Generation of Reactive Oxygen Species (ROS) and Genotoxicity by Metabolites of Polychlorinated Biphenyls (PCBs)

Gabriele Ludewig, Anandi Srinivasan, Parvaneh Espandiari, Larry W. Robertson

Graduate Center for Toxicology, 306 Health Sciences Research Building, University of Kentucky Medical Center, Lexington, KY 40536-0305, USA

Introduction

PCBs are persistent contaminants in our environment and bioaccumulate in the fat tissue of animals and humans. This raises concern about the long term health risks associated with the exposure to PCBs and related compounds. Commercial PCB mixtures are complete carcinogens, inducing particularly hepatocellular carcinoma in rats and mice, but the mechanisms by which they do so have not been determined.

Chronic exposure to PCBs produces chromosome aberration, including chromosome breaks, and sister chromatid exchanges, reviewed in [1]. One agent that forms this type of genotoxic lesions is oxygen radicals. Moreover, it is generally accepted that PCBs act as promoters in rodent liver carcinogenesis, again a process where oxygen radicals are believed to be involved. We have shown previously that lower halogenated PCBs can be metabolized to mono- and dihydroxylated compounds [2]. Further enzymatic [3] or non-enzymatic oxidation of these compounds can lead to the generation of semiquinones and quinones with the production of superoxide (see abstract of Dr. Robertson). Superoxide can generate other, even more reactive oxygen species, like singlet oxygen, hydroxyl anions and hydroxyl radicals. PCBs could be involved in carcinogenesis by generating reactive oxygen species. We have addressed these mechanisms of carcinogenicity by measuring superoxide production with the nitroblue tetrazolium (NBT) assay and strand break induction in supercoiled plasmid DNA *in vitro*. Employment of various scavengers was used to determine the type of reactive oxygen species involved in strand break induction. Measuring *in vitro* genotoxicity and cytotoxicity of a series of metabolites with differed chlorination or/and positioning of the hydroxyl groups provides insight into a structure activity relationship for these two activities.

Materials and Methods

Measurement of superoxide production (NBT assay). 100 nmols of a dihydroxybiphenyl was added to 1 ml of 2 mM phosphate buffer, pH 7.4, containing 5 mM CTAB and 10 mM NBT with/without 100 μM copper(II). The rate of NBT reduction at 37° C was determined spectrophotometrically at 540 nm in the absence and presence of superoxide dismutase (SOD, 60 U/ml).

Measurement of strand break induction. Mostly supercoiled plasmid DNA (Bluescript SK-, about 3 kb size; 1 μg/15 μl) was exposed to the test compound and modifiers for 1 hour at 37°C. The

DNA was immediately submitted to gel electrophoresis (0.8% agarose gel, 1xTAE buffer, 60 V) for 2 hours. The gels were stained with ethidium bromide (0.5 µg/ml water). Pictures were taken with a video camera coupled to a transilluminator and used for documentation. The relative amounts of plasmid DNA with no strand breaks (supercoiled), single strand breaks (open circle), and double strand breaks (linear form) was determined by densitometry.

Measurement of cytotoxicity. Yeast cells (*Saccharomyces cerevisiae*) and bacterial cells (*E. coli, strain JM109*) were used to determine the cytotoxicity of the various chlorinated dihydroxybiphenyls. Yeast cells in YPD medium (yeast extract, peptone, dextrose) or bacteria in LB medium were grown in microtiter plates in the presence of a 2x dilution series of the test compounds. After 24 hrs growth turbidity was determined at 550 nm.

Results and Discussion

All chlorinated dihydroxybiphenyls tested resulted in a reduction of NBT. Figure 1 shows the results with 4-chloro-2',5'-dihydroxybiphenyl. The presence of superoxide dismutase during the reaction reduced the amount of NBT reduction to about half, confirming the involvement of superoxide in the NBT reduced. This result indicates that ortho- and para-dihydroxybiphenyls oxidize in aqueous solution in the presence of oxygen with the production of superoxide.

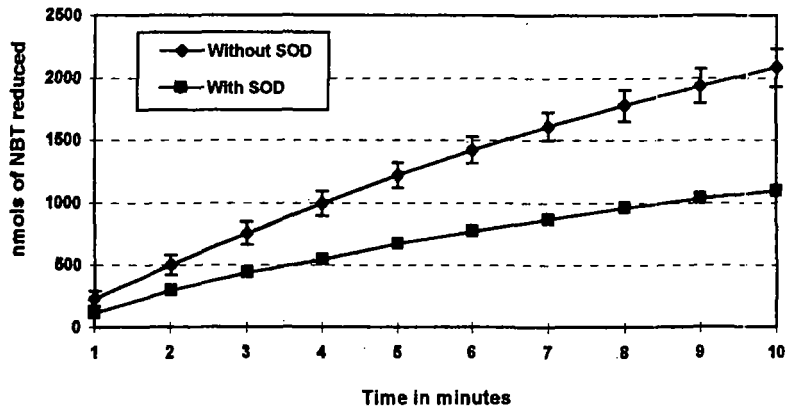

Figure 1: Reduction of nitroblue tetrazolium by 4-chloro-2',5'-dihydroxybiphenyl in the absence (diamond) and presence (square) of superoxide dismutase.

Strand break induction in supercoiled plasmid DNA was only observed when Cu(II) was present. This is in agreement with previous reports that superoxide does not induce strand breaks. Copper is probably not only essential for the production of hydroxyl radicals, but may also be involved in the oxidation of the dihydroxy metabolite to the corresponding semiquinone and quinone.

Using 4-Cl-2',5'-dihydroxybiphenyl as model compound and 100 µM Cu(II) we observed an increase in strand break induction with increasing concentrations of the PCB metabolite (Figure 2). At low concentrations of the test compound the supercoiled form disappeared with a corresponding increase in the open circle form due to single strand breaks. At even higher concentrations of the PCB metabolite linear plasmid DNA appeared, indicating the induction of

double strand breaks.

To learn more about the mechanism of strand break induction by this compound, we included various chelators, enzymes, cofactors, and scavengers (Table 1). The divalent cation chelator EDTA and the Cu(I) chelator bathocuproine strongly inhibited strand break induction by 4-Cl-2',5'-dihydroxybiphenyl, again pointing at the crucial role of copper in strand break induction by ROS. The cofactor glutathione also prevented strand break induction, probably by acting as a redox partner/radical scavenger. As expected, the enzyme SOD had no effect, whereas catalase reduced strand break induction. The superoxide scavenger tiron had no effect at the concentration

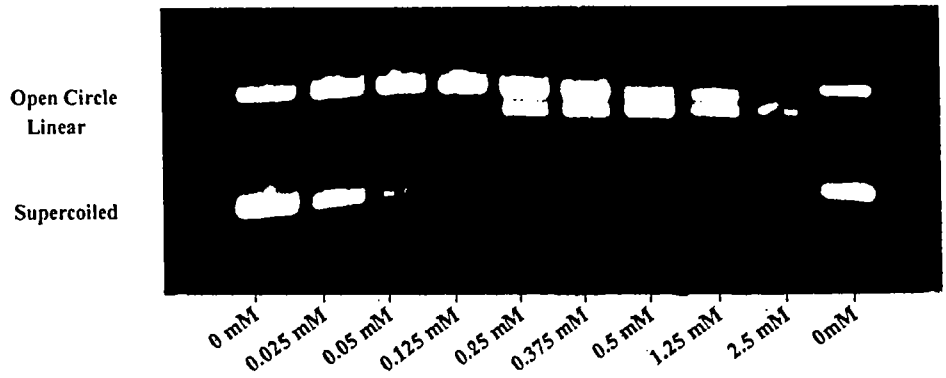

Figure 2:Effect of different concentrations of 4-Cl-2',5'-dihydroxybiphenyl on strand break induction in the presence of 100 μM Cu(II).

tested nor did the hydroxyl radical scavenger Na-benzoate. The hydroxyl radical scavenger DMSO and thiourea, however, protected the DNA from strand break induction, as did the singlet oxygen scavengers Na-azide and Tris. Both, hydroxyl radical and singlet oxygen, seem to be directly involved in the induction of strand breaks, whereas superoxide is only indirectly involved as a precursor.

Table 1: Effect of modifiers on strand break induction by 4-Cl-2',5'-dihydroxybiphenyl.

substance	effect as/on	concentration tested	effect on DNA
Cu(II)	redox partner	100 μM	essential
GSH	redox partner	100 μM	protective
EDTA	chelator	100 μM	complete protection
bathocuproine	Cu(I) chelator	0.1 - 200 mM	protection
SOD	superoxide radical	1.2 U/μl	no effect
tiron	superoxide radical	100 μM	no effect at this conc.
catalase	H_2O_2	5 U/μl	protection
DMSO	hydroxyl radical	7-53%	protection at 53%
Na-benzoate	hydroxyl radical	0.1 - 200 mM	no protection
thiourea	hydroxyl radical	0.1 - 200 mM	protection
Na-azide	singlet oxygen	0.1 - 200 mM	protective at >25 mM
Tris	singlet oxygen	5 - 50 mM	protective

An important piece of information is whether a structure activity relationship can be seen for a specific activity in a series of compounds. We compared para-dihydroxybiphenyls who had chlorines in the 2-, 3-, 4-, or 3,4- positions. All of these compounds produced strand breaks with similar strength, however, the ortho and meta chloro-substituted biphenyls seemed to be slightly more active than the para chloro-substituted. This is in agreement with our previously reported finding about *in vitro* 8-oxo-dG (8-oxo-deoxyguanosine) formation by various para-dihydroxybiphenyls, where the order was 2 > 2,5 > 3 > 4 [4]. The position of the dihydroxy groups also influenced the activity in the strand break assay, with 2',5'-dihydroxy being more active than the 3',4'- or 2',3'- metabolites. This is also in agreement with our previous findings in the 8-oxo-dG assay [4].

The cytotoxicity experiments with yeast and bacteria revealed that the number and position of chlorines as well as the position of the two hydroxyl groups also have an influence on this activity. The para-dihydroxy metabolites (2,5-) were less toxic than either of the ortho-dihydroxy metabolites (2,3- ;3,4-). Chlorines in para- and/or meta- position increased the toxicity compared to chlorines in the ortho position. When the compounds are ordered according to increasing cytotoxicity, an inverse relationship was seen compared to strand break induction and the previously reported formation of 8-oxo-dG by these compounds. This suggests that cytotoxicity and ROS production and ROS-mediated genotoxicity are not related and probably due to opposite physico-chemical properties of the compounds tested.

To summarize the results, we found that lower chlorinated dihydroxybiphenyls 1) oxidize with the formation of superoxide, 2) result in strand break induction in the presence of Cu(II), 3) cause strand break induction via hydroxyl radical and singlet oxygen, and 4) are cytotoxic to bacteria and yeast. We have further found that 5) structure activity relationships exist, with ortho chlorinated metabolites being more genotoxic, but less cytotoxic, than their corresponding para chlorinated metabolites. Para-dihydroxy metabolites are more genotoxic, and less cytotoxic, than the corresponding ortho dihydroxy biphenyls. These results support the involvement of reactive oxygen species in the genotoxicity of PCBs and provide one explanation for the carcinogenic effects of these industrial pollutants.

Acknowledgments
Funded by DAMD17-96-1-6162 and P42 ES 07380.

References

1. Silberhorn, E.M., Glauert, H.P., and Robertson, L.W; *Critical Reviews in Toxicology* **1990**, 20, 439-496.
2. McLean, M.R., Bauer, U., Amaro, A.R., and Robertson, L.W.; *Chem. Res. Toxicol.* **1996**, 9, 158-164.
3. Oakley, G.G., Robertson, L.W., and Gupta, R.C.; *Carcinogenesis* **1996**, 17, 109
4. Oakley, G.G., Devanaboyina, U., Robertson, L.W., and Gupta, R.C.; *Chem. Res. Toxicol.* **1996**, 9, 1285.