SHORT COMMUNICATION

Metabolism of phenylhydroquinone by prostaglandin (H) synthase: possible implications in o-phenylphenol carcinogenesis

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o-Phenylphenol (OPP) and its sodium salt sodium ortho-phenylphenate (NaOPP) are broad spectrum fungicides and antibacterial agents. Both are urinary bladder and renal carcinogens in the Fischer 344 rat. OPP is converted by mixed-function oxidases in the liver to phenylhydroquinone (PHQ). Since appreciable amounts of prostaglandin (H) synthase (PGS) are found in rat bladder and kidney-medullary papilla, the target sites of OPP- and NaOPP-induced tumors, we hypothesized that a secondary PGS-mediated activation of PHQ to phenylbenzoquinone (PBQ) may occur in the bladder and kidney. We have studied the metabolism of PHQ by PGS in the presence of arachidonic acid and hydrogen peroxide as co-factors. These studies showed that PHQ is indeed metabolized to a product having identical spectral and electrochemical properties to PBQ. The disappearance of PHQ with time was stoichiometric to the formation of PBQ. Less than 10% of PHQ was converted to PBQ in the absence of enzyme, indicating that auto-oxidation may play only a minor role in the conversion of PHQ to PBQ. Similar results were obtained when PGS was replaced with either myeloperoxidase or horseradish peroxidase and hydrogen peroxide as co-factor. These studies suggest that the peroxidative metabolism of PHQ by PGS to the reactive PBQ could play an important role in OPP-induced urinary bladder and kidney carcinogenesis in rats.

Due to their widespread use and potential for human exposure, extensive toxicological testing has been performed on both OPP and NaOPP. At high doses, a variety of acute toxic effects are observed, including hepatic and renal toxicity (3). Chronic studies in mice using both dietary and dermal exposures to OPP have been failed to detect treatment-related increases in neoplasia (2,4–6). However, OPP and NaOPP are potent carcinogens to the rat bladder and kidney. Hiraga and Fujii (7) reported increased frequencies of transitional cell carcinomas of the renal pelvis, renal papilla and urinary bladder in Fischer 344/DuCrj rats when NaOPP was administered in the diet. A number of follow-up studies have reported similar increased frequencies of urinary bladder tumors in Fischer rats following treatment with either OPP or NaOPP (8–11). The mechanisms involved in OPP- and NaOPP-induced bladder cancer, however, remain unclear.

Metabolic studies have shown that at doses approximating those causing tumorigenicity in rats (> 500 mg/kg), the conversion of OPP to phenylhydroquinone (PHQ; 2,5-dihydroxybiphenyl) occurs (12–14), a process that is mediated by the cytochrome P450 monoxygenases (15). PHQ can be further oxidized by hepatic microsomal enzymes to a protein-binding species, presumably 2-phenyl-1,4-benzoquinone (PBQ) (12,14). PBQ has also been observed in trace amounts in the urine (13).

The mechanisms involved in OPP toxicity and carcinogenicity may be similar to those involved in the myelotoxicity and leukomogenesis of benzene (16). Recent studies in our laboratory have shown that myelotoxicity of benzene is most likely mediated by a two-step process (17,18). The formation of phenolic metabolites occurs within the liver by cytochrome P450, followed by the transport of these phenolic metabolites to bone marrow, where they are activated to toxic quinones by peroxidase enzymes. In the case of OPP it is likely that it is being converted into PHQ in the liver (12,14) and a secondary peroxidase-mediated activation probably occurs in the kidney and urinary bladder of the rats.

Prostaglandin (H) synthase (PGS) is present in many mammalian cells and is present at high levels in platelets, lungs, kidney medulla and urinary bladder (19–23). The majority of the PGS in urinary bladder appears to be localized in the transitional epithelium. A broad spectrum of xenobiotics has been known to be metabolized by peroxidase activity of PGS, including aromatic amines and nitrofurans, which are carcinogenic to the bladder (20,21,23–26). Similarly nephrotoxicity induced by acetaminophen and phenacetin has been attributed to the metabolism of these compounds by kidney medullary PGS (27,28). Furthermore, several phenolic compounds have been shown to act as reducing co-substrates for the peroxidase activity of PGS (29,30). We propose that the PGS found in the rat urinary bladder transitional epithelium and kidney medullary papilla, the target sites of OPP and NaOPP, is responsible for the activation of PHQ to reactive intermediates in the bladder and the kidney.

To test this hypothesis we have studied whether or not PHQ can be metabolized by peroxidase activity of PGS using arachidonic acid and hydrogen peroxide as co-factors. We have also investigated whether other peroxidases such as myeloperoxidase (MPO) and horseradish peroxidase (HRP) can carry out a similar reaction.

Phenol, PHQ and PBQ were purchased from Aldrich Chemical Co. (Milwaukee, WI). Arachidonic acid (AA), OPP, glutathione, indomethacin, H2O2 and HRP (type VI) were purchased from Sigma Chemical Company (St Louis, MO). PBS was purchased from Oxford Biomedical Research Inc. (Oxford, MI). Human MPO was purchased from Calbiochem Corporation (La Jolla, CA). Activities of MPO and PBS were determined prior to each
Quinones are usually the major reactive products formed during the oxidation of a variety of monohydric and polyhydric phenols. Most quinones are yellow in color and can be detected spectrophotometrically due to their ability to absorb strongly in the visible region (usually between 350 and 400 nm). We therefore examined the spectral changes of PHQ following incubation with PGS and H$_2$O$_2$ (Figure 1). PHQ (0.2 mM) had an optical density (OD) of 1.06 at its absorption maximum, 300 nm. In the presence of PGS and H$_2$O$_2$, the OD of PHQ at 300 nm decreased considerably in a time-dependent fashion and the absorbance maximum shifted slightly to 290 nm (OD at 15 min: 0.88). During this time, the OD between 350 and 400 nm increased and an additional peak appeared with an absorbance maximum of 371 nm which increased with time. In the absence of PGS, only a small decrease in OD of PHQ at 300 nm and a corresponding small increase in OD at 371 nm was observed, which is presumably due to auto-oxidation of PHQ. These results therefore show that the peroxidase activity of PGS metabolizes PHQ to a quinone-like product. If PHQ is solely converted to PBQ, then one would expect an isobestic point during the formation of PBQ from PHQ. The lack of such an isobestic point, however, indicated that there may be other minor oxidation products of PHQ. One would expect this because peroxidatic oxidation of hydroquinones to corresponding quinones is generally believed to occur through a semiquinone intermediate.

The apparatus employed in the HPLC studies consisted of a Beckman (model 100 A) pump and an amperometric detector (Bioanalytical Systems LC-4A) equipped with glassy carbon working electrode (BAS-TL5A) and Ag/AgCl reference electrode, a Rheodyne injector (model 7125) with a 100 /l injection loop and a Supelco C-18 column (5 /im; 25 cm X 4.6 mm). The mobile phase for the analysis of PHQ consisted of 0.1 M ammonium acetate (pH 4) containing 20% acetonitrile at a flow-rate of 2 ml/min. The mobile phase for the analysis of PBQ consisted of 0.1 M ammonium acetate (pH 4) containing 40% acetonitrile at a flow-rate of 2 ml/min. The mobile phase was purged with nitrogen gas (to displace any dissolved oxygen). The operating potential for PHQ and PBQ was determined by plotting the hydrodynamic voltammograms (HDV) as described previously for other phenolic and quinone compounds (32). The HDV for PHQ was determined by serial injections of standard PHQ over a range of +0.2 to +1.0 V using the oxidative mode of the electrochemical detector versus an Ag/AgCl reference electrode. Similarly, the HDV for PBQ was determined by serial injection of standard PBQ over a range of 0.0 to −0.4 V using the reductive mode of electrochemical detector. Each relative current ratio $\Phi$, was calculated as the ratio of the current produced (peak height) at a given potential to the diffusion-limited plateau current (maximum peak height). PHQ had a maximum response at +0.9 V and +1.0 V, whereas PBQ had a maximum response at −0.3 V. We employed +0.9 V and −0.3 V as the working potentials for PHQ and PBQ in our experiments. We have also determined the HDV for the oxidation product formed during PGS-catalyzed PHQ metabolism, which eluted with a similar retention time to that of PBQ. The oxidation product of PHQ has identical electrochemical properties to that of PBQ, indicating that PHQ is metabolized to PBQ by PGS and AA (data not shown).

PGS possesses two distinct enzymatic activities: the cyclooxygenase activity converts AA to the 15-hydroperoxy-9,11-endoperoxide (PGG$_2$), while the peroxidase activity reduces
the hydroperoxy group of PGG_{2} to the corresponding 15-hydroperoxy-9,11-endoperoxide (PGH_{2}) (33–36). Our results show that PGS-dependent metabolism \textit{in vitro} occurs readily in the presence of AA, resulting in the formation of PBQ as the major metabolite (Figure 2). Metabolism of PGS-dependent PHQ metabolism also occurs in the presence of H_{2}O_{2} (Figure 3), indicating that PHQ metabolism by PGS is peroxidative in nature.

Figure 2(A) shows the metabolism of PHQ (0.2 mM) mediated by PGS in the presence of AA. A linear rate of PHQ removal was seen for at least 2 min. Approximately 22% of PHQ was metabolized within 5 min. Further incubation for up to 10 min resulted in little increase in PHQ removal. The rate of auto-oxidation of PHQ in the absence of enzyme is minimal when compared to that PHQ metabolized in the presence of enzyme. Figure 2(B) shows PBQ formation during the metabolism of PHQ by PGS. It can be seen that a stoichiometric conversion of PHQ to PBQ by PGS occurred in the presence of AA.

Figure 3(A) illustrates the time course for the metabolism of PHQ (0.2 mM) by PGS (100 U) in the presence of H_{2}O_{2} (0.2 mM). The disappearance of PHQ was linear for the first 2 min and only minor increases in PHQ removal occurred upon further incubation. Approximately 33% of PHQ was metabolized within 2 min. There was practically no removal of PHQ in the absence of enzyme in the same time period (2 min). The metabolism of PHQ by PGS in the presence of H_{2}O_{2} is slightly faster and higher when compared to the metabolism of PHQ by PGS in the presence of AA. This was expected, since the added AA first has to be metabolized to its endoperoxide derivative PGG_{2} by the cyclo-oxygenase activity of PGS.

Figure 3(B) shows PBQ formation during PHQ metabolism by PGS and H_{2}O_{2} under conditions identical to those described for Figure 3(A). About 55 ± 8 nmol of PBQ was formed within 2 min. When compared to the amount of PHQ removed (66 ± 8 nmol; Figure 3A) at the same time point under identical conditions, it is evident that most of the PHQ metabolized is stoichiometrically converted to PBQ.

Table I shows the effect of various modulators on the PHQ metabolism to PBQ by PGS and AA in order to determine the participation of the active enzyme in the process of metabolism. In order to determine this we used indomethacin as a specific inhibitor of cyclooxygenase activity of PGS. Addition of indomethacin (10 \textmu M) to the incubation mixture 30 s prior to the addition of AA resulted in ~ 75% inhibition of PBQ formation mediated by PGS. We have also tested the ability of quinone scavenger glutathione to inhibit PBQ formation from PHQ metabolized by PGS. A complete disappearance (100%) of PBQ was noted in the presence of GSH (0.2 mM). This appears to

<table>
<thead>
<tr>
<th>Test agent</th>
<th>PBQ formed (nmol)</th>
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<tbody>
<tr>
<td>None</td>
<td>45</td>
</tr>
<tr>
<td>Glutathione (0.2 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Indomethacin (0.01 mM)</td>
<td>12</td>
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Incubation conditions were the same as described in the text except that the test agents were added to the reaction mixtures 30 s before the addition of AA. The reactions were performed for 5 min.

The given values have been adjusted for auto-oxidation in the absence of enzyme.
be due to the conjugation of GSH with PBQ since GSH had little effect on PHQ removal by PGS and AA (data not shown). In support of this we detected the appearance of a minimum of two and a maximum of five additional peaks, depending upon incubation conditions during the metabolism of PHQ by PGS under oxidative mode conditions described above for PHQ (data not shown).

We next determined whether other peroxidases, such as MPO and HRP, are capable of metabolizing PHQ. Figure 4 shows that MPO is capable of metabolizing PHQ in the presence of H$_2$O$_2$ stoichiometrically to PBQ. Similar results were obtained when MPO was replaced with HRP (data not shown). These results thus indicate that the mechanism of PHQ metabolism to PBQ by these three peroxidases (PGS, HRP and MPO) may be similar and that the major metabolite formed is PBQ. This is consistent with reports of other polyhydroxyphenols which are co-oxidized by peroxidases (22, 27, 29, 30) and suggests that PHQ is metabolized by higher oxidation states of PGS and other peroxidases, presumably by acting as a reducing co-factor.

In summary, we have demonstrated that PHQ, the primary metabolite of OPP, is metabolized by the peroxidase activity of PGS, an enzyme shown to be present in urinary bladder and kidney medulla. Our results also show that PHQ is metabolized by other peroxidases, including HRP and MPO. These results suggest that PGS-dependent PHQ metabolism is a potential pathway for the generation of PBQ in urinary bladder and kidney whether OPP or its sodium salt is administered. Morimoto et al. (37) recently demonstrated that a single intravesical administration of PBQ into urinary bladder of F344 rats results in DNA damage and bladder epithelial hyperplasia. We therefore propose that PGS-dependent metabolism of PHQ to PBQ is an important pathway in producing the genetic and cellular damage necessary for OPP- and NaOPP-induced urinary bladder and kidney carcinogenesis.

Acknowledgements

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Co-oxidation of phenylhydroquinone by prostaglandin synthase


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