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Human Red Blood Cells as A Natural Flavonoid Reservoir

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Quercetin is rapidly and avidly taken up by human red blood cells (RBC) via a passive diffusion mechanism, driven by flavonoid binding to haemoglobin and resulting in an almost quantitative accumulation of the flavonoid. Heamoglobin-free resealed ghosts accumulated quercetin exclusively in the membrane fraction. Cell-associated quercetin was biological active and could be quantitatively utilised to support the reduction of extracellular oxidants mediated by a transplasma-membrane oxido-reductase. Additional experimental evidence revealed that quercetin uptake declined in the presence of albumin and that, under these conditions, the amount of cell-associated quercetin is enhanced by increasing the RBC number. Quercetin release from flavonoid-preloaded RBC was observed only in the presence of albumin (or in human plasma) and this response was progressively inhibited upon incubation in solutions containing albumin previously exposed to increasing concentrations of quercetin and cleared of the unbound fraction of the flavonoid. Furthermore, exposure to quercetin pre-saturated albumin promoted accumulation of the flavonoid in fresh RBC and this response was a direct function of the extent of albumin saturation. These results, indicating a flow of quercetin from albumin to haemoglobin, and *vice versa*, are therefore consistent with the possibility that human RBC play a pivotal role in the distribution and bioavailability of circulating flavonoids.

Keywords: Quercetin; Trans-plasma membrane oxidoreductase; Ferricyanide; Human erythrocytes

Abbreviations: RBC, Red blood cells; FIC, Ferricyanide; FOC, Ferrocyanide; Hb, Haemoglobin; DMSO, Dimethyl sulfoxide

INTRODUCTION

Flavonoids are polyphenol compounds widely distributed in plant foods ingested by humans. Recent studies demonstrate that their bioavailability is much greater than previously believed.[1] Quercetin, the major representative of the flavonoid subclass of flavonols,[2] is efficiently adsorbed and its elimination half-life period is about 24 h.[3] Quercetin displays a strong antioxidant activity, based on its ability to chelate transition metals and to scavenge reactive oxygen species,[4,5] and thus prevents the deleterious effects mediated by various sources of free radicals in different biological systems.[1] An additional mechanism whereby quercetin may exert beneficial effects was recently identified in our laboratory.[6] We found that the flavonoid is efficiently taken up by human red blood cells (RBC) and acts as a substrate for a trans-plasma membrane oxidoreductase, which transfers electrons from intracellular substrates to extracellular electron acceptors. This observation on the one hand indicates that quercetin enhances the ability of RBC to maintain a reduced extracellular environment and, on the other hand, suggests that RBC may represent an important compartment for quercetin transport in the blood stream. Under this perspective, RBC may represent a natural flavonoid reservoir and this possibility was tested in the present study.

We report that quercetin is taken up by passive diffusion and binds to haemoglobin in RBC, so that large amounts of the flavonoids accumulate in a very short time. Quercetin is retained by the cells in a biologically active form that can be quantitatively consumed upon supplementation of an extracellular oxidant. In its absence, quercetin is released only in an albumin-containing medium. Conversely, incubation

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of RBC with quercetin bound to albumin promotes the intracellular accumulation of the flavonoid that once again is biologically active and capable of reducing extracellular oxidants.

MATERIALS AND METHODS

Materials

Quercetin, 1,10-phenanthroline, Na-acetate, ethyl acetate, NADH, ATP and 3-phosphoglyceric phosphokinase were purchased from Sigma–Aldrich Chemie (Steinheim, Germany).

$K_3Fe(CN)_6$, $FeCl_3$, citric acid and NaH_2PO_4 were Carlo Erba products (Milan, Italy). The Amicon Ultra-4 filter apparatus was from Millipore (Bedford, MA, USA).

Methods

Human erythrocytes and erythrocyte ghost preparation. Human venous blood (in heparin) from healthy volunteers was obtained by venipuncture. The erythrocytes were used immediately after sampling. The blood was centrifuged at 1861.5*g* for 10 min at 4°C. After removal of plasma, buffy coat, and the upper 15% of the packed red blood cells, the RBC were washed twice with cold PBS (150 mM NaCl, 5 mM Na_2HPO_4, in deionised water, adjusted to pH 7.4) and then resuspended as described below.

Clear resealed erythrocyte ghosts were prepared following a method previously described by Steck and Kant.[7] The packed ghosts were kept on ice and used immediately after preparation. Resealed ghosts prepared in this manner contained no visible haemoglobin and 5–10% of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity[8] present in the same concentration of leaky ghosts. GAPDH activity is a marker of the inner face of the RBC membrane[7] and would be exposed only in ghosts that had not resealed. In five experiments, the protein concentration of packed RBC ghosts was 7.8 ± 0.93 mg/ml which was taken to correspond to 100% ghosts. The protein concentration was evaluated using the Coomassie Brilliant Blue G-250 assay.[9]

Incubations of human RBC with quercetin. Twenty mM stock solution of quercetin was prepared in dimethyl sulfoxide (DMSO) and then diluted 1:2 with PBS. Packed RBC (at haematocrit reported in the Legends to the figures) were incubated in PBS at 37°C for 5 min in the presence of quercetin at the concentration reported in the Legends to the figures. After this time, the suspensions were immediately centrifuged at 1861.5*g*, the RBC were washed twice with at least 50 volumes of PBS and then subjected to the subsequent analyses.

Measurement of ferricyanide reduction. Ferricyanide (FIC) reduction was estimated as reported by Avron and Shavit.[10] After exposure to the flavonoid, RBC were washed twice with PBS and resuspended (10% v/v) in PBS + 2.5 mM adenosine containing 1 mM FIC (potassium salt), dissolved immediately prior to use. Incubation with PBS + 5 mM glucose provided similar results (not shown).[11] The suspensions were incubated for 30 min at 37°C and then centrifuged at 1861.5*g* at 4°C. The resulting supernatants were assayed for their ferrocyanide (FOC) content using 1, 10-phenanthroline as an indicator and measuring A_{510} ($\varepsilon = 10,500\ M^{-1}\,cm^{-1}$).

Extracellular and intracellular content of quercetin. The extracellular concentration of the flavonoid was measured in the medium at the end of the RBC exposure to quercetin. The assay was performed spectrophotometrically by measuring the absorbance at the wavelength corresponding to the maximal peak of the absorption spectrum (quercetin 370 nm). The absorbance of control sample supernatants was subtracted from the absorbance of the supernatants obtained from the treated samples. The percentage of haemolysis was evaluated in the same samples by measuring the haemoglobin (Hb) concentration vs. the total Hb content. The extent of lysis was not different from controls and never higher than 0.5%.

Intracellular quercetin content was measured as described by Ferrali *et al.*,[12] with minor modifications. Briefly, the RBC suspension was centrifuged and washed twice, as described above. The packed cells were then resuspended with three volumes of PBS and lysed by freezing and thawing. Quercetin was extracted 3 times with 400 µl of cold ethyl acetate. The clear upper phase was measured spectrophotometrically at 370 nm.

The extra/intracellular concentrations of the flavonoid were obtained from the corresponding calibration curve performed in PBS or ethyl acetate, respectively.

Quercetin binding to haemoglobin. The cytosolic fraction was centrifuged (4,000*g*) using a swinging bucked rotor for 15 min through an Amicon Ultra-4 centrifugal filter device excluding molecules larger than 30,000 Da. Quercetin content was measured spectrophotometrically in the ultrafiltrates. Proteins retained in the filter units were recovered and extracted with ethyl acetate for quercetin analysis.

Saturation of albumin with quercetin. Thirty five mg/ml albumin (corresponding to 530 µM) were incubated for 10 min (37°C) in PBS containing increasing concentrations of quercetin. The samples were then cleared of unbound quercetin by centrifugation through the Amicon Ultra-4 centrifugal filter devices. The concentrated fractions recovered in the filter unit were washed twice and then diluted to the initial concentration with PBS.

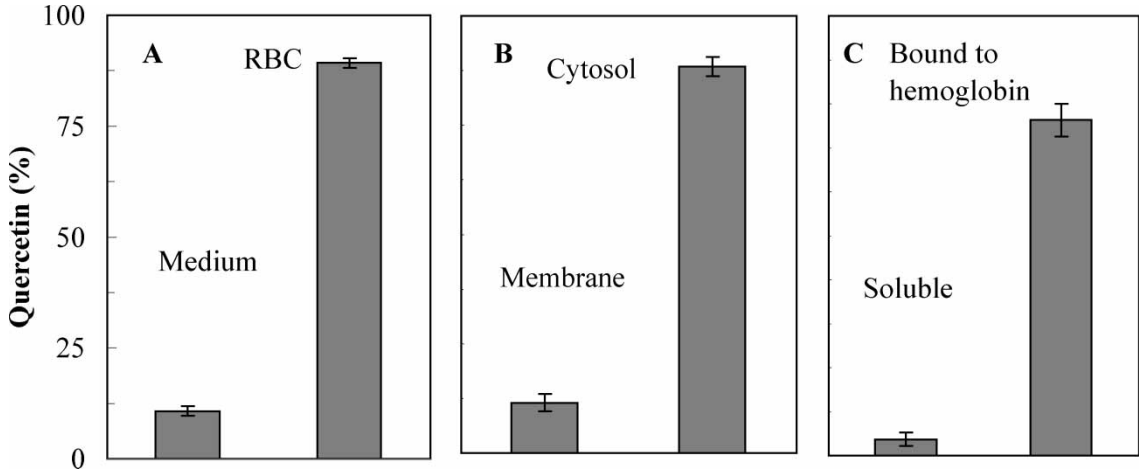

FIGURE 1 *Quercetin uptake and distribution in human erythrocytes.* (A) Human RBC (10% haematocrit) were incubated for 5 min with 50 μM quercetin. The cell suspension was then centrifuged and the supernatant was directly assayed for its flavonoid content. The RBC were washed, lysed and quercetin was finally extracted and measured as described in the "Method Section". (B) An aliquot of the haemolysate was centrifuged to separate the membranes from the cytosol. Quercetin was then extracted and determined in both fractions. (C) An aliquot of the cytosolic fraction was applied to Amicon Ultra-4 centrifugal filter devices and centrifuged. The quercetin content was directly analysed in the clear ultrafiltrates and in the concentrated fractions after ethyl acetate extraction, as detailed in the "Method Section". Data reported in A, B and C represent the means ± SD of at least five separate experiments.

Albumin concentration was evaluated before and after ultrafiltration using the Coomassie Brilliant Blue G-250 assay.[9]

RESULTS

Uptake and Subcellular Distribution of Quercetin in Human RBC

Human RBC were incubated with 50 μM quercetin and the flavonoid content was immediately determined in both the cells and extracellular milieu. As shown in Fig. 1A, as much as 85% of the initial amount of the flavonoid was taken up by the cells after only 5 min of incubation. More than 85% of the erythrocyte-associated quercetin was in the cytosolic fraction, whereas less than 15% was bound to the membranes (Fig. 1B). Further analyses of the cytosolic fraction revealed that a small amount of the flavonoid was in a soluble form, or bound to small molecules (<30,000 Da) and that the remaining fraction was protein-bound (>30,000 Da, Fig. 1C). Interestingly, the results of experiments in which hemoglobin-free, resealed erythrocyte-ghosts were incubated with 50 μM quercetin demonstrated that about 85% of the flavonoid was taken up by

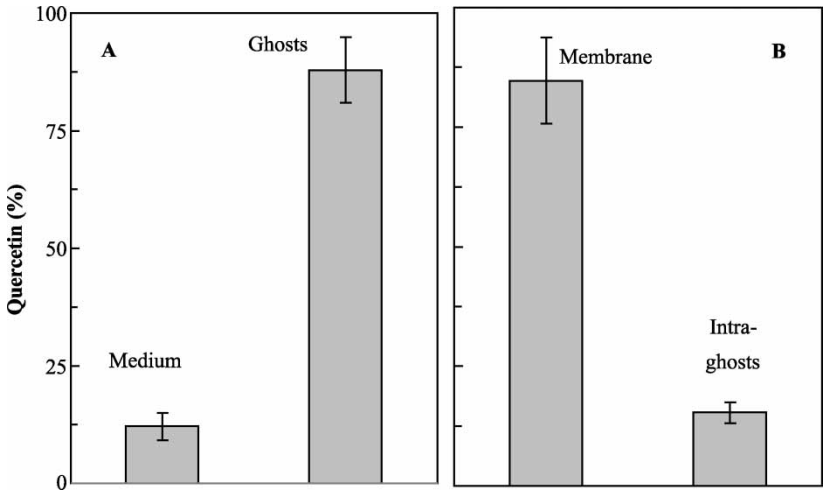

FIGURE 2 *Quercetin uptake and distribution in human resealed ghosts.* (A) Clear (haemoglobin-free) resealed ghosts (50% suspension in PBS) were incubated with 50 μM quercetin and processed as detailed in the legend to Fig. 1A. (B) The ghost lysates were processed as detailed in Fig. 1B. Data reported in A and B are the means ± SD of at least three independent determinations.

the ghosts (Fig. 2A) and largely associated with the membrane fraction (Fig. 2B).

Thus, these results strongly suggest that RBC accumulate large amounts of quercetin because this lipophylic flavonoid readily penetrates the plasma membrane and then binds to proteins. Since haemoglobin is the most represented protein of RBC, a pivotal role of haemoglobin in quercetin binding can be inferred. Under these conditions, the intracellular concentration of unbound quercetin is constantly very low, so that the flavonoid is almost quantitatively taken up by the cells.

Quercetin is Retained by RBC in a Biological Active Form

Since most of the intracellular quercetin appears to be bound to haemoglobin, it is important to know whether the flavonoid is stored in a biologically active form and, if this is the case, whether it can be released from haemoglobin to exert its biological functions. Our previous findings[6] indicating that intracellular quercetin promotes the reduction of extracellular oxidants mediated by a RBC plasma-membrane oxidoreductase on the one hand indicate that indeed the flavonoid is biologically active and on the other hand provide the means for assessing whether quercetin can be entirely released by haemoglobin to exert its biological effects. As shown in Table I, exposure of quercetin-preloaded cells to FIC, while causing reduction of the oxidant (see below), resulted in the complete disappearance of the reduced form of flavonoid from the cells. Quercetin was retained by the RBC upon post-incubation in the absence of FIC.

Quercetin is Taken up by RBC also in the Presence of Albumin

The effect of albumin on quercetin uptake was next determined. RBC were incubated in PBS with 50 μM quercetin and increasing amounts of albumin. As illustrated in Fig. 3A, the intracellular content of quercetin was an inverse function of the extracellular levels of albumin. Consistently, the FIC-reducing activity was also found to progressively decline in the presence of increasing levels of albumin (Fig. 3B). It is important to note, however, that even in the presence of albumin levels exceeding its physiological concentration, quercetin was nevertheless taken up in significant amounts to support reduction of extracellular oxidants.

TABLE I Effect of FIC exposure on RBC intracellular content of quercetin

Treatment	Intracellular quercetin (μM)
Before exposure to 1 mM FIC	410 ± 18
After exposure to 0 mM FIC	400 ± 16
After exposure to 1 mM FIC	8 ± 4

Human RBC (10% haematocrit) were incubated for 5 min with 50 μM quercetin. The RBC were then analysed for their flavonoid content either immediately or after a 30 min incubation in the absence or presence of 1 mM FIC. The data reported are the means ± SD of three independent experiments.

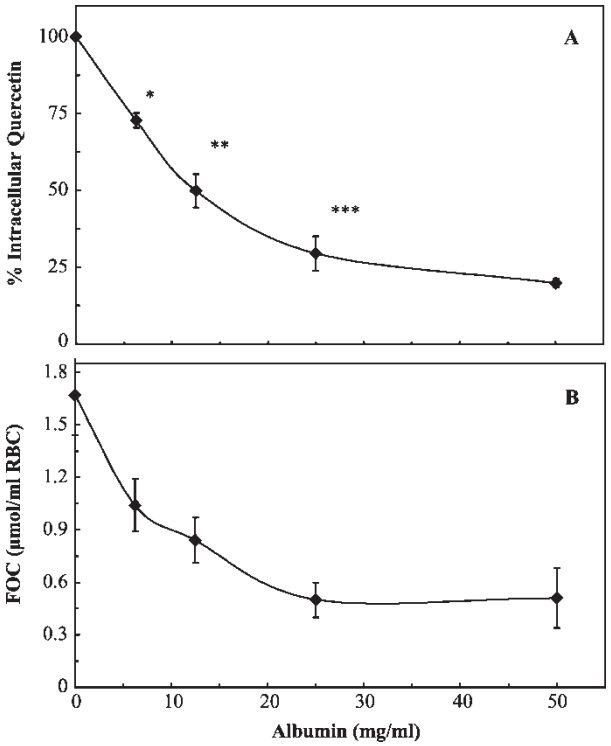

FIGURE 3 *Effect of increasing concentrations of albumin on the intracellular accumulation of quercetin and quercetin-dependent FIC-reducing activity.* Human RBC (10% haematocrit) were incubated for 5 min with 50 μM quercetin and increasing concentrations of albumin. (A) Aliquots of the RBC suspensions were centrifuged and the pellets analysed for their quercetin content. The data are expressed as a percentage of the intracellular quercetin content determined after incubation in the absence of albumin. (B) Aliquots of the RBC suspensions (see above) were centrifuged and the cells analysed for their ability to reduce extracellular FIC, as detailed in the "Method Section". Quercetin-dependent FIC-reducing activity was determined upon subtraction of basal FIC-reducing activity detected in the untreated samples. Data reported in A and B are the means ± SD of at least five independent determinations. *$p < 0.01$ as compared with the control samples; **$p < 0.05$ as compared with the samples incubated with 6.25 mg/ml albumin; ***$p < 0.01$ as compared with the samples incubated with 12.5 mg/ml albumin (Mann–Withney *U*-test).

The same experiments were performed using RBC suspensions at different densities incubated with 50 μM quercetin in the absence or presence of an albumin concentration normally found in the plasma (35 mg/ml). The results illustrated in Fig. 4 demonstrate that, in the presence of a physiological concentration of albumin, the RBC accumulation of the flavonoid is significantly reduced (A) but nevertheless takes place in amounts sufficient to support reduction of extracellular oxidants (B). This is also apparent using a 40% haematocrit mimicking the physiological conditions.

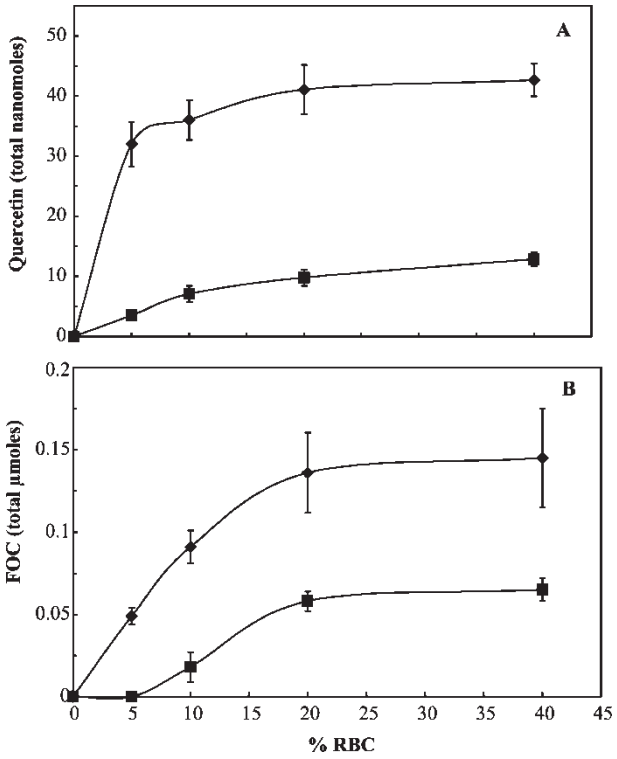

FIGURE 4 *Effect of increasing RBC densities on the intracellular accumulation of quercetin and quercetin-dependent FIC-reducing activity in an albumin-containing medium*. Human RBC at different haematocrit were incubated for 5 min with 50 μM quercetin in the absence (◆) or presence (■) of 35 mg/ml albumin. (A) Aliquots (1 ml) of the RBC suspensions were processed as detailed in Fig. 3A. Results are expressed as nanomoles of quercetin recovered in each sample. (B) Aliquots (0.5 ml) of the RBC suspensions were processed as detailed in Fig. 3B. Results are expressed as μmoles of FOC, measured in each sample, after subtraction of the μmoles of FOC detected in the untreated samples. Data reported in A and B are the means ± SD of at least five separate experiments.

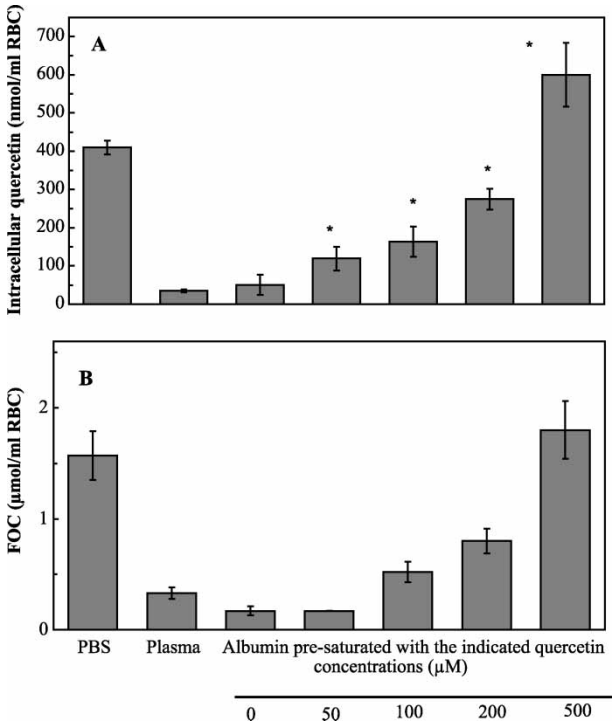

FIGURE 5 *Release of quercetin from quercetin pre-loaded RBC as a function of saturation of quercetin-binding sites in albumin*. Human RBC (10% haematocrit) were incubated for 5 min with 50 μM quercetin. The RBC suspensions were then washed and re-suspended in human plasma or in PBS containing albumin (35 mg/ml) previously exposed to 0–500 μM quercetin and cleared of unbound quercetin. The cells were incubated in these media for 30 min. (A) Aliquots of the RBC suspensions were processed as detailed in Fig. 3A. (B) Aliquots of the RBC suspensions were processed as detailed in Fig. 3B. Data reported in A and B are the means ± SD of at least five independent determinations. *$p < 0.05$ compared with the samples incubated with pure albumin (Mann–Withney *U*-test).

Quercetin is Released by RBC only in an Albumin Containing Medium

An additional question addressed in the present study was whether quercetin, once taken up by the RBC, is subsequently released in the extracellular milieu. For this purpose, quercetin-preloaded RBC (5 min) were post-incubated in fresh PBS for increasing time intervals, both in the absence or presence of albumin. We found no evidence of quecetin efflux after up to 3 h of incubation in PBS without albumin and at this time point the ability of RBC to reduce FIC was basically identical to that observed after quercetin loading (not shown). In marked contrast, quercetin content declined rapidly in the presence of albumin (35 mg/ml). As shown in Fig. 5, the amount of cell associated quercetin was indeed very low after 30 min (A) and this event was paralled by a dramatic reduction in FIC reduction (B). These responses were reproduced by replacing the albumin-containing solution with human plasma. Interestingly, but not unexpectedly, the ability of albumin to promote the efflux of quercetin sharply declined using protein preparations previously exposed to quercetin and cleared of unbound quercetin. Indeed, as reported in the Fig. 5A, the efflux of quercetin from RBC was an inverse function of the degree of albumin "saturation".

RBC take up Quercetin from an Albumin Containing Medium

The question of whether quercetin can be released by albumin to allow flavonoid accumulation in RBC was finally addressed. For this purpose RBC were exposed to albumin solutions (35 mg/ml) pre-incubated with increasing quercetin concentrations and cleared of unbound quercetin. As shown in Fig. 6, the intracellular accumulation of the flavonoid (A) and the ability of RBC to reduce FIC (B) were a direct function of the levels of "albumin saturation". These results indicate that indeed quercetin is released by albumin and that the flavonoid is subsequently taken up by the RBC.

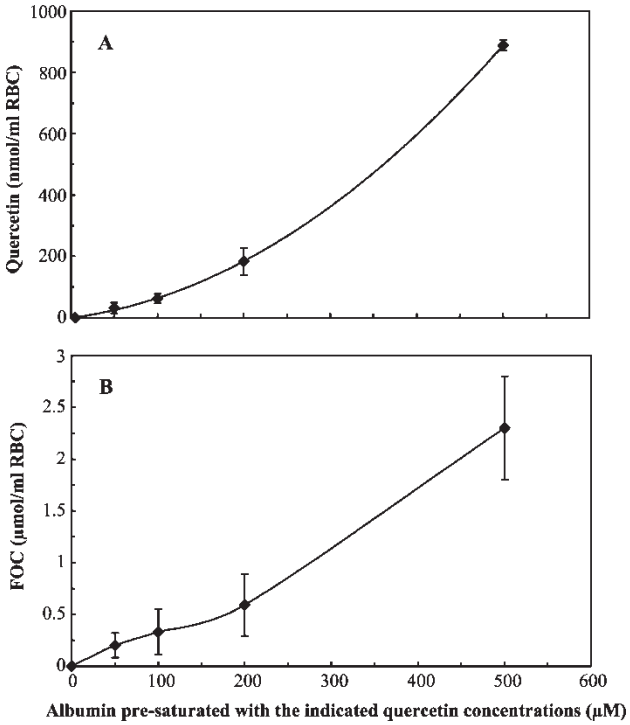

FIGURE 6 *Intracellular accumulation of quercetin and quercetin-dependent FIC-reducing activity in RBC exposed to the flavonoid in a medium containing quercetin- bound albumin*. Preparations of albumin (35 mg/ml) previously "saturated" with increasing concentrations of quercetin and cleared of the unbound fraction of the flavonoid were utilized to re-suspend human RBC at a 10% haematocrit. The RBC suspensions were maintained at 37°C for 5 min. (A) Aliquots of the RBC suspensions were processed as detailed in Fig. 3A. (B) Aliquots of the RBC suspensions were processed as detailed in Fig. 3B. Data reported in A and B are the means ± SD of at least five independent determinations.

DISCUSSION

Previous work from this laboratory[6] showed that human RBC accumulate large amounts of quercetin and, in keeping with these findings, the results reported in the present study demonstrate that as much as 85% of the initial amount of the flavonoid (50 μM) is taken up by the cells after only 5 min of incubation (Fig. 1). Further analyses revealed that a small amount of the cell-associated quercetin was stored in the membrane compartment and that about 90% of the cytosolic fraction was bound to haemoglobin. In addition, quercetin was effectively taken up also by haemoglobin-free resealed erythrocyte ghosts and, under these conditions, it was almost quantitatively sequestered in the membrane fraction (Fig. 2). These results, along with the observation that the flavonoid is retained within the RBC in a biologically active form [Ref. [6] and Table I], rule out the possibility that quercetin is taken up by an active transport mechanism or accumulate intracellularly because converted to a chemical entity unable to cross the plasma membrane. Rather, it would appear that quercetin readily penetrates the plasma membrane by passive diffusion, binds to haemoglobin and thus allows a continuous flux of the flavonoid leading to its almost quantitative internalisation.

This conclusion has important implications and raises the possibility that circulating RBC may represent a reservoir of the flavonoid. The results discussed below are consistent with this notion.

Quercetin is a polydentate molecule with a multiplicity of potential binding sites provided by the numerous phenolic groups (for hydrogen bonding) and by sites for hydrofobic interactions.[13] These features are responsible for the extensive binding of quercetin to albumin[14] and albumin was indeed capable of reducing in a concentration-dependent fashion the uptake of the flavonoid in human RBC (Fig. 3). Interestingly, however, the amount of cell-associated quercetin was enhanced in an albumin-containing medium at increasing RBC densities (Fig. 4). Quercetin accumulated in a biologically active form also under physiological conditions represented by 35 mg/ml albumin and a 40% haematocrit.

Thus, quercetin uptake takes place also in the presence of albumin and promotes the RBC-mediated reduction of extracellular oxidants. It was next important to determine whether the flavonoid was released by RBC and to assess the role of albumin in this response. The evidence obtained indicates that quercetin avidly binds to haemoglobin since it was effectively retained within the RBC unless albumin was added to the extracellular milieu (not shown and Fig. 5). Quercetin release from flavonoid-preloaded RBC was effectively elicited by albumin or by incubation in human plasma.

The observation that the effects of albumin on quercetin release were progressively inhibited using albumin solutions previously exposed to increasing concentrations of quercetin, is consistent with the notion that the rate of flavonoid efflux is driven by the availability of binding sites in albumin. These results suggest that, "*in vivo*", albumin binding of endogenous (e.g. hormones, etc.) or exogenous (e.g. drugs, etc.) substances might prevent quercetin release from RBC. This event is expected to enhance the reducing potential of RBC and may also be followed by subsequent release of quercetin upon albumin desaturation.

The last information provided by this study is that quercetin saturated albumin promotes flavonoid accumulation in RBC (Fig. 6). As a corollary, it might be speculated that endogenous/exogenous substances might displace quercetin from albumin and therefore favour its accumulation in RBC. Once again, this would enhance the reducing potential of RBC and provide a source of the flavonoid to be subsequently released upon albumin desaturation.

In conclusion, the results obtained indicate that biologically active quercetin can flow from albumin to haemoglobin, and *vice versa*, via a mechanism finely tuned by the extent of albumin saturation. Recent studies have demonstrated that, in humans, quercetin as well as other flavonoids are readily adsorbed and that their bioavailability is much greater than previously believed.[1] The reported concentrations of flavonoids in human plasma vary from 0.3 to 3 μM; it is presumable that the amount of circulating flavonoids is even greater if the RBC associated flavonoids are also taken into account. The half-life of quercetin is about 24 h[15] and this slow elimination rate was explained by its particularly high affinity for plasma albumin.[16] Our results raise the possibility that this effect may also result from the slow release of quercetin from the RBC paralleling the albumin de-saturation taking place during the distribution process. The data presented in this study also suggest that concomitant assumption of quercetin and drugs avidly binding to albumin might significantly enhance the accumulation of the flavonoid in RBC. This event is likely to reduce the ascorbic acid content in RBC since these cells mainly take up the vitamin as dehydroascorbic acid[17–27] and flavonoids inhibit its uptake.[28–30] A parallel increase in the distribution process of the vitamin, or in its elimination, is therefore to be expected. As a final note, although the trans-plasma membrane oxidoreductase present in human RBC mainly utilise ascorbic acid as an intracellular substrate,[31,32] electron transfer to extracellular acceptors under putative "*in vivo*" conditions of high quercetin/low ascorbic acid accumulation in RBC is most likely maintained. Indeed, as we previously showed,[6] and confirmed in the present study, quercetin is an excellent substrate of this enzyme and allows efficient reduction of extracellular oxidants.

Acknowledgements

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