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ESR DETECTION OF FREE RADICAL INTERMEDIATES DURING AUTOXIDATION OF 5-HYDROXYPRIMAQUINE

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Autoxidation of 5-hydroxyprimaquine, a putative metabolite of the antimalarial primaquine, was studied by oxygen consumption and ESR spectroscopy. 5-Hydroxyprimaquine underwent fast autoxidation under mild conditions (pH 7.4–8.5, 25°C, and presence of 1 mM diethylenetriamine pentaacetic acid); each mol of the drug consumed 0.75 mol of oxygen and formed 0.5 mol of hydrogen peroxide. Direct-ESR experiments demonstrated that 5-hydroxyprimaquine autoxidation was accompanied by generation of a drug-derived free radical that is oxygen sensitive. Generation of hydroxyl radical was also established by spin-trapping experiments in the presence of 5,5-dimethyl-1-pyrroline N-oxide. The effect of antioxidant enzymes on hydroxyl radical adduct yield and analysis of autoxidation stoichiometry suggest that the main route for hydroxyl radical generation is the iron-catalyzed reaction between the drug-derived free radical and hydrogen peroxide.

KEY WORDS: Primaquine putative metabolites, 5-hydroxyprimaquine, semiquinone-imine, hydroxyl radical, spin-trapping, antimalarial.

INTRODUCTION

Primaquine possesses a broad spectrum of antimalarial activity and has become the drug of choice for radical cure of *Plasmodium vivax* infections. Its clinical utility, however, is compromised by toxic effects on erythrocytes, particularly on those deficient in the enzyme glucose-6-phosphate dehydrogenase.[1] Toxic effects of primaquine have been attributed to its conversion to hydroxylated metabolites that could generate active oxygen species by autoxidation and/or redox-cycling of their corresponding quinone-imine derivatives.[1-4] However, there was no direct evidence for such a mechanism; also, it was recently reported that some semiquinone-imine radicals do not react with oxygen to generate oxygen-derived free radicals.[5,6] The recent identification of 5-hydroxyprimaquine as a primaquine metabolite in rat and rhesus monkey[4,7] and the availability of the compound through the WHO, lead us to examine autoxidation of 5-hydroxyprimaquine by oxygen consumption and ESR techniques. Our results demonstrate that indeed, 5-hydroxyprimaquine autoxidation is accompanied by generation of both a drug-derived free radical and hydroxyl radical.

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MATERIALS AND METHODS

5-Hydroxyprimaquine, 2 HBr* was obtained from WHO and was synthesized by Prof. A. Strother, University of Loma Linda, CA, U.S.A. as part of the programme SWG-CHEMAL, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Catalase was from Boehringer, Mannhein GmbH, GFR. DMPO, DTPA and SOD were from Sigma Chemical Co. DMPO was purified by charcoal filtration.[8] Stock solutions (5–10 mM) of 5-hydroxyprimaquine were made in deoxygenated milli-Q water and kept under nitrogen in tubes capped with rubber septa; the tubes were kept on ice throughout (about 4 h from preparation), and aliquots were removed with a Hamilton syringe for the experiments. Milli-Q water was used throughout.

Oxygen consumption was measured polarographically with an oxygen monitor (Gilson 5/6 Oxygraph) at 25°C; the saturating oxygen concentration at this temperature, which corresponds to the full response of the electrode, was taken as 0.24 mM.[9] The final volume of the reaction mixture was 1.8 ml.

ESR spectra were recorded at room temperature (24°C ± 1) on a Bruker ER 200 D-SRC spectrometer. The final volume of the reaction mixtures was 100 μl, and the samples were transferred to ESR containers immediately after 5-hydroxyprimaquine additions. The ESR containers used were small flat cells or gas permeable tubing to control the sample atmosphere (see Results).[10] The gas permeable tubing (internal diameter 0.8 mm, wall thickness 0.05 mm) was from Zeus Industrial Products, Inc., Raritan, NJ. 4-Hydroxy-2,2,6,6-tetramethyl-piperidinooxy radical (TEMPOL, $g \approx 2.0056$)[11] was used as standard for g value determination.

RESULTS

5-Hydroxyprimaquine was readily oxidized when the concentrated (5 mM) stock

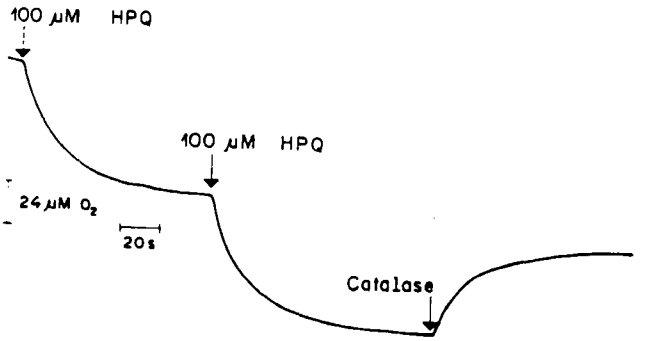

FIGURE 1 Oxygen consumption by 5-hydroxyprimaquine in 0.1 M borate buffer containing 1 mM DTPA, pH 8.3 at 25°C. The arrows indicate additions of aliquots that will give the specified final concentrations of 5-hydroxyprimaquine and catalase (100 μg/ml). Three independent determinations comparable to the shown experiment give the molar ratios of 1 5-hydroxyprimaquine: 0.75 oxygen: 0.5 hydrogen peroxide.

*Abbreviations and trivial names used: 5-hydroxyprimaquine, 5-hydroxy-6-methoxy-8-(4 amino-1-methylbutilamino) quinoline; DMPO; 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetriamine-pentaacetic acid; SOD, superoxide dismutase.

TABLE I
Effect of various agents on oxygen consumption by 5-hydroxyprimaquine[a]

Agent	Oxygen Consumption	
	Initial Rates ($\mu M\,s^{-1}$)	Total Consumption Oxygen (μM)
None	22	230
SOD (100 μg/ml)	18	221
Catalase (100μg/ml)	21	153
DMPO (100 mM)	20	230

[a]The reaction mixture was incubated at 25°C and contained 300 μM 5-hydroxyprimaquine and 1 mM DTPA in 0.1 M borate buffer, pH 8.5.

solution in water under nitrogen (pH ~ 2.0) was diluted with aerated buffers in the presence of the metal chelator DTPA. As shown in Figure 1, addition of 100 μM 5-hydroxyprimaquine to aerated buffer led to a fast oxygen consumption that faded off with consumption of 75 μM oxygen. This suggests almost complete oxidation of the substrate and indeed, addition of a new aliquot of 5-hydroxyprimaquine led to a new burst in oxygen uptake (Figure 1). Part of the consumed oxygen was reduced to hydrogen peroxide as demonstrated by oxygen liberation upon catalase addition to the reaction mixture; oxidation of 100 μM 5-hydroxyprimaquine produced 50μM hydrogen peroxide (Figure 1). Essentially the same results were obtained with other buffers, although inital rates of oxygen consumption were dependent on the buffer pH. For instance, the measured rates for 100 μM 5-hydroxyprimaquine were 3.0 $\mu M\,s^{-1}$ and 3.8 $M\,s^{-1}$ in PBS, pH 7.4 and borate buffer, pH 8.3, respectively. Catalase and SOD had little effect upon the initial rate of oxygen consumption although, as expected from Figure 1, catalase inhibited the total consumed oxygen (Table I). These results suggest a minor role for both hydrogen peroxide and superoxide anion in propagating 5-hydroxyprimaquine autoxidation.[12]

Parallel direct-ESR experiments demonstrated that 5-hydroxyprimaquine autoxidation is accompanied by generation of a free radical specie (Figure 2). The detected radical has a g value of 2.0079 and can be tentatively ascribed to a phenoxyl-type free radical;[13] a possible structure for the 5-hydroxyprimaquine free radical is shown in Figure 4A. Higher concentrations of the radical were obtained under low oxygen tensions (Figure 2), indicating its reactivity towards oxygen.[6] In these experiments the samples were contained in gas permeable tubing under a stream of air (Figure 2A) or nitrogen (Figure 2B) during the scanning of the ESR spectra.[10]

Autoxidation of 5-hydroxyprimaquine also led to generation of hydroxyl radicals as demonstrated by spin-trapping experiments in the presence of DMPO (Figure 3). Incubation of 5-hydroxyprimaquine in the presence of DMPO led to detection of the DMPO-hydroxyl radical adduct (a_N = 14.9; a_H = 14.9)[14] but no DMPO-superoxide radical adduct was observed (Figure 3A). This could be explained by the low stability of the DMPO-superoxide radical adduct, a specie that can decay to the DMPO-hydroxyl radical adduct. However, during the autoxidation of 5-hydroxyprimaquine most of the detected DMPO-hydroxyl adduct appears to be a direct product of hydroxyl radical and DMPO; indeed, in the presence of DMSO, the DMPO-methyl radical adduct (a_N = 16.6, a_H = 23.7)[14] was observed (Figure 3B). In the presence of SOD the yield of the DMPO-hydroxyl radical adduct was inhibited by about 60%

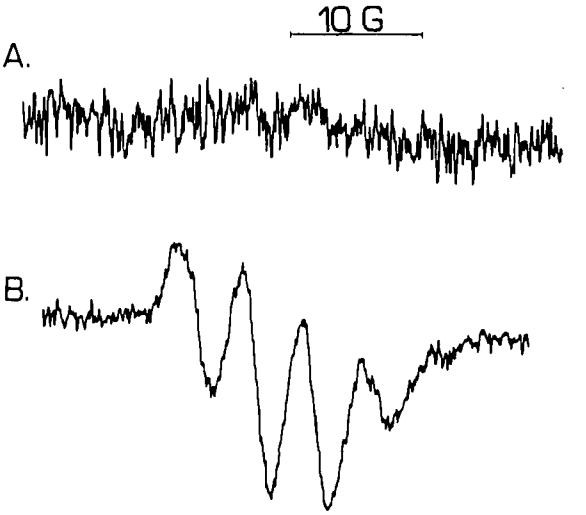

FIGURE 2 ESR spectra obtained during autoxidation of 5-hydroxyprimaquine (1 mM) in 0.1 M borate buffer containing 1 mM DTPA, pH 8.3 at 25°C under a stream of: (A) air; (B) nitrogen. Instrumental conditions: microwave power 20 mw; modulation amplitude, 1G; time constant 0.5 s; scan rate, 0.2 G/s; gain, 2×10^6 for (A) and 8×10^5 for (B).

(Figure 3C) whereas in the presence of catalase the adduct was barely detected (Figure 3D).

It is important to note that there was no correlation between oxygen consumption (Table I) and the appearance of the DMPO adducts (Figure 3). To obtain reproducible results and to better analyze the spin-trapping experiments, they were performed with 300 μM 5-hydroxyprimaquine in a flat cell (Figure 3). Under these experimental conditions all the dissolved oxygen was consumed in less than one minute (Figure 1, Table I) and the flat cell container precluded further aeration of the system. In spite of this, DMPO-adduct concentrations stopped increasing and started to decay, after ten minutes of reaction. Increases in the concentration of the DMPO-hydroxyl radical adduct with time can be followed in Figure 3 by the changes in the peak heights with the scanning of the ESR-spectra that started 1.5 min after 5-hydroxyprimaquine additions (Figure 3). These reproducible observations indicate that autoxidation products of 5-hydroxyprimaquine can generate hydroxyl radical in the absence of oxygen.

DISCUSSION

To accommodate the described results we suggest the schematic reactions shown in Figure 4. Reactions (1)–(3) (Figure 4B) account for the expected stoichiometry where one 5-hydroxyprimaquine reacts with one oxygen producing one quinone-imine and one hydrogen peroxide. However, the stoichiometry obtained from oxygen consumption studies (Figure 1, 4C) indicates that: (1) part of the substrate is being consumed by reaction with the produced quinone-imine[15] [reaction (4), Figure 4B], and (2) part of the formed hydrogen peroxide is reacting with the semiquinone-imine [reaction (5),

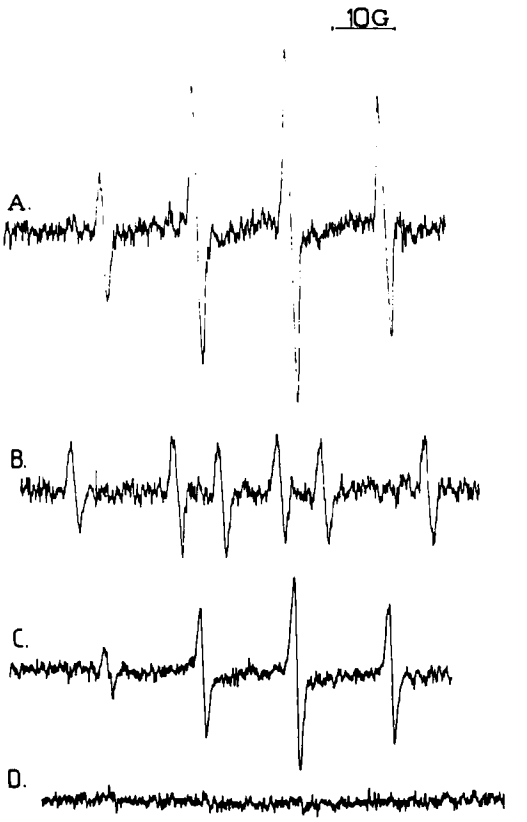

FIGURE 3 ESR spectra of DMPO radical adducts obtained after a 1.5 min incubation at 25°C of 5-hydroxyprimaquine (300 μM) and DMPO (100 mM) in 0.1 M borate buffer containing 1 mM DTPA, pH, 8.5: (A) without additions; (B) in the presence of DMSO (10%, v/v); (C) in the presence of SOD (100 μg/ml); and (D) in the presence of catalase (100 μg/ml). Instrumental conditions: microwave power 20 nw; modulatyion amplitude, 1G; time constant, 0.2 s for (A) and (B) and 0.5 s for (C) and (D); scan rate 0.5 G/s for (A) and (B) and 0.2 G/s for (C) and (D); gain 1.25 × 10^6.

Figure 4B], probably through an iron mediated process,[15,16] to generate the detected hydroxyl radical (Figure 3). The main role of the semiquinone-imine and hydrogen peroxide in generating hydroxyl radical is also supported by the effect of catalase completely inhibiting formation of the DMPO-hydroxyl radical adduct (Figure 3D), without affecting the oxygen consumption rate (Table I). In agreement, the less pronounced effect of SOD (Figure 3C) can be ascribed to displacement of reaction (2) (Figure 4B), that leads to a decrease in the available semiquinone-imine.[15] The observed lack of correlation between hydroxyl radical yield and oxygen consumption (Table I, Figure 3), further emphasizes the importance of hydrogen peroxide and semiquinone-imine in generating hydroxyl radical, because both species should be present after uptake of all of the dissolved oxygen (Figure 1,4C). Although the semiquinone-imine radical has not been completely characterized due to the low resolution of the ESR spectrum (Figure 2), its structure (Figure 4A) is supported by

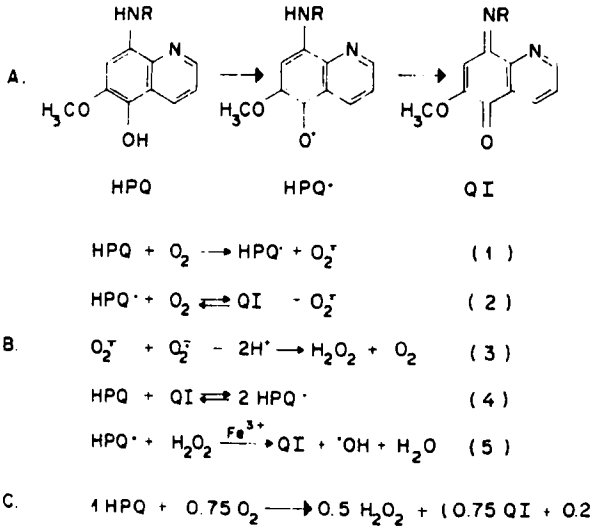

FIGURE 4 Schematic representation of 5-hydroxyprimaquine autoxidation: (A) compound structures; (B) suggested reaction sequence (hydrogens and charges are not balanced to avoid changing the basic abbreviation used for 5-hydroxyprimaquine, 5-HPQ); (C) molar ratio obtained from oxygen consumption studies (Figure 1).

experiments where the autoxidized 5-hydroxyprimaquine is reduced back by NADPH-ferredoxin reductase.*

In conclusion, our results present direct evidence for generation of free radicals from autoxidation of a putative metabolite of primaquine supporting the view that toxic effects of the drug may be mediated by its hydroxylated metabolites.[1-4]

Acknowledgments

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*Unpublished experiments.

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