCBQ and other halogenated quinones such as 2,5-dichloro-1,4-benzoquinone could enhance the decomposition of organic hydroperoxides such as *t*-butylhydroperoxide and formation of alkoxyl radicals in a similar mechanism [23]. Further studies led us to detect and identify a novel carbon-centered quinone ketoxy radical intermediate, the spin isomer of the proposed oxygen-centered quinone enoxy radical. These latest data provided direct experimental evidence to further support and expand our previously proposed mechanism for metal-independent decomposition of hydroperoxides by halogenated quinones [24, 25].

4. CONCLUSIONS

These findings represent a novel mechanism of $^{\bullet}$OH and alkoxyl radical formation not requiring the involvement of redox-active transition metal ions, and may partly explain the potential carcinogenicity of not only PCP, but also other widely used polyhalogenated aromatic compounds such as 2,4,6- and 2,4,5-trichlorophenol, hexachlorobenzene, Agent Orange (the mixture of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-D)) and the brominated flame-retardant 3,3',5,5'-tetrabromobisphenol A, since these compounds can be metabolized *in vivo* [26, 27], or dechlorinated chemically [28-31] to tetra-, di- or mono-halogenated quinones. Recently, H_2O_2 has been increasingly favored as an environmentally safe oxidant for remediation of the environmental pollutants such as chlorophenols [29-31]. One recent study showed 2,6-dichloro-1,4-benzoquinone could be detected within the first minutes and then undergone further transformations during the oxidative mineralization of 2,4-6-trichlorophenol by H_2O_2 with the catalysis of iron complexes [31]. Another study showed [20] that H_2O_2 could accelerate the rate of TCBQ decomposition by two orders of magnitude, and the rate of this reaction was too fast to measure except at acidic pH. It was suggested that peroxide-dependent decomposition pathway for halogenated quinones may be important in systems where hydroperoxide is either used or produced. However, the exact molecular mechanisms underlying such further transformations are not clear. Our findings may provide a new perspective to better understand such transformation mechanisms during waste water treatment or remediation process in which halogenated quinones are formed.

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