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FORMATION OF METHEMOGLOBIN AND PHENOXYL RADICALS FROM p-HYDROXYANISOLE AND OXYHEMOGLOBIN

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The reaction of p-hydroxyanisole with oxyhemoglobin was investigated using electron spin resonance spectroscopy (ESR) and visible spectroscopy. As a reactive reaction intermediate we found the p-methoxyphenoxyl radical, the one-electron oxidation product of p-hydroxyanisole. Detection of this species required the rapid flow device elucidating the instability of this radical intermediate. The second reaction product formed is methemoglobin. Catalase or SOD had no effect upon the reaction kinetics. Accordingly, reactive oxygen species such as hydroxyl radicals or superoxide could not be observed although the spin trapping agent DMPO was used to make these short-lived species detectable. When the sulfhydryl blocking agents N-ethylmaleimide or mersalyl acid were used, an increase of the methemoglobin formation rate and of the phenoxyl radical concentration were observed. We have interpreted this observation in terms of a side reaction of free radical intermediates with thiol groups.

KEY WORDS: ESR, 4-Hydroxyanisole, methemoglobin formation, free radicals, phenoxyl radical.

INTRODUCTION

4-Hydroxyanisole (4-methoxyphenol) is used in the treatment of malignant melanoma. The clinical use of this compound, however, is limited by undesirable side effects such as S-phase toxicity, tyrosinase-catalyzed oxidative cell damage, surface effects on cell membranes[1], and methemoglobin formation (P.A. Riley, pers. comm.). Since methemoglobin formation is a one-electron transfer step compounds which initiate oxidation of oxyhemoglobin to methemoglobin are supposed to mediate one-electron transfer. In the case of 4-hydroxyanisole a variety of intermediates other than the one-electron oxidation product can be expected, since 4-hydroxyanisole can be oxidized to several compounds such as phenoxyl radicals, o-semiquinones and o-quinones.[2] Free radicals and methemoglobin are usually involved in the O_2-coupled oxidation of reducing agents reacting with oxyhemoglobin. Such compounds have recently been investigated in our previous studies where we used several aliphatic hydroxylamines[3–5] as initiators of methemoglobin formation. We were able to detect transient free radical species using ESR and a flow system or low temperature. Based on these findings we assume that free radicals are also involved in a reaction of p-hydroxyanisole with oxyhemoglobin and that some of the observed toxic side effects can be explained by a free radical mechanism. Shiga *et al.*[6] reported on the oxidation of p-hydroxyanisole to the transient phenoxyl radical with a methemoglobin-

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hydrogen peroxide system but no investigations on the effect of oxyhemoglobin on p-hydroxyanisole have been reported previously.

MATERIALS AND METHODS

4-Hydroxyanisole was obtained from Sigma Chem. Co. Bovine hemoglobin was prepared in a procedure described by Eyer *et al.*[7] for human hemoglobin. Bovine red cells were washed five times with twice the amount of 0.2 M phosphate buffer, pH 7.4. The cells were sonicated in distilled water and 10 g of Celite was added to 250 ml of the hemolysate. The mixture was stirred for 20 min and then centrifuged for 30 min at 15,000 g. Purified hemoglobin was prepared by chromatography: 10 ml of the hemolysate was applied to a column (26 mm I.D.) containing 50 g of $DEAE_{52}$ cellulose (Serva) preequilibrated with 10 mM Tris/HCl pH 8.3 and eluted with 0.1 M Tris/HCl pH 7.0. The fractions were tested for catalase[8] and SOD activity[9] and only those with a catalase activity $k < 1$ and no detectable SOD activity were pooled. If necessary, the chromatographic procedure was repeated. The pooled fractions were dialyzed overnight against 0.2 M phosphate buffer, pH 7.4, using dialysis tubing from Union Carbide, type 36DMX100 FT9, in order to remove glutathione. Oxyhemoglobin was determined at its absorption maximum at 540 nm, the methemoglobin content by the absorbance increase at 540 nm after the addition of cyanide.[10] The ESR experiments were carried out in a Bruker ER 200 D-SRC 9/2.7 spectrometer operating at 9.6 GHz with 100 kHz modulation frequency equipped with a rectangular TE_{102} microwave cavity. For the measurements of the g-values at room temperature, 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) was used as an internal standard ($g = 2.0055$).[11] For the flow experiments a quartz mixing flat-cell was chosen. Computer simulations of ESR spectra and multi-scan experiments were carried out with the Bruker data system ESP 1600. For the spectrophotometric measurements, a Hitachi model 150-20 UV-VIS spectrometer was used. The rate constants were calculated from initial reaction rates at different concentrations of oxyhemoglobin and p-hydroxyanisole measured at 540 nm in 0.2 M phosphate buffer at 25°C.

RESULTS

ESR Experiments at Room Temperature

Figure 1a shows the ESR spectrum obtained when a hemolysate solution (containing 6.87 mM oxyhemoglobin) was mixed with a solution of 0.25 M p-hydroxyanisole (in 0.1 M phosphate buffer, pH 7.4) at a total flow rate of 50 ml/min. Both solutions contained 1 mM DETAPAC in order to eliminate undesirable side effects from free iron ions. The same ESR spectrum was obtained with purified, catalase and SOD-free oxyhemoglobin but due to the limited amount and its lower concentration, the spectral resolution could not be optimized (spectrum not shown). The hyperfine coupling constants were determined by computer simulation of the spectrum shown above. The best fit is shown in Figure 1b, which was simulated with the parameters $a_H^{(2)} = 5.05$ G and $a_H^{(3)} = 2.05$ G. The g-value was 2.0044. These values are typical for the p-methoxyphenoxyl radical, the one-electron oxidation product of p-hydroxy-

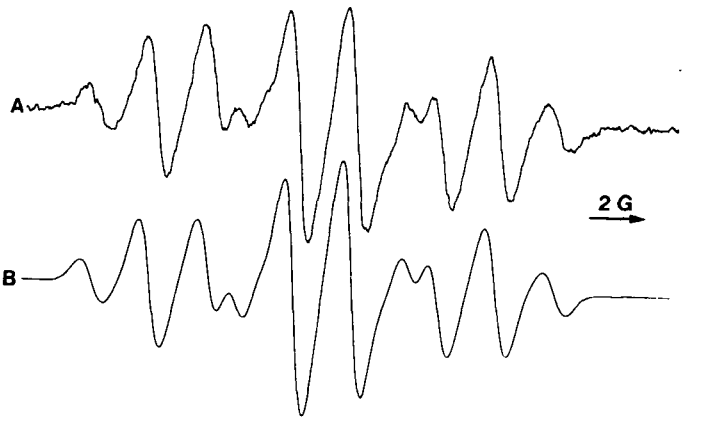

FIGURE 1 ESR spectrum of the p-methoxyphenoxyl radical (CH_3O-C_6H_4-$O^{\cdot}$). a) Two components were mixed in a flow-cell: 1.) A hemolysate containing 6.87 mM oxyhemoglobin and 1 mM DETAPAC in 0.1 M phosphate buffer pH 7.4 with a flow rate of 10 ml/min and 2.) a solution containing 0.25 M p-hydroxyanisole and 1 mM DETAPAC in 0.1 M phosphate buffer with a flow rate of 40 ml/min. The spectrometer settings were: scan range, 25 G; modulation amplitude, 1 G; receiver gain, 1.0×10^5; microwave power, 20 mW; time constant, 0.16 sec; scan rate, 35.7 G/min, 5 scans. b) Computer simulation of the ESR spectrum shown in a). The parameters used were: $a_H^{(2)} = 5.05$ G and $a_H^{(3)} = 2.05$ G; linewidth $\Delta_H = 1.0$ G; 100% Gaussian line shape.

anisole.[12] When the flow was stopped, the ESR signal disappeared rapidly, and only a broad asymmetric absorption remained, possibly a mixture of several quinoid oxidation products similar to those reported by Tomasi in a tyrosinase system.[2] The concomitant disappearance of oxyhemoglobin and the formation of methemoglobin was monitored by spectrophotometry (see below).

Spectrophotometric Measurements

Figure 2 shows the visible spectrum between 450 nm and 650 nm. In order to follow the methemoglobin formation in the above mentioned reaction, fifteen repetitive scans of a mixture of 1.3 mM HbO_2 and 33.33 mM p-hydroxyanisole were taken at intervals of 95 sec. Three isosbestic points indicate that of the hemoglobin derivatives only oxyhemoglobin and methemoglobin are present in the reaction mixture. Intermediary hemoglobin species were not detected.

Since thiol groups on the globin moiety have been reported to interfere with free radical reactions,[13] the effect of sulfhydryl blocking agents on the reaction kinetics became of interest.

Figure 3 shows the effect of sulfhydryl-blocking agents on the methemoglobin formation rate in the presence of p-hydroxyanisole. The dependence of the reaction rate on the concentration of oxyhemoglobin and p-hydroxyanisole was found to be almost linear. A rate constant of approximately 4.6 min^{-1} M^{-1} was calculated for the reaction of oxyhemoglobin with p-hydroxyanisole at pH 7.4 in 0.2 M phosphate buffer (line marked "▲"). When sulfhydryl blocking agents such as N-ethylmaleimide (5 mM, line marked "□") or mersalyl acid (5 mM, line marked "◆") were added to the reaction mixture, we observed an increase of the rate constant of about 50%. As shown in the

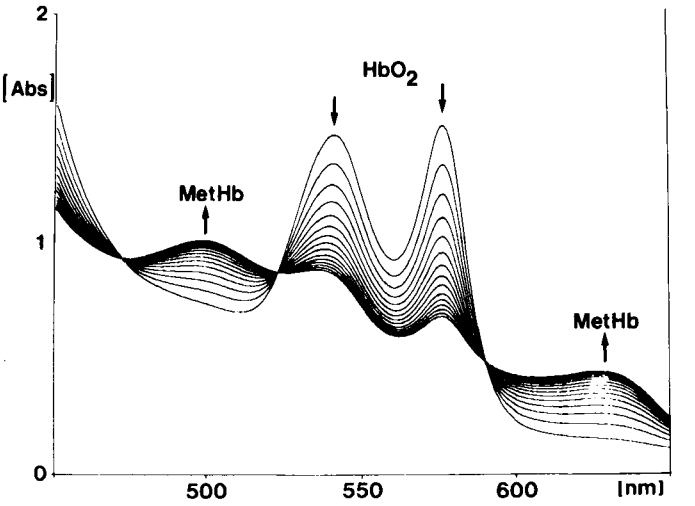

FIGURE 2 The reaction of p-hydroxyanisole with oxyhemoglobin followed in the visible range between 450 nm and 650 nm 15 repetitive scans of the reaction mixture containing 1.3 mM oxyhemoglobin and 33.33 mM p-hydroxyanisole were taken at intervals of 95 seconds.

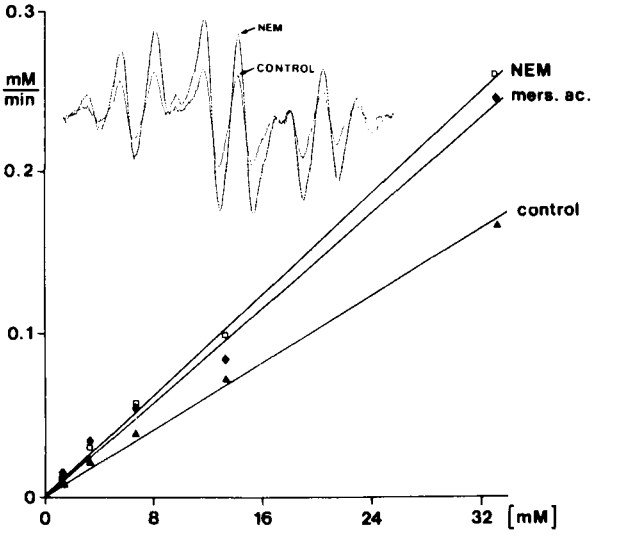

FIGURE 3 The dependence of the reaction rate upon the concentration of p-hydroxyanisole at a fixed concentration of oxyhemoglobin of 1.3 mM. Line marked "▲", oxyhemoglobin; line marked "□", oxyhemoglobin + 5 mM N-ethylmaleimide; line marked "◆", oxyhemoglobin + 5 mM mersalyl acid. The lines are a least square fit with correlation coefficients between 0.9954 and 0.9998, the standard deviation of individual points varying between 2.35% and 13.7% of the mean value. The inset shows the increase of the ESR intensity of the p-methoxyphenoxyl radical in the presence of N-ethylmaleimide (NEM). Spectrum without NEM (solid line) and in the presence of 10 mM NEM (dotted line) and in the presence of 10 mM NEM (dotted line). The spectra represent the sum of 19 single scans. The spectrometer settings were: scan range, 20 G; modulation amplitude, 1 G; receiver gain, 1.0×10^5; microwave power, 20 mW; time constant, 0.32 sec; scan rate, 28.6 G/min.

inset, the intensity of the ESR spectrum of the p-methoxyphenoxyl radical also increased when N-ethylmaleimide was added. This effect is indicative of the involvement of a side reaction of the p-methoxyphenoxyl radical with reactive sulfhydryl groups of hemoglobin itself, since other sulfhydryl containing compounds such as glutathione have been removed during the purification of the oxyhemoglobin solution. The involvement of hydrogen peroxide or superoxide radicals in the methemoglobin formation could be ruled out since neither catalase nor superoxide dismutase showed any effect on the reaction kinetics.

Spin Trapping Experiments with DMPO

The concomitant oxidation of p-hydroxyanisole to its phenoxy radical and of oxyhemoglobin to methemoglobin requires the simultaneous reduction of the hemoglobin-bound dioxygen. In order to investigate whether free oxygen radicals are involved in this process, we performed the reaction of oxyhemoglobin with p-hydroxyanisole in the presence of the spin trapping agent DMPO. Since no ESR spectrum was observed, the involvement of superoxide or hydroxyl radicals can be excluded. These findings are in agreement with our spectrophotometric results where superoxide dismutase showed no effect. Likewise, a Fenton type reaction of intermediary hydrogen peroxide to hydroxyl radicals can be excluded. The only remaining possibility for partially reduced oxygen intermediates are therefore the ferryl type species discussed below.

DISCUSSION

Our results clearly show the formation of methemoglobin from oxyhemoglobin and p-hydroxyanisole and the concomitant formation of a free radical reaction intermediate, p-methoxyphenoxyl.

The formation of this free radical species can be assumed to be the first reaction step:

$$[Hb^{2+}O_2] + CH_3O\text{-}C_6H_4\text{-}OH \rightarrow [MetHb^{3+}\text{-}O_2^{2-}] + CH_3O\text{-}C_6H_4\text{-}O^{\cdot} + H^+$$

As a reaction intermediate we assume the perferryl species $[MetHb^{3+}\text{-}O_2^{2-}]$ to be formed. Here, the oxygen is in the same oxidation state as in hydrogen peroxide but is bound to the heme iron. The release into the solution in the form of free hydrogen peroxide seems unlikely because catalase was found in our study to have no effect on the reaction rate. Instead, we propose the reaction of the perferryl species with excess p-hydroxyanisole thereby forming additional phenoxyl radicals as was observed by Shiga[6,14] in a methemoglobin-H_2O_2 system:

$$[MetHb^{3+}\text{-}O_2^{2-}] + 2CH_3O\text{-}C_6H_4\text{-}OH \rightarrow 2\ CH_3O\text{-}C_6H_4\text{-}O^{\cdot} + MetHb^{3+} + 2\ OH^-.$$

This reaction represents a secondary pathway to the formation of the p-hydroxyanisole derived phenoxyl radicals, their decay leading to a variety of secondary oxidation products such as dimers and quinone species.[15] The most dangerous decay route of the observed phenoxyl radical is hydrogen abstraction from biologic membranes

and proteins. One aspect of this reaction type is the reaction with reactive sulfhydryl groups on the globin moiety which has recently been reported by Maples *et al*[16]:

$$HbSH + R^{\cdot} \rightarrow HbS^{\cdot} + RH$$

In our studies we provided indirect evidence that part of the free radical intermediates will react with these internal thiol groups since the use of sulfhydryl blocking agents resulted in an increase of the p-methoxyphenoxyl radical concentration. The fact that in addition to the increase of the phenoxyl radical concentration we also found a higher methemoglobin formation rate suggests that a link exists between both processes. Most probably apart from an interference with SH-groups, the phenoxyl radicals will also react with oxyhemoglobin or the perferryl species thereby contributing to the methemoglobin formation. These secondary reactions of the detected radical are of toxicological interest since apart from its stimulation of methemoglobin formation it may also alter functional proteins and membrane structures. This would limit the clinical use of this compound for the treatment of malignant melanoma.

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References

1. Riley, P.A. (1984) Hydroxyanisole: the current status. In: Hydroxyanisole – Recent Advances in Anti-Melanoma Therapy (Ed. P.A. Riley), pp. 1–10. IRL Press, Oxford.
2. Tomasi, A. (1984) Radical Activation of 4-Hydroxyanisole by Mushroom Tyrosinase. In: Hydroxyanisole – Recent Advances in Anti-Melanoma Therapy (Ed. P.A. Riley), pp. 15–24. IRL Press, Oxford.
3. Stolze, K. and Nohl, H. (1989) Detection of Free Radicals as Intermediates in the Methemoglobin Formation from Oxyhemoglobin Induced by Hydroxylamine. *Biochemical Pharmacology* **38**, 3055–3059.
4. Stolze, K. and Nohl, H. (1990) Free Radical Intermediates in the Oxidation of N-Methylhydroxylamine and N,N-Dimethylhydroxylamine by Oxyhemoglobin. *Free Radical Research Communications*, **8**, 123–131.
5. Stolze, K. and Nohl, H. (1990) EPR Studies on the Oxidation of Hydroxyurea to Paramagnetic Compounds by Oxyhemoglobin. *Biochemical Pharmacology*, in press.
6. Shiga, T. (1973) Generation of Phenoxy Radicals by Methemoglobin-Hydrogen Peroxide Studies by Electron Paramagnetic Resonance. *Archives in Biochemistry and Biophysics*, **154**, 540–547.
7. Eyer, P., Hertle, H., Kiese, M. and Klein, G. (1975) Kinetics of Ferrihemoglobin Formation by some Reducing Agents, and the Role of Hydrogen Peroxide. *Molecular Pharmacology*, **11**, 326–334.
8. Nohl, H. and Hegner, D. (1978) Evidence for the Existence of Catalase in the Matrix Space of Rat Heart Mitochondria. *FEBS Letters*, **89**, 126–130.
9. Nohl, H., Hegner, D. and Summer, K.H. (1981) The Mechanism of Toxic Action of Hyperbaric Oxygenation on the Mitochondria of Rat Heart Cells. *Biochemical Pharmacology*, **30**, 1753–1757.
10. Grisk, A. (1969) Praktikum der Pharmakologie und Toxikologie. VEB Gustav Fischer Verlag, Jena.
11. Brière, R., Lemaire, H. and Rassat, A. (1965) Nitroxydes XV: Synthèse et étude de radicaux libres stables pipéridiniques et pyrrolidinique. *Bulletin of the Society of Chemistry France*, 3273–3283.
12. Nilges, M.J., Swartz, H.M. and Riley, P.A. (1989) Identification by Electron Spin Resonance of Free Radicals Formed during the Oxidation of 4-Hydroxyanisole Catalyzed by Tyrosinase. *Journal of Biological Chemistry*, **259**, 2446–2451.
13. Maples, K.R., Jordan, S.J. and Mason, R.P. (1989) *In Vivo* Rat Hemoglobin Thiyl Free Radical Formation Following Administration of Phenylhydrazine and Hydrazine-based Drugs. *Drug Metabl. Disp.* **16**, 799–803.

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References

1. Riley, P.A. (1984) Hydroxyanisole: the current status. In: Hydroxyanisole – Recent Advances in Anti-Melanoma Therapy (Ed. P.A. Riley), pp. 1–10. IRL Press, Oxford.
2. Tomasi, A. (1984) Radical Activation of 4-Hydroxyanisole by Mushroom Tyrosinase. In: Hydroxyanisole – Recent Advances in Anti-Melanoma Therapy (Ed. P.A. Riley), pp. 15–24. IRL Press, Oxford.
3. Stolze, K. and Nohl, H. (1989) Detection of Free Radicals as Intermediates in the Methemoglobin Formation from Oxyhemoglobin Induced by Hydroxylamine. *Biochemical Pharmacology* **38**, 3055–3059.
4. Stolze, K. and Nohl, H. (1990) Free Radical Intermediates in the Oxidation of N-Methylhydroxylamine and N,N-Dimethylhydroxylamine by Oxyhemoglobin. *Free Radical Research Communications*, **8**, 123–131.
5. Stolze, K. and Nohl, H. (1990) EPR Studies on the Oxidation of Hydroxyurea to Paramagnetic Compounds by Oxyhemoglobin. *Biochemical Pharmacology*, in press.
6. Shiga, T. (1973) Generation of Phenoxy Radicals by Methemoglobin-Hydrogen Peroxide Studies by Electron Paramagnetic Resonance. *Archives in Biochemistry and Biophysics*, **154**, 540–547.
7. Eyer, P., Hertle, H., Kiese, M. and Klein, G. (1975) Kinetics of Ferrihemoglobin Formation by some Reducing Agents, and the Role of Hydrogen Peroxide. *Molecular Pharmacology*, **11**, 326–334.
8. Nohl, H. and Hegner, D. (1978) Evidence for the Existence of Catalase in the Matrix Space of Rat Heart Mitochondria. *FEBS Letters*, **89**, 126–130.
9. Nohl, H., Hegner, D. and Summer, K.H. (1981) The Mechanism of Toxic Action of Hyperbaric Oxygenation on the Mitochondria of Rat Heart Cells. *Biochemical Pharmacology*, **30**, 1753–1757.
10. Grisk, A. (1969) Praktikum der Pharmakologie und Toxikologie. VEB Gustav Fischer Verlag, Jena.
11. Brière, R., Lemaire, H. and Rassat, A. (1965) Nitroxydes XV: Synthèse et étude de radicaux libres stables pipéridiniques et pyrrolidinique. *Bulletin of the Society of Chemistry France*, 3273–3283.
12. Nilges, M.J., Swartz, H.M. and Riley, P.A. (1989) Identification by Electron Spin Resonance of Free Radicals Formed during the Oxidation of 4-Hydroxyanisole Catalyzed by Tyrosinase. *Journal of Biological Chemistry*, **259**, 2446–2451.
13. Maples, K.R., Jordan, S.J. and Mason, R.P. (1989) *In Vivo* Rat Hemoglobin Thiyl Free Radical Formation Following Administration of Phenylhydrazine and Hydrazine-based Drugs. *Drug Metabl. Disp.* **16**, 799–803.

14. Shiga, T. (1975) Electron Spin Resonance Study on Peroxidase- and Oxidase-Reactions of Horse Radish Peroxidase and Methemoglobin. *Archives in Biochemistry and Biophysics*, **167**, 469–479.
15. Nilges, M.J. and Swartz, H.M. (1984) Quinone and Semiquinone Intermediates in the Reaction of 4-Hydroxyanisole and Tyrosinase. In: Hydroxyanisole – Recent Advances in Anti-Melanoma Therapy (Ed. P.A. Riley), pp. 25–34. IRL Press, Oxford.
16. Maples, K.R., Eyer, P. and Mason, R.P. (1990) Aniline-, Phenylhydroxylamine-, Nitrosobenzene-, and Nitrobenzene-Induced Hemoglobin Thiyl Free Radical Formation *In Vivo* and *In Vitro*. *Molecular Pharmacology*, **37**, 311–318.

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