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Understanding the mechanisms for metabolism-linked hemolytic toxicity of primaquine against glucose 6-phosphate dehydrogenase deficient human erythrocytes: Evaluation of eryptotic pathway

Shobana Ganesan[a,b,1], Narayan D. Chaurasiya[a], Rajnish Sahu[a], Larry A. Walker[a,b], Babu L. Tekwani[a,b,*]

[a] National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, United States
[b] Department of Pharmacology, School of Pharmacy, University of Mississippi, University, MS 38677, United States

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ABSTRACT

Therapeutic utility of primaquine, an 8-aminoquinoline antimalarial drug, has been limited due to its hemolytic toxicity in population with glucose 6-phosphate dehydrogenase deficiency. Recent investigations at our lab have shown that the metabolites generated through cytochrome P_{450}-dependent metabolic reactions are responsible for hemotoxic effects of primaquine, which could be monitored with accumulation of methemoglobin and increased oxidative stress. The molecular markers for succeeding cascade of events associated with early clearance of the erythrocytes from the circulation were evaluated for understanding the mechanism for hemolytic toxicity of primaquine. Primaquine alone though did not induce noticeable methemoglobin accumulation, but produced significant oxidative stress, which was higher in G6PD-deficient than in normal erythrocytes. Primaquine, presumably through redox active hemotoxic metabolites generated *in situ* in human liver microsomal metabolism-linked assay, induced a dose-dependent methemoglobin accumulation and oxidative stress, which were almost similar in normal and G6PD-deficient erythrocytes. Primaquine alone or in presence of pooled human liver microsomes neither produced significant effect on intraerythrocytic calcium levels nor affected the phosphatidyl serine asymmetry of the normal and G6PD-deficient human erythrocytes as monitored flowcytometrically with Annexin V binding assay. The studies suggest that eryptosis mechanisms are not involved in accelerated removal of erythrocytes due to hemolytic toxicity of primaquine.

1. Introduction

Primaquine, regarded as the drug of choice for radical cure (complete elimination of the dormant liver stages of the parasite from the body) of *Plasmodium vivax* malaria, has limited utility due to its narrow therapeutic index, rapid clearance, and hemolytic toxicities (Tekwani and Walker, 2006; Vale et al., 2009; Fernando et al., 2011). Primaquine is the only approved drug that kills the key parasite stages necessary for the survival of malaria. For *Plasmodium falciparum* (Pf) malaria, this is the mature (stage 5) gametocytes (Pfg), which transmit the infection (Bousema and Drakeley, 2011). For *P. vivax* (Pv) and *Plasmodium ovale* (Po), this is the sleeping liver stage or hypnozoite, which emerges weeks to months after the initial infection and causes relapse (Wells et al., 2010). The major drawback of primaquine is its hemotoxicity, namely methemoglobinemia and hemolysis in individuals who suffer from glucose-6-phosphate dehydrogenase (G-6-PD) deficiency (Taylor and White, 2004; Youngster et al., 2010). Due to their oxidant nature, primaquine metabolites oxidize hemoglobin and generate reactive oxygen species thus leading to depletion of protective thiols (Bloom et al., 1983; Summerfield and Tudhope, 1978). These events finally lead to a dose related hemolytic anemia. The severity of hemolysis is determined by the dose of primaquine, extent of G6PD-deficiency, and also on the patient's physiological condition (Clyde, 1981; Youngster et al., 2010).

Many studies have been done to better understand the nature of the hemolysis and reasons for primaquine sensitivity in G6PD-deficient individuals (Beutler et al., 1954; Bowman et al., 2004, 2005a,b; Flanagan et al., 1958; Jansson et al., 1980). Initial studies demonstrated the increased susceptibility of the sensitive individuals to Heinz body formation as compared to non-sensitive individuals (Beutler et al., 1954). Further studies demonstrated the

* Corresponding author at: National Center for Natural Products Research School of Pharmacy, University of Mississippi, University, MS 38677, United States. Tel.: +1 662 915 7882; fax: +1 662 915 7062.
E-mail address: btekwani@olemiss.edu (B.L. Tekwani).
[1] Present address: OSU/OHSU College of Pharmacy, 3303 SW Bond Ave, Portland, OR 97239, United States.

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acute fall in reduced glutathione level in primaquine sensitive as compared to non-sensitive individuals (Flanagan et al., 1958). All these studies helped in concluding the importance of G6PD and the role of reduced glutathione as an antioxidant in human red blood cells (Bloom et al., 1983).

Erythrocytes are believed to be devoid of classical apoptotic pathway of cell death, due to the absence of mitochondria and nucleus, the key organelles involved in apoptosis. But recent studies have shown that the stimulation of cation channels (Ca^{2+} channels) in erythrocytes, either by oxidative stress, osmotic shock or energy depletion, can lead to cellular shrinkage, exposure of phosphatidylserine on the cell surface, and finally cause apoptosis (Lang et al., 2006, 2008). This process has been termed as "eryptosis". Eryptosis has some similar characteristics to apoptosis such as cellular shrinkage, phosphatidylserine exposure, ceramide exposure and activation of cation channels in erythrocytes (Lang et al., 2008). All these changes lead to faster removal of the cells by the macrophage system, in liver and spleen. Intraerythrocytic proliferation of malaria parasite is accompanied with formation of new permeation pathways, due to the requirement of additional nutrients and disposal of waste metabolites (Föller et al., 2009). Activation of cation channels (primarily calcium and sodium), which are essential for the intracellular growth of the pathogen, *P. falciparum*, is believed to cause apoptosis in erythrocytes (Föller et al., 2009).

Oxidative stress causes activation of similar channels in non-infected erythrocytes, which leads to hemolysis in G6PD-deficient erythrocytes (Duranton et al., 2002). Entry of calcium leads to activation of intraerythrocytic scramblase, resulting in bidirectional movement of phospholipids and breakdown of phosphatidylserine asymmetry (Lang et al., 2008). Opening of Ca^{2+}-permeable cation channels in erythrocytes have been shown to trigger apoptosis during oxidative stress (Lang et al., 2006). Phosphatidylserine exposure has been shown to enhance the removal of erythrocytes from the circulation. Eryptosis has been suggested to be an alternative pathway or as a non-immune intravascular hemolysis (Lang et al., 2008). The exposed phosphatidylserine is then identified by the macrophage system leading to engulfment and degradation of the erythrocytes (Duranton et al., 2002). Oxidized glutathione has been shown to be a strong intracellular mediator of non-selective cation channel activation during oxidative stress (Koliwad et al., 1996).

Current study is the follow-up of our recent studies on cytochrome P_{450}-dependent hemotoxic effects of primaquine on human erythrocytes (Ganesan et al., 2009). Multiple cytochrome P_{450} isoforms were reported to contribute to generation of toxic metabolites and hemotoxicity of primaquine. These studies have been further extended to evaluate the effect of primaquine on normal and G6PD-deficient human erythrocytes. Accumulation of methemoglobin and generation of oxidative stress by primaquine, in presence of human liver microsomes, were monitored as biochemical markers for hemotoxic response (Ganesan et al., 2009). The microsomal metabolism-linked assay was employed to investigate eryptosis mechanism for hemotoxicity of primaquine on normal and G6PD-deficient human erythrocytes.

2. Materials and methods

2.1. Chemicals and reagents

The ROS probe 2,7-dichlorofluorescein diacetate (DCFDA) was obtained from Molecular Probes/Invitrogen, Eugene OR, USA. 8-(4-Amino-1-methylbutylamino)-6-methoxyquinoline diphosphate salt (primaquine), glucose-6-phosphate dehydrogenase (G6PD), glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide phosphate (β-NADP), magnesium chloride, and ionomycin were purchased from Sigma–Aldrich (St. Louis, MO, USA); Fluo-8 NW calcium detecting kit was purchased from AAT Bioquest (Sunnyvale, CA, USA); Annexin (ApopNexin™ FITC) was purchased from Chemicon International (CA, USA). The pooled human (mixed sexes) liver microsomes were procured from Celsis (Chicago, IL, USA).

2.2. Procurement of human blood and preparation of erythrocytes for hemotoxicity assays

Normal blood was drawn from healthy volunteers in tubes containing citrate phosphate under IRB approval and stored at 4 °C. G6PD-deficient blood was obtained from a Caucasian individual. The G6PD blood was genotyped at the lab of Professor Jeff Friedman, The Scripps Research Institute, La Jolla, CA, USA. The mutation was found to be A439P in exon 11 as found in US White population (Beutler et al., 1992). Both normal and G6PD-deficient human bloods were analyzed for G6PD activity and reduced glutathione levels as reported earlier (Ganesan et al., 2009). The G6PD activity in deficient blood was only 10% as compared to the normal blood, while total reduced glutathione contents were only marginally lower in G6PD-deficient as compared to the normal human erythrocytes. Both normal and G6PD-deficient bloods were centrifuged at 1000 × *g*, buffy coats were removed and the erythrocyte pellets were washed twice with 0.9% saline and suspended at 50% hematocrit in phosphate buffered saline (110 mM sodium chloride, 20 mM disodium hydrogen phosphate and 4 mM potassium dihydrogen phosphate, pH 7.4) with 10 mM glucose (PBSG).

2.3. In vitro metabolism-linked hemotoxicity assays

An *in vitro* metabolism-linked hemotoxicity assay as described earlier was employed (Ganesan et al., 2009). This assay was adapted from an earlier microsomal incubation test described by Bloom et al. (1983). This assay used pooled human liver microsomes, which allowed *in situ* metabolism of primaquine leading to generation and direct interaction of potential reactive metabolites, with the normal or G6PD-deficient human erythrocytes. Different biochemical endpoints, which have been linked to oxidative hemolytic toxicity, such as accumulation of methemoglobin and real-time generation of reactive oxygen intermediates were monitored (Ganesan et al., 2009). Changes in intraerythrocytic Ca^{2+} levels and Annexin-V binding were measured as the markers of apoptosis response (Lang et al., 2008).

2.3.1. Methemoglobin formation assay

The reaction mixture for the *in vitro* methemoglobin toxicity assay contained 100 μl of erythrocytes (suspended in PBSG with 50% hematocrit), 100 μl of cofactor mix (0.8 μmol $NADP^+$, 5 μmol glucose-6-phosphate, 3 μmol $MgCl_2$ and 0.2 unit glucose-6-phosphate dehydrogenase) (also referred as NADPH regeneration cocktail), 100 μl of KCl (31 μmol), 25 μl of pooled human liver microsomes (15 nmol/mg protein of the relevant CYP) and 5 μl of primaquine (concentration as mentioned) or control to yield the final desired concentration and 100 mM potassium phosphate buffer (pH 7.4) to make the final volume of the reaction mix to 500 μl. The reaction mixtures with appropriate erythrocyte controls without drugs and without microsomes were also set up simultaneously. Each assay was set up at least in duplicates. The reaction mixtures were incubated at 37 °C in a metabolic water bath for 1 h with constant shaking at 80 rpm. After 1 h the reaction mixtures were chilled on ice and methemoglobin levels were measured with a CO-Oximeter (IL-682).

2.3.2. Reactive oxygen species (ROS) formation oxidative stress kinetics assay

Accelerated generation and accumulation of reactive oxygen intermediates (superoxide radical, hydroxyl radical and hydrogen peroxide) are mainly responsible for oxidative stress and damage to the erythrocytes (Sivilotti, 2004). The intraerythrocytic formation of ROS was monitored in real-time with 2′7′-dichlorofluorescein diacetate (DCFDA), a fluorescent ROS probe. Human erythrocytes collected in citrate phosphate anticoagulant were used. The erythrocytes were washed twice with 0.9% saline and suspended in PBSG at a hematocrit of 10%. A 60 mM stock of DCFDA was prepared in DMSO and added to the erythrocytes suspension in PBSG (10% hematocrit) to obtain the final concentration of 600 μM. Erythrocytes suspension containing 600 μM of DCFDA was incubated at 37 °C for 20 min and centrifuged at 1000 × *g* for 5 min. The pellet of DCFDA loaded erythrocytes was suspended in PBSG to 50% hematocrit and used for kinetic ROS formation assay. The metabolism-linked assay was directly set up in a clear flat-bottom 96 well microplate. The reaction mixture contained 40 μl of DCFDA loaded erythrocytes, 40 μl NADPH regeneration cocktail (0.8 μmol $NADP^+$, 5 μmol glucose-6-phosphate, 3 μmol $MgCl_2$ and 0.2 units glucose-6-phosphate dehydrogenase), 40 μl KCl (31 μmol), 10 μl of pooled human liver microsomes, 2 μl of primaquine (concentration as mentioned) and potassium phosphate buffer (100 mM, pH 7.4), to make up the final volume to 200 μl. The controls without drug (with and without microsomes) were also set up simultaneously. Each assay was set up at least in duplicate. The plate was immediately placed in a microplate reader programmed to kinetic measurement of fluorescence (excitation 488 nm and emission 535 nm) for 2 h with 5 min time intervals.

2.4. Fluo-8 NW assay to monitor intraerythrocytic Ca^{2+} influx

The Ca^{+2} influx by untreated and treated (Primaquine alone and in presence of pooled human liver microsomes) was assayed as described earlier (Lang et al., 2003). Normal and G6PD-deficient erythrocytes were washed with phosphate buffer saline glucose (PBSG) and suspended in HBSS at 0.3% hematocrit (1X Hank's with 20 mM HEPES buffer, pH 7.0). Fluo-8 NW dye loading solution was prepared according to the protocol supplied by the manufacturer (ABD Bioquest). Fluo-8 dye loading solution was added to an equal volume of the suspension of erythrocytes. The