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Breaking the dogma: PCB-derived semiquinone free radicals do not form covalent adducts with DNA, GSH, and amino acids

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Abstract

Covalent bond formations of free radical metabolites with biomolecules like DNA and proteins are thought to constitute a major mechanism of toxicity and carcinogenesis. Glutathione (GSH) is generally accepted as a radical scavenger protecting the cell. In the present study, we investigated a semiquinone radical (SQ^{•-}) metabolite of the semivolatile 4-chlorobiphenyl, using electron paramagnetic resonance spectroscopy, and oxygen consumption. Proton nuclear magnetic resonance (¹H NMR) and liquid chromatography–mass spectrometry (LC-MS) were also employed to elucidate the radical interaction with DNA, amino acids, and GSH. We found that DNA and oligonucleotides stabilized SQ^{•-} by electron delocalization in the π -stacking system, resulting in persistent radical intercalated, rather than forming a covalent bond with SQ^{•-}. This finding was strongly supported by the semiempirical calculation of the semioccupied molecular orbital and the linear combination of the atomic orbitals, indicating 9.8 kcal mol⁻¹ energy gain. The insertion of SQ^{•-} into the DNA strand may result in DNA strand breaks and interruption of DNA replication process or even activate radical mediated secondary reactions. The presence of amino acids resulted in a decrease of the electron paramagnetic resonance (EPR) signal of SQ^{•-} and correlated with their isoelectric points. The pH shifts the equilibrium of the dianions of hydroquinone and influenced indirectly the formation of SQ^{•-}. Similar findings were observed with GSH and Cys. GSH and Cys functioned as indirect radical scavengers; their activities depend on their chemical equilibria with the corresponding quinones, and their further reaction via Michael addition. The generally accepted role of GSH as radical scavenger in biological systems should be reconsidered based upon these findings, questioning the generally accepted view of radical interaction of semiquinones with biologically active compounds, like DNA, amino acids, proteins, and radical scavengers like GSH.

Keywords

Electron paramagnetic resonance; Glutathione; Prostaglandin H synthase-2; Polychlorinated biphenyls; Reactive oxygen species; Semiquinone free radicals; Carcinogenesis; DNA

Introduction

It is generally accepted that free radicals can cause carcinogenesis. The free radicals generated by a biotransformation of xenobiotics are of particular interest as proximate carcinogens, since they react with DNA and other biomolecules readily to form adducts (Myers 1982). Free radicals have a wide range of reactivity (Buettner 1993), with lifetimes ranging from nanoseconds to years (Nonhebel and Walton 1974; Mill et al. 1980). The decrease of the Gibbs free energy (ΔG), referred to as energy minimization, is the driving force for their reactivity. Free radicals can undergo diverse reaction pathways: (i) redox reactions by electron or hydrogen transfers, (ii) termination by binding covalently to another free radical forming a closed-shell molecule, (iii) covalent binding with a non-radical, forming a new radical species, (iv) intramolecular delocalization of the free electron forming a relatively stable species, or (v) intermolecular by a non-covalent, coordinate, or ligand bond (e.g., π -interaction with an aromatic ring). In the present study, we investigated the molecular interaction of DNA with semiquinones ($SQ^{\bullet-}$), a group of free radicals generated by comproportionation of hydroquinone (H_2Q) and quinone (Q). $SQ^{\bullet-}$ and Q have been extensively studied as the ultimate carcinogens causing cytotoxicity, mutagenicity, and DNA strand breaks (Morrison et al. 1985; Dwivedy et al. 1992; Shen et al. 1998; Bolton and Thatcher 2008; Lehmann et al. 2007). In addition, we investigate the reaction with glutathione (GSH), a generally accepted radical scavenger, and amino acids as monomers of the proteins.

Our model compound is from the group of environmental pollutants, the polychlorinated biphenyls (PCBs) that persist in our environment in spite of manufacturing cessation in the 1970s. Semivolatile PCBs have triggered our research interest, as a number of studies have shown that lower chlorinated, so-called semivolatile PCBs are genotoxic (Oakley et al. 1996a, b; Srinivasan et al. 2001; Zettner et al. 2007; Ludewig et al. 2008). In our previous studies, we demonstrated that metabolites of 4-chlorobiphenyl (4-CB), the major congener determined in indoor air samples (Davis et al. 2002), function as reducing co-substrates in prostaglandin H synthase (PGHS) metabolism. This resulted in the generation of reactive Q metabolites, undergoing Michael additions with nucleophilic species (Wangpradit et al. 2009). The subsequent PGHS metabolism is correlated with elevated downstream production of prostaglandins which may disrupt the homeostasis and result in the inflammation responses in tissues (Kobayashi and Narumiya 2002). The $SQ^{\bullet-}$ intermediates of 4-CB such as 4-chlorobiphenyl-2',5'-semiquinone (4-CB-2',5'- $SQ^{\bullet-}$) are formed transiently in situ. Therefore, it has been suggested that $SQ^{\bullet-}$ binds covalently to DNA resulting in DNA damage as well (Oakley et al. 1996a, b; Arif et al. 2003). However, mechanisms of DNA- $SQ^{\bullet-}$ interaction remain elusive. As it is thought that a mutation resulting from these reactions can adversely affect the cell cycle and progressively lead to malignancy (Harman 1962), it is particularly important to understand the chemical nature of

the interaction to develop a prevention treatment or decelerate the adverse reactions. Amino acids as the other major target source of radical stress were also investigated. A major focus was given to the GSH thought to be the protector of the cell from radical attack.

It is our hypothesis that $SQ^{\bullet-}$, such as 4-CB-2',5'- $SQ^{\bullet-}$, do not form direct covalent products by radical reactions with DNA, GSH, and amino acids. To test our hypothesis, we employed a direct approach observing the radical interaction with the DNA, GSH, and amino acids in electron paramagnetic resonance (EPR) spectroscopy, the state-of-art technique to monitor real-time formation, and interactions of the radicals. In addition, we monitored the oxygen consumption of the adduct formation to support our findings. The adducts were identified by proton nuclear magnetic resonance (1H NMR) and high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS). Semiempirical *in silico* computations were performed to visualize and explain the processes energetically. 4-CB-2',5'- $SQ^{\bullet-}$ was generated by an enzymatic reaction catalyzed by human recombinant prostaglandin H synthase-2 (hPGHS-2) with deoxynucleosides, oligonucleotides, and DNA. The use of hPGHS-2 was shown in our previous study to be an excellent catalyst generating high concentration of the $SQ^{\bullet-}$ over a time span of several hours in the medium (Wangpradit et al. 2010).

The present study provides fundamental findings relevant to the fields of toxicology, environmental science, medicinal and radical chemistry, and chemical carcinogens: (i) elucidating for the first time the guest-host interactions of *in situ* generated $SQ^{\bullet-}$ with DNA and amino acids by a direct and real-time monitoring approach, and (ii) insights in the protection of the cells by GSH function as radical scavenger toward radical stress.

Materials and methods

Materials

4-Chlorobiphenyl-2',5'-hydroquinone (4-CB-2',5'- H_2Q) was supplied by Dr. Hans-Joachim Lehmler, the University of Iowa. The corresponding quinone, 4-CB-2',5'-Q (4-chlorobiphenyl-2',5'-benzoquinone), was synthesized using the Meerwein arylation as described by Amaro et al. (1996). Human recombinant PGHS-2 (hPGHS-2) and arachidonic acid (AA) (Cayman Chemical Company, Ann Arbor, MI). Hematin (MP Biomedicals, Solon, OH); salmon sperm DNA (SA Biosciences, Frederick, MD); glutathione (GSH), cysteine (Cys), glycine (Gly), histidine (His), lysine (Lys), arginine (Arg), and methionine (Met) (Research Products International Corp., Mt. Prospect, IL); and deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), and thymidine (dT) (Acros Organic, Morris Plains, NJ) were purchased from the sources indicated. Four oligonucleotides with 20 bases each, (AAAA)₅, (CCCC)₅, (GGGG)₅, and (TTTT)₅, were supplied by Integrated DNA Technology, Iowa, IA. Dimethyl sulfoxide (DMSO) (Fisher Chemical, Chicago, IL) was used as a vehicle for 4-CB-2',5'- H_2Q . The deuterated solvents, acetonitrile- d_3 (ACN- d_3) and deuterium oxide (D_2O), were purchased from Acros organics, NJ. Each experiment contained less than 2 % DMSO. Potassium arachidonate (KAA) was prepared by adding equal molar amounts of arachidonic acid (AA) to potassium hydroxide in aqueous solution.

Methods

EPR determination of 4-CB-2',5'-SQ[•] by hPGHS-2

EPR spectroscopy: EPR spectroscopy was determined using Bruker EMX spectrometer equipped with a high-sensitivity cavity and an Aqua-X sample system for EPR measurements. Typical parameters to obtain EPR spectra (room temperature) were as follows: 3510 G center field, 15 G scan width, 9.854-GHz microwave frequency, 20-mW power, 2×10^5 receiver gain, 100-kHz modulation frequency, and 1.0 G modulation amplitude, with the conversion time and time constant both being 40.96 ms with 5X-scans for each 1024-point spectrum. The spectra were recorded with the 20 scan accumulation mode for 30 min.

In the presence of salmon sperm DNA: Two hundred units of hPGHS-2 was added to the solutions containing 0.02, 0.2, 2, and 7.5 mg of salmon sperm DNA in 0.1 M phosphate buffer (pH 7.4). The reactions were initiated by addition of 4-CB-2', 5'-H₂Q dissolved in DMSO to the concentration of 100 μ M in 1 mL solution. The control experiment was conducted under the same condition without an addition of salmon sperm DNA. The reaction mixtures were immediately transferred into the EPR spectrometer and measured as previously described.

In the presence of deoxynucleosides and oligonucleotides: Two hundred units of hPGHS-2 was added to the phosphate buffer solutions (pH 7.4) containing 100 μ M of dA, dC, dG, and dT for the deoxynucleosides, and 50 μ M of (AAAA)₅, (CCCC)₅, (GGGG)₅, and (TTTT)₅ for the oligonucleotides. The reactions were initiated by addition of 4-CB-2',5'-H₂Q dissolved in DMSO to the concentration of 100 μ M in 1 mL solution.

In the presence of amino acids: Two hundred units of hPGHS-2 was added to the solutions containing 100 μ M of Gly, Lys, Arg, Cys, His, and Met in 0.1 M phosphate buffer (pH 7.4). The reactions were initiated by addition of 4-CB-2', 5'-H₂Q dissolved in DMSO to the concentration of 100 μ M in 1 mL solution.

In the presence of GSH: Two hundred units of hPGHS-2 was added to the solutions containing 5, 10, 25, 50, and 100 GSH μ M in 0.1 M phosphate buffer (pH 7.4). The reactions were initiated by addition of 4-CB-2',5'-H₂Q dissolved in DMSO to the concentration of 100 μ M in 1 mL solution.

Oxygen consumption—Oxygen uptake for each reaction was determined using Clark electrode YSI model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH). All experiments were conducted at room temperature. One hundred millimolars of Tris-HCl buffer (pH 8), and 100 units of hPGHS-2 were loaded in the chamber of the oxygen monitor and aerated by stirring for 5 min. The electrode was then placed in contact with the solution, and all air bubbles were removed. After 5-min equilibration, 4-CB-2',5'-H₂Q was introduced through the access slot to a final concentration of 50 μ M. Amino acids, GSH, or DNA were added to a final concentration of 100 μ M immediately after the addition of 4-CB-2',5'-H₂Q. The total volume after addition of study compounds was 3.0 mL. Each experiment was monitored for 60–90 min and repeated two

times. The solution without amino acids, GSH, or DNA was used as a control. The reaction mixtures after monitoring oxygen consumption were kept in -80°C freezer for LC-MS quantification of the adducts.

Quantification of the GS adducts—Quantification of the GS-adducts was performed on a Thermo LCQ deca mass spectrometer (Thermo Scientific, CA) interfaced with Dionex Ultimate 3000 LC (Dionex Corp., Sunnyvale, CA). Chromatographic separations were performed at 5°C on a 2.1 mm i.d. $\times$ 15 cm, 5 μM , Supelco Discovery C-18 column coupled to Supelguard column, 2.1 mm i.d. $\times$ 2 cm, 5 μM (Sigma-Aldrich, MO). The mobile phases used were 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) delivered at a total flow rate of 200 $\mu\text{L}/\text{min}$. The gradient profile was programmed as follows: 90 % A/10 % B isocratic held from 0 to 2 min, and gradient B up to 100 % from 2 to 30 min. The solutions were injected using a 200 vial autosampler equipped with a 20- μL loop. The LC was coupled with a photodiode array (PDA) detector followed by mass spectrometer (MS) operated in positive electrospray ionization (ESI) mode. The detection wave-length was 280 nm. MS data were acquired over single ion monitoring (SIM) mode at m/z ranges of 523.5–528.5 (mono-GS adducts), 828.5–833.5 (di-GS adducts), and 1133.5–1138.5 (tri-GS adducts).

NMR determination of adduct formation

4-CB-2',5'-Q with dG: The reaction was carried out by addition of dG dissolved in D_2O to a solution of 4-CB-2',5'-Q dissolved in ACN-d_3 . The final concentrations of dG and 4-CB-2',5'-Q were 600 μM and 4 mM, respectively. The solvent ratio of ACN-d_3 : D_2O was 2:3. This reaction was incubated at room temperature for 24 h before NMR determination. The reaction of 4-CB-2',5'-SQ $^{\bullet}$ with dG was prepared by addition of 600 μM dG to the mixture of 2 mM of 4-CB-2',5'-Q and 4-CB-2',5'-H $_2$ Q.

4-CB-2',5'-Q with GSH: The reaction was carried out by addition of GSH dissolved in D_2O to a solution of 4-CB-2', 5'-Q dissolved in ACN-d_3 . The final concentrations of GSH and 4-CB-2',5'-Q were 30 mM and 4 mM, respectively. The solvent ratio of ACN-d_3 : D_2O was 2:3. This reaction was incubated at room temperature for 5 min before NMR determination. The reaction of 4-CB-2',5'-SQ $^{\bullet}$ with GSH was prepared by the addition of 30 mM GSH to the mixture of 2 mM of 4-CB-2',5'-Q and 4-CB-2',5'-H $_2$ Q.

NMR spectroscopy: All products were characterized by the Avance-600 Bruker NMR spectrometer (Billerica, MA) operating at 600 MHz using a mixed solvent system, ACN-d_3 / D_2O (2:3 by volume). Among the solvent systems researched for these studies (DMSO-d_6 / CD_3OD , DMSO-d_6 /Dioxane- d_4 , Dioxane- d_4 / CD_3OD , and ACN-d_3 / D_2O), ACN-d_3 / D_2O was found to be the most optimal in terms of solubility of the component molecules and solution conditions for NMR studies. A 1.7-mm inverse microprobe was used for all the one dimensional ^1H NMR experiments. The residual HOD signal was saturated with a weak radio frequency field (rf-field) to improve the signal-to-noise-ratio of the resonances of interest. The NMR data was collected with a 64k time domain data points, with 32 scans and a relaxation delay of 5 s. All the NMR data were processed with TOPSPIN 1.3 suite of

software programs. One-dimensional ^1H NMR data were processed with zero filling to 128k data points and 0.2-Hz exponential line broadening.

Semiempirical computations of adduct formations—Semiempirical studies were carried out to determine the structures and energies of the complexes formed when 4-CB-2',5'-SQ $^{\bullet-}$ was added to the different deoxynucleosides, dA, dG, dC, and dT as well as with dinucleotides d(ApA), d(GpG), d(CpC), and d(TpT). For efficient computational purposes, we deleted one of the phosphate groups from the end of the dinucleotides which we assumed have little effect on the binding. Initial structures of these deoxynucleosides and dinucleotides were taken from the protein data bank (PDB) ID 3OOR.¹ Subsequent 4-CB-2',5'-SQ $^{\bullet-}$ was orientated forming complexes between nucleic acid base pairs, so-called sandwich complexes. These complexes were then energetically minimized using molecular mechanics followed by semiempirical Austin Model 1 (AM1) calculations with standard VSTO-3G (5d,7f) basis functions using Gaussian 03 (Frisch et al. 2004). The optimized structures and binding enthalpies of the semioccupied molecular orbitals (SOMO) were obtained from these studies by a linear combination of the atomic orbitals (LCAO) of the hypothetical complexes formed. The complexation energy was determined by subtraction of the energy of the free bases and semiquinone from that of the complex ($E_{\text{complexation}} = E_{\text{complex}} - (E_{\text{free bases}} + E_{\text{free SQ}})$). The SOMO were presented as closed spheres and mesh images using Gaussview (Gaussian, Inc.).

Results and discussion

Formation of 4-CB-2',5'-SQ $^{\bullet-}$ can be accomplished by the autoxidation of 4-CB-2',5'-H $_2$ Q and/or comproportionation of the 4-CB-2',5'-Q and 4-CB-2',5'-H $_2$ Q (20–22). However, the signal intensity of 4-CB-2',5'-SQ $^{\bullet-}$ from these reactions was considerably lower than the one-electron oxidation of 4-CB-2',5'-H $_2$ Q by hPGHS-2 (20). Hence, hPGHS-2 was used as a catalyst to oxidize 4-CB-2',5'-H $_2$ Q for all the EPR studies.

The intensity of 4-CB-2',5'-SQ $^{\bullet-}$ spectrum observed in EPR depends on both formation and loss of the 4-CB-2',5'-SQ $^{\bullet-}$ by its reaction with other molecules. Distortion of the redox equilibrium where 4-CB-2',5'-SQ $^{\bullet-}$ is an intermediate can lead to the decrease of 4-CB-2',5'-SQ $^{\bullet-}$ intensity as well. The latter case generally results from adduct formation of 4-CB-2',5'-Q, the oxidized form of 4-CB-2',5'-SQ $^{\bullet-}$, with a nucleophile. This reaction can initially shift the equilibrium to the formation of 4-CB-2',5'-Q in order to compensate for a shift in the redox system.

Salmon sperm DNA, oligonucleotides, and deoxynucleosides

In the presence of salmon sperm DNA, the intensities of 4-CB-2',5'-SQ $^{\bullet-}$ spectra increased with increasing concentrations of salmon sperm DNA compared to the control (Fig. 1a). Addition of salmon sperm DNA to the oxygen monitor chamber did not consume more oxygen compared to the control (Fig. 1b). These findings indicated no adduct formation of 4-CB-2',5'-SQ $^{\bullet-}$ and 4-CB-2',5'-Q with DNA.

¹<http://www.rcsb.org/pdb/explore/explore.do?structureId=3OOR>

As an increased signal intensity of 4-CB-2',5'-SQ^{•-} in the presence of DNA was not predicted from previous observations, we investigated the effects of each base on the EPR signal intensity of the semiquinone radical, by the addition of (AAAA)₅, (GGGG)₅, (TTTT)₅, and (CCCC)₅ to the incubation mixture. Figure 2a demonstrates that EPR signal of 4-CB-2',5'-SQ^{•-} is enhanced in the presence of the oligonucleotides compared to control. Interestingly, addition of pyrimidine oligonucleotides, (TTTT)₅ and (CCCC)₅, resulted in greater enhancements. In addition, the purine oligonucleotides, (AAAA)₅ and (GGGG)₅, showed slightly increased signal intensities compared to the control. However, there was no significant difference in the 4-CB-2',5'-SQ^{•-} intensities observed for single deoxynucleosides, dA, dC, dG, and dT, compared to the control (Fig. 2b).

DNA is a non-radical compound, structured by a double helix phosphate backbone attached to four nucleosides connected as base pairs by hydrogen bonds. Within these nucleoside base pairs, planar molecules can reversibly insert, a process called intercalation. This host-guest interaction with aromatic guest molecules is a 3D π -stacking association that gains a lower Gibbs enthalpy by the extension or delocalization of the aromatic systems. We elucidated this by computations of the SOMO simulating the complex formations by LCAO. Figure 3 shows the optimized structures and the closed-shell models of the complexes of 4-CB-2',5'-SQ^{•-} and the dinucleotides, d(ApA), d(GpG), d(TpT), and d(CpC). Computations of the SOMOs of the complexes of 4-CB-2', 5'-SQ^{•-} with two single deoxynucleosides, dA, dG, dT and dC, were also carried out. The LCAO of the single nucleotides resulted in all cases in very little π -stacking or sandwiching and this even while they were artificially brought into close proximity allowing orbital interactions (−384.0, −182.6, and −107.9 kcal/mol for dT, dG, and dA, respectively). The LCAO of the dinucleosides with the 4-CB-2',5'-SQ^{•-} on the other hand resulted in strong π -stacking interactions (−588.6, −317.5, and −158.8 kcal/mol for d(TpT), d(GpG), and d(ApA), respectively). The coordination by the phosphate group allows a stronger interaction; even the alignment of the axis is less favorable compared to free base pairs, due to the twist. Our computations demonstrate a successful LCAO resulting in an energy gain of 9.8 and 9.5 kcal mol^{−1} by delocalization for both purine and pyrimidine base pairs. This explains the increase of the 4-CB-2',5'-SQ^{•-} intensity; half-life was increased due to stabilization. In addition, the computations demonstrated that the complexes formed by the pyrimidine bases (T and C) show a stronger LCAO compared to purine (A and G). This is in agreement with the observation of a stronger increase in EPR signal intensity for the 4-CB-2',5'-SQ^{•-} in the presence of pyrimidine base pairs. We explain this finding on the basis of the differences in the stacking interaction between purine and pyrimidine oligonucleotides; steric hindrance results in a lower percentage of effective π -stacking in purine systems. This is in agreement with previous studies that demonstrated a stabilization in the alignment of an intercalator with the axes of the base pairs (Li et al. 2009). The twist of the DNA backbone affects the intercalation due to the difference in the alignment axis of the upper and the lower base pairs. Therefore, the stacking cannot uniquely determine the configuration of the final intercalation and by that the Gibbs energy (G). Computations support our hypothesis that the stability of the non-covalent adducts is enhanced by strong π -stacking effects and delocalization of the free electron due to the formation of new molecular orbitals (MOs) in the formed complexes by LCAO. The energy gain of 9.8 and 9.5 kcal mol^{−1} by π -stacking is small compared to the formation of a C–O (78 kcal/mol) or a

C–C (80 kcal/mol) covalent bond. However, the decreased steric hindrance of the aromatic rings by approaching with their planes is favorable and results in stacking. This is why π -stacking energy contribution to the stability of the nucleic acids in general is larger than the hydrogen bonding between the nucleic acid base pairs (Blanksby and Ellison 2003). This explains the signal increase of the $SQ^{\bullet-}$ in the presence of DNA in general. We would like to emphasize that these delocalized guest radicals inside the DNA may initiate a variety of reactions themselves, or promote them.

Glutathione (GSH)

The signal intensities of 4-CB-2',5'- $SQ^{\bullet-}$ decreased with increasing concentrations of GSH (Fig. 4a). The lineshapes of 4-CB-2',5'- $SQ^{\bullet-}$ spectra varied as a consequence of the combination of different 4-CB-2',5'- $SQ^{\bullet-}$ species, a quartet of an original 4-CB-2',5'- $SQ^{\bullet-}$, triplets of the mono-SG adducts, and doublets of a di-SG adducts. The covalent products GS-4-CB-2',5'-Q and GS₂-4-CB-2',5'-Q were characterized using ¹H NMR spectroscopy. Figure 5 shows the differences of ¹H NMR spectral signatures of the adducts from the reactants. A tri-SG or tri-NAC adduct was not observed either in EPR, NMR, or MS (15). Nevertheless, the 4-CB-2',5'- $SQ^{\bullet-}$ signal was totally diminished in the presence of 100 μ M GSH (Fig. 4a). The formation of GS substituted 4-CB-2',5'- $SQ^{\bullet-}$ may derive from the following pathways: (i) the direct covalent binding between 4-CB-2',5'- $SQ^{\bullet-}$ and a glutathionyl radical ($GS^{\bullet}$) generated from an oxidation of GSH by PGHS-2 (Eling et al. 1986), and (ii) Michael addition of 4-CB-2',5'-Q with GSH. Comparable to the comproportionation of 4-CB-2',5'-Q with the 4-CB-2',5'-H₂Q to form 4-CB-2',5'- $SQ^{\bullet-}$ (Song et al. 2008), the GS-4-CB-2',5'-Q and GS₂-4-CB-2',5'-Q undergo comproportionation to form GS-4-CB-2',5'- $SQ^{\bullet-}$ and GS₂-4-CB-2',5'- $SQ^{\bullet-}$ or undergo another hPGHS-catalyzed oxidation to the semiquinone metabolites of the GS-adducts.

To gain a deeper understanding of the observations, we investigated the reaction pathway by monitoring the formation of the adducts by oxygen consumption. The consumption of O₂ was conducted without an addition of hPGHS-2 in order to avoid an enzymatic oxidation of GSH to $GS^{\bullet}$ by hPGHS-2 that may distort the stoichiometry of the adduct formation. The consumption of oxygen was expected to result solely from the autoxidation of 4-CB-2',5'-H₂Q, and reoxidation of the GS-4-CB-2',5'-H₂Q adducts. Figure 4b depicts two major findings: (i) the increased O₂ consumption with increasing concentrations of GSH, and (ii) an increased lag phase with increasing GSH concentration.

The first stage of O₂ consumption began with autoxidation of 4-CB-2',5'-H₂Q. The second stage indicated adduct formation of 4-CB-2',5'-Q with GSH, a process that pulled the equilibrium to the right, thus expediting the consumption of O₂. Autoxidation of 4-CB-2',5'-H₂Q (50 μ M) consumed 22 μ mol of O₂ in Tris-HCl buffer pH 8, at 80 min. The oxidation of 4-CB-2',5'-H₂Q consumed 1 mol of O₂ for approximately 1.3, 1.4, and 1.7 mol of GSH in the presence of 50, 100, and 200 μ M GSH, respectively. The overall stoichiometry of this reaction could be explained by Scheme 1. The mono-SG adduct formed in this reaction may undergo further oxidation to form the di-SG adduct. However, LC-MS data revealed that mono-SG adducts were the major adducts formed in all incubations, approximately 97 % in the presence of 50 and 100 μ M GSH, and 90 % in the presence of 200 μ M GSH. Therefore,

the stoichiometry of 4-CB-2',5'-H₂Q oxidation is likely to be 1:1:1 ratio of 4-CB-2',5'-H₂Q/O₂/GSH as shown in Fig. 6. The rest of GSH consumed in the oxidation may have resulted from the oxidation of GSH by H₂O₂.

The increased lag phase with increasing concentration of GSH is due to the formation of GS-4-CB-2',5'-H₂Q adducts via the Michael addition of 4-CB-2',5'-Q with GSH. This reaction is favored, thus diminishing the concentration of 4-CB-2',5'-Q. The GS-4-CB-2',5'-H₂Q can undergo a second round of autooxidation and Michael addition with GSH, resulting in the formation of GS₂-4-CB-2',5'-H₂Q. Lower level of 4-CB-2',5'-Q or GS-4-CB-2',5'-Q leads to a lower concentration of 4-CB-2',5'-SQ^{•-} and GS-4-CB-2',5'-SQ^{•-} as a consequence of the comproportionation reaction with the 4-CB-2',5'-H₂Q or GS-4-CB-2',5'-H₂Q. The formation of 4-CB-2',5'-SQ^{•-} via the 4-CB-2',5'-Q²⁻ (dianion) is the rate limiting reaction since the concentration of the dianion is relatively low in equilibrium.

Amino acids

Similar to DNA, it was found that certain amino acids covalently bind with quinones (Kalyanaraman et al. 1987; O'Brien 1991; Bolton et al. 2000). Semiquinone radicals were also thought to be reactive toward nucleophilic moieties of proteins, resulting in the radical-mediated protein damage (Flowers-Geary et al. 1996). Five amino acids from each group, Met (non-polar, hydrophobic), Lys and His (polar, basic), and Gly and Cys (polar, uncharged), were chosen to investigate the adduct formation in this study. Addition of these amino acids, except for Cys, resulted in decreased intensities of 4-CB-2',5'-SQ^{•-} (Fig. 7a). At a first glance, these observations could indicate the adduct formation between 4-CB-2',5'-SQ^{•-}/Q and amino acids. However, the oxygen consumption in the presence of these amino acids was not different from the control (Fig. 7b). Hence, the nucleophilic addition of 4-CB-2',5'-SQ^{•-}/Q with these nitrogen-containing amino acids can be excluded. A study by Amaro et al. (1996) illustrated the adduct formation of 4-CB-2',5'-Q with amino acids at very slow rates for His ($k=0.75 \text{ min}^{-1} \text{ M}^{-1}$), Gly ($k=0.62 \text{ min}^{-1} \text{ M}^{-1}$), and Lys ($k=0.64 \text{ min}^{-1} \text{ M}^{-1}$) at pH 7.4, supporting our findings that the adduct formation between 4-CB-2',5'-SQ^{•-}/Q and these amino acids was unlikely within the limited incubation time (30 min).

The formation of 4-CB-2',5'-SQ^{•-} is pH dependent; true autooxidation is mediated via the dianion of the hydroquinone (Song and Buettner 2010). Individual amino acids appear to influence the formation of 4-CB-2',5'-SQ^{•-} differently based upon their isoelectric points (pI). The EPR signal intensity of correlates with the pI: Lys (pI 7.22), Gly (pI 6.07), Met (pI 5.73), and His (pI 5.68). A higher EPR signal intensity of the 4-CB-2',5'-SQ^{•-} corresponds to a higher isoelectric point of the amino acid, representing a lower acidity. Therefore, we can speculate that an increase in the level of amino acid cation assists in generating more of the dianion of the hydroquinone.

The incubation with Cys completely diminished the EPR signal of 4-CB-2',5'-SQ^{•-} (Fig. 7a). In addition, the consumption of O₂ increased in the incubation of 4-CB-2',5'-H₂Q with Cys (Fig. 7b). These findings indicate the formation of covalent adducts and were consistent with the previous experiment with GSH.

Conclusions

We found that (i) additions of SSDNA, and oligonucleotides (TTTT)₅, (AAAA)₅, (GGGG)₅, and (CCCC)₅ to the incubation of 4-CB-2',5'-H₂Q with hPGHS-2 resulted in an increase of the EPR signal intensity of 4-CB-2',5'-SQ[•], while the deoxynucleotides (dT, dA, dG, dC) did not change the intensity of 4-CB-2',5'-SQ[•]; (ii) SSDNA, oligonucleotides, and deoxynucleotides did not influence the oxygen consumption; and (iii) SSDNA, oligonucleotides, and the deoxynucleotides did not form covalent bonds with 4-CB-2',5'-SQ[•] observed by ¹H NMR and LC-MS.

In a previous study by Zhao et al. (2004), the covalent product of 4-CB-2',5'-Q with dG was found in an incubation of 4-CB-2',5'-Q in the presence of calf thymus DNA. These inconsistent sets of data could be explained by (i) the different experimental setup and reaction conditions, and/or (ii) non-covalent adducts formed with a high stability comparable with the covalent bond. These non-covalent adducts may have similar behavior to the covalent adducts in chromatographic separation. The latter supports our hypothesis that the 4-CB-2',5'-SQ[•] forms a non-covalent adduct by π -stacking or intercalation with the aromatic base pairs of DNA. The *in silico* LCAO modulations of the complexes between 4-CB-2',5'-SQ[•] and dinucleotides were calculated and revealed that there are energy gains from these complexes. The π -stacking interaction results in a delocalization and stabilization of the free electron of the radical and subsequently decreased ΔG that favors the guest-host complex formation.

The biological implications of these persistent guest-host complexes formed by PCB-semiquinones are unknown and cannot be demonstrated clearly at this moment. It is a new insight that stable radicals can be located within the DNA by guest-host complexations; the non-covalent intercalation of the radical may change the DNA structure as well, the implications of which are also unknown. The change of the reactivity of DNA due to the intercalation of SQ[•] with long half-lives is a focus of ongoing studies. Aside from the undesired damaging effects of these xenobiotics, this might provide intriguing insights into the role of biogenic SQ[•] formed by tyrosinase. Current studies in our laboratory investigate the effect on the epigenetics in the presence of semiquinones on the specific methylations of CpG sites. A better understanding of the interactions of intercalating radicals with the DNA at the molecular level will certainly shed new insights into the carcinogenesis of xenobiotics and may result in novel intercalation-based therapeutics.

Studies with biological compounds containing thiol function, such as GSH and CYS, showed the following: (i) GSH and Cys diminish the EPR signal of 4-CB-2',5'-SQ[•], (ii) ¹H NMR and LC-MS measurement confirmed that GSH and Cys form mono- and di-substituted but no tri-substituted analogues, and (iii) GSH and Cys show stoichiometry of 1:1:1 with oxygen and 4-CB-2',5'-H₂Q in the overall reaction. The oxidation of GSH to glutathione disulfide (GSSG) by H₂O₂ may play a role in additional consumption of oxygen. The lag phase of oxygen consumption in the presence of GSH also increased with increasing concentration of GSH. We could explain this finding on the basis of the “capturing” of the 4-CB-2',5'-SQ[•] formed at relatively low concentrations in the metal-free buffer via the dianion of the hydroquinone. The resulting 4-CB-2',5'-Q readily reacted with GSH and Cys

forming the covalent adducts of GS and Cys substituted 4-CB-2',5'-H₂Q. By shifting the chemical equilibrium, the concentration of 4-CB-2',5'-Q was too low to form sufficient 4-CB-2',5'-SQ^{•-} by comproportionation with 4-CB-2',5'-H₂Q or its GS-substituted. By this, the potential of GSH and Cys as the radical scavengers is more of an indirect nature depending on the chemical equilibrium. The generally accepted role of GSH as radical scavenger in biological systems should be reconsidered based upon these findings.

However, we could not confirm the semiquinone radical reaction with nucleophilic moieties of proteins. No change of the oxygen consumption compared to the control was observed in the presence of the amino acids from all selected groups: Met (non-polar, hydrophobic), Lys and His (polar, basic), and Gly and Cys (polar, uncharged), with the exception of Cys. The EPR investigations, on the other hand, showed a diminished 4-CB-2',5'-SQ^{•-} concentration. We explain that the lower EPR signal intensity of the 4-CB-2',5'-SQ^{•-} corresponds to a higher isoelectric point of the amino acid present in the solution, referring to a lower acidity, and higher concentration of the basic dianion.

The findings presented here could change the scientific view of radical interaction of semiquinones in general with biologically active compounds like DNA, amino acids, and proteins. The role of GSH as radical scavenger in the cell and the influence of persistent coplanar radicals intercalating in the DNA, and their potential effects on replication, catalysis of other reactions, or influence of epigenetic signaling need to be investigated in much more detail in future studies.

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Abbreviations

4-CB-2',5'-Q	4-Chlorobiphenyl-2',5'-benzoquinone
4-CB-2',5'-H₂Q	4-Chlorobiphenyl-2',5'-hydroquinone
4-CB-2',5'-SQ^{•-}	4-Chlorobiphenyl-2',5'-semiquinone radical
AA	Arachidonic acid
DMSO	Dimethyl sulfoxide
EPR	Electron paramagnetic resonance
GSH	Glutathione, reduced form
H₂Q	Hydroquinones
hPGHS-2	Human recombinant prostaglandin H synthase-2

KAA	Potassium arachidonate
LCAO	Linear combination of the atomic orbitals
PCBs	Polychlorinated biphenyls
Q	Quinones
ROS	Reactive oxygen species
SOMO	Semioccupied molecular orbital
SQ[•]	Semiquinone free radicals

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Fig. 1.

a EPR spectra of 4-CB-2',5'-SQ[•] formed in the incubation of 100 μM 4-CB-2',5'-H₂Q with 200 units of hPGHS-2 in the presence of salmon sperm DNA (SSDNA). **b** Oxygen consumption of the incubation of 50 μM 4-CB-2',5'-H₂Q with hPGHS-2 in the presence of SSDNA and GSH

Fig. 2.

a EPR spectra of 4-CB-2',5'-SQ•- formed in the incubation of 100 μM 4-CB-2',5'-H₂Q with 200 units of hPGHS-2 in the presence of 50 μM oligonucleotides (20 bases): (AAAA)₅, (CCCC)₅, (GGGG)₅, and (TTTT)₅. **b** EPR spectra of 4-CB-2',5'-SQ•- formed in the incubation of 100 μM 4-CB-2',5'-H₂Q with 200 units of hPGHS-2 in the presence of 100 μM deoxynucleosides: dA, dC, dG, and dT

Fig. 3.

Semiempirical computations of the semioccupied molecular orbitals (SOMO) of the complexes of 4-CB-2',5'-SQ^{•-} with the dinucleotides, d(ApA), d(GpG), d(TpT), and d(CpC), presenting as the optimized structures (*left*), and the closed orbital shell (*right*)

Fig. 4.

a EPR spectra of 4-CB-2',5'-SQ[•] formed in the incubation of 100 μM 4-CB-2',5'-H₂Q with 200 units of hPGHS-2 in the presence of GSH. **b** Oxygen consumption of the incubations of 50 μM 4-CB-2',5'-H₂Q in the presence of GSH (no hPGHS-2 added)

Fig. 5.
 ^1H NMR spectra of the adducts from incubations of 4-CB-2',5'-SQ $^{\bullet-}$ /Q with dG, and GSH. The 4-CB-2',5'-SQ $^{\bullet-}$ was generated from the comproportionation of an equal molar of 4-CB-2',5'-H $_2$ Q and 4-CB-2',5'-Q

Fig. 6.
Mechanism of the adduct formation of 4-CB-2',5'-Q and GSH

Fig. 7.

a EPR spectra of 4-CB-2',5'-SQ[•] formed in the incubation of 100 μM 4-CB-2',5'-H₂Q with 200 units of hPGHS-2 in the presence of 100 μM amino acids: Gly, glycine; Lys, lysine; Cys, cysteine; His, histidine; Met, methionine. **b** Oxygen consumption of the incubation of 50 μM 4-CB-2',5'-H₂Q with hPGHS-2 in the presence of amino acids