Clotrimazole as an Amplifier of Hemin-Induced Damage to Human Erythrocytes. Antioxidant-Independent Protective Effects of Flavonoids

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Abstract—This study is focused on the effect of the antifungal drug clotrimazole (CLT), also possessing antimarial and anticanic activities, on hemin-induced hemolysis and changes in ion permeability and filterability of human erythrocytes. In the presence of 10 μM clotrimazole, the hemolytic response of erythrocytes to exogenous hemin at concentrations as low as 2–8 μM was considerably potentiated and their filterability, as measured by passing them through a 5-μm nuclepore filter, dropped sharply. Flavonoids quercetin (Q) and taxifolin (DHQ), unlike the standard antioxidant Trolox, abolished the effects of clotrimazole, suggesting that protection of hemin-treated erythrocytes by flavonoids is not related to their antioxidant properties.

Keywords: erythrocytes, clotrimazole, hemin, flavonoids.

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In the norm, the blood concentration of free hemin is kept very low (10–20 μM) owing to the hemin detoxifying system, which includes hemin-binding proteins (albumin and hemopexin) and heme oxygenase, an enzyme degrading hemin. However, elevated levels of toxic free hemin may be observed in malaria, intravascular hemolysis, as well as in hemoglobinopathies and membranopathies [1–3].

Hemin is known to be a source of reactive oxygen species (ROS), which determines, at least in part, its toxic properties [3, 4]. Hemin alters the membrane structure [5–7], causing either cell activation (endothelium, neutrophils) or damage to various tissues and cells (including erythrocytes) [1, 8–10].

In the early 1980s it was demonstrated that incubation of erythrocytes with exogenous hemin leads to leakage of intracellular potassium, an increase in their susceptibility to hypotonic lysis, and eventually lysis [11–13]. Exogenous hemin at a concentration as high as 20–40 μM was shown to decrease erythrocyte deformability in vitro [14–17].

In many pathological conditions, exogenous antioxidants, such as dietary flavonoids, are used so that to reduce oxidative stress caused by excessive accumulation of free radicals [18, 19]. Antioxidant activity of flavonoids is contributed by both their metal-binding and free radical-scavenging properties [20, 21]. Being polyphenolic compounds, flavonoids can donate hydrogen and inhibit ROS production at three stages: superoxide formation, hydroxyl radical generation in the Fenton reaction, and lipid peroxidation [20, 22]. The antioxidant activity of phenolic compounds is determined by their ability to intercalate into the membrane lipid bilayer, distributing and orienting themselves within the bilayer [23]. Recently, evidence has emerged that the protective effect of flavonoids on the cell membrane may be independent of their antioxidant activity [23–25].

Free hemin plays a major role in the pathogenesis of severe forms of malaria [26, 27]. When growing within human erythrocytes, malaria parasites digest hemoglobin (which serves as an amino acid source for them) and thereby release free hemin. In severe malaria, the deformability of the entire erythrocyte population, including uninfected cells, is known to be reduced in proportion to disease severity. Reduced cell deformability and related hemolysis contribute to anemia development and poor outcome of the disease [28–30].

The mechanisms that protect the malaria parasite against hemin toxicity include hemin conversion by polymerization to insoluble and much less toxic hemozoin as well as degradation of the hemin molecule mediated by reduced glutathione (GSH). Many antimalarial drugs, such as quinine and chloroquine, are thought to kill malaria parasites by inhibiting hemin crystallization and degradation [31, 32].

Clotrimazole (CLT) is an imidazole derivative used mainly as an antifungal agent [33]. It is also a well-known Gardos channel blocker, efficacious in therapy

1 The article was translated by the authors.
of severe sickle-cell anemia and talassemia [34]. In addition, CLT possesses antitumor properties related to its ability to inhibit Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum [35] as well as to its effect on certain glycocolytic enzymes [36]. At concentrations of 2–5 μM, CLT inhibits in vitro growth of \textit{Plasmodium falciparum} in the infected cells and alters the erythrocyte morphology [37]. Although CLT is thought to be a promising antimalarial drug and can enhance the effect of artemisinin, the most powerful antimalarial drug in use today [32], the mechanism of its action on erythrocyte homeostasis remains obscure.

Recent data provide evidence that CLT forms a complex with hemin, which resists GSH-mediated degradation and markedly increases hemin-induced hemolysis but virtually does not change the oxidative effects of hemin [38, 39].

In this study, we examined the effect of CLT on hemin-induced hemolysis and changes in ion permeability and filterability of human erythrocytes. In the presence of 10 μM clotrimazole, the hemolytic response of erythrocytes to exogenous hemin at concentrations as low as 2–8 μM was considerably potentiated, and their filterability, as measured by passing them through a 5-μm nuclepore polycarbonate membrane filter, dropped sharply.

The flavonoids quercetin (Q) and taxifolin (DHQ), unlike the standard antioxidant Trolox, abolished the effects of clotrimazole, suggesting that protection of hemin-treated erythrocytes by flavonoids is not related to the antioxidant properties thereof.

**MATERIALS AND METHODS**

**Preparation of erythrocyte suspension.** The blood used in the experiments was collected from healthy donors who gave an informed consent. Erythrocytes were sedimented by centrifugation, washed twice with physiological saline and once with isotonic solution (U = 300 mOsm/kg) containing (mM): 10 HEPES (pH 7.4), 5 KCl, 0.8 MgSO\(_4\), 1.5 CaCl\(_2\), and 5 glucose. At each washing step, the supernatant and the topmost erythrocyte layer enriched in platelets and leukocytes were removed. The washed erythrocytes were resuspended in the same HEPES-buffered solution to a hematocrit (Hct) of 40–60%. The resulting suspension contained no more than 0.5 leukocyte per μl; the erythrocyte density distribution was assessed by direct counting ([43]. Mixtures of dimethylphthalate and dibutyl phthalate were added to the suspension sample to a final concentration of 10 μM, and the suspension was gently agitated for 1–2 min. The interval from the moment when we started to prepare the emulsion to the moment it was added to a suspension sample was not longer than 20 min.

**Preparation of solutions of Trolox, quercetin, and taxifolin.** Trolox, quercetin, and taxifolin were dissolved in ethanol to a concentration of 20 mM and added to the erythrocyte suspension to a final concentration of 100 μM. This resulted in the final concentration of ethanol in the suspension below 0.5%.

**Hemolysis measurements.** Samples of erythrocyte suspension were incubated at room temperature with hemin added to a desired final concentration. At various time intervals, cells were sedimented by centrifugation and the supernatant was mixed with Drabkin’s solution at a ratio of 2 : 1 or 5 : 1 (depending on the extent of hemolysis). Thereupon, the mixture (300 μl) was transferred into the wells of a microplate absorbance spectrophotometer (Thermomax, Molecular Devices). The optical density was measured at \(\lambda = 490\) nm. One aliquot of the suspension was brought to complete hemolysis (25 μl suspension + 975 μl Drabkin’s solution); the optical density of its supernatant times a factor of 40 was taken as corresponding to 100% hemolysis [41].

**Filterability measurements.** Erythrocyte filterability was assessed with a Hanss filtrometer in our modification (IDA-01 filtrometer) [42]. In this instrument, aliquots (250 μl) of buffer and the erythrocyte suspension are allowed to pass through a membrane filter and their passage times (\(t_b\) and \(t_s\), respectively) are recorded at an accuracy of 0.1 s with a sensor mounted into the lid closing the filled column. The sensor is a system of three needle electrodes of different lengths connected to an electronic time recorder. Zero time corresponds to the moment when fluid begins to come out of the filter; the recording is automatically stopped when a volume of 250 μl has passed. Filters used were Nuclepore polycarbonate membranes with pore size of 5 μm. The filterability was defined as the \(t_b/t_s\) ratio, with \(t_b\) corresponding to the passage time of the buffer used for resuspension.

**Erythrocyte density distribution measurements.** The erythrocyte density distribution was assessed using the phthalate method of Danon and Marikovsky [43]. Mixtures of dimethylphthalate and dibutyl phthalate were added to the suspension sample to a final concentration of 20 mM hemin in 5 mM NaOH) and then centrifuged to sediment insoluble material. The hemin concentration in 5 mM NaOH was determined spectrophotometrically at 385 nm using an extinction coefficient \(\varepsilon = 58.4\) M\(^{-1}\) cm\(^{-1}\) [40].