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# Reactive Oxygen-dependent DNA Damage Resulting from the Oxidation of Phenolic Compounds by a Copper-Redox Cycle Mechanism[1]

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## Abstract

**Recently, copper has been shown to be capable of mediating the activation of several xenobiotics producing reactive oxygen and other radicals. Since copper exists in the nucleus and is closely associated with chromosomes and DNA bases, in this study we have investigated whether the activation of 1,4-hydroquinone (1,4-HQ) and a variety of other phenolic compounds by copper can induce strand breaks in double-stranded $\phi$X-174 RF I DNA ($\phi$X-174 relaxed form I DNA). In the presence of micromolar concentrations of Cu(II), DNA strand breaks were induced by 1,4-HQ and other phenolic compounds including 4,4′-biphenol, catechol, 1,2,4-benzenetriol, 2-methoxyestradiol, 2-hydroxyestradiol, diethylstilbestrol, butylated hydroxytoluene, butylated hydroxyanisole, *tert*-butylhydroquinone, ferulic acid, caffeic acid, chlorogenic acid, eugenol, 2-acetamidophenol, and acetaminophen. Structure-activity analysis shows that in the presence of Cu(II), the DNA cleaving activity for phenolic compounds with a 1,4-hydroquinone structure, such as 1,2,4-benzenetriol and *tert*-butylhydroquinone is greater than those with a catechol group (catechol, 2-hydroxyestradiol and caffeic acid). Those compounds having one phenol group, such as eugenol, 2-acetamidophenol, and acetaminophen, are the least reactive. In addition, the induced DNA strand breaks could be inhibited by bathocuproinedisulfonic acid, a Cu(I)-specific chelator, or catalase indicating that a Cu(II)/Cu(I) redox cycle and $H_2O_2$ generation are two major determinants involved in the observed DNA damage. Using reactive oxygen scavengers, it was observed that the DNA strand breaks induced by the 1,4-HQ/Cu(II) system could not be efficiently inhibited by hydroxyl radical scavengers, but could be protected by singlet oxygen scavengers, suggesting that either singlet oxygen or a singlet oxygen-like entity, possibly a copper-peroxide complex, but not free hydroxyl radical probably plays a role in the DNA damage. The above results would suggest that macromolecule-associated copper and reactive oxygen generation may be important factors in the mechanism of 1,4-HQ and other phenolic compound-induced DNA damage in target cells.**

## Introduction

Phenolic compounds are widely distributed in nature in both the plant and animal kingdoms (1, 2). They are also prevalent as environmental pollutants and drugs (3, 4). Some phenolic compounds have been shown to be cytotoxic and/or genotoxic in a variety of biological systems (1–4). It is generally appreciated that the toxicity associated with some phenolic compounds, if not all, is mediated by their further oxidative activation (4–10).

There are a number of both enzymatic and nonenzymatic mechanisms by which xenobiotics can be activated to reactive intermediates (11). Recently, studies have also provided evidence that metals, particularly copper, are capable of mediating the activation of several compounds, such as benzoyl peroxide, quercetin, and dietary flavonoids, by a redox mechanism leading to the formation of reactive oxygen and other organic radicals (12, 13). We have also previously shown that copper can induce the oxidation of 1,4-HQ,[3] a benzene metabolite, producing 1,4-BQ and $H_2O_2$ through a semiquinone intermediate (14).

Copper is an essential trace element, which is distributed throughout the body (15). Besides forming the essential redox-active center in a variety of metalloproteins, such as ceruloplasmin, Cu,Zn superoxide dismutase, cytochrome C oxidase, dopamine $\beta$-hydroxylase, tyrosinase, lysyl oxidase, and ascorbate oxidase (15), copper has also been found in the nucleus and to be closely associated with chromosomes and DNA bases, particularly guanine (16–21). DNA-associated copper has been suggested to be involved in maintaining normal chromosome structure and in gene-regulatory processes (22–24). Since copper is closely associated with DNA, we therefore decided to investigate the DNA damage induced by copper-mediated activation of 1,4-HQ and a variety of other phenolic compounds including phenol, 4,4′-biphenol, catechol, 1,2,4-BT, 17$\beta$-estradiol, 2-methoxyestradiol, 2-OH-$E_2$, 4-OH-$E_2$, diethylstilbestrol, BHT, BHA, *t*BHQ, ferulic acid, caffeic acid, chlorogenic acid, eugenol, 2-acetamidophenol, acetaminophen, and BP-7,8-diol.

## Materials and Methods

**Reagents.** Cupric sulfate was obtained from Fisher Scientific Co. (Fair Lawn, NJ). 1,2,4-BT, *t*BHQ, ferulic acid, caffeic acid, and chlorogenic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2-OH-$E_2$ and 4-OH-$E_2$ were products of Steraloids Inc. (Wilton, NH). BP-7,8-diol was purchased from NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, MO). Phenol was obtained from BRL (Bethesda, MD). Dulbecco's phosphate-buffered saline (pH 7.4) was purchased from GIBCO (Grand Island, NY). Supercoiled $\phi$X-174 RF I double-stranded DNA was obtained from New England Biolabs (Beverly, MA). All other chemicals were products of Sigma Scientific Co. (St. Louis, MO).

**$O_2$ Consumption and $H_2O_2$ Generation.** $O_2$ consumption was monitored with a Clark oxygen electrode (YSI-53; Yellow Springs, OH) upon mixing the phenolic compounds with Cu(II) in PBS at 37°C. $H_2O_2$ generation was indirectly determined by $O_2$ production upon adding catalase (500 units/ml) to the reaction mixture of phenolic compounds and Cu(II).

**Reduction of Cu(II) to Cu(I).** Cu(I) generation was determined by using the Cu(I) reagent, bathocuproinedisulfonic acid as described previously (14).

**Assay for DNA Strand Breaks.** DNA strand breaks were measured by the conversion of supercoiled $\phi$X-174 RF I double-stranded DNA to open circular and linear forms as described previously (25). Briefly, 0.2 $\mu$g of DNA was incubated with phenolic compounds, Cu(II), or other chemicals in PBS at 37°C at a final volume of 24 $\mu$l in 1.5 ml brown microcentrifuge tubes. Following incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, and electrophoresed in a horizontal slab gel apparatus (Owl Scientific, MA) in Tris/acetate gel buffer. After electrophoresis, gels were then stained with 0.5 $\mu$g/ml solution of ethidium bromide for 30 min, followed by another 30 min destaining in water, and exposed to UV light at 254 nm. The gels were then photographed and the negatives scanned with a Beckman DU-7 spectrophotometer at 561 nm. The percentage of DNA in each form was calculated by integrating the area under the peaks.

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[3] The abbreviations used are: 1,4-HQ, 1,4-hydroquinone; 1,4-BQ, 1,4-benzoquinone; 1,2,4-BT, 1,2,4-benzenetriol; 2-OH-$E_2$, 2-hydroxyestradiol; 4-OH-$E_2$, 4-hydroxyestradiol; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; *t*BHQ, *tert*-butylhydroquinone; BP-7,8-diol, benzo(*a*)pyrene-7,8-dihydrodiol; PBS, Dulbecco's phosphate-buffered saline; BCS, bathocuproine-disulfonic acid; $\phi$X-174 RF I, $\phi$X-174 relaxed form I.

Table 1 *Effects of various inhibitors and incubation conditions on the reactive oxygen-dependent DNA strand breaks initiated by a copper-induced oxidation of 1,4-HQ*[a]

| Treatment | Reduction of CU(II) to CU(I)[b] | Oxygen consumption[c] | $H_2O_2$ Generation[d] | DNA strand breaks | | |
|---|---|---|---|---|---|---|
| | | | | SC (%) | OC (%) | L (%) |
| Control | | | | 95 | 5 | 0 |
| 1,4-HQ/Cu(II) | + | + | + | 0 | 89 | 0 |
| +BCS | + | – | – | 92 | 8 | 0 |
| +Catalase | + | + | – | 90 | 10 | 0 |
| +Boiled catalase | + | + | + | 0 | 96 | 4 |
| +SOD | + | + | + | 0 | 92 | 8 |
| +Boiled SOD | + | + | + | 0 | 91 | 9 |
| +Anaerobic con. | + | – | – | 80 | 20 | 0 |
| +Mannitol[e] | + | + | + | 0 | 91 | 9 |
| +PBN[e] | + | + | + | 0 | 88 | 12 |
| +Sodium azide[f] | + | + | + | 51 | 49 | 0 |
| +4-oxo-TEMP[f] | + | + | + | 41 | 59 | 0 |

[a] DNA was incubated with 10 μM 1,4-HQ plus 10 μM Cu(II) in the presence of various inhibitors or under anaerobic conditions in PBS, at 37°C for 30 min. The percentage of each form of DNA was determined as described in "Materials and Methods." The concentration of BCS was 40 μM; the activity of catalase or SOD was 100 units/ml; the concentration of mannitol, PBN, sodium azide, or 4-oxo-TEMP was 100 mM. The sequence of addition to DNA was inhibitors, Cu(II) and 1,4-HQ.
[b] Reduction of Cu(II) to Cu(I) was determined as described in "Materials and Methods."
[c] $O_2$ consumption was recorded continuously for 10 min after mixing 1,4-HQ and Cu(II) in PBS at 37°C.
[d] $H_2O_2$ generation was indirectly determined by $O_2$ release upon adding catalase to the mixture of 1,4-HQ and Cu(II).
[e] Hydroxyl radical scavengers. PBN, *N-tert*-Butyl-α-phenylnitrone.
[f] Singlet oxygen scavengers. 4-oxo-TEMP, 2,2,6,6-tetramethyl-4-piperidone.

## Results and Discussion

**Reactive Oxygen-dependent DNA Strand Breaks Initiated by Copper-induced Oxidation of 1,4-HQ.** Previously, Cu(II) has been shown to be capable of mediating the oxidation of 1,4-HQ producing 1,4-BQ and $H_2O_2$ by a copper-redox mechanism (14). Therefore, in this study we first assessed the role of Cu(II)/Cu(I) redox cycle and reactive oxygen generation in 1,4-HQ/Cu(II)-induced DNA damage. As shown in Table 1, in the presence of 10 μM Cu(II), 10 μM 1,4-HQ induced extensive DNA strand breaks as indicated by the disappearance of the supercoiled (SC) form and the formation of both open circular (OC) and linear (L) forms. 1,4-HQ or Cu(II) alone elicited no damage to DNA. The inhibition by BCS indicates that a Cu(II)/Cu(I) redox cycle is critical to the 1,4-HQ/Cu(II)-induced DNA damage (Table 1). BCS is a Cu(I)-specific chelator which can strongly bind Cu(I) forming a redox-inert complex (26). In the presence of BCS, oxidation of 1,4-HQ to 1,4-BQ and $H_2O_2$ was blocked (14). The oxidation of 1,4-HQ by Cu(II) generates nearly the same concentration of $H_2O_2$ as the initial 1,4-HQ concentration used (14). Catalase but not Cu, Zn superoxide dismutase inhibited the 1,4-HQ/Cu(II)-induced DNA strand breaks (Table 1), indicating that $H_2O_2$ rather than superoxide is necessary for eliciting DNA strand breaks. Anaerobic conditions also significantly protected DNA from the 1,4-HQ/Cu(II)-induced DNA strand breaks (Table 1), suggesting that $O_2$ is required for the 1,4-HQ/Cu(II) system to generate a DNA cleaving species. In order to examine the involvement of reactive oxygen species in the 1,4-HQ/Cu(II)-induced DNA strand breaks, the effects of several reactive oxygen scavengers were examined (Table 1). Mannitol and *N-tert*-butyl-α-phenylnitrone are generally regarded as hydroxyl radical scavengers (27, 28), while sodium azide and 2,2,6,6-tetramethyl-4-piperidone are viewed as singlet oxygen scavengers (27, 29). The ineffectiveness of hydroxyl radical scavengers on the 1,4-HQ/Cu(II)-induced DNA strand breaks (Table 1) suggests that free hydroxyl radicals are not involved in the observed DNA strand breaks. In contrast, the singlet oxygen scavengers exhibited some inhibitory effect on the 1,4-HQ/Cu(II)-mediated DNA strand breaks (Table 1), indicating that either singlet oxygen or a singlet-like entity, possibly a copper-peroxide complex (27), probably plays a role in the DNA strand breaks.

**Reactive Oxygen-dependent DNA Damage Initiated by Copper-induced Activation of other Phenolic Compounds.** Since a copper redox cycle and reactive oxygen generation have been implicated in the 1,4-HQ/Cu(II)-induced DNA damage, we next examined whether the copper redox mechanism and reactive oxygen are also involved in the DNA damage mediated by copper-induced activation of other phenolic compounds. Phenol, 4,4′-biphenol, catechol and 1,2,4-BT are benzene metabolites (30). Among these metabolites, 1,2,4-BT/Cu(II) exhibited the strongest DNA cleaving activity followed by catechol/Cu(II) (Table 2). 4,4′-biphenol/Cu(II) only elicited slight DNA strand breaks. Phenol/Cu(II) at the used concentrations was not capable of inducing DNA strand breaks (Table 2). Consistent with their capabilities to induce DNA damage, 1,2,4-BT/Cu(II) and catechol/Cu(II) also induced significant $O_2$ consumption and $H_2O_2$ generation (Table 2).

Examining the DNA strand breaks induced by estrogen-derived

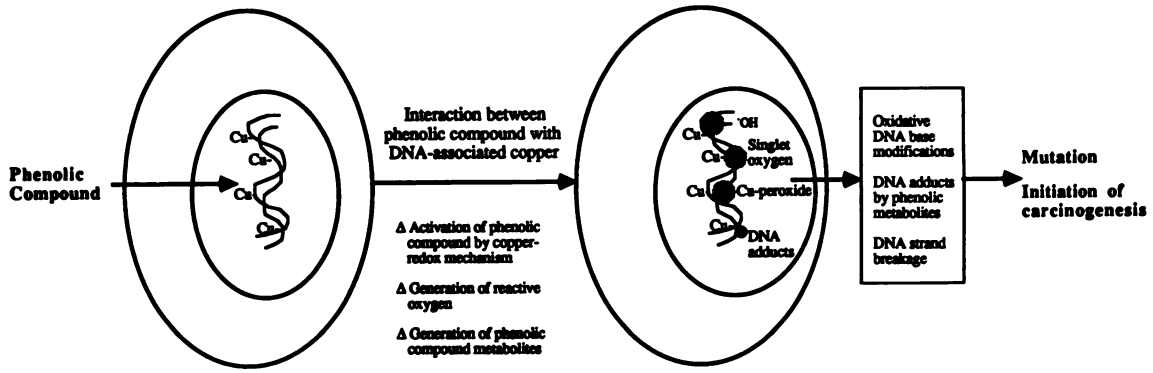
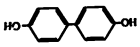
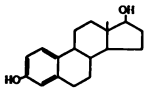
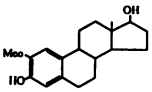
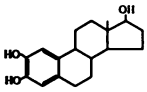
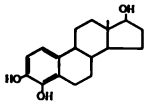
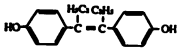
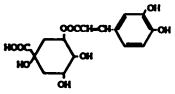



Fig. 1. Proposed model illustrating the potential role for DNA-associated copper in DNA damage mediated by copper-induced activation of phenolic compounds.

Table 2 *Oxygen consumption, $H_2O_2$ generation and reactive oxygen-dependent DNA cleavage initiated by copper-induced activation of phenolic compounds*[a]

| Phenolic compound[c] | Structure | $O_2$ consumption/ $H_2O_2$ generation[d] | DNA cleavage[b] | | |
|---|---|---|---|---|---|
| | | | | Inhibition by BCS | Inhibition by catalase |
| Phenol |  | – | – | | |
| 4,4′-Biphenol |  | | + | + | + |
| Catechol |  | + | +++ | + | + |
| 1,2,4-BT |  | + | ++++ | + | + |
| E2 |  | – | – | | |
| 2-M-$E_2$ |  | | + | + | + |
| 2-OH-$E_2$ |  | + | +++ | + | + |
| 4-OH-$E_2$ |  | – | – | | |
| DES |  | | + | + | + |
| BHT |  | | + | + | + |
| BHA |  | | ++ | + | + |
| *t*BHQ |  | + | ++++ | + | + |
| Ferulic acid |  | | + | + | + |
| Caffeic acid |  | + | +++ | + | + |
| Chlorogenic acid |  | | + | + | + |
| Eugenol |  | | + | + | + |
| 2-Acetamidophenol |  | | + | + | + |
| Acetaminophen |  | | + | + | + |
| BP-7,8-diol |  | – | – | | |

[a] DNA was incubated with various phenolic compounds plus 10 μM Cu(II) in the presence or absence of 40 μM BCS or 100 units/ml catalase in PBS, at 37°C for 60 min. The sequence of addition to DNA was BCS or catalase, Cu(II), and phenolic compounds.

[b] DNA cleaving activity was classified as follows: –, no change as compared to control; +, slightly greater conversion of supercoiled DNA to open circular form than control; ++, complete conversion of supercoiled DNA to open circular. +++, conversion of supercoiled DNA to both open circular and linear forms; ++++, disappearance of all three forms of DNA and replaced by smear.

[c] The concentration of estradiol$_2$, 4-OH-$E_2$, 2-M-$E_2$, 2-OH-$E_2$, DES, or BP-7,8-diol was 40 μM; the concentration of all of the other phenolic compounds was 100 μM. DES, diethylstilbestrol, 2-M-$E_2$, 2-methoxyestradiol.

[d] $O_2$ consumption was recorded continuously for 15 min after mixing phenolic compounds with Cu(II) in PBS at 37°C. $H_2O_2$ generation was indirectly determined by $O_2$ generation upon adding catalase (500 units/ml) to the reaction mixture of phenolic compounds and Cu(II). Only the $O_2$ consumption by those compounds that show DNA cleaving activity of –, +++, or ++++ was determined.

phenolic metabolites, it was found that 2-OH-$E_2$/Cu(II) induced extensive DNA strand breaks as well as significant $O_2$ consumption and $H_2O_2$ generation (Table 2). 2-methoxyestradiol/Cu(II) only induced slight DNA strand breaks. Interestingly, 4-OH-$E_2$/Cu(II) did not exhibit any DNA cleaving activity, suggesting that 4-OH-$E_2$ is not a good redox cycling compound as compared to 2-OH-$E_2$ in this metal-driven experimental system. Diethylstilbestrol, a bridged hydroquinone molecule, in the presence of Cu(II) exhibited a slight DNA cleaving activity (Table 2).

BHT and BHA are phenolic antioxidants, which have also been shown to be tumor promoters and animal carcinogens (11, 31). Demethylation of BHA produces *t*BHQ (31). In the presence of Cu(II), *t*BHQ induced extensive DNA degradation, while BHA and BHT exhibited less DNA cleaving activities (Table 2). Cu(II) was also observed to strongly mediate the oxidation of *t*BHQ through a semiquinone intermediate, producing *tert*-butyl(1,4)paraquinone and $H_2O_2$ (Table 2).[4]

Ferulic acid and caffeic acid are widely distributed in plants (1). These two phenolic compounds have been shown to be genotoxic, with caffeic acid being more genotoxic than ferulic acid (1). In this study, caffeic acid/Cu(II) also exhibited a stronger DNA cleaving capability than did ferulic acid/Cu(II). In the presence of Cu(II), other phenolic compounds including chlorogenic acid, eugenol, 2-acetamidophenol, and acetaminophen induced only slight DNA strand breaks, while BP-7,8-diol, the proximate carcinogenic metabolite of benzo-

[4] Personal observations.

(*a*)pyrene, elicited no damage to DNA, which may be due to the steric structure of the 7,8-hydroxyl groups similar to that of 4-OH-$E_2$ (Table 2).

As shown in Table 2, for all of the phenolic compounds that can induce DNA strand breaks in the presence of Cu(II), the DNA damage could be inhibited by BCS or catalase, indicating that a copper redox cycle and $H_2O_2$ generation are two major determinants involved in the observed DNA damage. Therefore, the reactive oxygen species generated from the further interaction between copper and $H_2O_2$ may be responsible for the DNA damage mediated by copper-induced activation of these phenolic compounds.

Structure-activity analysis of the data in Table 2 shows that in the presence of Cu(II), the DNA cleaving activity for phenolic compounds with a 1,4-hydroquinone structure, such as 1,2,4-BT and *t*BHQ, is greater than those with a catechol group (catechol, 2-OH-$E_2$ and caffeic acid). Those compounds having one phenol group, such as eugenol, 2-acetamidophenol, and acetaminophen, are the least reactive. Another interesting comparison is the relationship exhibited between phenols and their demethylated metabolites such as BHA *versus* *t*BHQ and ferulic acid *versus* caffeic acid. On the basis of this comparison, it is possible that the cytochrome P-450 catalyzed demethylation of eugenol would result in a metabolite which exhibits significant DNA cleaving activity in the presence of Cu(II).

**Potential Role for DNA-associated Copper in Phenolic Compound-induced Genotoxic Injury.** Since copper is capable of mediating activation of a variety of phenolic compounds, it is reasonable to propose that the DNA-associated copper in cells may have the potential to activate phenolic compounds via a copper-redox reaction, producing reactive oxygen and electrophilic phenolic intermediates (Fig. 1). The interaction of phenolic compounds with DNA-associated copper may finally result in a spectra of lesions in DNA, including oxidative DNA base modifications, DNA strand breaks, and DNA adducts of phenolic intermediates. As illustrated in Fig. 1, these lesions in DNA might contribute to mutations in DNA and to the initiation of carcinogenesis induced by phenolic compounds.

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