Biochemical Pharmacology, Vol. 32, No. 23, pp. 3571–3575, 1983.
Printed in Great Britain.

0006–2952/83 $3.00 + 0.00
Pergamon Press Ltd.

A MECHANISM FOR PRIMAQUINE MEDIATED OXIDATION OF NADPH IN RED BLOOD CELLS

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(*Received* 6 *May* 1983; *accepted* 7 *July* 1983)

Abstract—The incubation of NADPH with primaquine results in the formation of free radicals which were demonstrated by the electron spin resonance (ESR) technique of spin trapping using 5,5-dimethyl-l-pyrroline-*N*-oxide (DMPO) as the spin trap. The free radicals formed were identified as the superoxide (DMPO–OOH) and hydroxyl (DMPO–OH) spin adducts of DMPO. Copper/zinc superoxide dismutase inhibited the formation of DMPO–OOH while it only partly inhibited the formation of DMPO–OH which could be totally inhibited by catalase. This indicates that the formation of hydroxyl radicals is not totally arising from the Haber–Weiss reaction. However since the formation of hydroxyl radicals is dependent on hydrogen peroxide, a non-metal catalysed reduction of hydrogen peroxide is postulated for their formation. Oxygen consumption during the reaction between primaquine and NADPH was found to be consistent with the spin trapping experiments and the rate of production of DMPO–OH indicates the formation of 1:1 catalytic complex between the two reactants. Quenching of the fluorescence of NADPH at 460 nm in the presence of primaquine indicates the formation of a charge transfer complex. When red blood cells are incubated with primaquine a hydroxyl spin adduct (DMPO–OH) is observed. The formation of this radical is probably the main cause of primaquine mediated toxicity.

The toxicity of the antimalarial drug, primaquine has been described both *in vitro* utilising glucose-6-phosphate deficient red blood cells [1] and *in vitro* in normal red blood cells [2]. The formation of H_2O_2 by the reaction of primaquine with oxyhemoglobin is thought to be a major reaction leading to primaquine toxicity [3]. Also primaquine causes an increase of flux through the hexose monophosphate shunt [4]. This has been assumed to provide reducing equivalents for the removal of H_2O_2 by glutathione peroxidase. Consequently cells which are deficient in glucose-6-phosphate dehydrogenase were unable to detoxify H_2O_2 via the glutathione peroxidase pathway [5]. However, *in vitro* lysis may also be induced by primaquine in normal as well as in glucose-6-phosphate deficient red blood cells [6]. Also primaquine has been found to stimulate flux through the hexose monophosphate shunt in red blood cells containing methemoglobin [7]. In these cells there is no accumulation of H_2O_2 in the presence of primaquine [3]. Recently primaquine mediated toxicity in red blood cells has been shown to be independent of H_2O_2, oxyhemoglobin and glutathione turnover and that oxidation of NADPH, as a result of which stimulation of the hexose monophosphate shunt occurs, to produce H_2O_2 is the key reaction [8]. We have therefore investigated the reaction between primaquine and NADPH and the effect of primaquine on red blood cells using the electron paramagnetic resonance technique for spin trapping. This technique has previously been used to demonstrate free radical production from intact erythrocytes [9–11]. The free radicals formed by primaquine are defined in this investigation. A mechanism for their formation is proposed.

MATERIALS AND METHODS

Reagents

Primaquine and chloroquine salts were obtained from Sigma Chemical Co., Dorset, England. Catalase (activity 25000 U/mg protein) was supplied by Boehringer Chemical Corp., Sussex, England. The spin trap 5,5-dimethyl-l-pyrroline-*N*-oxide (DMPO) was prepared and purified by the method of Bonnet *et al.* [12]. Human copper/zinc superoxide dismutase was prepared from outdated blood [13]. Human hemoglobin type IV obtained from Sigma Chemical Co. was reduced with sodium dithionite and saturated with oxygen prior to use. The oxyhemoglobin formed was separated from excess dithionite by gel filtration on Sephadex G-25. Purified oxyhemoglobin was checked for methemoglobin content. Oxyhemoglobin concentration was determined at 413 nm using an extinction of 125,000/M/cm^{-1} [14].

Erythrocyte preparations

Fresh human blood was drawn into a 3.8% sodium citrate solution. Plasma and white cells were removed by centrifugation and erythrocytes were washed four times with phosphate-buffered saline (9 parts 0.154 M NaCl and 1 part 100 mM phosphate buffer, pH 7.4). A 10% hematocrit suspension in Krebs–Ringer phosphate buffer, pH 7.4 was prepared as stock solution and stored on ice. Carbon monoxyhemoglobin erythrocytes were prepared by slow bubbling of a 10% hematocrit suspension of erythrocytes with carbon monoxide. Treated erythrocytes were sedimented and the carbon monoxide saturated supernatant replaced with aerated Krebs–Ringer phosphate buffer, pH 7.4.

Electron spin resonance (ESR) spectroscopy

Spectra were recorded on a Varian E 104 X-band spectrometer with an E900-3 data acquisition system. ESR spectra were recorded with field set 3385G, modulation frequency 100kHz, modulation amplitude 1.0 G, microwave power 10 mW, microwave frequency 9.478 kHz and computer averaging 16 scans from $t = 0.5$ to $t = 16.5$ min to give a qualitative and comparative estimate of spin adduct production. A quantitative measure of spin adduct production was obtained by setting a field for the top of a peak of the spin adduct ESR spectrum and following the signal intensity with time. Each observation was reproduced three to four times. α,α-Diphenyl-β-picrylhydrazyl (DPPH) was used as g factor standard. Spin adduct concentrations were calculated from a calibrated double integral.

Fluorescence spectroscopy

Spectra were recorded on a Perkin-Elmer 650-10s fluorescence spectrometer fitted with a Perkin-Elmer 56 chart recorder.

Oxygen uptake

Oxygen consumption was monitored using a Clark-type oxygen electrode YSI 5331 (Yellow Springs Instruments, Ohio, U.S.A.).

RESULTS

The reactions of 1 mM primaquine with 1 mM NADPH in the presence of DMPO results in the production of two spin adducts labelled a and b in Fig. 1A. Simulation of component a in Fig. 1A gives the following ESR spectral parameters: g = 2.0051, $a_N = a\beta_H = 14.9$G (Fig. 1B) whilst simulation of component b in Fig. 1A gives the following ESR spectral parameters: g = 2.0061, $a_H = 14.3$G, $a_H = 11.7$G and $a\beta_H = 1.25$G (Fig. 1C). The two simulated components can be combined (Fig. 1D) to reproduce the experimental spectrum Fig. 1A. The parameters obtained for component a in Fig. 1A are identical with those previously reported for the hydroxyl spin adduct of DMPO, 5,5-dimethyl-2-hydroxypyrrolidino-1-oxyl(DMPO–OH) and the parameters obtained for component b in Fig. 1A are identical with those reported for the superoxide spin adduct of DMPO, 5,5-dimethyl-2-hydroperoxypyrrolidino-1-oxyl(DMPO–OOH) [15]. The reaction between primaquine and NADPH appears to be unique in producing free radical spin adducts. Very little free radical production is observed when NADH is substituted for NADPH and no free radical production is observed when the related antimalarial drug chloroquine is substituted for primaquine (Fig. 2). An increase in oxygen uptake by primaquine in the presence of NADPH (Fig. 3) confirms that oxygen centred free radicals are formed from the reaction between primaquine and NADPH. The effect of the catalase and copper/zinc superoxide dismutase on spin adduct formation is shown in Fig. 4. Catalase completely inhibited DMPO–OH formation and had no effect on superoxide spin adduct production. However, in the presence of copper/zinc superoxide dismutase while all the DMPO–OOH formation is abolished about 50% of the hydroxyl spin adduct

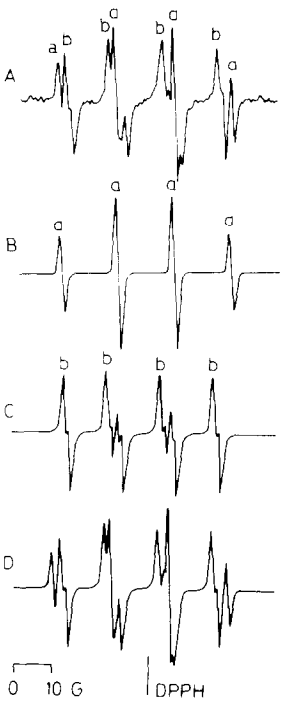

Fig. 1. (A) Spin trapping of the free radicals formed in the presence of 100 mM DMPO from the reaction between 1 mM NADPH and 1 mM primaquine in 50 mM phosphate buffer pH 7.4 containing 1 mM DTPA; (B) computer simulation of spin adduct a; (C) computer simulation of spin adduct b and (D) computer simulation of composite spin adducts a and b.

remains. These results indicate that not all the hydroxyl radical formed is arising from the interaction between superoxide radicals and hydrogen peroxide (Haber–Weiss reaction) [16]. The rate of this reaction is increased in the presence of iron complexes [17–19]. The iron chelator, diethylenetriaminepentaacetic acid (DTPA), present in the reaction medium

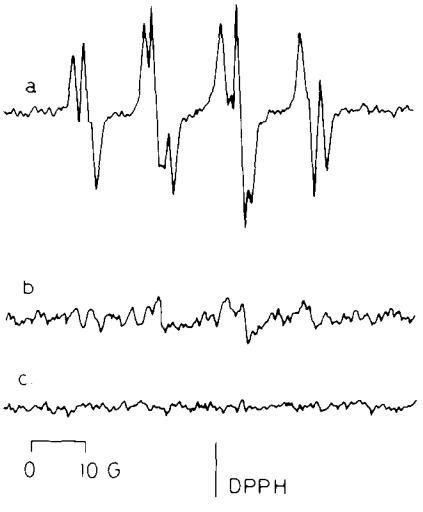

Fig. 2. Spin trapping of the free radical formed from the reactions between (a) NADPH and primaquine (b) NADH and primaquine and (c) NADPH and chloroquine reaction conditions as in Fig. 1.

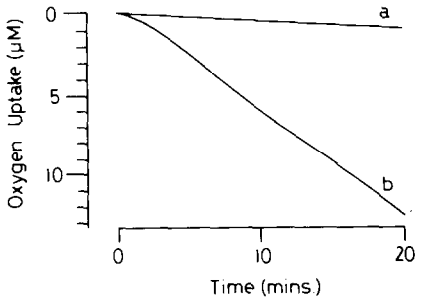

Fig. 3. Oxygen consumption by (a) 1 mM primaquine in 50 mM phosphate buffer pH 7.4 containing 1 mM DTPA and (b) plus 1 mM NADPH.

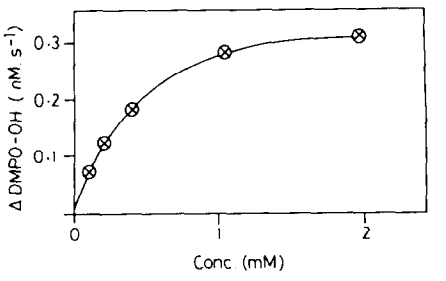

Fig. 5. Effect of primaquine concentration on the initial rates of production of DMPO–OH. Reaction conditions as in Fig. 1.

is probably acting as the catalyst. Iron-DTPA can catalyse an increase in the formation of hydroxyl radicals from superoxide radicals and H_2O_2 generated by the xanthine–xanthine oxidase reaction [20]. In contrast to the observation of Finkelstein *et al.* [21] no decomposition of the superoxide spin adduct to a hydroxyl spin adduct was observed indicating that the DMPO–OH spin adduct observed in the presence of copper/zinc superoxide dismutase is originating from the reduction of H_2O_2 in a reaction that is not metal-ion catalysed. Copper/zinc superoxide dismutase may be enhancing this reaction by accelerating the formation of H_2O_2 from the dismuation of superoxide radicals ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$).

The rate of DMPO–OH production increases with increasing primaquine concentration in the presence of 1 mM NADPH (Fig. 5). Maximum DMPO–OH production is achieved at approx. 1 mM concentration indicating a 1:1 catalytic complex between NADPH and primaquine. Fluorescence spectra of NADPH in the presence of primaquine (Fig. 6) shows quenching of NADPH fluorescence at 460 nm. At equimolar NADPH:primaquine concentration, the intensity of the 460 nm band is decreased by about 50% as compared to the control without primaquine. The disappearance of the fluorescence band at 460 nm indicates that the formation of a proximate NADPH/primaquine pair, as is expected from a charge transfer complex.

Incubation of red blood cells with primaquine resulted in the formation of a spin adduct having the same parameters as those of DMPO–OH (Fig. 7a) chloroquine treated cells exhibited no free radical formation (Fig. 7b). DMPO–OH production from primaquine-treated carbonmonoxyhemoglobin-containing erythrocytes was lower than that observed with oxyhemoglobin containing erythrocytes (Fig. 7c). This suggests an involvement of oxyhemoglobin in free radical production by primaquine, however, when primaquine is incubated with oxyhemoglobin no detectable spin adduct production was observed over a reaction time of 60 min. Increasing the oxyhemoglobin concentration from 50 μM to 200 μM still resulted in no detectable free radical production. The spin adduct formed from the interaction between primaquine and oxyhemoglobin containing red blood cells was confirmed to be due to hydroxyl radicals

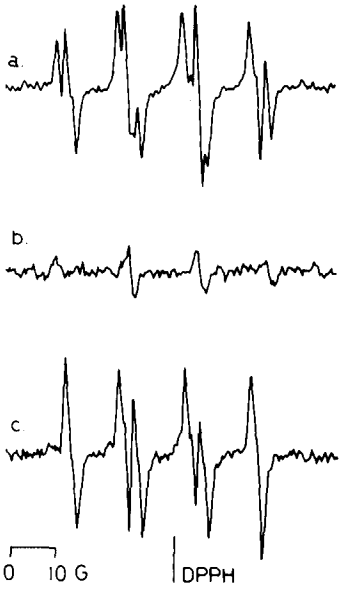

Fig. 4. Effect of copper/zinc superoxide dismutase and catalase on free radical production from the reaction between NADPH and primaquine. Reaction conditions as in Fig. 1. (a) control, (b) +100 μg/ml copper zinc superoxide dismutase and (c) +250 U/ml catalase.

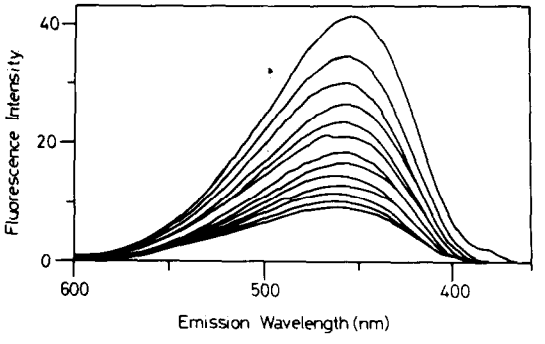

Fig. 6. Fluorescence emission spectrum of 200 μM NADPH in 50 mM phosphate buffer, pH 7.4 in the presence of 300 μM (bottom spectrum) to no added primaquine (top spectrum) by 25 μM increments.

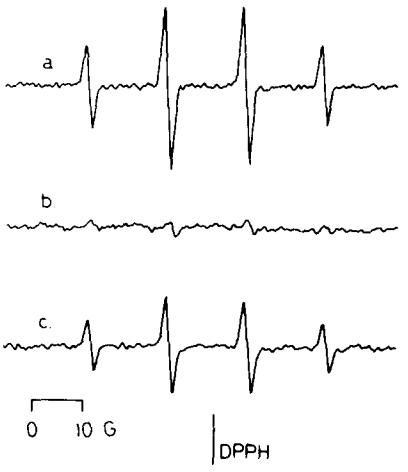

Fig. 7. Spin trapping of the free radicals formed in the presence of 100 mM DMPO from 1% red blood cells in Krebs–Ringer phosphate buffer, pH 7.4 containing 1 mM DTPA after treatment with (a) 100 μM primaquine; (b) 100 μM chloroquine and (c) carbon monoxide treated erythrocytes in the presence of 100 μM primaquine.

from the scavenging effect of various concn of mannitol (Fig. 8).

DISCUSSION

The mechanism of action of primaquine in red blood cells is as yet undefined. The oxidation of NADPH has been implicated as an important reaction in the mechanism [8]. The results obtained show that primaquine does react preferentially with NADPH in a 1:1 complex to produce superoxide and hydroxyl radicals. The reaction of NADH with primaquine resulted in a very reduced level of free radical production indicating that the phosphate group on the nucleotide facilitates the formation of the primaquine:pyridine nucleotide complex. Complex formation was found to occur to the same extent in HEPES and in phosphate buffer indicating that any involvement of the phosphate buffer is minimal. The electronic effects of the chloro substituents probably hinder the approach of NADPH to the quinoline ring as chloroquine and NADPH gave no detectable free radical spin adducts.

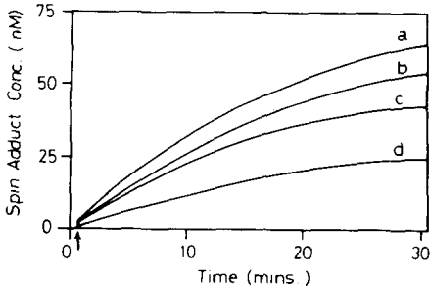

Fig. 8. Effect of various concentrations of mannitol on DMPO–OH production from 100 μM primaquine treated 1% red blood cells in Krebs–Ringer phosphate buffer, pH 7.4 containing 1 mM DTPA. (a) control (b) 10 mM (c) 20 mM and (d) 50 mM.

The formation of hydroxyl radicals is totally dependent on the reduction of H_2O_2 as catalase abolished the signal. The effect of copper/zinc superoxide dismutase on the reaction between primaquine and NADPH suggests that not all the H_2O_2 is formed from the dismutation reaction. Hydrogen peroxide formation can also result from superoxide radicals mediated oxidation of the complex. An analogous reaction involving oxidation of the lactate dehydrogenase–NADH complex by superoxide radicals has been reported by Bielski and Chan [22].

A mechanism for the oxidation of NADPH by primaquine is postulated in Table 1 on the basis of the observations made. No evidence was found to suggest electron transfer from NADPH to primaquine to give $NADP^+$ and the dihydro derivative of primaquine, PQH_2. Therefore the formation of an NADPH:primaquine complex is considered to be the initiation reaction. Four propagation routes are proposed following the initiating reaction. Reactions 2 and 3 lead to superoxide radical formation whilst reactions 6 and 8 lead to H_2O_2 formation. The NADPH:primaquine complex is proposed as the initial reductant of oxygen (reaction 2) producing superoxide and primaquine $PQH^\cdot$ radicals, PQH. The $PQH^\cdot$ free radical would be expected to be unstable by analogy with similar pyridinyl radicals [23] and so evades detection by ESR. $PQH^\cdot$ is most likely to decay by reduction of oxygen (reaction 3) rather than by disproportionation (reaction 7). Reduction of oxygen leads to the formation of superoxide radicals and primaquine.

Hydrogen peroxide can be formed either from the dismutation of superoxide radicals (reaction 8) or from the oxidation of the NADPH:primaquine complex by superoxide radicals (reaction 6). The termination reaction lead to the formation of hydroxyl radicals. These radicals can be formed either by the Haber–Weiss reaction (reaction 4) or by the reduction of hydrogen peroxide by $PQH^\cdot$ (reaction 5). The Haber–Weiss reaction is probably being catalysed by iron-DTPA formed by the chelation of spurious iron by the DTPA present in the reaction medium [20], whilst semiquinone reduction of hydrogen peroxide has been demonstrated to produce hydroxyl radicals [24].

Table 1. Mechanism for primaquine catalysed oxidation of NADPH

Initiation reaction
[1] $NADPH + PQ \rightarrow (NADP...H...PQ)$

Propagation Reactions
[2] $(NADP...H...PQ) + O_2 \rightarrow NADP^+ + PQH\cdot + O_2^-$
[3] $PQH\cdot + O_2 \rightarrow PQ + H^+ + O_2^-$
[4] $O_2^- + H_2O_2 \rightarrow OH^- + O_2 + OH^\cdot$
[5] $PQH^\cdot + H_2O_2 \rightarrow PQ + H_2O + OH^\cdot$
[6] $(NADP...H...PQ) + O_2^- + 2H^+ \rightarrow NADP^+ + PQH\cdot + H_2O_2$

Termination reactions
[7] $2PQH^\cdot \rightarrow PQM_2 + PQ$
[8] $2O_2^- + 2H^+ + H_2O_2 + O_2$

Abbreviation: PQ = primaquine.

Primaquine treated red blood cells have been demonstrated in this investigation to produce hydroxyl radicals. This is consistent with the prepared mechanism as also the earlier observation [4] that primaquine causes an increase in the level of hexose monophosphate shunt activity. Primaquine mediated toxicity is therefore ultimately due to the production of hydroxyl radicals. These highly reactive radicals are known to react with many cellular components at rates approaching diffusion control [25]. These reaction are known to initiate the peroxidation of membrane lipids, the polymerisation of proteins and the denaturation of enzymes [26].

Acknowledgements—JVB thanks the Medical Research Council (Grant No. G8205693CA) for support. AS is supported by NIH Grant HL 19532. We thank Dr. H. A. O. Hill for use of the ESR facility and Professor R. J. P. Williams FRS, for reading the manuscript.

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