Free Rad. Res. Comms., Vol. 15, No. 5, pp. 285–296
Reprints available directly from the publisher
Photocopying permitted by license only

HYDROXYLATION OF PHENOL TO HYDROQUINONE CATALYZED BY A HUMAN MYELOPEROXIDASE-SUPEROXIDE COMPLEX: POSSIBLE IMPLICATIONS IN BENZENE-INDUCED MYELOTOXICITY

VANGALA V. SUBRAHMANYAM, PREMA KOLACHANA and MARTYN T. SMITH†

Department of Biomedical and Environmental Health Sciences, School of Public Health, University of California at Berkeley, Berkeley, CA 94720, USA

(*Received July 5th, 1991, in revised form August 21st, 1991*)

Benzene, a known human myelotoxin and leukemogen is metabolized by liver cytochrome P-450 monooxygenase to phenol. Further hydroxylation of phenol by cytochrome P-450 monooxygenase results in the formation of mainly hydroquinone, which accumulates in the bone marrow. Bone marrow contains high levels of myeloperoxidase. Here we report that phenol hydroxylation to hydroquinone is also catalyzed by human myeloperoxidase in the presence of a superoxide anion radical generating system, hypoxanthine and xanthine oxidase. No hydroquinone formation was detected in the absence of myeloperoxidase. At low concentrations superoxide dismutase stimulated, but at high concentrations inhibited, the conversion of phenol to hydroquinone. The inhibitory effect at high superoxide dismutase concentrations indicates that the active hydroxylating species of myeloperoxidase is not derived from its interaction with hydrogen peroxide. Furthermore, catalase a hydrogen peroxide scavenger, was found to have no significant effect on hydroxylation of phenol to hydroquinone, supporting the lack of hydrogen peroxide involvement. Mannitol (a hydroxyl radical scavenger) was found to have no inhibitory effect, but histidine (a singlet oxygen scavenger) inhibited hydroquinone formation. Based on these results we postulate that a myeloperoxidase-superoxide complex spontaneously rearranges to generate singlet oxygen and that this singlet oxygen is responsible for phenol hydroxylation to hydroquinone. These results also suggest that myeloperoxidase dependent hydroquinone formation could play a role in the production and accumulation of hydroquinone in bone marrow, the target organ of benzene-induced myelotoxicity.

KEY WORDS: Benzene, hydroquinone, myeloperoxidase, phenol, singlet oxygen, superoxide anion radial.

ABBREVIATIONS: CAT, Catechol; H_2O_2, Hydrogen peroxide; $HO^{\cdot}$, Hydroxyl radical; HQ, Hydroquinone; HRP, Horseradish peroxidase; HX, Hypoxanthine; MPO, Myeloperoxidase; $O_2^{\cdot -}$, Superoxide anion radical; $^1O_2$ = Singlet oxygen; XO, Xanthine oxidase.

INTRODUCTION

The hematotoxic and leukemogenic effects of benzene are believed to occur following its metabolism to one or more reactive species.[1-3] However, the exact site(s) of benzene metabolism and the nature of the enzymes involved are not clear. Several investigators

†To whom correspondence should be addressed at: Department of Biomedical and Environmental Health Sciences, School of Public Health, University of California, Berkeley, CA-94720, USA. Tel: (510)-642-8770, Fax: (510)-642-5815.

believe that the liver is the major site of benzene metabolism.[4-7] The hepatic metabolism of benzene has been shown to be mediated by the cytochrome P-450 monooxygenase (a hemeprotein enzyme and specifically cytochrome P-450 IIE1), which is present at high concentrations in this organ.[8,9] Epoxidation of benzene is believed to be the first step in benzene metabolism by cytochrome P-450.[10] Benzene epoxide rapidly undergoes rearrangement to phenol. Cytochrome P-450 has also been shown to hydroxylate phenol[11,12] to hydroquinone (HQ) and catechol (CAT) in a ratio of 20 : 1.[12] Benzene epoxide may also be hydrated by epoxide hydrolase to form benzene dihydrodiol.[13,14] Subsequent action of benzene dihydrodiol dehydrogenase may result in the formation of CAT.[14]

Phenol, HQ and CAT have been detected (concentrations ranging from 0.01 to 0.1 mM) in bone marrow following benzene administration to laboratory animals.[15] Bone marrow also contains some cytochrome P-450, which is believed to contribute to some extent to the formation of phenolic metabolites from benzene in bone marrow.[16-18] It is not known, however, whether enzyme(s) other than cytochrome P-450 can contribute to the formation of phenolic metabolites from benzene.

Myeloperoxidase (MPO; donor: hydrogen peroxide oxidoreductase, E.C. 1.11.1.7) another hemeprotein enzyme is present in high concentrations in the neutrophils of blood and bone marrow.[19,20] This enzyme has been shown to metabolize benzene's phenolic metabolites to reactive metabolites[3,21-23] in the presence of hydrogen peroxide (H_2O_2) as cofactor. Phenol metabolism by human MPO or horseradish peroxidase (HRP) results in the formation of dimeric metabolites 2,2′-biphenol and 4,4′-biphenol.[21,24-26] No HQ or CAT formation occurred in this system.[26] However, some investigators[27-31] found that phenol hydroxylation to HQ and CAT can occur by HRP in the presence of dihydroxyfumaric acid (DHFA) as cofactor. Dordick *et al.*[31] proposed that this was due to the complexation of superoxide anion radical ($O_2^{\cdot -}$) generated during the autooxidation of DHFA with ferric heme of HRP. The resultant $O_2^{\cdot -}$-complex of HRP (also known as oxyperoxidase or compound III) decays to the native enzyme releasing hydroxyl radicals ($HO^{\cdot}$). These authors proposed that either $HO^{\cdot}$ or oxidants with similar reactivity are involved in phenol hydroxylation in the DHFA-HRP system. Myeloperoxidase has also been shown to form compound III in the presence of $O_2^{\cdot -}$ in purified form[32] and in intact polymorphonuclear leukocytes.[33] Here we report that human MPO, in the presence of hypoxanthine (HX) and xanthine oxidase (XO), a $O_2^{\cdot -}$ generating system,[34] catalyzes the hydroxylation of phenol to HQ. The possible role of this hydroxylation in benzene metabolism and toxicity is discussed.

MATERIALS AND METHODS

Reagents

Phenol, HQ, CAT, HX, xanthine, H_2O_2 (30%), superoxide dismutase (SOD) and XO were purchased from Sigma Chemical Company (St. Louis, MO). Human MPO isolated from polymorphonuclear leukocytes, with a stated purity of 98% was purchased from Calbiochem (La Jolla, CA). Activity of MPO was determined prior to each experiment using the guaiacol method described by Klebanoff *et al.*[35] One unit (U) of MPO is defined as the amount of enzyme that will decompose 1 μmol of H_2O_2 per minute at 25 °C, pH 7. All other chemicals or solvents used were of the highest grade commercially available.

Preparation of stock solutions

Phenol (100 mM stock) and mannitol (0.5 M stock) were prepared in deionized distilled H_2O and sodium phosphate buffer (0.1 M; pH 7.4) respectively. Histidine (0.5 M stock) and HX (50 mM stock) were prepared by dissolving initially in an aliquot of sodium hydroxide (1 M) and then made up to their respective stock concentrations by the addition of sodium phosphate buffer (0.1 M; pH 7.4). Stock solutions of all enzymes were prepared in sodium phosphate buffer (0.1 M; pH 7.4) and 20 μl aliquots were added to the incubation mixtures to give their final concentrations respectively, wherever indicated.

Incubations and analysis

A typical reaction mixture consisted of phenol (1 mM), HX (1 mM) and MPO (0.1 U) in 1 ml of sodium phosphate buffer (0.1 M; pH 7.4). Reactions were initiated with XO (50 mU) and incubated up to 1 h at 37 °C. Reactions were terminated with 100 μl of 70% of perchloric acid. Aliquots of incubation mixtures were analyzed for HQ formation by HPLC using amperometric detection as described previously.[21,23]

Spectral studies

Optical absorption spectra were recorded by using a Perkin-Elmer (PE) Lambda 38 UV/vis spectrophotometer connected to PE 3600 data station and PE 660 printer.

RESULTS

Early studies by Mason *et al.*[27] and more recent ones by Dordick *et al.*[31] have shown that DHFA and HRP-catalyzed phenol hydroxylation produces HQ and CAT. We, therefore, investigated whether or not these two metabolites were formed following incubation of phenol with HX/XO/MPO. Figure 1 shows the time course for the formation of HQ and CAT in these incubations. HQ formation (Figure 1A) was entirely dependent on the presence of MPO, but CAT formation (Figure 1B) was independent of MPO. Both the formation of HQ and CAT was inhibited in the absence of either HX or XO indicating that at least $O_2^{\cdot -}$ and/or H_2O_2 formation is required for the hydroxylation of phenol to HQ and CAT. Because MPO is also required for the hydroxylation of phenol to HQ, it is clear that higher oxidation states of MPO formed from the complexation with $O_2^{\cdot -}$ or H_2O_2 are involved in this hydroxylation.

Next the effects of various scavengers of active oxygen species, such as SOD, catalase, mannitol and histidine, on the HX/XO/MPO-catalyzed hydroxylation of phenol to HQ were studied. As shown in Table I, low concentrations of SOD (20 U and 50 U) significantly stimulated the HX/XO/MPO-catalyzed hydroxylation of phenol to HQ (by approximately 5 and 30% respectively). However, a higher concentration of SOD (125 U), inhibited HX/XO/MPO-mediated hydroxylation of phenol to HQ by approximately 50%. Large amounts of catalase (12 000 U) had no significant effect, indicating that H_2O_2 is not involved in the HX/XO/MPO-mediated phenol hydroxylation. This data suggested that the higher oxidation states of MPO involved in phenol hydroxylation may be formed from a direct reaction of $O_2^{\cdot -}$ with MPO but not from a reaction involving H_2O_2. This suggested that compound III is

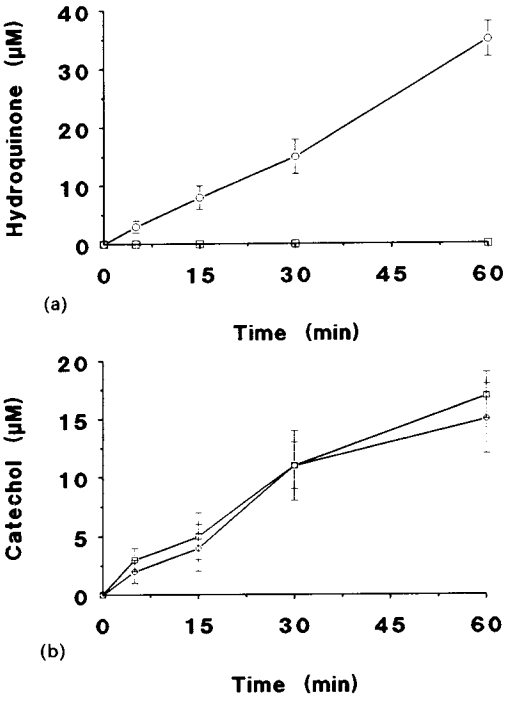

FIGURE 1 Time course for HX/XO/MPO-mediated hydroxylation of phenol to HQ and CAT. See Materials and methods for exact incubation conditions.

(*A*): *HQ formation*: ○ = +MPO; □ = −MPO.
(*B*): *CAT formation*: ○ = +MPO; □ = −MPO.

probably the active species involved in phenol hydroxylation. Table I also shows that mannitol (50 mM), a $HO^{\cdot}$ scavenger,[36] did not have any inhibitory effect on the hydroxylation of phenol by HX, XO and MPO indicating that $HO^{\cdot}$ is unlikely to be the active hydroxylating species. On the other hand, histidine (50 mM), a singlet oxygen ($^1O_2$) scavenger,[36] inhibited the HX/XO/MPO-dependent phenol hydroxylation by > 90% indicating that the active oxygen species involved in phenol hydroxylation to HQ may be $^1O_2$. Lower concentrations of histidine (0.1–10 mM), however, were found to have no significant effect on HX/XO/MPO-catalyzed hydroxylation of phenol to HQ, indicating that histidine is not simply acting as a substrate for compound III of MPO.

Figure 2 shows that phenol hydroxylation to HQ by the HX/XO/MPO system can occur at significant rates even when the phenol concentration is lowered considerably to toxicologically relevant levels. Thus, when 100 μM phenol was used at least 5 to 10 μM HQ could be detected in our incubations. This indicates that the MPO-dependent hydroxylation of phenol to HQ in bone marrow may play a significant role in HQ accumulation in bone marrow, the target organ for benzene toxicity.

In order to obtain additional evidence for a definite role of MPO compound III involvement in phenol hydroxylation to HQ, we determined the effect of phenol on spectral changes, in the Soret region, of native human MPO incubated with HX and

TABLE I
Human myeloperoxidase-catalyzed phenol hydroxylation to hydroquinone: Effect of various scavengers of active oxygen species

Scavenger	Hydroquinone (μM)
Complete[a]	36 ± 3[b]
+ Superoxide dismutase (20 U)	41 ± 3[c]
(50 U)	49 ± 2[c]
(125 U)	16 ± 2[c]
+ Catalase (12 000 U)	34 ± 4[d]
+ Mannitol (50 mM)	43 ± 2[c]
+ Histidine (50 mM)	4 ± 2[c]

[a] Complete system consisted of phenol (1 mM), HX (1 mM), MPO (0.1 U) and XO (50 mU) in 1 ml of sodium phosphate buffer (0.1 M; pH 7.4) and incubated at 37 °C for 1 h. Appropriate scavengers were added before the addition of XO. The reactions were terminated with 100 μl of 70% perchloric acid and analyzed for HQ by HPLC as described in Materials and Methods.

[b] Mean ± SE of five experiments.

[c] Significantly different from complete system at $P < 0.05$; P values were determined using two-tailed Student's t test.

[d] Not different from complete system $P > 0.05$.

XO. The Soret absorption maximum of native human MPO was found to be 430 nm in accordance with previous observations.[33,37] These studies have shown that addition of xanthine and XO to MPO results in a rapid disappearance of the 430 nm peak of the native enzyme, with the simultaneous-appearance of a peak at 450 nm, corresponding to compound III.[33,37] Figure 3 shows that a similar spectral change is observed when human MPO is incubated with HX and XO. Maximal compound III formation occurred within 3 min after addition of XO (spectrum 3; Figure 3). Addition of phenol to the reaction mixture resulted in an immediate decay of compound III to native enzyme (spectrum 4; Figure 3), which is fully recovered. If phenol

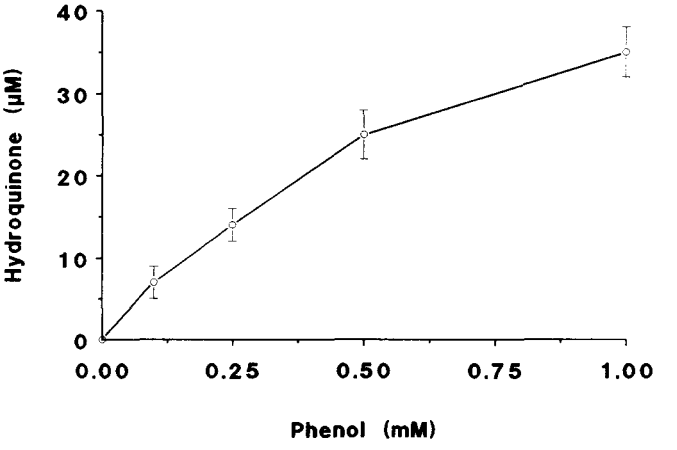

FIGURE 2 Effect of phenol concentration on HX/XO/MPO-dependent phenol hydroxylation. The amount of hydroquinone formed (y-axis) at different phenol concentrations (x-axis) is shown. See Materials and Methods for exact incubation conditions.

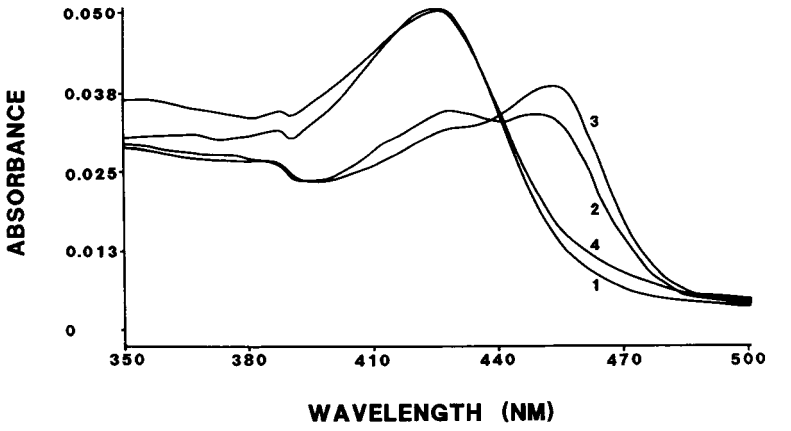

FIGURE 3 Effect of phenol on the stability of MPO-Compound III. Spectrum #1 in the figure is of native human MPO (0.5 U) in 1 ml of sodium phosphate buffer (0.1 M; pH 7.4). Compound III was then prepared by adding HX (0.5 mM) and XO (1.2 mU) to MPO. Two repetitive scans (spectra #2 and 3; time: 80 s each) were taken, at the time which maximal compound III formation occurred. Addition of phenol to the reaction mixture, at this point, resulted in an immediate complete conversion of compound III back to native enzyme (spectrum #4).

was not added, decay of compound III to native enzyme was slower (1 h) and only 50% of the native enzyme was recovered maximally. This indicated that compound III formation is followed by an inactivation of the enzyme, but the presence of phenol protects the enzyme from inactivation because the reactive oxygen, generated during the decay of compound III, is incorporated into phenol.

Further evidence for inactivation of MPO was obtained when HQ formation was determined while adding phenol after MPO had been incubated with HX and XO. Figure 4 shows that the hydroxylating ability of MPO under these conditions decreased and was completely inhibited after 15 min. These results when compared with the results shown in Figure 1A, where HQ formation was linear for at least 60 min, indicate that the active oxygen complex of MPO was turned over continuously while hydroxylating phenol. In the absence of phenol, rapid inactivation of the enzyme occurs due to its reaction with reactive oxygen species generated during the decay of compound III.

DISCUSSION

Peroxidases catalyze the oxidation of a wide variety of compounds by H_2O_2. Early work by Chance[38] and George[39] with HRP established that the reaction proceeds by the general mechanism:

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I}$$

$$\text{Compound I} + RH_2 \longrightarrow \text{Compound II} + RH^{\cdot}$$

$$\text{Compound II} + RH_2 \longrightarrow \text{Peroxidase} + RH^{\cdot}$$

where peroxidase represents the native ferric enzyme, Compounds I and II are the

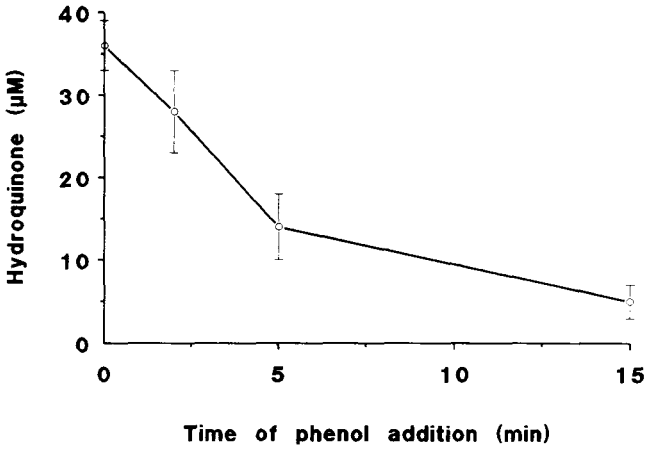

FIGURE 4 Inactivation of MPO during its incubation with HX and XO: Effect of pre-incubation of MPO with HX and XO on HQ formation from phenol. MPO (0.1 U) was pre-incubated with HX (1 mM) and XO (50 mU) in 1 ml of sodium phosphate buffer (0.1 M; pH 7.4) at 37 °C for various time periods as indicated in the figure. Phenol (1 mM) was then added to these reaction mixtures and further incubated for 1 h. The reactions were terminated with 100 μl 70% perchloric acid and HQ formation was analyzed by HPLC as described in Materials and Methods.

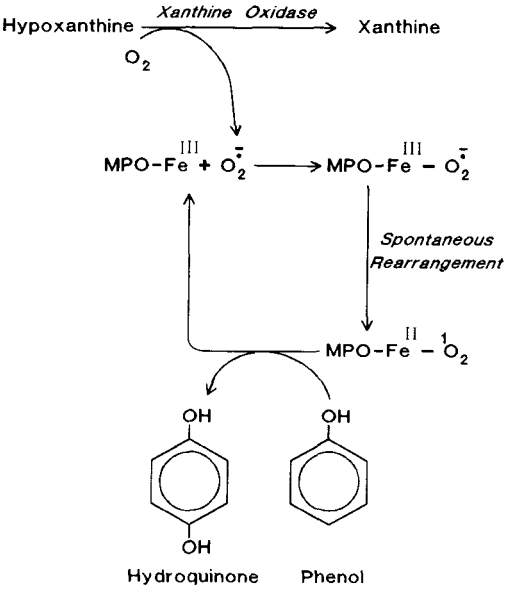

FIGURE 5 Scheme for the postulated mechanism of HX/XO/MPO-mediated phenol hydroxylation to HQ.

oxidized forms of the enzyme, RH_2 the reducing substrate and $RH^{\cdot}$ the substrate derived free radical. The free radicals derived from organic compounds dismutate or dimerize.[40] Similarly, phenol oxidation by HRP/H_2O_2 or MPO/H_2O_2 results in the formation of dimers and polymers.[21,26]

A third peroxidase derivative called compound III (oxyperoxidase) formation was first reported by Keilin and Mann[41] in the reaction of HRP with a large excess of H_2O_2. Subsequent studies[42–44] demonstrated that compound II is a necessary intermediate in the formation of compound III of peroxidase in the presence of H_2O_2. A direct formation of compound III can also occur from native ferric peroxidase by reaction with $O_2^{\cdot -}$. Mason *et al.*[28] and Dordick *et al.*[31] demonstrated that $O_2^{\cdot -}$ generated by the autooxidation of DHFA binds with HRP and results in the formation of compound III. Compound III generated by incubation of DHFA with HRP can react with a variety of phenolic compounds including phenol.[27–31,45,46] These studies have shown that phenol is hydroxylated to HQ and CAT by HRP-compound III. Here we report that a similar hydroxylation of phenol to HQ can occur when phenol is incubated with HX and XO, another $O_2^{\cdot -}$ generating system, in the presence of MPO. No HQ formation occurred in the absence of MPO. Catechol was also formed, but this was not dependent on the presence of MPO. Spectral studies demonstrated that MPO-compound III was involved in the hydroxylation of phenol to HQ. We suggest that this HQ formation from phenol by MPO-compound III (MPO-$O_2^{\cdot -}$ complex) may play an important role in benzene myelotoxicity.

Phenol, HQ and CAT accumulate in the bone marrow of experimental animals following benzene administration.[18] The accumulation of phenol in bone marrow is transient, disappearing within a few hours, but HQ and CAT persist for at least 9 h after a single dose of benzene. These observations coupled with the results presented in this manuscript indicate that phenol could be metabolized in bone marrow to HQ by a MPO-$O_2^{\cdot -}$ complex. However, our study does not explain the mechanism of CAT accumulation in bone marrow, which may be catalyzed by anon-protein bound free iron-dependent Fenton reaction.

Our results also show that SOD at high concentrations inhibits HX/XO/MPO-mediated phenol hydroxylation to HQ, although it increases the hydroxylation at lower SOD concentrations. SOD was expected to increase the dismutation of $O_2^{\cdot -}$ to H_2O_2.[34] However, recent studies by Marquez and Dunford[37] showed that DHFA-dependent MPO-compound III formation is not inhibited by low concentrations of SOD indicating that MPO has a higher affinity for $O_2^{\cdot -}$ than SOD. But at higher concentrations SOD can effectively compete with MPO for $O_2^{\cdot -}$ and increase its dismutation to H_2O_2. Since high SOD concentration results in the inhibition of HX/XO/MPO-dependent phenol hydroxylation, it is unlikely that H_2O_2 is involved in this hydroxylation. This is also supported by the fact that catalase, which decomposes H_2O_2, had no significant effect on HX/XO/MPO-mediated hydroxylation of phenol to HQ. Furthermore, we did not detect any HQ or CAT formation, by a direct incubation of phenol with MPO and H_2O_2 (unpublished observations). In addition, incubation of MPO and phenol with glucose and glucose oxidase a system which generates H_2O_2 by a direct two-electron reduction of dioxygen without the intermediate $O_2^{\cdot -}$ formation,[47] also does not result in phenol hydroxylation (unpublished observations).

The Compound III of peroxidase can be generated by incubation of peroxidase with excess H_2O_2.[42–44] However, no HQ or CAT formation occurred when phenol was incubated with MPO and excess H_2O_2. The reason for this may be due to the fact that

compound III formation in this system occurs via compound II.[42–44] Since phenol can readily donate electrons to compound II and in the process be oxidized to dimers and other polymeric products,[21,24–26] it would be expected that compound III formation is prevented in MPO/H_2O_2 system in the presence of phenol. We also found that phenol hydroxylation does not occur even if phenol was added after compound III formation by treatment of MPO with a large excess of H_2O_2. It is possible that the decay of MPO compound III formed in the presence of H_2O_2 could be more rapid than the decay of compound III formed in the presence of HX and XO, resulting in the inactivation of the enzyme.

It is unlikely that MPO-$O_2^{\cdot -}$ complex can directly hydroxylate phenol to HQ since low concentrations of SOD significantly stimulated the HX/XO/MPO-mediated phenol hydroxylation. The recent work of Marquez and Dunford,[37] which shows that SOD enhances the rate of decay of MPO compound III to native ferric peroxidase, may be relevant in this regard. It is, therefore, possible that compound III may undergo rearrangements to the active hydroxylating species and that SOD enhances the rate of this rearrangement. However, at higher concentrations, SOD can effectively compete with MPO for $O_2^{\cdot -}$ and prevent the formation of MPO-$O_2^{\cdot -}$ complex.[37] This may explain why SOD at lower concentrations stimulates but inhibits at higher concentrations, the HX/XO/MPO-dependent phenol hydroxylation to HQ.

Early studies performed by Mason *et al.*[27] indicated that DHFA/HRP-dependent hydroxylation of phenol results in both HQ and CAT formation. Later studies by Dordick *et al.*[31] confirmed this and further showed that mannitol, a $HO^{\cdot}$ scavenger, significantly inhibits phenol hydroxylation. However, the results presented in this manuscript show that mannitol does not inhibit HX/XO/MPO-mediated phenol hydroxylation to HQ. Similar results were obtained with HX/XO/HRP indicating that the effect of mannitol was not specific to the type of peroxidase used. This indicated to us that the mechanism of aromatic hydroxylation catalyzed by peroxidases in the presence of DHFA is different from that catalyzed in the presence of HX and XO. In addition, studies by Dordick *et al.*[31] indicate that DHFA itself may reduce compound III, thereby complicating the analysis of the reaction mechanism. Hydroquinone formation from phenol may, therefore, occur via other active oxygen species. Our data shows that histidine, a $^1O_2$ scavenger, effectively inhibits phenol hydroxylation in the HX/XO/MPO system, indicating that $^1O_2$ may be involved in phenol hydroxylation to HQ. Imidazole containing compounds, including histidine, have also been proposed to alter the interactions of higher oxidation states of peroxidase heme-iron with the surrounding apoprotein, due to their hydrogen bonding properties with the amino acids distal to the heme-iron.[23,48] However, histidine was also found to inhibit phenol hydroxylation by HX/XO/Fe^{2+}-EDTA or HX/XO/Fe^{3+}-EDTA (unpublished observations). Although HX/XO/iron-EDTA systems are known to generate $HO^{\cdot}$,[49,50] formation of other active oxidants may also occur.[51] Therefore, the inhibitory effects of histidine on MPO-dependent phenol hydroxylation to HQ are unlikely to be due to its interactions with the polar environment surrounding the heme-prosthetic group. Another possibility is that histidine could serve as a substrate for compound III of MPO, but this appears unlikely since inhibition of phenol hydroxylation to HQ occurred with only very high concentrations of histidine (50 mM) and lower concentrations (0.1 mM to 10 mM) had no significant effect. Since, $^1O_2$ scavenging effects of histidine are usually observed at high concentrations, it is likely that histidine is inhibiting HX/XO/MPO-dependent phenol hydroxylation by scavenging the $^1O_2$ generated from this reaction.

In summary, our results demonstrate that phenol hydroxylation by a human MPO-$O_2^{\cdot -}$ complex can occur readily. Hydroquinone is the only hydroxylated product detected under these conditions. Significant phenol hydroxylation to HQ occurred at toxicologically relevant concentrations of phenol, indicating that this property of MPO may play a significant role in benzene metabolism to HQ in addition to cytochrome P-450, especially outside the liver. The mechanism of this hydroxylation of phenol to HQ is not clear, but appears not to be due to the addition of $HO^{\cdot}$ to phenol. Involvement of other active oxygen species such as $^1O_2$ should also be considered. Preliminary studies with methylene blue plus light, a $^1O_2$ generating system, show that hydroxylation of phenol results in HQ as the major and CAT as the minor products (V.V. Subrahmanyam and M.T. Smith, manuscript in preparation). In contrast, H_2O_2/Fe^{2+}-EDTA a $HO^{\cdot}$ generating system results in CAT as the major and HQ as the minor products of phenol hydroxylation. The formation of $^1O_2$ by HX/XO/MPO system may occur by a spontaneous rearrangement of Compound III (MPO-$O_2^{\cdot -}$ complex), as shown in Figure 5. This $^1O_2$ may preferentially attack the *para* position of phenol resulting in HQ formation. This, however, should not be interpreted as that $^1O_2$ does not attack *ortho* position of phenol. It may be that this reaction is too slow to result in sufficient levels of CAT to be detected above background levels that are formed in the absence of MPO. It is interesting to note that cytochrome P-450 catalyzed hydroxylation of phenol also results in HQ as the predominant metabolite with little or no CAT formation.[11,12] It is, therefore, possible that the active oxygen species involved in phenol hydroxylation by MPO and cytochrome P-450 could be similar.

Acknowledgements

Supported by NIH grants P42ES04705 and P30ES01896. Dr. P. Kolachana is a trainee of the Health Effects Component of the University of California Toxic Substances Program.

References

1. K.R. Cooper and R. Snyder (1988) Benzene metabolism (Toxicokinetics and the molecular aspects of benzene toxicity). In *Benzene Carcinogenicity* (ed. M. Aksoy), CRC Press: Boca Raton, Fl., pp. 33–58.
2. G.F. Kalf (1987) Recent advances in the metabolism and toxicity of benzene. *CRC Critical Reviews in Toxicology* **18,** 141–159.
3. M.T. Smith, J.W. Yager, K.L. Steinmetz and D.A. Eastmond (1989) Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environmental Health Perspectives* **82,** 23–39.
4. L.S. Andrews, E.W. Lee, C.M. Witmer, J.J. Kocsis and R. Snyder (1977) Effects of toluene on the metabolism, disposition and hematopoietic toxicity of benzene. *Biochemical Pharmacology*, **26,** 293–300.
5. M. Ikeda and H. Ohtsuji (1971) Phenobarbital-induced protection against toxicity of toluene and benzene in rat. *Toxicology and Applied Pharmacology*, **20,** 30–43.
6. T. Nakajima, T. Okina and A. Sato (1987) Kinetic studies on benzene metabolism in rat liver—possible presence of three forms of benzene metabolizing enzymes in liver. *Biochemical Pharmacology*, **36,** 2799–2804.
7. D. Sammett, E.W. Lee, J.J. Kocsis and R. Snyder (1979) Partial hepatectomy reduces both metabolism and toxicity of benzene. *Journal Toxicology and Environmental Health*. **5,** 785–792.
8. L.M. Gonasun, C.M. Witmer, J.J. Kocsis and R. Snyder (1973) Benzene metabolism in mouse liver microsomes. *Toxicology and Applied Pharmacology*, **26,** 398–406.
9. D.R. Koop, C.L. Laetham, G.G. Schneir (1989) Identification of ethanol-inducible P-450 isozyme 3a (P450 IIE1) as a benzene and phenol hydroxylase. *Toxicology and Applied Pharmacology*, **98,** 278–288.

10. D. Jerina, D.B. Witkop, P. Zaltzman-Nirenberg, S. Udenfriend (1968) Role of the arene-oxide-oxepin system in the metabolism of aromatic substrates. 1. In vitro conversion of benzene oxide to a premercapturic acid and a dihydrodiol. *Archives of Biochemistry and Biophysics.* **128,** 176–183.
11. A. Tunek, K.L. Platt, M. Przybylski and F. Oesch, (1981) Multistep metabolic activation of benzene. Effects of superoxide dismutase on covalent binding to microsomal macromolecules, and identification of glutathione conjugates using high pressure liquid chromatography and field desorption mass spectrometry. *Chemico-Biological Interactions.* **33,** 1–17.
12. T. Sawahata and R.A. Neal (1983) Biotransformation of phenol to hydroquinone and catechol by rat liver microsomes. *Molecular Pharmacology.* **23,** 453–460, 1983.
13. A. Tunek, T. Olofsson and M. Berlin (1979) Toxic effects of benzene and benzene metabolites on granulopoietic stem cells and bone marrow cellularity in mice. *Toxicology and Applied Pharmacology.* **59,** 149–156.
14. R.E. Billings (1985) Mechanisms of catechol formation from aromatic compounds in isolated hepatocytes. *Drug Metabolism and Disposition.* **13,** 287–290.
15. L.S. Andrews, H.A. Sasame and J.R. Gillette (1979) ^{3}H-Benzene metabolism in rabbit bone marrow. *Life Sciences.* **25,** 567–572.
16. L. Gollmer, H. Graf and V. Ullrich (1984) Characterization of the benzene monooxygenase system in rabbit bone marrow. *Biochemical Pharmacology.* **33,** 3597–3602.
17. G.G. Schneir, C.L. Laetham, and D.R. Koop (1989) Identification and induction of cytochrome P-450, P-450 IIE1 and P-450 IA1 in rabbit bone marrow. *Journal of Pharmacology and Experimental Therapeutics,* **251,** 790–796.
18. D.E. Rickert, J.S. Baker, J.S. Bus, C.S. Barrow and R.D. Irons (1979) Benzene disposition in the rat after exposure by inhalation. *Toxicology and Applied Pharmacology.* **49,** 417–423.
19. S.T. Test and S.J. Weiss (1986) The generation and utilization of chlorinated oxidants by human neutrophils. *Advances in Free Radical Biology and Medicine,* **2,** 91–116.
20. K. Kariya, E.W. Lee, M. Hirouchi, M. Hosokawa and M. Sayo (1987) Purification and some properties of peroxidases of rat bone marrow. *Biochimica et Biophysica Acta.* **99,** 95–101.
21. D.A. Eastmond, M.T. Smith, L.O. Ruzo and D. Ross (1986) Metabolic activation of phenol by human myeloperoxidase and horseradish peroxidase. *Molecular Pharmacology,* **30,** 674–679.
22. A. Sadler, V.V. Subrahmanyam and D. Ross (1988) Oxidation of catechol by horseradish peroxidase and human leukocyte peroxidase: Reactions of o-benzoquinone and o-benzosemiquinone. *Toxicology and Applied Pharmacology.* **98,** 62–71.
23. V.V. Subrahmanyam, P. Kolachana and M.T. Smith (1991) Metabolism of hydroquinone by human myeloperoxidase: mechanisms of stimulation by other phenolic compounds. *Archives of Biochemistry and Biophysics.* **286,** 76–84.
24. D.J. Danner, P.J. Brignac, D. Archeneaux and V. Patel (1973) The oxidation of phenol and its reaction product by horseradish peroxidase and hydrogen peroxide. *Archives of Biochemistry and Biophysics,* **156,** 759–763.
25. T. Sawahata and R.A. Neal (1982) Horseradish peroxidase- mediated oxidation of phenol. *Biochemical and Biophysical Research Communications,* **109,** 988–994.
26. V.V. Subrahmanyam and P.J. O'Brien (1985) Phenol oxidation products, formed by a peroxidase reaction, that bind to DNA. *Xenobiotica,* **15,** 873–885.
27. H.S. Mason, I. Onopryenko and D. Buhler (1957) Hydroxylation: the activation of oxygen by peroxidase. *Biochimica et Biophysica. Acta.,* **24,** 225–226.
28. H.S. Mason (1957) The transfer of oxygen by peroxidase. In *Proceedings of the International Symposium on Enzyme Chemistry,* Maruzen: Tokyo, pp. 220–224.
29. B. Halliwell and S. Ahluwalia (1976) Hydroxylation of *p*-coumaric acid by horseradish peroxidase: the role of superoxide and hydroxyl radicals. *Biochemical Journal,* **153,** 513–518.
30. B. Halliwell (1977) Generation of hydrogen peroxide, superoxide and hydroxyl radicals during the oxidation of dihydroxyfumaric acid by peroxidase. *Biochemical Journal,* **163,** 441–448.
31. J.S. Dordick, A.M. Klibanov and M.A. Marletta (1986) Horseradish peroxidase catalyzed hydroxylations: mechanistic studies. *Biochemistry,* **25,** 2946–2951.
32. D. Metodiewa and H.B. Dunford (1989) The reactions of horseradish peroxidase, lactoperoxidase and myeloperoxidase with enzymatically generated superoxide. *Archives of Biochemistry and Biophysics,* **272,** 245–253.
33. C.C. Winterbourn, R.C. Garcia and A.W. Segal (1985) Production of the superoxide adduct of myeloperoxidase (compound III) by stimulated human neutrophils and its reactivity with hydrogen peroxide and chloride. *Biochemical Journal,* **228,** 583–592.

34. J.M. McCord and I. Fridovich (1969) Superoxide dismutase, an enzymatic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244,** 6049–6055.
35. S.J. Klebanoff, A.M. Waltersdorph and H. Rosen (1984) Antimicrobial activity of myeloperoxidase, in *Methods in Enzymology* Vol. 105, (ed. Packer, L.) Academic Press, New York, pp. 399–403.
36. B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*, Clarendon Press: Oxford.
37. L.A. Marquez and H.B. Dunford (1990) Reaction of compound III of myeloperoxidase with ascorbic acid. *Journal of Biological Chemistry*, **265,** 6074–6078.
38. B. Chance (1952) The kinetics and stoichiometry of the transition from the primary to the secondary peroxidase peroxide complexes, *Archives of Biochemistry and Biophysics*, **41,** 416–424.
39. P. George (1952) The chemical nature of the second hydrogen peroxide compound formed by cytochrome c peroxidase and horseradish peroxidase. *Biochemical Journal*, **54,** 267–276.
40. B.C. Saunders, A.G. Holmes-Siedle and B.P. Stark (1964) *Peroxidase: The properties and uses of a versatile enzyme and some related catalysts*, Butterworth: Washington.
41. D. Keilin and T. Mann (1937) On the haematin compound of peroxidase. *Proceedings of the Royal Society of London, Series B.*, **122,** 119–133.
42. R. Nakajima and I. Yamazaki (1987) The mechanism of oxyperoxidase formation from ferryl peroxidase and hydrogen peroxide, *Journal of Biological Chemistry*, **262,** 2576–2581.
43. B. Chance (1952) The spectra of the enzyme-substrate complexes of catalase and peroxidase, *Archives of Biochemistry and Biophysics*, **41,** 404–415.
44. P. George (1953) The third intermediate compound of horseradish peroxidase and hydrogen peroxide, *Journal of Biological Chemistry*, **201,** 427–434.
45. B. Halliwell (1978) Lignin Synthesis: The generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols, *Planta (Berl)*, **140,** 81–88.
46. B. Halliwell and J.D. Rycker (1978) Superoxide and peroxidase-catalyzed reactions: Oxidation of dihydroxyfumarate, NADH and dithiothreitol by horseradish peroxidase, *Photochemistry and Photobiology*, **28,** 757–763.
47. R. Bentley (1963) Glucose Oxidase, in *The Enzymes* Vol. 7, 2nd ed., (eds. P.D. Boyer, H. Lardy and K. Myrback) Academic Press, New York, pp. 567–586.
48. Che-Fu-Koo and I. Fridovich (1987) Stimulation of the activity of horseradish peroxidase by nitrogenous compounds. *Journal of Biological Chemistry*, **263,** 3811–3817.
49. R. Richmond, B. Halliwell, J. Chauhan and A. Darbre (1981) Superoxide-dependent formation of hydroxyl radicals: detection of hydroxylation of aromatic compounds, *Analytical Biochemistry*, **118,** 328–335.
50. O.I. Aruoma, B. Halliwell and M. Dizdaroglu (1989) Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase, *Journal of Biological Chemistry*, **264:** 13024–13028.
51. C.C. Winterbourn and H.C. Sutton (1986) Iron and xanthine oxidase catalyze formation of an oxidant species distinguishable from $HO^{\bullet}$: Comparison with Haber-Weiss reaction. *Archives of Biochemistry and Biophysics*, **244,** 27–34.

Accepted by Prof. B. Halliwell