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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JANUARY 1997

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Oxidative DNA Damage Induced by Activation of Polychlorinated Biphenyls (PCBs): Implications for PCB-Induced Oxidative Stress in Breast Cancer

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Received June 24, 1996[⊗]

We have previously reported that mono- and dichlorinated biphenyls (PCBs) can be metabolized to dihydroxy compounds and further oxidized to reactive metabolites which form adducts with nitrogen and sulfur nucleophiles including DNA [Amaro et al. (1996) *Chem. Res. Toxicol.* **9**, 623−629; Oakley et al. (1996) *Carcinogenesis* **17**, 109−114]. The former studies also demonstrated that during the metabolism of PCBs superoxide may be produced. We have therefore examined the abilities of PCB metabolites to induce free radical-mediated oxidative DNA damage using a newly developed, highly sensitive, ^{32}P-postlabeling assay for 8-oxodeoxyguanosine (8-oxodG) [Devanaboyina, U., and Gupta, R. (1996) *Carcinogenesis* **17**, 917−924]. The incubation of 3,4-dichloro-2′,5′-dihydroxybiphenyl (100 μM) with calf thymus DNA (300 μg/mL) in the presence of the breast tissue and milk-associated enzyme, lactoperoxidase (10 mU/mL), and H_2O_2 (0.5 mM) resulted in a significant increase in free radical-induced DNA damage (253 8-oxodG/10^6 nucleotides) as compared to vehicle-treated DNA (118 8-oxodG/10^6 nucleotides). Substituting $CuCl_2$ (100 μM) for lactoperoxidase/H_2O_2, however, resulted in a substantial increase in 8-oxodG content (2669 8-oxodG/10^6 nucleotides). $FeCl_3$ was ineffective, suggesting that $CuCl_2$ but not $FeCl_3$ mediates oxidation of PCB dihydroxy metabolites, resulting in oxidative DNA damage. The addition of catalase (100 U/mL) and sodium azide (0.1 M) reduced the effect of $CuCl_2$ (849 and 896 8-oxodG/10^6 nucleotides, respectively), while superoxide dismutase (600 U/mL) moderately stimulated and glutathione (100 μM) substantially stimulated 8-oxodG formation (3014 and 4415 8-oxodG/10^6 nucleotides, respectively). The effect of various buffers as well as the effects of PCB structure on Cu(II)-mediated oxidative DNA damage were examined. These results demonstrate that free radicals and oxidative DNA damage are produced during oxidation of lower chlorinated biphenyls. The relevance of the results is discussed in view of the recent report that increased oxidative DNA base damage is detected in the DNA of human breast tumor tissue.

Introduction

Polychlorinated biphenyls (PCBs)[1] are environmental contaminants that, due to their persistence and lipophilicity, bioaccumulate in the food chain and are concentrated in fatty tissues, including breast tissue. In animals and/or humans chronic exposure to PCBs produces a variety of effects including decreased body weight (wasting syndrome), chloracne, edema, liver hypertrophy, porphyria, estrogenic activity, immunosuppression, neurotoxicity (*1*), and liver abnormalities such as adenofibrosis, neoplastic nodules, and hepatocellular carcinoma (*2*). Although not conclusive, recent epidemiology studies have suggested a link between the accumulation of PCBs in human breast tissue with an increased risk of breast cancer (*3, 4*). Previously, Wasserman et al. (*5*) reported an increase in concentration of PCBs in malignant breast tissue compared to the surrounding normal breast and adipose tissue. In a separate pilot study, Falck et al. (*6*) reported elevated levels of PCBs in mammary adipose tissue from women with breast cancer as compared to women without breast cancer.

It is well documented that PCBs are present in human breast milk. The results of these studies demonstrate clear differences in the composition of PCBs in commercial PCB mixtures like Aroclors and Clophens compared to PCBs of breast milk. For example, PCB 28 (2,4,4′-trichlorobiphenyl) is a minor component (0.04%) of Aroclor 1260 but is present in breast milk at a much higher concentration (8.8% of total PCBs) (*7*). In fact many of the lower chlorinated biphenyls appear to be selectively retained in breast tissue and milk (*8*, *9*).

Lower halogenated PCBs can be metabolized by cytochrome P450s, thus potentially activating them to carcinogenic metabolites. We have recently reported that mono- and dichlorinated biphenyls can be metabolized to hydroquinones which can be further oxidized to reactive metabolites, notably semiquinones, that react with O_2 to form superoxide and quinones (*10*). These PCB-derived quinones react with nitrogen and sulfur nucleophiles to form the 1,4-Michael addition products. In addition, they are capable of redox cycling,[2] increasing oxidative stress in biological systems. It is especially

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⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1996.
[1] Abbreviations: PCBs, polychlorinated biphenyls; SOD, superoxide dismutase; PHQ, phenylhydroquinone; GSH, glutathione; 8-oxodG, 8-oxodeoxyguanosine.

[2] Unpublished data.

S0893-228x(96)00103-8 CCC: $12.00

alarming that some lower halogenated biphenyls are found in surprisingly high amounts in the breast, where they are available for metabolism by cytochrome P450-mediated oxidations within the breast tissue. In an analogous paradigm, the carcinogenicity of the endogenous hormone 17β-estradiol, is attributable to the ability of its catechol metabolites to induce free radical damage. This damage was detected as DNA strand breaks and the formation of 8-oxodeoxyguanosine (8-oxodG). It was proposed that these arose via a redox cycling mechanism (*11*−*16*).

The highest monooxygenase activity is found in the liver. Normally, the hydroxy metabolites produced in the liver undergo phase II metabolism (conjugation) and are excreted in the feces and urine. Therefore, it was assumed that potentially dangerous metabolites of lower halogenated PCBs would be eliminated immediately. However, a recent investigation demonstrated that hydroxylated metabolites of PCBs are retained in human plasma (*17*).

There are numerous enzymatic and nonenzymatic mechanisms by which catechol/hydroquinone-like xenobiotics can be further oxidized to reactive intermediates. For example, hydroxylated PCB metabolites can be further oxidized by peroxidases to quinones via semiquinone intermediates (*18*). In addition, studies have demonstrated the copper-dependent oxidation of chemically similar structures, e.g., hydroquinone and 2-hydroxyestradiol, metabolites of benzene and 17β-estradiol, respectively, to reactive intermediates that induce oxidative DNA damage (*19*, *20*). This pattern of genotoxicity is similar to that reported in the DNA of human breast tumor tissue (*21*).

It has been suggested that the link between PCBs and breast cancer involves their estrogenic activity (*3*). We propose an alternative mechanism that does not directly involve the estrogen receptor. Our preliminary findings that PCB metabolites are capable of redox cycling *in vitro* has led us to consider that certain PCB metabolites (Figure 1) may cause increased oxidative DNA damage via enzymatic and nonenzymatic mechanisms. Therefore, we have begun to investigate pathways through which hydroxylated PCBs may cause oxidative DNA damage.

Materials and Methods

Lactoperoxidase from bovine milk (EC 1.11.1.7), superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6) glutathione, RNase A (Type III-A), RNase T1, and micrococcal nuclease were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium azide and cupric chloride were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sources of materials used in the ^{32}P-postlabeling analysis have been described elsewhere (*22*). [γ-^{32}P]ATP was synthesized as described by Johnson and Walseth (*23*), with modifications (*24*). Commercial calf thymus DNA (type I; Sigma Chemical Co.) was further purified by a rapid, solvent extraction procedure using sequential treatments with RNases A and T1 (100 μg/mL and 25 U/mL, respectively) and proteinase K (150 μg/μL), followed by solvent extractions and ethanol precipitation, as described (*22*), except that the antioxidant butylated hydroxytoluene was added prior to extractions with phenol-containing solvents (*25*). **Caution:** *Synthetic PCBs and metabolites should be considered potentially toxic and hazardous and therefore should be handled in an appropriate manner.*

Synthesis of Lower Chlorinated Dihydroxybiphenyls. PCB hydroxy metabolites (Figure 1) were synthesized as previously described (*26*). The 3,4-dichloro-2′,5′-dihydroxybiphenyl was purified by crystallization to >99% purity and characterized

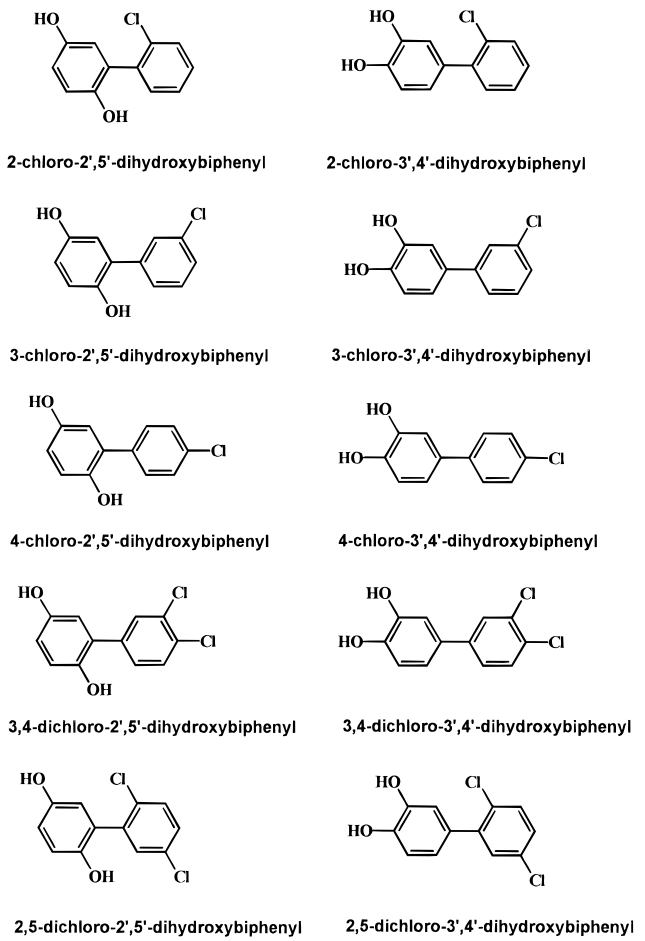

Figure 1. Structures of the PCB dihydroxy metabolites.

by GC/MS on a Finnigan INCOS 50 using a fused silica capillary column (DB-5MS 15 m × 0.25 mm and OV-1, 25 m, J&W Scientific, Folsom, CA). For GC/MS the 3,4-dichloro-2′,5′-dihydroxybiphenyl was silylated with BSTFA/pyridine (1:1). The melting point was determined on a MEL-TEMP apparatus and is uncorrected.

3,4-Dichloro-2′,5′-dihydroxybiphenyl was synthesized from the reduction of the respective quinone (*27*) with sodium dithionite in 78% yield: white solid from $CHCl_3$/petroleum ether 1:3, mp 105−106 °C; IR (KBr) 3276, 3033, 1600, 1550, 1500, 1453, 1375, 1244, 1190, 1128, 1033 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6) δ = 6.72 (dd, J = 8.6, 3.0 Hz, 1H, 4′-H), 6.82 (dd, J = 3.0, 0.5 Hz, 1H, 6′-H), 6.84 (dd, J = 8.5, 0.4 Hz, 1H, 3′-H), 7.53 (dd, J = 8.4, 2.0 Hz, 1H, 6-H), 7.57 (dd, J = 8.6, 0.6 Hz, 1H, 5-H), 7.79 (dd, J = 2.0, 0.4 Hz, 1H, 2-H), 7.92, 8.22 (2bs, 2H, OH); MS (70 eV): m/z (%) = 398 (59) {M$^+$}, 383 (12) {M$^+$ − CH_3}, 348 (45) {M$^+$ − CH_3Cl}, 73 (100).

Lactoperoxidase-Catalyzed Oxidation of 3,4-Dichloro-2′,5′-dihydroxybiphenyl. 3,4-Dichloro-2′,5′-dihydroxybiphenyl (50 μM) was incubated with lactoperoxidase (1.25 U/mL) and ethanol (0.5%) at 37 °C in the presence of sodium citrate (100 mM, pH 7.4). The reaction was monitored with a Shimadzu MPS-2000 UV−vis spectrophotometer over the range of 500−200 nm. After a base line was obtained, the reaction was initiated by the sequential addition of H_2O_2 (5.0 μM) every 2 min.

Reactions of PCB Metabolites with Calf Thymus DNA. The purified DNA (300 μg/mL) was incubated with 3,4-dichloro-2′,5′-dihydroxybiphenyl (100 μM) or other lower (mono-, di-, and tri-) chlorinated dihydroxybiphenyls in a reaction mixture containing ethanol (0.5%), Tris-HCl (10 mM, pH 7.4), and one of the following oxidative systems: lactoperoxidase (0.01 U/mL)/H_2O_2 (0.5 mM), $CuCl_2$ (100 μM), or $FeCl_3$ (100 μM) at 37 °C for 60 min. After incubation, DNA was purified by ethanol precipitation. Experiments with reactive oxygen scavengers were carried out as described above except that the reaction

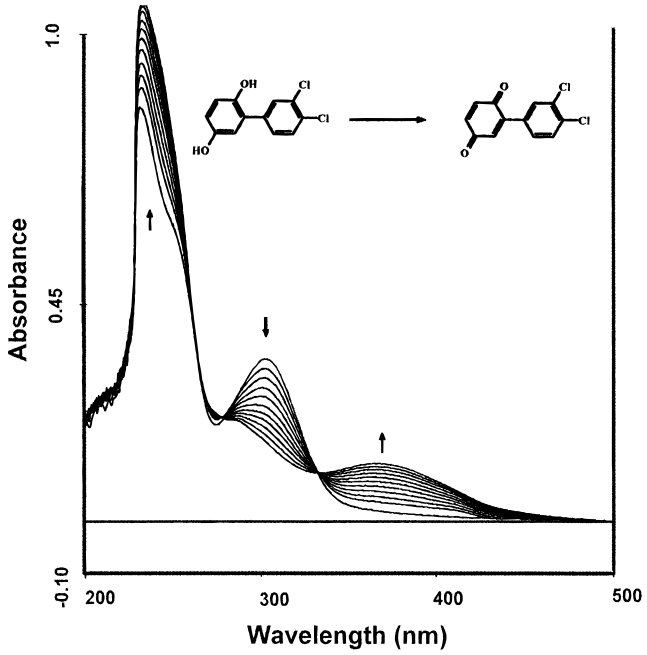

Figure 2. Absorption spectra of 3,4-dichloro-2′,5′-dihydroxybiphenyl oxidized to the corresponding quinone, 2-(3′,4′-chlorophenyl)-1,4-benzoquinone. 3,4-Dichloro-2′,5′-dihydroxybiphenyl (50 μM) was placed in a cuvette containing lactoperoxidase (1.25 U/mL), H_2O_2 (5.0 μM), ethanol (0.5%), and sodium citrate (100 mM, pH 7.4). The reaction was monitored with a Shimadzu MPS-2000 UV−vis spectrophotometer over the range of 500−200 nm. The reaction was initiated by the addition of H_2O_2 (5.0 nmol). Additional H_2O_2 (5.0 nmol) was added, and the absorption was recorded every 2 min for an additional 20 min.

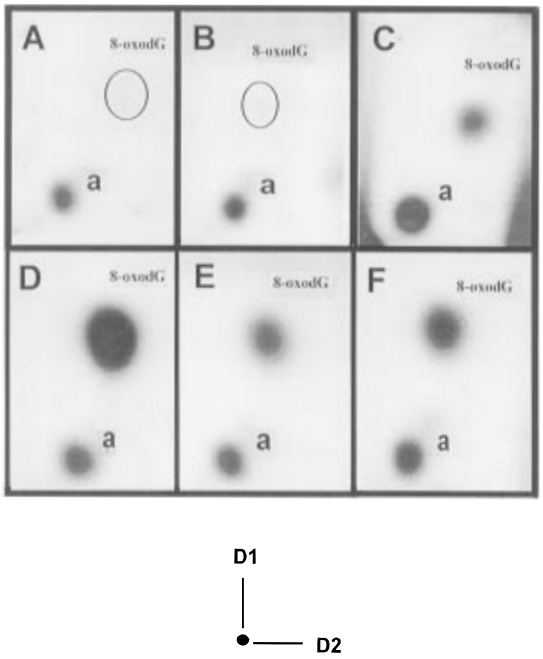

Figure 3. ^{32}P-Postlabeling maps of 8-oxodG formation in the presence of 3,4-dichloro-2′,5′-dihydroxybiphenyl with calf thymus DNA: (A) control, ethanol (1%); (B) 3,4-dichloro-2′,5′-dihydroxybiphenyl/$FeCl_3$; (C) 3,4-dichloro-2′,5′-dihydroxybiphenyl/lactoperoxidase (10 mU/mL) and H_2O_2 (0.5 mM); (D) 3,4-dichloro-2′,5′-dihydroxybiphenyl/$CuCl_2$; (E) 3,4-dichloro-2′,5′-dihydroxybiphenyl/$CuCl_2$ with catalase (100 U/mL); (F) 3,4-dichloro-2′,5′-dihydroxybiphenyl/$CuCl_2$ with sodium azide (0.1 M). 3,4-Dichloro-2′,5′-dihydroxybiphenyl (100 μM) was incubated with Tris-HCl (10 mM), calf thymus DNA (150 μg), lactoperoxidase (10 mU/mL) and H_2O_2 (0.5 mM) or $CuCl_2$ (100 μM) or $FeCl_3$ (100 μM) in the presence or absence of catalase and sodium azide. After 60 min, the DNA was purified by ethanol precipitation. 8-OxodG adducts were labeled as described in Materials and Methods. Chromatography was on PEI−cellulose sheets: D1, 1.1 M formic acid with a 8 cm Whatman 17 paper wick attached to chromatogram; D2, 0.6 M ammonium formate (pH 6) with a 5 cm Whatman 1 paper wick. The autoradiography exposure time was 6 h at −80 °C. The amount of DNA analyzed was 36 ng. (a) represents a contaminant in the commercial [^{32}P]-P_i preparation.

was performed in the presence of 100 μM glutathione, 600 U/mL of superoxide, 100 U/mL of catalase, or 0.1 M sodium azide. Controls were carried out by omitting cupric choride and/or the PCB metabolite or in the presence of glutathione omitting the PCB metabolite from the reaction mixture.

Statistics. All results are expressed as the mean ± SD of at least three experiments. *P* values for significance were determined using the two-tailed Student's *t* test.

Analysis of 8-OxodG. 8-OxodG in DNA was analyzed by a newly developed ^{32}P-postlabeling assay (*25*). Briefly, DNA was enzymatically hydrolyzed to deoxyribonucleoside 3′-monophosphates and labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled nucleotides were hydrolyzed to 5′-monophosphates with nuclease P1. Separation of labeled p8-oxodG was accomplished with two-dimensional PEI−cellulose TLC. 8-OxodG levels were determined as relative adduct labeling (RAL) by measurement of the radioactivity in p8-oxodG and normal nucleotide spots as described (*25*), except that the nucleotide radioactivity was determined using a Packard Instant Imager Model A202400. An appropriate blank region of the chromatogram was also counted and the radioactivity subtracted from 8-oxodG spot radioactivity.

Results

Absorption Spectral Changes during Lactoperoxidase/H_2O_2 Oxidation of 3,4-Dichloro-2′,5′-dihydroxybiphenyl. We have previously shown that horseradish peroxidase, myeloperoxidase, and prostaglandin H synthase can oxidize a number of PCB catechols and hydroquinones to their corresponding quinones (*18*, *28*). Lactoperoxidase, found in the mammary glands, is very similar to these peroxidases. As shown in Figure 2, the addition of lactoperoxidase results in the conversion of 3,4-dichloro-2′,5′-dihydroxybiphenyl to the corresponding 2-(3′,4′-dichlorophenyl)-1,4-benzoquinone as limiting increments of H_2O_2 are sequentially added. The original hydroquinone absorbance (300 nm) decreased while a concomitant bathochromic shift from 300 to 365 nm occurred during the reaction, indicating the biosynthesis of the corresponding quinone. We have shown that superoxide is produced during this reaction, as evidenced by nitroblue tetrazolium (NBT) reduction (*29*).

Formation of 8-OxodG in DNA Mediated by Lactoperoxidase Oxidation of 3,4-Dichloro-2′,5′-dihydroxybiphenyl. The oxidation of 3,4-dichloro-2′,5′-dihydroxybiphenyl with lactoperoxidase/H_2O_2 in the presence of calf thymus DNA resulted in increased formation of 8-oxodG as compared to vehicle-treated DNA. Measurement of the 8-oxodG content was over 2-fold higher in the PCB-treated sample (253 ± 89 versus 118 ± 57 8-oxodG/10^6 nucleotides; Table 1).

Effects of $CuCl_2$ or $FeCl_3$ on the Formation of 8-OxodG. When $CuCl_2$ was substituted for lactoperoxidase/H_2O_2 in the reaction mixture described above, the result was a significant increase in 8-oxodG formation (Table 1). Replacing $CuCl_2$ with $FeCl_3$ in the presence of 3,4-dichloro-2′,5′-dihydroxybiphenyl reduced 8-oxodG formation to control levels (Table 1). DNA incubated with $CuCl_2$ and vehicle (0.5% ethanol) or 3,4-dichloro-2′,5′-dihydroxybiphenyl without $CuCl_2$ did not exhibit elevated levels of 8-oxodG as compared to DNA treated with vehicle alone.

Effects of Reactive Oxygen Scavengers in 3,4-Dichloro-2′,5′-dihydroxybiphenyl/$CuCl_2$ Induced

Table 1. 3,4-Dichloro-2′,5′-dihydroxybiphenyl (PCB Metabolite)-Mediated Formation of 8-Oxodeoxyguanosine in DNA in the Presence or Absence of Various Scavengers of Reactive Oxygen Species[a]

treatment	8-oxodG/10^6 nucleotides
DNA + vehicle	118 ± 57
DNA + PCB metabolite + lactoperoxidase	253 ± 89[b]
DNA + PCB metabolite + $FeCl_3$	122 ± 15
DNA + PCB metabolite + $CuCl_2$	2669 ± 305[c]
DNA + PCB metabolite + $CuCl_2$ + catalase	849 ± 115[c]
DNA + PCB metabolite + $CuCl_2$ + sodium azide	896 ± 134[c]
DNA + PCB metabolite + $CuCl_2$ + glutathione	4415 ± 1086[c]
DNA + $CuCl_2$ + glutathione	966 ± 197[c]
DNA + PCB metabolite + $CuCl_2$ + SOD	3014 ± 615[b]

[a] The conditions of the reaction and method of adduct analysis are described in Materials and Methods. Each value is a mean of 3−6 replicates processed in parallel from 3−6 separate experiments ± SD. [b] Significantly different from the control value ($P < 0.05$). [c] Significantly different from the control value ($P < 0.01$).

8-OxodG Formation. Recently, we have observed that PCB metabolites are capable of redox cycling *in vitro* with the generation of $O_2^{\bullet-}$. Therefore, we examined the role of PCB dihydroxy-mediated superoxide and its dismutation product, H_2O_2, in the Cu(II)-induced formation of 8-oxodG. The addition of catalase or sodium azide, a singlet oxygen scavenger, inhibited Cu(II)/3,4-dichloro-2′,5′-dihydroxybiphenyl-generated 8-oxodG formation (Figure 3). However, the addition of superoxide dismutase had a minimal effect (Table 1), indicating that H_2O_2 but not $O_2^{\bullet-}$ may be involved in Cu(II)/PCB dihydroxy-generated 8-oxodG formation.

Due to high intracellular levels of GSH (0.1−10 mM) (*30*) and its ability to detoxify xenobiotics, we studied its effect on Cu(II)/PCB dihydroxy-generated 8-oxodG formation. GSH (100 μM) incubated with Cu(II)/3,4-dichloro-2′,5′-dihydroxybiphenyl in the presence of calf thymus DNA resulted in a significant increase in oxidative DNA damage (4415 ± 1086 8-oxodG/10^6 nucleotides) compared with vehicle-treated control (118 ± 57 8-oxodG/10^6 nucleotides) (Table 1). In the absence of 3,4-dichloro-2′,5′-dihydroxybiphenyl, GSH/$CuCl_2$ caused a considerable accumulation of 8-oxodG (966 ± 197, Table 1) most likely due to formation of thiyl radicals accompanied by generation of superoxide and H_2O_2 (*31*, *32*).

Structure−Activity Relationships of Several PCB Metabolites on Copper-Mediated 8-OxodG Formation. Since a Cu(II)/3,4-dichloro-2′,5′-dihydroxybiphenyl system demonstrated significant oxidative DNA damage detected by the generation of 8-oxodG, we examined the capacity of other PCB cytochrome P450-mediated metabolites to induce oxidative DNA damage in the presence of $CuCl_2$. The PCB metabolites with an *ortho*-substituted chlorine in the nonhydroxylated ring (2-chloro-2′,5′-dihydroxy- and 2,5-dichloro-2′,5′dihydroxybiphenyl) had higher activity as compared to the coplanar PCBs, while increasing the chlorine content in the nonhydroxylated ring decreased the formation of 8-oxodG (Figure 4).

Among the *para* dihydroxy metabolites, 2-chloro-2′,5′-dihydroxybiphenyl exhibited the strongest 8-oxodG-forming activity, followed by 2,5-dichloro-2′,5′dihydroxy-, 3-chloro-2′,5′dihydroxy-, 4-chloro-2′,5′-dihydroxy-, 3,4,5-trichloro-2′,5′-dihydroxy-, and 3,4-dichloro-2′,5′-dihydroxybiphenyl, respectively (Figure 4). Investigating the *ortho* dihydroxy metabolites, we found that all the corresponding catechol-like metabolites had 5−7 times less 8-oxodG-forming activity as compared to the *para* dihydroxys (Figure 4), suggesting the *ortho* dihydroxy metabolites

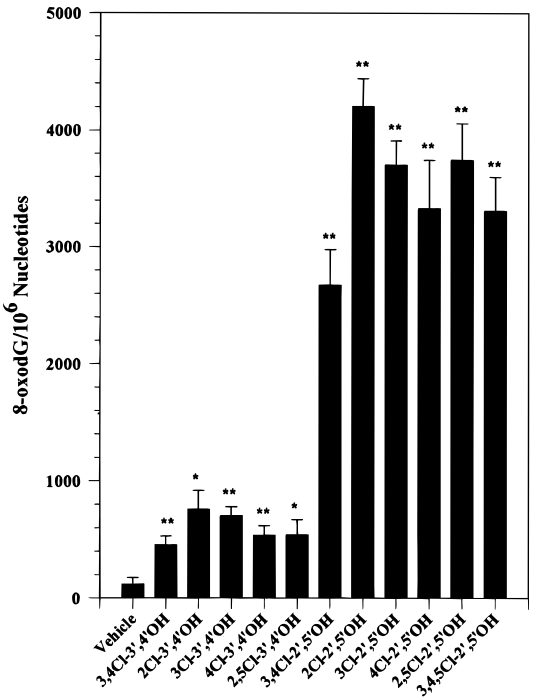

Figure 4. Analysis of 8-oxodG formation with mono-, di-, and trichlorobiphenyl *ortho* and *para* dihydroxy metabolites. Lower chlorinated dihydroxybiphenyls (100 μM) were incubated with Tris-HCl (10 mM), calf thymus DNA (150 μg), and $CuCl_2$ (100 μM) for 60 min at 37 °C. 8-OxodG measurement conditions were the same as described in the legend to Figure 3. 8-OxodG levels were determined as relative adduct labeling (RAL) by measurement of the radioactivity in p8-oxodG and normal nucleotide spots by a Packard InstantImager, Model A202400. Values are expressed as the mean ± standard deviation of 3−4 replicates run in parallel. (*) Significantly greater than control, $P < 0.05$; (**) significantly greater than control, $P < 0.01$.

are not oxidized by Cu(II) as well as the *para* dihydroxys in this nonenzymatic system. Interestingly, the redox potential coupling $Q^{\bullet}/QH_2$ for the 4-chloro-2′,5′-dihydroxy- and 3,4-dichloro-2′,5′-dihydroxybiphenyl (−0.36 and −0.328 V, respectively) is lower than that of the corresponding *ortho* dihydroxys, 4-chloro-3′,4′-dihydroxy and 3,4-dichloro-3′,4′-dihydroxybiphenyl (−0.223 and −0.194 V, respectively) (*10*), indicating that the former compounds are more readily oxidized nonenzymatically by copper(II) ions.

Effects of Various Buffers on the 3,4-Dichloro-2′,5′-dihydroxybiphenyl/$CuCl_2$-Induced 8-OxodG Formation. Previous studies have reported Tris-HCl has an inhibitory effect on Cu(II)/hydroquinone-induced DNA strand breaks and chromium/H_2O_2-induced 8-oxodG formation (*33*, *34*). Therefore, we determined the effects of various buffers on Cu(II)/PCB dihydroxy-induced 8-oxodG formation. Concentrations of 10 mM (pH 7.4), sodium succinate, sodium phosphate, and sodium citrate reduced the formation of 8-oxodG as compared to the levels found with 10 mM Tris-HCl. Sodium citrate exhibited the strongest inhibitory effect (sodium citrate > sodium phosphate > sodium succinate, Figure 5) probably due to its ability to chelate Cu(II) and prevent the copper-mediated oxidation of the PCB dihydroxy metabolite. The ability of copper to bind to phosphate (*35*) may suggest a competitive inhibitory mechanism with sodium phosphate.

Discussion

The data presented in this paper provide evidence that peroxidase or Cu(II) mediated oxidation of PCB dihy-

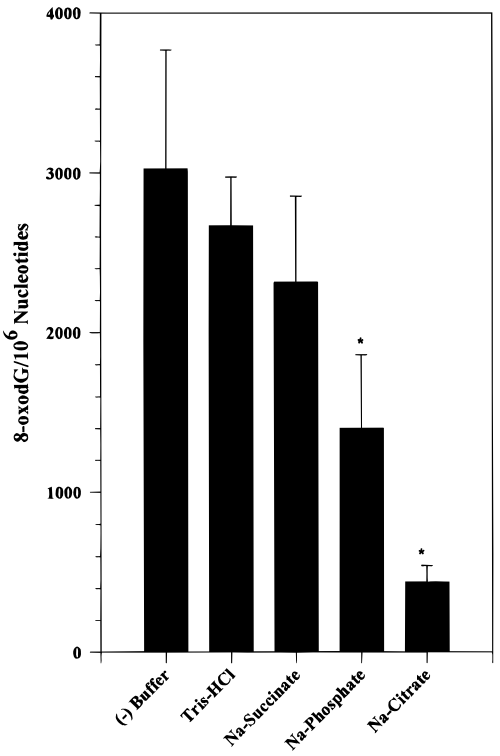

Figure 5. Comparison of various buffers on the $CuCl_2$/3,4-dichloro-2′,5′-dihydroxybiphenyl-mediated formation of 8-oxodG. Experimental conditions were the same as described in legend to Figure 4 except that Tris-HCl (10 mM) was replaced with a 10 mM concentration of the buffer indicated. (*) Significantly less than control, $P < 0.05$.

droxy metabolites results in oxidative DNA damage. Using UV−visible spectroscopy, we have demonstrated that lactoperoxidase, found in high concentration in the mammary glands and milk, catalyzes the oxidation of PCB dihydroxy compounds to the corresponding quinones via semiquinone intermediates (Figure 2). Lactoperoxidase is activated by H_2O_2 to compound I which is reduced to the compound II enzyme intermediate before returning to the native enzyme. During one enzymatic cycle, lactoperoxidase can catalyze the oxidation of two PCB dihydroxy metabolites to semiquinones which readily react with O_2 to generate superoxide anion radicals (*36*). The formation of semiquinones may contribute to oxidative stress in biological systems by propagating the formation of superoxide and its dismutation product H_2O_2.

An alternative nonenzymatic mechanism of PCB oxidation involves Cu(II). Copper is an essential micronutrient and widely distributed in mammalian tissues. Approximately 20% of the total copper is stored in the nucleus (*37*). This makes the nucleus a major target for copper-catalyzed oxidations within the cell. Copper(II) has been shown to bind preferentially to guanine residues at the N-7 position placing copper in close proximity to the major site of oxidative modification in DNA, the C-8 position of guanine (*38*, *39*). The direct correlation between oxygen free radical-mediated 8-oxodG formation and carcinogenesis is due to its ability to induce mutagenic events during DNA synthesis (*40*).

Our results show that the formation of 8-oxodG by PCB dihydroxy metabolites was significantly increased in the presence of Cu(II) (Table 1). However, Fe(III)/3,4-dichloro-2′,5′-dihydroxybiphenyl did not induce the formation of 8-oxodG above control levels (Table 1). This indicates there is not an iron-mediated oxidation of PCBs and the subsequent production of 8-oxodG as demonstrated with copper. Similar results have been observed with phenylhydroquinone (PHQ), a metabolite of the fungicide *o*-phenylphenol. Nagai et al. (*41*) demonstrated that copper, but not iron, catalyzed the oxidation of PHQ with the formation of 8-oxodG. Both the generation of 8-oxodG and DNA cleavage by PHQ were inhibited by catalase and sodium azide (*42*); however, PHQ/Cu(II)-induced DNA damage was unaffected by SOD (*43*). Tsou et al. (*34*) demonstrated that sodium azide, Tris-HCl, and catalase, but not SOD, significantly reduced $CrCl_3/H_2O_2$-induced formation of 8-oxodG. In the present study, Cu(II)/PCB-induced oxidative DNA damage was inhibited by catalase and sodium azide. However, the addition of SOD did not block 8-oxodG formation, indicating superoxide is not involved under these experimental conditions (Table 1). H_2O_2 and singlet oxygen or putative copper−peroxide complexes (*44*) are implicated as intermediates due to the capacity of catalase and sodium azide to inhibit 8-oxodG formation (Table 1). In studies examining DNA cleaving activity with copper/hydrazine, hydroquinone, or 2-hydroxyestradiol, the reactive oxygen scavengers sodium azide, Tris-HCl, and catalase exhibited inhibitory effects (*20*, *33*, *45*). Interestingly, SOD did not inhibit Cu(II)-induced DNA damage, but it did have an inhibitory effect on DNA cleaving activity with Mn(III)/hydrazine (*45*). Zhang et al. (*46*) have suggested the lack of effect of SOD with Cu(II)/trihydroxybenzene to a trihydroxybenzene−$Cu-O_2$ complex that allows for an inner sphere transfer of two electrons precluding superoxide release. A similar reaction forming a hypothetical ternary complex (PCB−$Cu-O_2$) would change the radical-propagated reaction observed with lactoperoxidase/PCB dihydroxy compounds to a unified two electron transfer that is not accessible to SOD.

Due to the inhibitory effect on 8-oxodG formation and DNA strand breaks induced by various compounds in conjunction with transition metals, Tris-HCl is believed to be a singlet oxygen and radical scavenger (*47*). However, in the present investigation Tris-HCl failed to inhibit Cu(II)/PCB dihydroxy-induced 8-oxodG formation (Figure 5). Also in a recent study employing Cu(II) and 2-hydroxyestradiol, a catechol metabolite of estradiol, Tris-HCl did not inhibit 8-oxodG formation (*48*).

The majority of studies discussed in the preceding paragraphs, which examined DNA strand breaks, used a phosphate containing buffer. However, upon the addition of Tris-HCl, DNA cleaving activity was diminished. Our findings show that sodium phosphate decreased 8-oxodG by 50% as compared to Tris-HCl. This suggests two different mechanisms may be involved in the formation of DNA strand breaks and 8-oxodG.

Structure−activity analysis of the data shown in Figure 4 demonstrates that *para* dihydroxys have 5−7 times higher 8-oxodG forming activity than do the catechols, the *ortho* dihydroxy metabolites. Structurally similar compounds with a 1,4-hydroquinone structure, including the genotoxic metabolites of benzene, 1,4-hydroquinone and 1,2,4-trihydroxybenzene, and the synthetic antioxidant 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), which can be demethylated to 2-*tert*-butyl-(1,4)-hydroquinone (TBHQ), were reported to have higher DNA cleaving activity than *ortho* dihydroxy compounds such as the benzene metabolite catechol, the 17β-estradiol metabolite 2-hydroxyestradiol, and the plant product caffeic acid (*49*).

Numerous studies have examined the effect of glutathione on copper-mediated oxidative DNA damage with equivocal results. Reed and Douglas (*32*, *50*) have

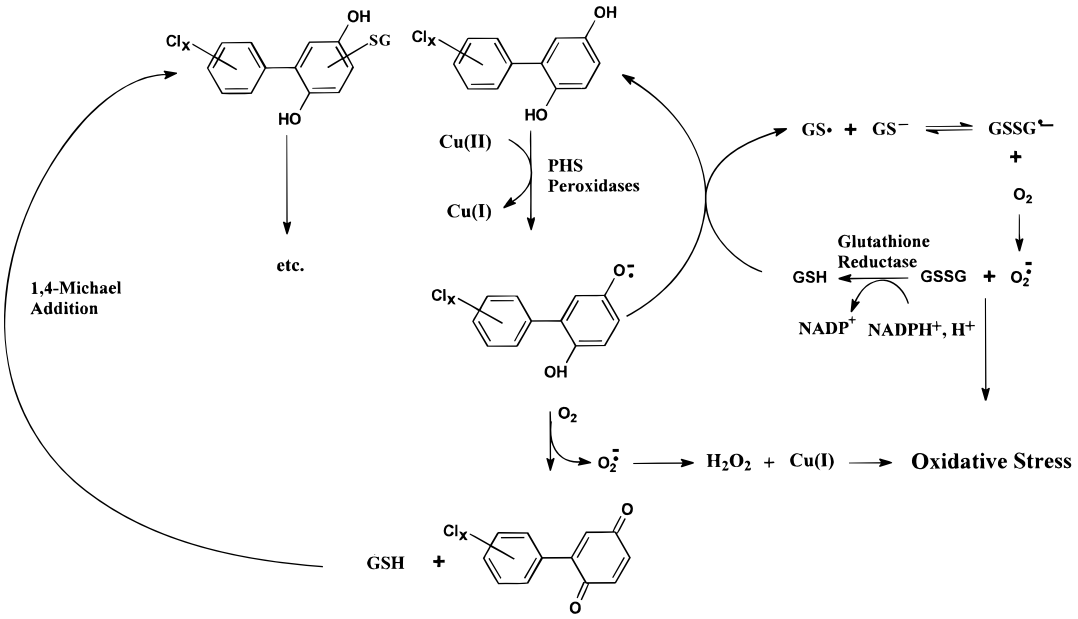

Figure 6. Activation of PCB *para* dihydroxys as representative PCB metabolites and the reaction sequence catalyzed by copper. Cu(II) oxidizes the PCB metabolite to a semiquinone intermediate. Oxygen reacts with the semiquinone intermediate to produce the quinone and superoxide. Alternatively, in the presence of reduced glutathione, the thiyl radical forms, reducing the semiquinone back to the PCB hydroquinone. The glutathione thiyl radical reacts with another glutathione molecule to form the glutathione disulfide radical anion, which reacts with O_2 to form oxidized glutathione and superoxide. Superoxide dismutates to hydrogen peroxide, which reacts with Cu(I) utilizing Fenton chemistry to produce hydroxyl radicals.

demonstrated DNA cleaving activity by GSH/copper(II) ions, while, Milne et al. (*51*) found that GSH was inhibitory in a $CuCl_2/H_2O_2$/ascorbate system. The inhibitory effect was attributed to stabilization of Cu(I), preventing Cu(I) from reacting with H_2O_2 in a typical Fenton type reaction. In contrast to the latter study, the present study demonstrates the addition of GSH to Cu(II)/PCB dihydroxy metabolites produced the highest yield of 8-oxodG adducts (Table 1). This suggests an additional thiol-driven redox cycling of PCB metabolites occurring in conjunction with copper redox cycling, increasing production of reactive oxygen species. We propose the reaction sequence outlined in Figure 6 as a possible mechanism for PCB-induced oxidative damage. As predicted by this scheme, one electron oxidation of PCB dihydroxy metabolites catalyzed by Cu(II) or peroxidases yields a semiquinone free radical and Cu(I) (*19*, *52*). The intermediate reacts with molecular oxygen to give the quinone and superoxide anion radical (*10*, *53*) as shown by peroxidase-catalyzed oxidation of PCB dihydroxy compounds (Figure 2). Alternatively, in the presence of GSH, the thiyl radical forms, reducing the semiquinone back to the PCB hydroquinone (*54*, *55*). The thiyl radical reacts with glutathione anion to form glutathione disulfide radical anion, which in the presence of O_2 forms GSSG and superoxide (*56*). In support of this hypothesis, estradiol and phenol are reported to form glutathione thiyl free radicals in the presence of a peroxidase-catalyzed oxidation (*57*, *58*) via a phenoxyl radical mechanism. Additionally, this peroxidase-catalyzed oxidation of phenol in the presence of deoxyguanosine 3′-monophosphate and Fe(III)−EDTA produced 8-oxodG, with the highest yield of adducts derived from thiol-dependent generation of oxygen radicals (*58*). Alternatively, PCB dihydroxy compounds can form 1,4-Michael addition products with GSH that may participate in copper-mediated redox cycling, resulting in GSH depletion and oxidative stress. We have recently reported that the oxidation product of 4-chloro-2′,5′-dihydroxybiphenyl, 2-(4′-chlorophenyl)-1,4-benzoquinone, formed glutathionyl−hydroquinone adducts (*10*) which are susceptible to reoxidation and the formation of quinone thioethers (*59*).

In summary, this study provides the first experimental evidence that PCB dihydroxy metabolites are activated (via enzymatic and nonenzymatic mechanisms) to reactive intermediates that produce oxidative DNA damage. Certainly, G to T transversions induced by 8-oxodG formation may lead to oncogene activation (*60*) or tumor suppressor gene inhibition and contribute to the carcinogenic potential of PCBs. These results provide a possible mechanismto support the hypothesis that environmental carcinogens, such as PCBs, may contribute to human breast cancer.

Acknowledgment. This work is supported by NIH Grant CA 57423, ACS Grant CN-67A, and DAMD 17-96-1-6162. G.G.O. is supported by NIEHS Training Grant ES 07266. The authors thank Parvaneh Espandiari for many helpful discussions during the course of this work and Wendell Neeley for technical assistance. Content of the information does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred.

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