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PROTECTIVE EFFECTS OF RUTIN AGAINST HEMOGLOBIN OXIDATION

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Abstract—A prooxidant drug, primaquine (PQ) was used to produce oxidative stress in human red blood cells (RBC) *in vitro*. Rutin, a plant flavonoid, did not prevent PQ-induced cell lysis but protected against hemoglobin (Hb) oxidation inside RBC. After PQ removal, rutin failed to reduce preformed met-Hb indicating that the rutin protective effect manifests only in the presence of PQ. Since H_2O_2 was proved to mediate PQ-induced Hb oxidation, authentic Hb was studied for its reaction with H_2O_2 and rutin in solution. Rutin partially protected oxy-Hb against H_2O_2-induced oxidation and heme loss. Rutin was also shown to delay H_2O_2-induced met-Hb oxidation to ferryl-Hb. Rutin directly reduced ferryl-Hb to met-Hb in stoichiometric (1 : 1) reaction characterized by a rate constant of 100 to 130/M/sec. It is assumed that by reducing ferryl-Hb, rutin prevents oxy-Hb from reacting with ferryl-Hb (comproportionation reaction), thus preventing half of the oxy-Hb molecules from being converted to met-Hb. This mechanism is consistent with 50% inhibition by rutin (at the maximum of its activity) of PQ-induced oxy-Hb oxidation in RBC. The present results demonstrate new antioxidant properties of rutin that may be useful in diminishing oxidative damage to pathological red blood cells.

Key words: methemoglobin; ferrylhemoglobin; oxyhemoglobin; primaquine; hydrogen peroxide

Activated oxygen species are thought to be involved in the mechanism of RBC§ damage in β-thalassemia, sickle cell anemia and glucose-6-phosphate dehydrogenase deficiency [1–4]. This raises the possibility of using antioxidants to prevent oxidative alterations in those pathological RBC. To test antioxidant effects, models simulating oxidative stress *in vitro* are needed. In most cases, prooxidant drugs capable of penetrating into the cell and causing fluxes of oxygen radicals are used [3–6].

PQ, an antimalarial drug causing eventual hemolysis, is known to produce multiple oxidative effects in RBC *in vitro* [4, 6–8]. Among other effects, PQ causes Hb oxidation to a met-form that is reportedly mediated by H_2O_2 [6–8]. In addition, PQ was shown to induce intracellular excess of O_2^- [4], thus providing a suitable model for antioxidant testing.

Plant polyphenolic flavonoids have been extensively studied for their capacity to inhibit radical reactions in chemical and biological systems [9]. Rutin, quercetin-rutinoside (Fig. 1) has been characterized as an O_2^- scavenger and iron chelator [10, 11]. It was also shown that rutin may be oxidized by H_2O_2 in a stoichiometric reaction catalysed by horseradish peroxidase [12]. However, studies on rutin interaction with RBC [13] did not reveal whether rutin is able to react with intracellularly

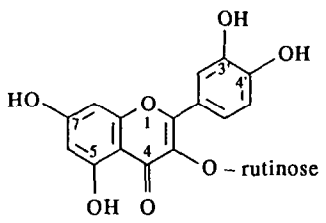

Fig. 1. Chemical structure of rutin.

produced oxidants and thus protect against Hb oxidation.

Recently, a number of phenolic antioxidants (non-flavonoids) were shown to reduce ferryl-Hb (or ferryl-Mb) to their met-forms [14, 15]. Ferryl-Hb (Hb^{IV}-OH) was implicated in the mechanisms of oxidation induced in RBC by oxidant drugs and peroxides [5, 16, 17]. We tested the possibility that rutin as an iron reductant can react with ferryl-Hb formed during H_2O_2-induced Hb oxidation.

In this study we demonstrate that rutin protects against Hb oxidation promoted by PQ in human RBC. We also present evidence that this protective effect may be due to ferryl-Hb reduction by rutin.

MATERIALS AND METHODS

PQ (8-[4-amino-1-methylbutylamino]-6-methoxyquinoline) diphosphate and hemoglobin (product H-2500) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Rutin (3′,4′,5,7-tetrahydroxyflavone-3-rutinoside), NF grade was

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§ Abbreviations: Hb, hemoglobin; Mb, myoglobin; NF, U.S. National Formulary; PQ, primaquine; RBC, red blood cells.

obtained from R. W. Greef & Co. (Old Greenwich, CT, U.S.A.). Catalase was obtained from Boeringher Mannheim, (Germany).

Blood samples were collected in EDTA-treated tubes. Only samples with normal hematological indices were used throughout the experiments. Plasma, platelets and buffy coat were removed by consecutive centrifugations. PBS consisted of NaCl, 75 mM and Na_2HPO_4/NaH_2PO_4, 75 mM was used for washings and further cell incubation.

A typical sample contained PBS, 145 mM; glucose, 5.5 mM; PQ, 2.25 mM; rutin at indicated concentrations, and 20 μL of RBC in total volume of 1 mL. In some cases, PBS was substituted for glucose. Rutin was prepared by dissolving the powder in DMSO followed by dilution with PBS to obtain a required rutin concentration in 25% DMSO solution. The final concentration of DMSO in the samples was 0.25%, which did not interfere with results of measurements. RBC-containing samples were incubated at 37° in the dark for 2 hr with continuous shaking.

Rate of cell lysis and met-Hb content were measured after incubation as described previously [18]. The percentage of RBC lysis was calculated from the ratio of Hb released from the cells to the total Hb in the samples. Met-Hb as a percentage of total Hb was determined by the Evelyn and Malloy classical method [19] with minor modifications.

Spectrophotometric studies were carried out using a UVIKON-390 set. Met-Hb was prepared from 2 × crystallized bovine Hb by dissolving the commercial product in K–P_i buffer (0.1 M, pH 7.4). Oxy-Hb was prepared by reducing met-Hb with an equimolar amount of sodium dithionite followed by a 3 hr dialysis against a 100-fold excess of de-aerated K–P_i buffer. The final oxy-Hb product containing about 5–7% of met-Hb was found not to undergo oxidation for several hours when kept on ice.

To follow Hb oxidation and eventual heme loss, a 3-fold excess of H_2O_2 was added to the oxy-Hb preparation in the absence or presence of rutin. After a 60 min incubation at 24°, the mixtures were scanned at 500–800 nm. To distinguish between Hb derivatives, spectrophotometric equations giving relative concentrations of oxy-, met- and ferryl-Hb [16, 20] were used. After scanning, the samples were supplemented with KCN, 0.1% and $K_3Fe(CN)_6$, 0.1% to convert all Hb to its complex with CN^-. It was found that under such conditions all three forms of Hb (oxy-, met- and ferryl-) show the same spectral characteristics. To assess heme loss, absorbances at 408 nm (Soret band) were then measured. Results are expressed as a difference in absorbance at 408 nm between a control sample without H_2O_2 (A_0) and a sample with H_2O_2 (A_x) both containing rutin at the indicated concentrations.

Ferryl-Hb was obtained by adding a 10-fold excess of H_2O_2 to met-Hb (if not stated otherwise). Catalase (240 U/mL) was added to stop the reaction. Spectrophotometric scans between 500 and 800 nm were made to follow met-Hb oxidation to ferryl-form and reverse reduction of ferryl- to met-form. These two reactions were also studied in kinetic experiments by measuring changes in absorption at 556 nm (the point at which the two forms differ most). A difference in extinction coefficients between met- and ferryl-forms was found to be 3.6 mM/cm at 556 nm, which is consistent with the experience of others [21].

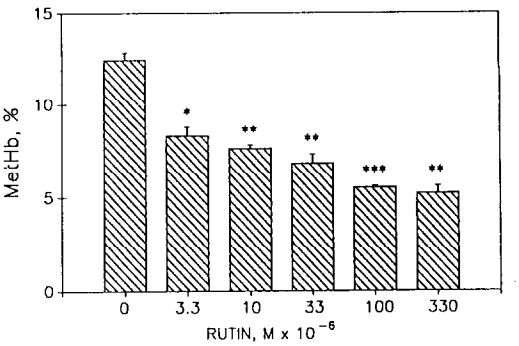

Fig. 2. Primaquine-induced methemoglobin formation as a function of rutin concentration. RBC were incubated for 120 min in the presence of PQ at 2.25 mM and rutin at the indicated concentrations. The bars from left to right represent results obtained in 7, 4, 3, 3, 4 and 2 experiments, respectively. The data are expressed as means ± SEM Statistical differences from the control (no rutin) are denoted with *($P < 2 \times 10^{-4}$), **($P < 5 \times 10^{-5}$) and ***($P < 10^{-6}$).

RESULTS

Experiments with RBC

In the presence of glucose and oxygen, PQ interaction with RBC resulted in met-Hb accumulation and cell lysis (Table 1) in accordance with earlier studies [6, 18]. When rutin was added to the samples, the rate of met-Hb production was significantly decreased whereas the rate of RBC lysis was practically unchanged (Table 1). Therefore we further investigated the rutin effect on oxy-Hb and not on the cell lysis.

Figure 2 shows that increasing concentrations of

Table 1. Methemoglobin content and RBC lysis in the presence of primaquine with and without rutin

	PQ 3 mM	PQ + RUT 3 mM	PQ + RUT 0.1 mM
MetHb (%)			
Mean ± SE	19.3 ± 0.6		9.8 ± 0.4
N	6		6
P*		$<10^{-6}$	
P**		$<10^{-7}$	
Lysis (%)			
Mean ± SE	29.7 ± 1.7		31.3 ± 2.5
N	6		6
P*		>0.6	
P**		>0.2	

RBC were incubated for 120 min.
N denotes number of experiments.
P* determined by *t*-test for independent samples.
P** determined by *t*-test for paired samples.

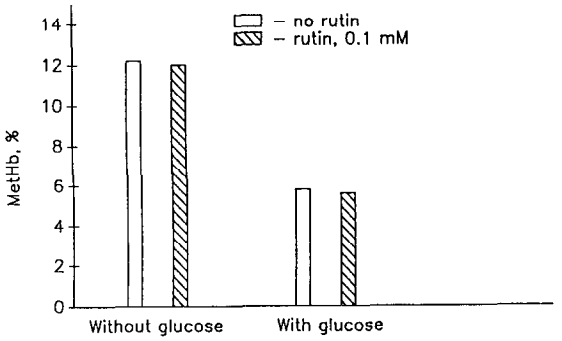

Fig. 3. Methemoglobin content in RBC after pre-incubation with primaquine followed by incubation with or without rutin. RBC were pre-incubated with PQ (2.25 mM) for 90 min without glucose. Then PQ was removed by triple washings and incubation continued for another 120 min without or with glucose, 5.5 mM. Means of duplicates are represented.

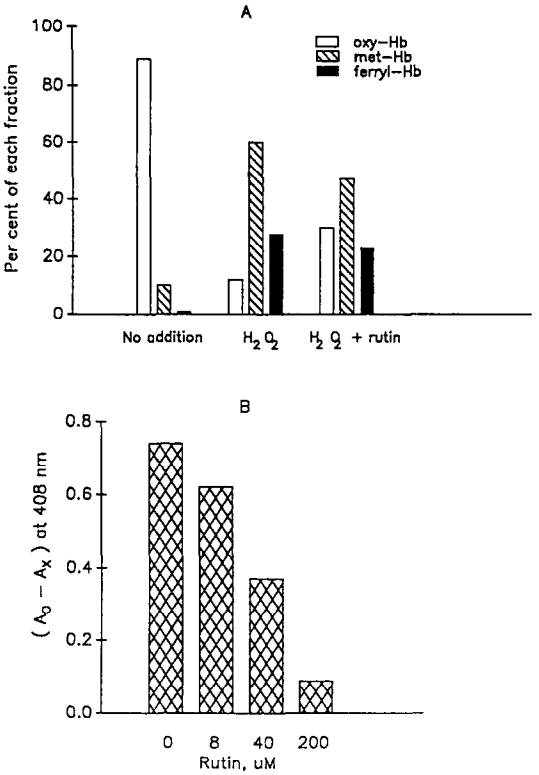

Fig. 4. Effect of rutin on Hb oxidation and heme loss. Hb preparation (total heme content 31 μM) was supplemented with H_2O_2 (100 μM) in the presence or absence of rutin and incubated for 60 min at 24°. Data obtained in a typical experiment are presented. (A) Per cent of oxy-Hb, met-Hb, and ferryl-Hb after incubation in the absence or presence of H_2O_2 with or without rutin (100 μM). (B) Difference in absorbance at 408 nm between a control sample (A_0) without H_2O_2 and a sample with H_2O_2 (A_x) reflecting the difference in Hb heme content between the two samples. See Materials and Methods for details.

rutin caused an increase in oxy-Hb protection. Even at the minimal concentration tested (3.3 μM), the rutin effect became statistically significant. Since this concentration of rutin was about three orders of magnitude lower than that of PQ (2.25 mM), the rutin protective effect could not be attributed to complexation and further inactivation of PQ by rutin. Figure 2 also shows that at the highest concentrations tested the rutin effect approximates its maximum, still leaving about half of the PQ-sensitive Hb unprotected. Notably, rutin alone without PQ did not affect the level of met-Hb which did not exceed 1% (not shown).

To test whether rutin accumulates in RBC and thus enhances their resistance to PQ, we used pre-incubation of RBC with rutin prior to addition of PQ. It was found that after PQ addition and 2 hr incubation the protective effect of rutin was about the same in samples pre-incubated either 0, 15 or 30 min with rutin. This observation indicates that rutin transport into the cells occurred immediately and may also suggest that the "targets" for rutin in the cell appeared only after PQ application.

The lower level of met-Hb in the samples containing rutin could result from (i) delay in met-Hb formation; (ii) partial reduction of met-Hb already formed by PQ attack; (iii) a combination of these two reactions. To test whether rutin is able to restore oxy-Hb after its oxidation by PQ, we pre-incubated RBC with PQ but without glucose to provoke some met-Hb formation but avoid cell lysis. Then the cells were thoroughly washed to remove PQ and incubated for 2 hr in the presence or absence of glucose with or without rutin. The results are presented in Fig. 3.

Pre-incubation of RBC with PQ (2.25 mM) for 90 min resulted in met-Hb accumulation up to 6.3% (not shown). After PQ removal, the process of oxy-Hb oxidation continued during the incubation period and resulted in 12.2% of met-Hb. Glucose prevented this oxy-Hb oxidation probably by supplying NADH and/or NADPH for met-Hb reducing enzymes. Rutin could neither reduce met-Hb nor prevent further oxy-Hb oxidation both in the presence or absence of glucose. The results are consistent with the above suggestion that the protective effect of rutin manifests itself only when PQ is present.

Experiments with Hb derivatives

Since H_2O_2 was implicated in PQ-induced oxidative effects [6–8, 22], we tested the possibility that rutin may facilitate H_2O_2 removal. For this purpose, we addressed the question whether rutin is able to protect Hb against H_2O_2-induced oxidation. Oxy-Hb incubated with 3-fold excess of H_2O_2 gave rise to rapid oxidation of Hb accompanied by a partial heme loss. Figure 4(A) demonstrates that in the presence of H_2O_2, oxy-Hb was converted to met- and ferryl-forms. Rutin (100 μM) partially prevented this oxidation and retained twice more Hb in oxy-form. Figure 4(B) shows that rutin also protected Hb against heme loss in a dose-dependant manner. The above experiments indicated that rutin is able

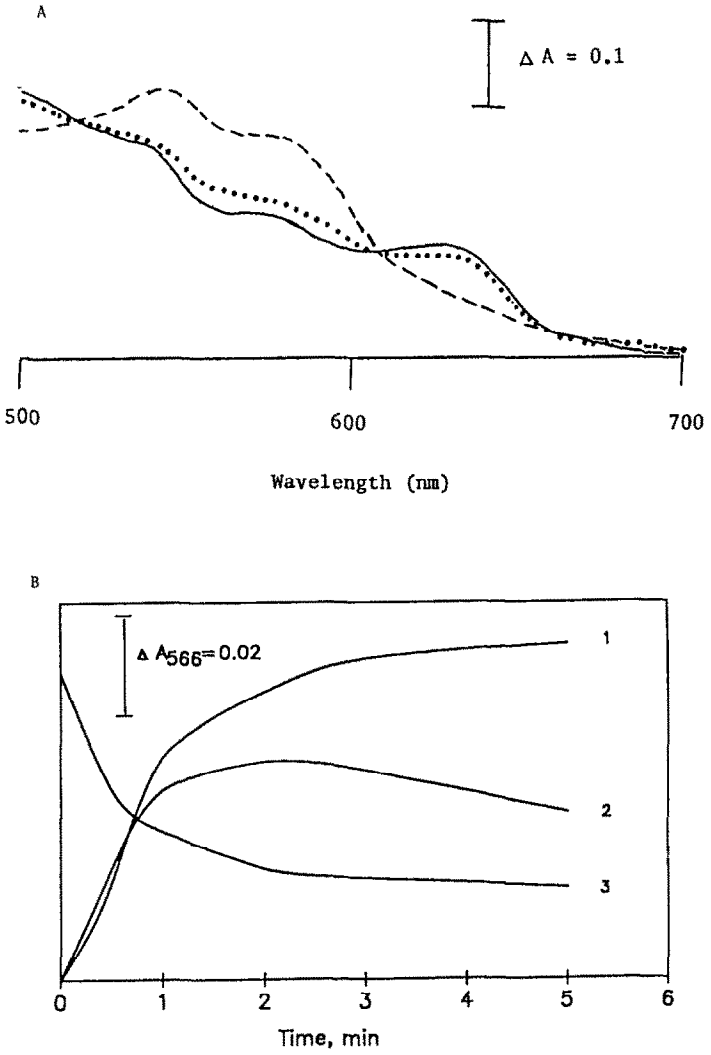

Fig. 5. Visible absorption changes during met-Hb ↔ ferryl-Hb conversion. (A) Spectra of met-Hb (– – –), ferryl-Hb (— — —) and the product of ferryl-Hb reduction by rutin (····). Met-Hb (30 μM) was converted to ferryl-Hb with H_2O_2 (100 μM). Catalase (240 U/mL) was added to remove excess H_2O_2. Rutin (200 μM) was then added to ferryl-Hb. The final spectrum was recorded after 10 min when no further spectral changes were detected. (B) Time course of the reactions as recorded by changes in absorbance at 556 nm. 1. met-Hb (30 μM) + H_2O_2 (100 μM); 2. met-Hb + H_2O_2 in the presence of rutin (200 μM); 3. ferryl-Hb + rutin (200 μM).

to stimulate H_2O_2 removal in the system containing Hb and its oxidized derivatives.

Since met-Hb was reported to remove H_2O_2 in a peroxidase-like reaction in RBC [8], we tested whether rutin may potentiate this function of met-Hb. Figure 5(A) shows spectral changes in met-Hb challenged with H_2O_2. The resulting spectrum represents a form with higher valence state, namely ferryl-Hb (Hb^{IV}-OH) that is fully consistent with published data [17, 21]. After catalase was added to remove excess H_2O_2, rutin rapidly reduced ferryl-Hb to met-form. Notably, the met-Hb spectrum induced by rutin did not completely fit the initial met-Hb spectrum before addition of H_2O_2 [Fig. 5(A)]. That may be due to globin radical formation [17, 21] leading to partial irreversibility of met- to ferryl-Hb transition.

We did not detect spectral changes upon addition of rutin alone to met-Hb *per se*. This is consistent with the absence of rutin effect on preformed met-Hb after PQ removal from RBC (Fig. 3).

Figure 5(B) demonstrates the time course of the above reactions. It may be seen that $t_{1/2}$ of both reactions (H_2O_2 + met-Hb and rutin + ferryl-Hb) is about 1 min. At that moment, 50% of Hb is present in met- and 50% in ferryl-form. In the presence of rutin, H_2O_2-induced met-Hb oxidation is reversed. Therefore this system (met-Hb + rutin) may function as a "pseudoperoxidase" capable of reducing more H_2O_2 than met-Hb alone.

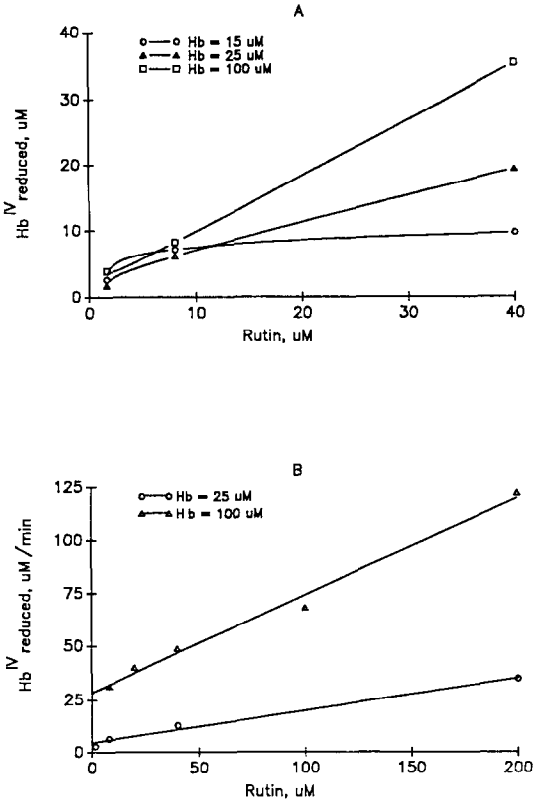

Fig. 6. Dependence of ferryl-Hb reduction (met-Hb formation) on rutin concentration. Met-Hb at concentrations of 15, 25 or 100 μM was supplemented with 10-fold excess of H_2O_2 to produce ferryl-Hb. After 2 min catalase (240 U/mL) was added to remove excess H_2O_2. Then rutin was added to the reaction mixture and decrease in A_{556} showing ferryl-Hb reduction to met-Hb formation (extinction coefficient 3.6 mM/cm). (A) Total amount of ferryl-Hb reduced. Decrease in A_{556} reached its plateau within 5 min and the difference between this value and the initial point (before addition of rutin) was measured. (B) Rate of ferryl-Hb reduction. Initial slopes of the curves were measured and expressed as μM of ferryl-Hb reduced per minute.

Experiments were carried out to test the stoichiometry of the rutin reaction with ferryl-Hb. Results [Fig. 6(A)] show that this reaction approximates 1 : 1 stoichiometry provided that ferryl-Hb is in excess to rutin. Three curves obtained for three different initial concentrations of ferryl-Hb reflect the same 1:1 ratio. At higher rutin concentrations (close to those of ferryl-Hb) the two lower curves decline from the above ratio, thus reflecting inability of rutin to completely reduce ferryl-Hb to met-Hb [see also Fig. 5(A)].

Kinetic data have been obtained in the same experiments. Initial rates of ferryl-Hb reduction were recorded immediately after addition of rutin and then plotted against rutin concentration. The results are presented in Fig. 6(B). The second order constant was calculated as 100/M/sec and 130/M/sec for two different concentrations of ferryl-Hb. Notably, the slopes of the two lines in Fig. 6(B) differ by factor of 4, reflecting the difference in initial concentrations of ferryl-Hb, 25 and 100 μM. For comparative purposes, uric acid (reported to be a powerful reductant of ferryl-Hb) was also tested. In our hands, the rate constant for reaction between uric acid and ferryl-Hb was found to be 140/M/sec which is consistent with results of others [21].

DISCUSSION

In this study, PQ was used to simulate oxidative stress in RBC under experimental conditions [18] allowing the quantification both of cytosolic (met-Hb) and membrane (lysis) oxidation. PQ-induced lysis and met-Hb formation were observed in consistence with earlier findings [6–8, 18]. Rutin did not protect the cell membrane against PQ-induced damage. Possibly, the intracellular distribution of rutin results in its preferential localization in the cytosolic and not in the membrane compartment. The lack of effect of pre-incubation indicates rapid rutin penetration into the cell interior. The presence of a hydrophilic rutinoside residue in the rutin molecule also speaks in favour of this assumption.

PQ was reported to react with oxy-Hb, producing H_2O_2 and met-Hb; both products have been identified in PQ-treated RBC [6, 7]. H_2O_2 is likely to promote further Hb oxidation because catalase was shown to inhibit PQ-induced metHb formation [22]. Notably, PQ failed to produce measurable amounts of H_2O_2 in RBC containing most of their Hb in met-form, thus suggesting that (i) oxy-Hb is needed for PQ-induced H_2O_2 production and (ii) met-Hb is important in H_2O_2 removal [8].

Rutin substantially protected oxy-Hb against oxidation in the presence of PQ but failed to restore oxy-Hb from preformed met-Hb. This indicated that rutin reacted with an intermediate product which was formed in the process of PQ-induced oxidation and disappeared after PQ withdrawal.

Ferryl-Hb (Hb^{IV}-OH) was reported to be formed in reaction between H_2O_2 and oxy-Hb (Hb^{II}-O_2) [3, 5, 16, 17, 21]:

$$H^+ + H_2O_2 + Hb^{II}\text{-}O_2 \rightarrow O_2 + H_2O + Hb^{IV}\text{-}OH. \quad (1)$$

Under excess oxy-Hb, its comproportionation with ferryl-Hb to form met-Hb has been proposed [21]:

$$Hb^{IV}\text{-}OH + Hb^{II}\text{-}O_2 \rightarrow 2Hb^{III} + O_2. \quad (2)$$

As a result of reactions (1) and (2), oxy-Hb converts to met-Hb, and the intermediate form, ferryl-Hb, disappears. The second reaction is suggested to occur at low fluxes of H_2O_2, whereas in the presence of excessive H_2O_2 met-Hb oxidation to ferryl-form prevails [21]:

$$Hb^{III} + H_2O_2 + H^+ \rightarrow H_2O + Hb^{IV}\text{-}OH. \quad (3)$$

The latter equation (3) reflects a reaction we used to obtain ferryl-Hb. Rutin actively reduced ferryl-Hb to met-form, thus restoring a substrate for the reaction with H_2O_2. As a result, more H_2O_2 is removed from the system and Hb oxidation is inhibited. We suggest that in this way rutin protected Hb against H_2O_2-induced oxidation and heme loss in our experiments.

Phenolic reductants, ascorbic acid and Trolox C,

have been shown to reduce ferryl-Mb in experiments similar to the above [14, 15]. We found a gradual loss of absorbance at 360 nm of rutin upon addition of H_2O_2 together with met-Hb (not shown). Spectral changes were indicative of rutin oxidation similar to that previously described by others as caused by lipoperoxides + cytochrome c [11] or by H_2O_2 + horseradish peroxidase [12]. In those papers, reaction products of rutin oxidation have not been identified although hydroquinone → quinone transformation was proposed for 3′ and 4′ catechol groups [12].

We assume that the reaction between rutin and ferryl-Hb may be described as:

$$\text{Rut-OH} + \text{Hb}^{\text{IV}}\text{-OH} \rightarrow \text{Rut} = \text{O} + \text{Hb}^{\text{III}} + \text{H}_2\text{O}. \quad (4)$$

Our data on stoichiometry of this reaction are consistent with equation (4). Considering three reactions [(1), (2) and (4)] simultaneously occur in RBC in the presence of PQ, we suggest that rutin competes with Hb-O_2 for Hb^{IV}-OH, thus saving one molecule of Hb^{III}. As a result, instead of two molecules of met-Hb formed from two molecules of oxy-Hb (without rutin), only one molecule of met-Hb is formed from one molecule of oxy-Hb (with rutin). This may be the base for approximately 50% inhibition by rutin of PQ-induced oxy-Hb oxidation in RBC.

Comparison of the reaction rate constants supports the above mechanism. The rate constant for reaction (2) (ferryl-Hb reduction by oxy-Hb) was reported to be 20/M/sec [21]. We used the same commercial Hb and obtained the similar rate constant for ferryl-Hb reduction by uric acid as reported by Giuvili and Davies who suggested the comproportionantion of oxy- and ferryl-Hb [21]. The rate constant for reaction (4) (ferryl-Hb reduction by rutin) as determined in this study is 100–130/M/sec. Accordingly, the two reactions run at equal rates when rutin concentration is 5–6 times less than that of oxy-Hb. In our RBC experiments, Hb concentration was 500 μM in total volume of the suspension. Rutin at 100 μM inhibited by 50% the rate of met-Hb formation thus corresponding to the ratio of the rate constants.

The above calculations do not consider real intracellular concentrations of the reactants since we do not know how much rutin is taken up by RBC. More work is needed to elaborate on the proposed mechanism of the observed rutin effects. It is of interest to understand how rutin at 3.3 μM protects *ca* 20 μM of Hb against PQ induced oxidation (Fig. 1). We have preliminary data indicating that rutin may enter redox cycling inside the cell: (i) oxidized rutin may be reduced by NADPH and (ii) glucose known to provide NADPH regeneration in RBC is essential for the rutin protection against PQ-induced oxidation. Those observations are part of our current work continuing these studies.

In conclusion, ferryl-Hb is suggested to be a putative rutin target in RBC: it represents a transitory Hb product capable of stoichiometric reduction by rutin with a rate constant 5–6 times higher than that of ferryl-Hb reduction by oxy-Hb. According to the proposed mechanism, half as much oxy-Hb converts to met-form; that is in line with a 50% inhibition of met-Hb accumulation in RBC treated with PQ. An essential element of the rutin reaction with ferryl-Hb is enhanced H_2O_2 removal by met-Hb in the presence of rutin. It is assumed that catalase, although capable of reacting with H_2O_2 about 10^5 times more rapidly than met-Hb, will not overshadow this mechanism, since all the above reactions occur inside the Hb moiety, thus favouring direct interactions between H_2O_2 and Hb heme.

The present work provides evidence for new antioxidant functions of the well-known flavonoid rutin (vitamin P). Studies are underway to evaluate whether rutin may protect pathological RBC against oxidative damage.

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REFERENCES

1. Scott MD, van den Berg J, Repka T, Rouyer-Fessard Ph, Hebbel RP, Beuzard Y and Lubin BH, Effect of excess of α-hemoglobin chains in cellular and membrane oxidation in model β-thalassemic erythrocytes. *J Clin Invest* **91**: 1706–1712, 1993.
2. Hebbel RP, Eaton JW, Balasingam M and Steinberg MH, Spontaneous oxygen radical generation by sickle erythrocytes. *J Clin Invest* **70**: 1253–1259, 1982.
3. Winterbourn CC, Benatti U and de Flora A, Contributions of superoxide, hydrogen peroxide and transition metal ions to auto-oxidation of the favism-inducing pyrimidine aglycone, divicine, and its reactions with haemoglobin. *Biochem Pharmacol* **35**: 2009–2015, 1986.
4. Grinberg LN, Shalev O, Goldfarb A and Rachmilewitz EA, Primaquine-induced superoxide production by β-thalassemic red blood cells. *Biochim Biophys Acta* **1139**: 248–250, 1992.
5. Minetti M, Mallozzi C, Scorza G, Scott MD, Kuypers FA and Lubin BH, Role of oxygen and carbon radicals in hemoglobin oxidation. *Arch Biochem Biophys* **302**: 233–244, 1993.
6. Cohen G and Hochstein P, Generation of hydrogen peroxide in erythrocytes by hemolytic agents. *Biochemistry* **3**: 895–900, 1964.
7. Thornalley P, Stern A and Bannister J, A mechanism for primaquine mediated oxidation of NADPH in red blood cells. *Biochem Pharmacol* **32**: 3571–3575, 1983.
8. Kelman SN, Sullivan SG and Stern A, Primaquine-mediated oxidative metabolism in the human red cell. Lack of dependence on oxyhemoglobin, H_2O_2 formation, or gluthathione turnover. *Biochem Pharmacol* **31**: 2409–2414, 1982.
9. Bors W, Heller W, Michel Ch and Saran M, Flavonoids as antioxidants: determination of radical scavenging efficiencies. *Methods Enzymol* **186**: 343–355, 1990.
10. Robak J and Gryglewski R, Flavonoids are scavengers of superoxide anions. *Biochem Pharmacol* **37**: 837–841, 1988.
11. Afanas'ev I, Dorozhko A, Brodski A, Kostyuk V and Potapovitch A, Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* **38**: 1763–1769, 1989.
12. Takanama U, Spectrophotometric study on the oxidation of rutin by horseradish peroxidase and characteristics of the oxidized products. *Biochim Biophys Acta* **882**: 445–451, 1986.

13. Maridonneau-Parini I, Braquet P and Garay R, Heterogenous effects of flavonoids on K^+ loss and lipid peroxidation induced by oxygen-free radicals in human red cells. *Pharm Res Commun* **18**: 61–72, 1986.
14. Giulivi C, Romero F and Cadenas E, The interaction of Trolox C, a water soluble vitamin E analog, with ferrylmyoglobin: reduction of the oxoferryl moiety. *Arch Biochem Biophys* **299**: 302–319, 1992.
15. Galaris D, Cadenas E and Hochstein P, Redox cycling of myoglobin and ascorbate: a potential protective mechanism against oxidative reperfusion injury in muscle. *Arch Biochem Biophys* **273**: 497–504, 1989.
16. van den Berg J, Op den Kamp J, Lubin BH, Roelofsen B and Kuypers F, Kinetics and site specificity of hydrogen peroxide-induced oxidative damage in red blood cells. *Free Rad Biol Med* **12**: 487–498, 1992.
17. Puppo A and Halliwell B, Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is hemoglobin a biological Fenton reagent? *Biochem J* **249**: 185–190, 1988.
18. Grinberg LN and Allakhverdiev A, Hemolytic effect of primaquine and protective effect of sodium formate *in vitro*. *Med Parazitol (Mosk)* **50(2)**: 54–57, 1981.
19. Evelyn KA and Malloy HT, Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood. *J Biol Chem* **126**: 655–662, 1938.
20. Winterbourn CC, Oxidative reactions of hemoglobin. *Methods Enzymol* **186**: 265–272, 1990.
21. Giulivi C and Davies KJA, A novel antioxidant role for hemoglobin. The comproportionation of ferrylhemoglobin with oxyhemoglobin. *J Biol Chem* **265**: 19,453–19,460, 1990.
22. Summerfield M and Tudhope G, Studies with primaquine *in vitro*: superoxide radical formation and oxidation of haemoglobin. *Br J Clin Pharmacol* **6**: 319–323, 1978.