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PRIMAQUINE-MEDIATED OXIDATIVE METABOLISM IN THE HUMAN RED CELL

LACK OF DEPENDENCE ON OXYHEMOGLOBIN, H_2O_2 FORMATION, OR GLUTATHIONE TURNOVER

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Abstract—Stimulation of the hexose monophosphate shunt by primaquine results from the oxidation of NADPH by primaquine. This conclusion was based on the observations that primaquine lowered cellular NADPH but not GSH and that, in red cells in which the GSH was unavailable for reaction, primaquine still stimulated the rate of the hexose monophosphate shunt. In a non-cellular system, primaquine interacted with NADPH, but not GSH, to produce H_2O_2. Stimulation of the hexose monophosphate shunt by primaquine does not primarily involve H_2O_2 accumulation since stimulation of the pathway by primaquine was also observed in red cells containing methemoglobin, a red cell preparation in which no H_2O_2 accumulates. Methemoglobin prevented the formation and/or accumulation of H_2O_2 in intact red cells incubated with primaquine as well as in a non-cellular system containing primaquine plus Fe^{2+}-EDTA as an H_2O_2 source. Methemoglobin probably acts by scavenging reactive intermediates since oxyhemoglobin was formed from methemoglobin in the non-cellular experiments. In the red cell, primaquine stimulated glucose-dependent conversion of methemoglobin to oxyhemoglobin.

The formation of H_2O_2 by reaction of primaquine with oxyhemoglobin is thought to be a major reaction leading to primaquine toxicity in the red cell [1].† Primaquine-mediated increase of flux through the hexose monophosphate shunt [6] is assumed to result from the removal of H_2O_2 by glutathione (GSH)‡ peroxidase. We have expressed doubts previously about the validity of this scheme since primaquine also stimulates flux through the hexose monophosphate shunt in red cells containing methemoglobin [7], a red cell preparation in which no H_2O_2 accumulates in the presence of primaquine [1]. Here we present evidence that primaquine-mediated oxidative metabolism is independent of oxyhemoglobin, H_2O_2 formation, or glutathione turnover. We hypothesize that oxidation of NADPH by primaquine may be a key reaction supporting oxidative damage.

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† In this report, we have concentrated on primaquine toxicity *in vitro*. Primaquine toxicity has been described both *in vivo* in glucose-6-phosphate dehydrogenase deficient red cells [2] and *in vitro* in normal red cells [3]. *In vivo* toxicity in glucose-6-phosphate dehydrogenase deficient red cells is achieved at much lower primaquine concentrations than are required for *in vitro* toxicity in either normal or deficient red cells [3]. The relationship between *in vivo* and *in vitro* oxidative effects of primaquine is not understood [3–5].

‡ Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; NEM, *N*-ethylmaleimide; and DEAE-cellulose, diethylaminoethyl-cellulose.

MATERIALS AND METHODS

Purified hemoglobin preparations. Human adult blood (3 ml) was drawn into 3.8% sodium citrate solution. Following centrifugation, the plasma and buffy coat were removed. The red cells were washed three times with phosphate-buffered saline (9 parts 0.154 M NaCl; 1 part 0.1 M KH_2PO_4/K_2HPO_4, pH 7.4) and lysed with 1 vol. of water. A small volume of concentrated potassium phosphate buffer, pH 7.4, containing EDTA was added to give a final concentration of 0.01 M phosphate and 0.3 mM EDTA. All subsequent procedures were carried out at 4°. Lysates were centrifuged at 27,000 *g* for 15 min to remove red cell membranes. The supernatant fraction was then applied to a Sephadex G-25 (Pharmacia, Piscataway, NJ) column which had been equilibrated previously with 0.3 mM EDTA, 0.01 M KH_2PO_4/K_2HPO_4, pH 7.4. The red eluate was collected and added to 1 vol. of DEAE-cellulose (Nutritional Biochemicals Corp., Cleveland, OH) suspension prepared by the method of Hennessey *et al.* [8]. After 20 min in the cold, the DEAE-cellulose–hemoglobin suspension was centrifuged at 2000 *g* for 15 min, and the supernatant fraction was subjected to a second treatment with DEAE-cellulose. The purified hemoglobin solution was found to be essentially free of superoxide dismutase and catalase by standard assays [9–11]. The visible spectrum of hemoglobin was monitored using a Cary 14 spectrophotometer to ensure that all of the hemoglobin was in oxyhemoglobin form and to determine the concentration of the hemoglobin solution. A small volume of concentrated potassium phosphate buffer, pH 7.4, containing EDTA was added to the

purified hemoglobin solution to give a final concentration of 0.125 M phosphate, 0.3 mM EDTA. The purified hemoglobin solution was stored at 4°. Less than 1% methemoglobin was present after 2 weeks of storage.

Methemoglobin was prepared by adding a small volume of a concentrated solution of $NaNO_2$ to an aliquot of the purified oxyhemoglobin solution to give a final concentration of 1% $NaNO_2$ (w/v). This solution was kept in the cold for at least 10 min before being applied to a Sephadex G-25 column which had been equilibrated previously with 0.375 mM EDTA, 0.125 M KH_2PO_4/K_2HPO_4, pH 7.4, in order to remove nitrite. The brown eluate was collected and the visible spectrum was observed to ensure complete conversion to methemoglobin and removal of all of the nitrite and to determine the concentration of the methemoglobin solution.

Measurement of primaquine-mediated H_2O_2 formation in non-cellular experiments. Primaquine-mediated H_2O_2 formation in non-cellular experiments was less than 10^{-5} M as measured by a YSI-Clark Oxidase Probe (Yellow Springs Instruments, Yellow Springs, OH). Therefore, the inactivation of catalase in the presence of 3-amino-1,2,4-triazole was used as the index of H_2O_2 formation [1]. This system is sensitive to small quantities of H_2O_2 even in the presence of other reaction routes for consumption of H_2O_2. In the presence of H_2O_2, ferricatalase is oxidized to Compound I [12]; if 3-amino-1,2,4-triazole is present, it forms an irreversible complex with Compound I with loss of catalase activity [13,14]. No inhibition of catalase activity was observed in the absence of 3-amino-1,2,4-triazole in any experiments.

Samples containing 132 μg/ml catalase (88,823 units/mg; Calbiochem, LaJolla, CA), 5 mM 3-amino-1,2,4-triazole, 0.375 mM EDTA and 125 mM KH_2PO_4/K_2HPO_4, pH 7.4, in a final volume of 0.5 ml were incubated at 37° for 2 hr in a shaking water bath. Other additions in individual experiments were present in the following final concentrations: primaquine, 0.8 mM; NADH, NADPH, NADP, GSH and GSSG, 0.1 mM; oxyhemoglobin, methemoglobin and bovine serum albumin, 50 μM; and $FeCl_2$ and $FeCl_3$, 0.1 or 0.2 mM. Iron salts were purchased from the Fisher Scientific Co. (Springfield, NJ); all other reagents were products of the Sigma Chemical Co. (St. Louis, MO). Catalase activity was determined at 2 hr as described by Beers and Sizer [11]. A 0.05-ml aliquot from each of the incubated samples was added to 4 ml of 0.05 M NaH_2PO_4/Na_2HPO_4, pH 6.8, and 2 ml of this mixture was added to a sample cuvette and 2 ml to a reference cuvette in a Cary 14 spectrophotometer. The contents of the sample cuvette were constantly mixed by a small magnetic stirring bar. 0.04 ml of 0.78 M H_2O_2, 0.05 M NaH_2PO_4/Na_2HPO_4, pH 6.8, was injected into the sample cuvette, and the decrease in absorbance at 240 nm was measured. The decrease in absorbance was linear with time and proportional to catalase activity up to 20 sec after injection.

Spectra of hemoglobin-containing samples were monitored after 2 hr of incubation. The presence of methemoglobin was confirmed by converting methemoglobin to oxyhemoglobin by reaction with sodium dithionite ($Na_2S_2O_4$) in the presence of excess catalase (modification of Van Slyke *et al.* [15]) and observing changes in the visible absorption spectra.

Red cell preparations and incubation conditions. Adult human blood was drawn daily into 3.8% sodium citrate solution. After centrifugation, plasma and white cells were removed, and red cells were washed three times with phosphate-buffered saline. A 25% (v/v) suspension of red cells was prepared in Krebs–Ringer phosphate buffer (100 parts 0.154 M NaCl; 4 parts 0.154 M KCl; 1.5 parts 0.11 M $CaCl_2$; 1 part 0.154 M $MgSO_4$; 21 parts 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4) with or without 5 mM D-glucose. Incubations of red cell suspensions were carried out at 37° in a shaking water bath. Primaquine diphosphate (Sigma Chemical Co.) was added where indicated at a final concentration of 1 mM. When primaquine was not added, sodium phosphate was added to control for the phosphate contained in the primaquine salt.

Red cells containing methemoglobin were prepared by mixing 1 volume of red cells with 1 volume of 1% $NaNO_2$ in phosphate-buffered saline, incubation at room temperature for 10 min, and washing the red cells five times with phosphate-buffered saline to remove the nitrite [16]. Such washing was efficient as shown by observing the visible spectra of lysates of washed cells (lack of methemoglobin nitrite) (also see Ref. 17). NO_2^- has been shown to react with oxyhemoglobin to form methemoglobin and NO^-_3 stoichiometrically with little formation of other hemoglobin- or nitrogen-containing derivatives [18]. Although H_2O_2 and other oxygen-containing intermediates may be formed as intermediates of the reaction between hemoglobin and NO_2^- [18], sufficient superoxide dismutase and catalase should be present to protect other cell components. No loss of catalase activity was observed in red cells incubated with NO_2^-.

Red cells treated with *N*-ethylmaleimide (NEM) (Sigma) were prepared by incubating a 25% red cell suspension with 2.5 μmoles NEM/ml red cells for 30 min at 37°. The red cells were then washed three times with Krebs–Ringer phosphate containing 5 mM D-glucose to remove the NEM. Titration with NEM indicated that 2.5 μmoles NEM/ml red cells was sufficient to bind all the GSH while causing between 10 and 45% inhibition of hexose monophosphate shunt activity (also see Ref. 19).

Measurement of primaquine uptake by red cells. A 25% red cell suspension was prepared in Krebs–Ringer phosphate and incubated with 1 mM primaquine in the presence and absence of 5 mM D-glucose for 1 hr at 37°. The suspension was then centrifuged at 4000 *g* for 10 min. The medium was removed and its absorbance was measured against water in a Cary 14 spectrophotometer at 265 nm. The concentration of primaquine in the red cells and medium of each sample was determined.

Measurement of cellular GSH. A 25% red cell suspension was prepared in Krebs–Ringer phosphate and incubated in the presence and absence of 1 mM primaquine and 5 mM D-glucose for 1 hr at 37°. Measurement of GSH was carried out by the method of Beutler [20].

Table 1. Primaquine-mediated H_2O_2 formation*

Catalase and aminotriazole incubated in the presence of:	% Catalase activity	
	Control	Incubated with primaquine
No additions	100	98
Oxyhemoglobin	100	52
Methemoglobin	100	100
$FeCl_2$ (0.1 mM)	95	58
$FeCl_2$ (0.1 mM) + oxyhemoglobin	32	23
$FeCl_2$ (0.2 mM) + methemoglobin	100	95
NADPH	96	63
NADH	92	63
GSH	100	100

* Catalase (0.55 μM) and 3-amino-1,2,4-triazole (5 mM) were incubated for 2 hr at 37° in a buffer containing EDTA (0.375 mM) and KH_2PO_4/K_2HPO_4 (125 mM), pH 7.4 (representative experiment). Formation of H_2O_2 was estimated by inhibition of catalase as described in Materials and Methods. Concentrations: oxyhemoglobin (0.05 mM), methemoglobin (0.05 mM), primaquine (0.8 mM), $FeCl_2$ (0.1 mM or 0.2 mM), NADPH (0.1 mM), NADH (0.1 mM), and GSH (0.1 mM).

Measurement of flux through the hexose monophosphate shunt. The flux of glucose through the hexose monophosphate shunt was determined using D-[1-^{14}C]glucose and measuring $^{14}CO_2$ evolution as previously described [7]. The flux was calculated after subtracting blank values and expressed as μmoles CO_2 produced per ml red cells per hr.

Measurement of cellular oxyhemoglobin and methemoglobin. A 25% red cell suspension containing either 100% oxyhemoglobin or 100% methemoglobin was prepared in Krebs–Ringer phosphate and incubated in the presence and absence of 1 mM primaquine and 5 mM D-glucose at 37°. Aliquots for hemoglobin analysis were taken at 0, 1, 2 and 5 hr. Oxyhemoglobin, methemoglobin and intact hemoglobin (defined as the sum, oxyhemoglobin plus methemoglobin) were measured by a modification of the technique of Harley and Mauer [21] as previously described [22]. A decrease of intact hemoglobin during incubation of red cells was taken as indirect evidence for an accumulation of hemoglobin metabolites. No loss of intact hemoglobin was observed in any experiment.

RESULTS

Primaquine-mediated H_2O_2 formation in non-cellular experiments. Primaquine interacted with oxyhemoglobin but not methemoglobin to produce H_2O_2 (Table 1). Similarly, Fe^{2+}-EDTA (or Fe^{3+}-EDTA, not shown) interacted with oxyhemoglobin but not methemoglobin to produce H_2O_2. When oxyhemoglobin was incubated with iron and/or primaquine, H_2O_2 formation was paralleled by significant methemoglobin formation as indicated by the changes in the visible spectra at 500, 541, 577 and 631 nm (Fig. 1a). The presence of a functional heme group in the methemoglobin product was demonstrated by reduction of methemoglobin by dithionite in the presence of excess catalase followed by conversion to oxyhemoglobin. After the addition of dithionite, the ratio of the magnitudes of the 541 nm to the 577 nm absorption peak heights approached that of oxyhemoglobin (Fig. 1a).

When methemoglobin was incubated with the H_2O_2 generating system, primaquine plus Fe^{2+}-EDTA, the presence of methemoglobin prevented the formation or accumulation of H_2O_2 (Table 1). In contrast to methemoglobin, albumin did not prevent H_2O_2 formation (not shown). The active involvement of methemoglobin in preventing formation or accumulation of H_2O_2 in a mixture of

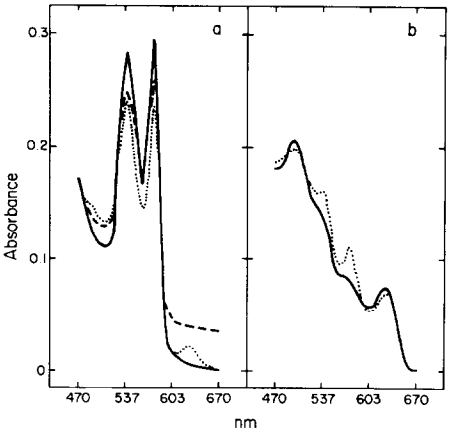

Fig. 1. Spectra of oxyhemoglobin and methemoglobin after incubation with primaquine and chelated iron. Oxyhemoglobin (0.05 mM) or methemoglobin (0.05 mM) was incubated for 2 hr at 37° in a medium containing catalase (0.55 μM), 3-amino-1,2,4-triazole (5 mM), EDTA (0.38 mM) and KH_2PO_4/K_2HPO_4 (125 mM), pH 7.4. Other additions were: primaquine (0.8 mM) and $FeCl_2$ (0.1 mM or 0.2 mM). Spectra were observed as described in Materials and Methods. (a) Oxyhemoglobin (———); oxyhemoglobin plus primaquine plus $FeCl_2$ (0.1 mM) (·····); the latter samples after addition of sodium dithionite and excess catalase (– – – –). (b) Methemoglobin (———); methemoglobin plus primaquine plus $FeCl_2$ (0.2 mM) (·····). Similar results were observed when $FeCl_2$ was replaced by $FeCl_3$.

Table 2. Uptake of primaquine by red cells*

Time	Additions	Compartment	Primaquine concentration (mM)
0	None	Medium	1.33
0	None	Cells	0.00
1 hr	None	Medium	0.41
1 hr	None	Cells	2.77
1 hr	Glucose	Medium	0.41
1 hr	Glucose	Cells	2.77

* The primaquine concentrations in cells and medium were determined after incubation of a 25% red cell suspension for 1 hr at 37° as described in Materials and Methods (representative experiment).

primaquine and iron, is shown by the formation of a significant amount of oxyhemoglobin, as indicated by the changes in the visible spectra at 500, 541, 577 and 631 nm (Fig. 1b).

Primaquine-mediated H_2O_2 formation was also observed when primaquine was incubated with either NADPH or NADH (Table 1). In contrast, no H_2O_2 formation was observed when primaquine was incubated with $NADP^+$, GSH or GSSG.

Uptake of primaquine by red cells. The uptake of primaquine in red cells incubated with and without glucose was determined by measuring primaquine concentration in the medium, and the results are shown in Table 2. Red cells incubated with primaquine established a 7-fold primaquine concentration gradient in 1 hr between the red cells and the medium whether or not glucose was present.

Role of GSH and NADPH. In previous studies we showed that primaquine lowered cellular NADPH levels [7]. As part of the present study we observed the effects of primaquine on cellular GSH, another major product of the hexose monophosphate shunt. Primaquine and/or glucose had no effect on GSH levels after 1 hr of incubation (data not shown).

We also observed previously that primaquine increased hexose monophosphate shunt activity [7]. To determine whether or not GSH was involved in the effect of primaquine on the hexose monophosphate shunt, we prepared red cells in which GSH was removed by pretreatment with NEM. Primaquine caused a significant increase in flux through the hexose monophosphate shunt in both normal and NEM-treated red cells (Table 3).

Table 3. Effect of primaquine on flux through the hexose monophosphate shunt in red cells pretreated with *N*-ethylmaleimide*

Treatment	CO_2 produced [μmoles·(ml red cells)$^{-1}$·hr^{-1}]
None	0.127 ± 0.015 (6)
Primaquine	0.210 ± 0.028† (6)
NEM	0.083 ± 0.014 (6)
NEM + primaquine	0.109 ± 0.018† (6)

* The flux through the hexose monophosphate shunt was measured after incubation of normal and NEM-treated red cells for 1 hr at 37° as described in Materials and Methods. Data are shown as the means ± S.E. with the number of observations in parentheses.

† $P < 0.005$, versus treatment without primaquine. The P values are based on paired *t*-tests. The differences observed are highly significant in spite of the large S.E. since most of the variance was between different paired experiments while the effect of primaquine was consistent.

Effect of primaquine on hemoglobin in red cells containing oxyhemoglobin or methemoglobin. When red cells containing 100% oxyhemoglobin were incubated with primaquine, 20% of the oxyhemoglobin was converted to methemoglobin in 5 hr as compared to 9% in control red cells (Fig. 2). Qualitatively similar results have been reported [23]. When red cells containing oxyhemoglobin were incubated with primaquine plus glucose, a protective effect of glucose against methemoglobin formation was seen at 1 hr but was no longer observable after 5 hr of incubation. When red cells containing 100% methemoglobin at zero time were incubated with or

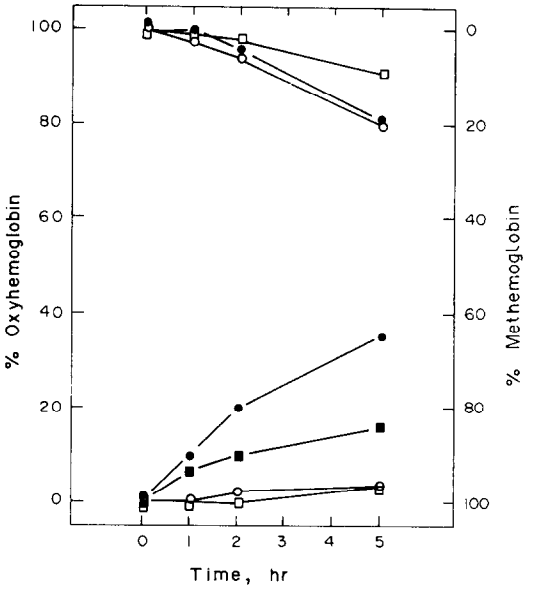

Fig. 2. Effect of primaquine on hemoglobin in red cells containing oxyhemoglobin or methemoglobin. The percentages of oxyhemoglobin and methemoglobin were determined after incubation of red cells for 0, 1, 2, and 5 hr at 37° as described in Materials and Methods. Concentrations: red cells (25%), primaquine (1 mM), and glucose (5 mM). Red cells were incubated with no additions (□), glucose (■), primaquine (○), and primaquine plus glucose (●).

without primaquine, only 3% of the total hemoglobin content was converted to oxyhemoglobin by 5 hr. If red cells containing 100% methemoglobin were incubated with glucose alone, red cell reductase activity converted 16% of the methemoglobin to oxyhemoglobin by 5 hr. In the presence of both primaquine and glucose, 35% of the methemoglobin was converted to oxyhemoglobin by 5 hr. Although primaquine alone did not cause conversion of methemoglobin to oxyhemoglobin, primaquine greatly increased the conversion of methemoglobin to oxyhemoglobin in the presence of glucose.

DISCUSSION

The key observations in studies of the oxidative effects of primaquine have been primaquine-mediated increase in flux through the hexose monophosphate shunt [6,7] and primaquine-mediated H_2O_2 formation in oxyhemoglobin-containing red cells [1]. The increased flux through the hexose monophosphate shunt, however, does not seem to be linked to removal of H_2O_2 since primaquine also stimulates flux through the hexose monophosphate shunt in red cells containing methemoglobin [7], a red cell preparation in which no H_2O_2 accumulates in the presence of primaquine [1]. Primaquine appears to increase flux through the hexose monophosphate shunt by a mechanism similar to that of methylene blue, which is specifically reduced in the red cell by NADPH [24]. It is the reduced form of methylene blue which then reacts with molecular oxygen to form H_2O_2 [25]. It is possible that primaquine is present in the red cell in various redox states, and that the increase in flux through the hexose monophosphate shunt is in large part due to the reduction of primaquine or its derived products by NADPH and/or GSH. We demonstrated previously that primaquine decreases cellular NADPH levels in the presence or absence of glucose [7]; here we report that primaquine had no consistent effect on cellular GSH levels in the presence or absence of glucose. Comparable results with GSH were obtained by investigators who carried out similar experiments in the presence of glucose [26,27]. In red cells treated with NEM, an agent which binds the sulfhydryl groups of GSH so that they are unavailable for reaction, primaquine still had a significant stimulatory effect on the hexose monophosphate shunt. It seems likely that primaquine interacts with NADPH (not GSH) and that this interaction explains the observed increase in flux through the hexose monophosphate shunt.

Primaquine-mediated H_2O_2 formation in the red cell has been ascribed to direct reaction of primaquine and oxyhemoglobin [1]. Primaquine in aqueous solution autoxidizes generating H_2O_2, superoxide and hydroxyl radical [28]. When oxyhemoglobin was added to the autoxidizing primaquine solution, methemoglobin was generated presumably by reaction of H_2O_2 and oxyhemoglobin. Methemoglobin generation could be inhibited by EDTA. Summerfield and Tudhope [28] made no attempt to estimate possible generation of H_2O_2 by reaction of primaquine and oxyhemoglobin. We studied primaquine-mediated H_2O_2 formation in non-cellular experiments by following inactivation of catalase in the presence of aminotriazole. This method has been used as a sensitive measure of low levels of H_2O_2 formation [1]. EDTA was added in all incubations and effectively inhibited H_2O_2 formation by primaquine "autoxidation". Primaquine was shown to react with oxyhemoglobin to produce H_2O_2 and methemoglobin. In contrast when methemoglobin was incubated in the presence of an H_2O_2 generating system, primaquine plus Fe^{2+}-EDTA, methemoglobin prevented formation or accumulation of H_2O_2. The active involvement of methemoglobin was shown by the lack of a similar effect with albumin and by the conversion of methemoglobin to oxyhemoglobin during the incubation. Methemoglobin may scavenge one-electron donors and thereby prevent formation of H_2O_2. Alternatively, methomoglobin may remove H_2O_2 itself by a peroxidatic mechanism [29, 30]. These results explain the observed lack of H_2O_2 formation in methemoglobin-containing red cells [1]. We also observed primaquine-mediated H_2O_2 formation in the presence of the reduced nucleotides, NADPH and NADH (but not GSH), showing the lack of necessity of oxyhemoglobin for intracellular H_2O_2 formation.

In red cells containing 100% methemoglobin, primaquine greatly stimulates the conversion of methemoglobin to oxyhemoglobin in the presence of glucose. It is likely that reducing equivalents for formation of oxyhemoglobin are provided by the hexose monophosphate shunt via the interaction between primaquine and NADPH.* Primaquine-stimulated conversion of methemoglobin to oxyhemoglobin in the presence of glucose may account for the observation that glucose limits methemoglobin accumulation and Heinz body formation in red cells exposed to 1 mM primaquine for 16 hr [23]. It has been hypothesized that an interaction between primaquine and NADPH is also responsible for primaquine-induced osmotic fragility and hemolysis [4, 35]. These observations indicate that the interaction between primaquine and NADPH modulates various aspects of primaquine toxicity. The synergism between primaquine and products of glucose metabolism is independent of primaquine uptake into the red cell since our results show that a 7-fold primaquine concentration gradient is established between the red cell and the medium in 1 hr, whether or not glucose is present.

Our results allow several hypotheses to be made concerning primaquine-mediated oxidative metabolism. Oxidation of NADPH by primaquine is independent of the ligand state of hemoglobin and results in increased flux of glucose through the hexose monophosphate shunt. In the oxyhemoglobin-containing red cell, primaquine and/or reactive intermediates formed from the reaction of primaquine with NADPH give rise to methemoglobin formation

* Ascorbate and methylene blue have been shown to catalyze formation of oxyhemoglobin from methemoglobin using reducing equivalents from the hexose monophosphate shunt [31–34].

and H_2O_2 formation. Methemoglobin, once formed, acts as a scavenger of reactive intermediates, preventing primaquine-induced accumulation of H_2O_2. Oxyhemoglobin is formed as a by-product of the protective effect of methemoglobin. The requirement of glucose metabolism for primaquine-induced oxyhemoglobin formation suggests that reducing equivalents from the hexose monophosphate shunt are transferred to methemoglobin by products of the reaction between NADPH and primaquine.

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