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STUDIES WITH PRIMAQUINE *IN VITRO*: SUPEROXIDE RADICAL FORMATION AND OXIDATION OF HAEMOGLOBIN

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1 The production of superoxide radicals from primaquine diphosphate in aqueous solution has been demonstrated, using as indicator the reduction of cytochrome C with inhibition of the reaction by superoxide dismutase.

2 Primaquine-mediated oxidation of haemoglobin to methaemoglobin was reduced by the addition of catalase and increased by superoxide dismutase. Mannitol, a hydroxyl radical scavenger, abolished the increase in methaemoglobin observed in the presence of superoxide dismutase. EDTA reduced the oxidation of haemoglobin with and without superoxide dismutase.

3 Although the oxidation of haemoglobin in the presence of primaquine includes the effects of hydrogen peroxide, superoxide and hydroxyl radicals and metal ions, the results indicate that hydrogen peroxide, rather than the superoxide radical, is the main oxidizing species. The increase in haemoglobin oxidation occurring with superoxide dismutase may result from the augmented rate of hydrogen peroxide formation from superoxide radicals.

Introduction

The occurrence of haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficient subjects following the ingestion of the 8-aminoquinoline, primaquine and other drugs has been well known for many years (Beutler, 1959, 1969). Although the biochemical reactions leading to such drug-induced haemolysis have not been identified with certainty, the drugs produce oxidant effects in the erythrocyte. Hydrogen peroxide has been shown to be formed in intact normal erythrocytes incubated with primaquine, menadione, phenylhydrazine and other haemolytic drugs (Cohen & Hochstein, 1964, 1965; Tudhope & Leece, 1971), and it has been postulated that hydrogen peroxide is the main agent producing oxidant damage in the erythrocyte (Cohen & Hochstein, 1964).

Following the demonstration in erythrocytes of the enzyme superoxide dismutase which catalyzes the formation of hydrogen peroxide from superoxide radicals (McCord & Fridovich, 1969a) and the recent interest in the biological importance of the superoxide and hydroxyl radicals (Fridovich, 1974), reconsideration is required of the active oxidative agent in drug-induced haemolysis and of the relative importance of the several enzymatic defences against oxidation in the erythrocyte. Superoxide radicals, formed during the monovalent reduction of molecular oxygen, have been detected during the autoxidation of haemoglobin (Misra & Fridovich, 1972; Wever, Oudega & Van Gelder, 1973; Winterbourn, McGrath & Carrel, 1976) and during the reaction between phenylhydrazine and oxyhaemoglobin (Misra & Fridovich, 1976). Superoxide dismutase has been shown to inhibit the formation of methaemoglobin from oxyhaemoglobin (Lynch, Lee & Cartwright, 1976).

This report describes experiments to investigate superoxide radical formation from primaquine and to study the importance of this radical as an oxidizing species in reactions involving primaquine and haemoglobin *in vitro*.

Methods

Haemoglobin was prepared from normal human blood, collected, using acid citrate dextrose as anticoagulant. Erythrocytes were washed four times with 0.15 M NaCl, haemolyzed with two volumes of distilled water and acidified to pH 5.8 with HCl. After centrifugation and removal of a small precipitate, the solution was neutralized to pH 7.0 with NaOH and again centrifuged. Haemoglobin was reduced by the addition of sodium dithionite to the supernatant solution which was then loaded on to a 36.0 x 2.6 cm column containing Sephadex G75 Superfine. Haemoglobin was eluted with 0.05 M phosphate buffer, pH 7.8, containing 0.15 M NaCl; before use of buffer, oxygen had been removed by displacement with nitrogen. The haemoglobin was stored in the

reduced form under nitrogen. This solution of haemoglobin was shown to be free from catalase and superoxide dismutase activities.

Catalase was prepared free from thymol using the same column and buffer but without employing nitrogen. The catalase solution was shown to be free from superoxide dismutase activity, using the assay of McCord & Fridovich (1969b).

Cytochrome C Type VI (horse heart), superoxide dismutase Type I (bovine erythrocyte) 2880 units/mg, catalase (beef liver) 20,000 units/mg, EDTA disodium salt, D-mannitol and primaquine diphosphate were obtained from Sigma London Chemical Company Limited.

Superoxide radical formation was measured at 37°C using cytochrome C as an indicating scavenger molecule (Cohen & Heikkila, 1974). The increase in absorbance at 550 nm, produced by the reduction of cytochrome C, was measured in glass cells of 1 cm light path on a Perkin Elmer recording spectro-6photometer fitted with a thermostatically controlled cell compartment. In a total volume of 3 ml, the cuvettes contained 2.5×10^{-5} M cytochrome C and 0.05 M phosphate buffer, pH 7.0. Primaquine diphosphate, superoxide dismutase and catalase were added as indicated. The reaction rates were measured during 12 min.

Oxidation of haemoglobin was studied at 37°C using incubation mixtures, of volume 3.0 ml, containing haemoglobin 1.7 mg/ml and primaquine 3.3×10^{-3}M in 0.05 M phosphate buffer, pH 7.0. Superoxide dismutase, catalase, mannitol and EDTA were added as indicated. The absorbance at 630 nm was measured immediately before and 100 min after the addition of primaquine. The subsequent increase in absorbance was expressed as a percentage of the increase occurring in the control mixture containing only primaquine and haemoglobin.

Results

Superoxide radical formation

The formation of superoxide radicals from primaquine diphosphate in aqueous solution was shown by the reduction of cytochrome C with inhibition of the reaction by superoxide dismutase. With increasing concentrations of primaquine there was an increase in the rate of reduction of cytochrome C (Figure 1). The addition of superoxide dismutase led to a maximum inhibition of the reduction of cytochrome C of 55–60% (Figure 2). Catalase, added to the reaction mixture in a range of concentrations 0.3–6.67 μg/ml, produced no effect on the reduction of cytochrome C in the presence of primaquine.

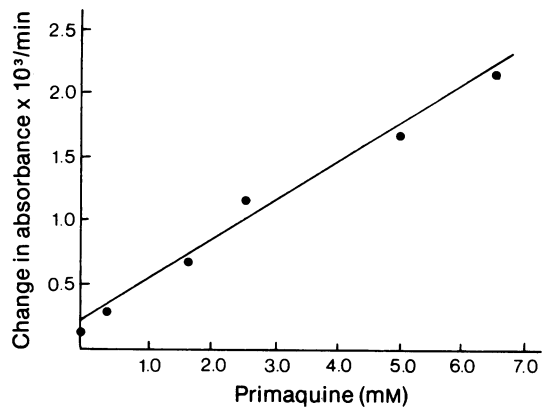

Figure 1 Rate of reduction of cytochrome C as a function of primaquine concentration. Cuvettes contained, in a total volume of 3 ml, 2.5×10^{-5} M ferricytochrome C, 0.05 M phosphate buffer, pH 7.0, and the indicated concentrations of primaquine diphosphate. The rate of reduction of cytochrome C at 37°C was measured by following the increase in absorbance at 550 nm over 12 min.

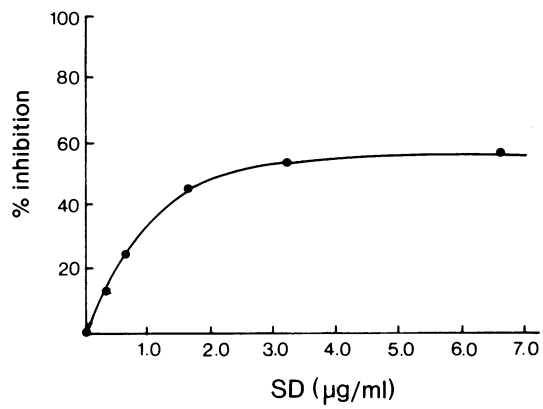

Figure 2 Inhibition by bovine erythrocyte superoxide dismutase (SD) of cytochrome C reduction in the presence of primaquine. Cuvettes contained, in a total volume of 3 ml, 2.5×10^{-5} M ferricytochrome C, 3.3×10^{-3} M primaquine diphosphate, 0.05 M phosphate buffer, pH 7.0, and the indicated concentrations of bovine erythrocyte superoxide dismutase. The percentage inhibition of the rate of cytochrome C reduction at 37°C measured at 550 nm is shown as a function of superoxide dismutase concentration. The mean results from three similar experiments are shown.

Oxidation of haemoglobin induced by primaquine

Methaemoglobin production. Haemoglobin 1.7 mg/ml and primaquine diphosphate 3.3×10^{-3} M in 0.05 M phosphate buffer, pH 7.0 were mixed in a glass cuvette

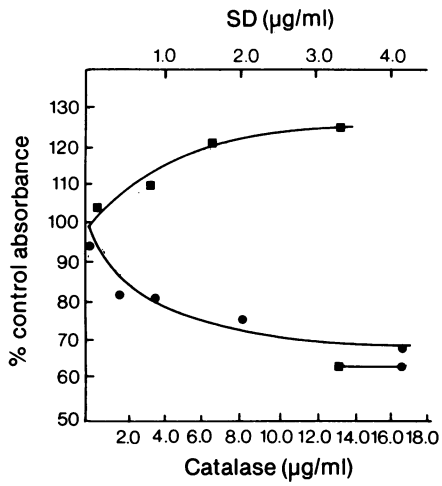

Figure 3 Influence of bovine erythrocyte superoxide dismutase (SD) and beef liver catalase on methaemoglobin formation in the presence of primaquine. Incubation mixtures contained, in a total volume of 3 ml, 1.7 mg/ml oxyhaemoglobin, 3.3×10^{-3} M primaquine diphosphate, 0.05 M phosphate buffer, pH 7.0, and the indicated amounts of superoxide dismutase or catalase. The increase in absorbance at 630 nm occurring during incubation for 100 min at 37°C in the absence of the enzymes was determined as the control value. The results, following the addition of superoxide dismutase (■), catalase (●), or both superoxide dismutase and catalase together (■——●) are expressed as percentages of the control value.

of 1 cm light path. The visible spectrum between 480 nm and 700 nm was recorded every 15 min using a Pye Unicam SP800 recording spectrophotometer. A steady increase in absorbance was observed at 630 nm, along with a decrease at 575 and 540 nm. Following the addition of an excess of sodium cyanide after 45 min, the previously observed increase in absorbance at 630 nm was decreased by approximately 80%. These results show that methaemoglobin is the major oxidation product of haemoglobin on interaction with primaquine and in subsequent experiments an increase in absorbance at 630 nm was used as a measure of haemoglobin oxidation.

The influence of superoxide dismutase, catalase, mannitol and EDTA on the oxidation of haemoglobin in the presence of primaquine (a) Superoxide dismutase, added to the incubation mixture, led to a marked increase in the oxidation of haemoglobin (Figure 3). No increase in oxidation occurred when the superoxide dismutase had been previously inactivated by boiling for 10 min.

(b) Catalase. The oxidation of haemoglobin was reduced to about 70% of the control value by the addition of catalase (Figure 3). When both superoxide dismutase and catalase were added together, the haemoglobin oxidation was the same as with catalase alone (Figure 3).

(c) Mannitol, a scavenger of hydroxyl radicals, when added to the incubation mixture of primaquine and haemoglobin produced no effect at two of the concentrations used; however at the intermediate concentration of mannitol there was consistently a slight reduction in haemoglobin oxidation (Table 1). When mannitol was added to the mixture containing superoxide dismutase, the results were the same as the control without the enzyme; the addition of mannitol prevented the increased oxidation of haemoglobin observed with superoxide dismutase (Table 1). Addition of mannitol to the incubation mixtures containing catalase or catalase and superoxide dismutase produced no change in the rate of oxidation of haemoglobin. In the absence of primaquine, mannitol produced negligible oxidation of haemoglobin in this incubation system.

Table 1 The effect of mannitol and of EDTA on the oxidation of haemoglobin in the presence of primaquine with and without superoxide dismutase. The increase in absorbance at 630 nm after the addition of primaquine is shown, expressed as a percentage of the increase occurring in the mixture with haemoglobin and primaquine only. Each result is the mean of three experiments.

	Mannitol (M)				*EDTA (M)*			
Incubation mixture	*0*	*3×10^{-3}*	*1.5×10^{-2}*	*4.5×10^{-2}*	*0*	*10^{-6}*	*10^{-4}*	*10^{-2}*
Primaquine + haemoglobin	100	96	82	99	100	68	80	83
Primaquine + haemoglobin + superoxide dismutase	124	98	105	100	124	88	86	96

(d) EDTA, as a chelating agent of metal cations, consistently reduced the rate of haemoglobin oxidation with or without the addition of superoxide dismutase (Table 1). When EDTA was added to mixtures containing catalase, no marked or consistent change occurred in the rate of haemoglobin oxidation.

Discussion

The physiological defences against the toxic effects of hydrogen peroxide include the enzymes, catalase and glutathione peroxidase; the action of the latter, requiring a constant supply of NADPH, is impaired in G6PD-deficient erythrocytes. The relative importance of catalase and of glutathione peroxidase has been uncertain but most of the evidence has suggested that glutathione peroxidase is the more important agent in removing the small amount of hydrogen peroxide generated in the erythrocyte as a result of the effect of oxidant drugs (Mills, 1957; Cohen & Hochstein, 1963; Tudhope and Leece, 1971). Nevertheless, in studies on the effects of drugs on erythrocytes *in vitro*, no constant relationship was found between the degree of inhibition of glutathione peroxidase activity and the extent of oxidative changes produced in the erythrocytes (Hopkins & Tudhope, 1974).

Erythrocytes contain the enzyme, superoxide dismutase, which catalyzes the reaction between superoxide radicals to form oxygen and hydrogen peroxide (McCord & Fridovich, 1969a).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow[\text{Dismutase}]{\text{Superoxide}} O_2 + H_2O_2$$

The discovery of this enzyme in erythrocytes has aroused interest in the possible formation of superoxide radicals during normal erythrocytic metabolism and from drugs. The importance of superoxide dismutase in the erythrocyte in the protection against the oxidative action of superoxide radicals produced by drugs has still to be elucidated.

Primaquine, incubated with normal erythrocytes *in vitro*, caused oxidation of haemoglobin (Tudhope & Leece, 1971) and changes in osmotic fragility (Miller & Smith, 1970; Tudhope & Leece, 1971); there was a direct relationship between Heinz body formation and increased mechanical fragility (Hopkins & Tudhope, 1974). The concentration of primaquine required to produce oxidant effects on erythrocytes *in vitro* is much higher than would result *in vivo* from therapeutic use of the drug. Metabolites of primaquine may cause the erythrocytic changes *in vivo*. Several types of drugs causing oxidant effects on erythrocytes are metabolized to hydroxylated derivatives which possess greater oxidant properties than the original compounds (Kiese, 1966; Fraser & Vesell, 1968). Hydroxylated derivatives of primaquine increased the mechanical fragility and the methaemoglobin content of G6PD deficient erythrocytes more than that of normal erythrocytes. Primaquine itself was inactive at the same concentration (Fraser and Vesell, 1968). However, in a study in man of possible metabolites of primaquine, the only compound which could be identified was 6-methoxy 8-aminoquinoline (Baty, Evans & Robinson, 1975).

Hydrogen peroxide has been detected in intact erythrocytes after the addition of primaquine (Cohen & Hochstein, 1964, 1965). The present study has shown that primaquine, incubated at 37°C in aqueous medium at pH 7.0 generated superoxide radicals. If this occurred within the erythrocyte, the superoxide radicals would be very rapidly eliminated by superoxide dismutase with the formation of hydrogen peroxide. Hydroxyl radicals could also be produced from the interaction of superoxide radicals and hydrogen peroxide.

$$O_2^{\cdot -} + H_2O_2 \rightarrow OH\cdot + OH^- + O_2$$

Thus, hydrogen peroxide previously shown to be formed in erythrocytes exposed to oxidant drugs may be the result of initial transient superoxide radical production.

Primaquine causes oxidation of haemoglobin. In the present study, this reaction was studied *in vitro* using pure haemoglobin. Paradoxically, destruction of superoxide radicals by superoxide dismutase markedly increased the rate of oxidation of haemoglobin. This increase was abolished by the addition of mannitol, a scavenger of hydroxyl radicals. These results suggest that hydroxyl radicals may be involved in the oxidation of haemoglobin by primaquine in the presence of superoxide dismutase. Hydrogen peroxide, a product of the dismutation reaction can react with superoxide radicals giving rise to hydroxyl radicals. Support for the involvement of hydrogen peroxide is afforded in these experiments by the reduction of oxidation of haemoglobin resulting from the addition of catalase. Superoxide radicals not only oxidize haemoglobin to methaemoglobin but also can produce the reverse reaction involving the reduction of methaemoglobin (Winterbourn *et al.*, 1976). The increased oxidation of haemoglobin observed with superoxide dismutase may be partly attributable to blocking the latter effect of superoxide radicals. As the addition of both superoxide dismutase and catalase together to the incubation mixture reduced the oxidation of haemoglobin only to 70% of the control value, it appears that a major part of the oxidation of haemoglobin in the presence of primaquine occurs without the action of superoxide radicals or hydrogen peroxide. This may be due to direct oxidation of haemoglobin by primaquine, present in relatively high concentration in the *in vitro* experiments. The implication of traces of heavy metal ions in catalyzing the reaction is suggested by the reduction in oxidation occurring when EDTA was added. The oxidation of haemoglobin to methaemoglobin in the presence of primaquine involves the composite effects of hydrogen

peroxide, superoxide and hydroxyl radicals and metal ions.

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