FEBS 19330 FEBS Letters 416 (1997) 123–129

Protection against oxidative damage of erythrocyte membrane by the flavonoid quercetin and its relation to iron chelating activity

M. Ferrali[a], C. Signorini[a], B. Caciotti[a], L. Sugherini[a], L. Ciccoli[a], D. Giachetti[b], M. Comporti[a,*]

[a] *Institute of General Pathology, University of Siena, Via Aldo Moro, I-53100 Siena, Italy*
[b] *Institute of General Biology, University of Siena, I-53100 Siena, Italy*

Received 28 July 1997; revised version received 5 September 1997

Abstract Incubation of glutathione (GSH) depleted mouse erythrocytes with the oxidants phenylhydrazine, acrolein, divicine and isouramil resulted in the release of free iron and in lipid peroxidation and hemolysis. The addition of the flavonoid quercetin, which chelates iron and penetrates erythrocytes, resulted in remarkable protection against lipid peroxidation and hemolysis. The protection seems to be due to intracellular chelation of iron, since a semi-stoichiometric ratio between released iron and the amount of quercetin necessary to prevent lipid peroxidation and hemolysis was found. Incubation of GSH depleted human erythrocytes with divicine and isouramil did not induce lipid peroxidation and hemolysis in spite of a substantial release of iron. However, divicine and isouramil produced alterations of membrane proteins, such as spectrin and band 3, as well as formation of senescent cell antigen. The addition of quercetin prevented these alterations.

Key words: Erythrocytes; Oxidizing agent; Quercetin; Iron chelation; Lipid peroxidation; Hemolysis; Senescent cell antigen

1. Introduction

It is well known that iron plays a central role in generating harmful oxygen species. Its redox cycling, in fact, promotes the Fenton reaction in which O_2^- reacts with H_2O_2 to produce the very reactive hydroxyl radical [1–4]. Normally iron is transported and kept in specific proteins (transferrin, ferritin, heme proteins) which prevent its reaction with reduced oxygen species [5]. Thus only when iron is released from these complexes in a free form can it be redox cycling active. A low molecular weight iron pool is present in the cell as a transit pool [6–8], and it is likely that components of this pool represent the iron species catalytically active in initiating free radical reactions.

Our previous studies [9–11] have shown that iron is released in a free (desferrioxamine (DFO) chelatable) form when mouse erythrocytes are incubated with a number of oxidizing agents such as phenylhydrazine, divicine, isouramil, acrolein, phenylhydroxylamine and others. Iron is released from hemoglobin [10] and the release is accompanied by methemoglobin formation [11]. If the erythrocytes are severely depleted of glutathione (GSH) (which occurs directly with acrolein [9,10,12] or after a short preincubation with GSH depletors such as diethylmaleate (DEM) with the other oxidants [11,12]), the release of iron is followed by peroxidation of membrane lipids and hemolysis. It seems therefore that the release of iron initiates a chain of reactions leading to lipid peroxidation and consequent hemolysis.

Iron acts from the inside of the cell; in fact, in experiments in which the released iron was chelated at the intracellular level (in DFO preloaded mouse erythrocytes) lipid peroxidation and hemolysis were prevented, which did not occur with extracellularly added DFO [11]. Moreover, only when DFO was present in the intracellular compartment in amounts at least equivalent to those of the released iron, lipid peroxidation and hemolysis were prevented [11].

As is known [13,14] and has been observed in our experiments [11] DFO penetrates cells in minimal amounts only (it cannot penetrate human or calf erythrocytes at all). Therefore, further studies were carried out with substances capable of penetrating the cell and chelating iron. One of these substances is the flavonoid quercetin, which can penetrate cells (as we have verified in preliminary experiments) and which, like its rutinoside rutin, can bind iron [15]. Rutin, on the other hand, penetrates cells to a small extent [16,17], probably because of its hydrophilicity and consequent negligible solubility in erythrocyte membranes. As phenolic compounds, flavonoids can scavenge free hydroxyl and peroxyl radicals [15,18] and can react with superoxide via a one electron transfer [15]; furthermore, as metal chelating agents, they can extract iron ions and hinder radical reactions otherwise set into motion by the metal redox cycling [15,19–22].

In the present work we have studied the effects of quercetin on the oxidative alterations of membrane lipids and proteins which are induced by the oxidizing agents mentioned above and which appear to be mediated by the release of iron. The results show that quercetin affords a remarkable protection and suggest that the protection is dependent on the intracellular chelation of the released iron.

2. Materials and methods

2.1. Materials

Desferrioxamine (Desferal, DFO) was kindly supplied by Ciba-Geigy (Basel, Switzerland). Quercetin was from Roth Gmbh and Co. (Karlsruhe, Germany); rutin was from Aldrich Chimica (Milan, Italy); the other flavonoids used were from Sigma. Divicine (2,6-diamino-4,5-dihydroxypyrimidine) and isouramil (6-amino-2,4,5-trihydroxypyrimidine) were prepared from vicine and convicine respectively, as previously reported [11]. [^{125}I]Protein A (specific activity 9.01 μCi/μg) was from NEN (Radiochemicals). The solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade.

2.2. Erythrocyte incubation

In the experiments in which mouse erythrocytes were used, male Swiss albino mice (Nossan, Correzzana, Milan, Italy) weighing 25–35

*Corresponding author. Fax: (39) (577) 227009.

PII S0014-5793(97)01182-4

g and maintained on a pellet diet (Nossan) were used. Blood was withdrawn from the abdominal aorta under ether anesthesia and heparinized. After centrifugation, the plasma and buffy coat were removed. The erythrocytes were washed three times with 0.123 M NaCl, 28 mM sodium phosphate/potassium phosphate buffer, pH 7.4, and resuspended in the same buffer as a 50% (v/v) suspension. Iron contamination was removed from the buffer as previously described [9]. The hemoglobin concentration, measured as in [23] and expressed per heme, was 7100±280 nmol/ml. In the experiments in which erythrocytes were depleted of GSH, the cells were preincubated with 1.5 mM DEM for 15 min at 37°C. DEM was dissolved in a small volume of dimethyl sulfoxide (DMSO); DMSO alone had no effect on the parameters examined. After preincubation the cells were sedimented, washed, resuspended as above, and used for the subsequent incubation. Phenylhydrazine, divicine and isouramil were added at a concentration of 1 mM; acrolein was added at a 6 mM concentration [9,10]. Quercetin was added at the indicated concentrations. The incubation was carried out aerobically at 37°C. At the end of the incubation, samples were withdrawn for the determination of 'free' iron (DFO chelatable), methemoglobin [24], GSH [25], malonaldehyde (MDA) [26] and hemolysis [11]. Additional samples were withdrawn for the electrophoretic analysis of ghost proteins. 'Free' iron was determined as a DFO-iron complex (ferrioxamine) as previously reported [9,10].

In the experiments in which human erythrocytes were incubated with divicine and isouramil, erythrocytes were prepared from heparinized blood of volunteers as above. The cells were washed and resuspended in the NaCl-sodium phosphate/potassium phosphate buffer mentioned above, as a 50% (v/v) suspension. The cells were preincubated with 100 μM quercetin for 15 min at 37°C and subsequently with 1 mM *N*-ethylmaleimide (NEM) for an additional 15 min, in order to obtain GSH depletion. When quercetin was not used preincubation was in the only buffer. The cells were then recovered, washed and resuspended as above. The incubation was carried out aerobically for 24 and 48 h, in the presence of antibiotics (20 units penicillin and 20 μg streptomycin/ml of buffer). Both divicine (0.5 mM) and isouramil (0.5 mM) were added to the experimental sample. Determinations of 'free' iron, methemoglobin, GSH, MDA and hemolysis were performed as above. Electrophoretic analysis of ghost proteins was also performed.

In the experiments concerned with the binding of autologous IgG to human erythrocytes, the erythrocytes were preincubated and incubated with divicine and isouramil as above. The incubation period was 3 h.

2.3. Experiments carried out in an attempt to extract the quercetin-iron complex from the erythrocytes incubated with phenylhydrazine

Attempts were made to separate quercetin from its complex with iron by HPLC, using a C_{18} reverse phase column and phosphate buffer (25 mM, pH 7.2) plus acetonitrile (40–60%) as eluent. Different eluents were also used but we failed to separate the two substances (standard solutions) in any instance.

In order to prove the intracellular formation of the quercetin-iron complex, GSH depleted mouse erythrocytes were incubated with phenylhydrazine (1 mM) as above in the presence of 50 μM quercetin. The cells were then lysed by freezing and thawing and the ghosts removed by centrifugation. The ghost-free lysate was extracted with ethyl acetate. As evidenced by an equally extracted standard of quercetin-iron complex in buffer (pH 7.4), this procedure failed to extract the complex, although the uncomplexed (free) quercetin was extracted and found to be, when referred to packed cells, 19.5±4.6 μM. Other extractions with different solvents equally failed to extract the complex, likely because of its increased hydrophilicity compared to free quercetin. In order to have an indirect measurement of the complex, the erythrocyte lysate was brought to strongly acid pH (pH 3), to produce the dissociation of the complex (as evidenced by the spectrophotometric change of a standard solution) and to measure free quercetin after extraction with ethyl acetate. However, the latter procedure, under these experimental conditions, resulted in the extraction of both free quercetin and hemoglobin products, which made any spectrophotometric measurement impossible. To remove these products, the concentrated ethyl acetate extract was applied to a silicic acid column (0.8×0.5 cm) and eluted with 3 ml of ethyl acetate. The hemoglobin products remained on the top of the column and the hemoglobin-free eluent was recovered. Free quercetin was measured spectrophotometrically using a standard quercetin-iron complex in buffer (pH 7.4), equally acidified (pH 3), extracted and applied to a silicic acid column. The recovery of quercetin as measured in this standard was low (nearly 40%), likely because quercetin is difficult to elute from silicic acid. When calculated on the basis of this recovery, the amount of quercetin present in the erythrocyte lysate and referred to packed cells was 80.0 μM.

2.4. Electrophoretic analysis of ghost proteins

Erythrocyte ghosts were prepared according to Dodge [27], resuspended in 7.5 mM sodium phosphate buffer, pH 7.4, and the protein concentration was determined [28]. The electrophoretic analysis was performed on acrylamide gel (SDS-PAGE) according to Laemmli [29]. Molecular weight markers (BDH) were used.

2.5. Autologous IgG binding to erythrocytes

IgG was prepared from the serum of the erythrocyte donors by precipitation in 40% ammonium sulfate according to Good et al. [30]. After dialysis, IgG in 50 mM NaCl/10 mM sodium phosphate, pH 7.35, was partially purified on a DEAE column and equilibrated with the same buffer, as reported by Levy et al. [31]. The recovered IgG was measured spectrophotometrically at 280 nm, using $E^{1\%}=14.0$ and adjusted at a concentration of 2 mg/ml.

The binding of autologous IgG to erythrocytes was determined with the use of protein A labelled with ^{125}I, according to the procedure reported in [32] with minor modifications.

3. Results

Fig. 1A shows that the addition of increasing amounts (5, 10, 25 μM) of $FeSO_4$ to quercetin (50 μM) in sodium phosphate buffer, pH 7.4, results in a progressive shift of the absorption maximum from 377 to 420–425 nm. Further addition of $FeSO_4$ does not modify the 420–425 peak. This suggests that at an iron ion concentration half that of quercetin a complex is formed in which the molar ratio quercetin:iron is 2:1. The addition of DFO (50 μM) to the complex (Fig. 1B) extracts iron from the iron-quercetin complex, as shown by the restoration of the quercetin spectrum.

Similar results were obtained using the other flavonoid kaempferol, which therefore seems able to chelate iron. On the other hand, other flavonoids such as catechin, hesperitin,

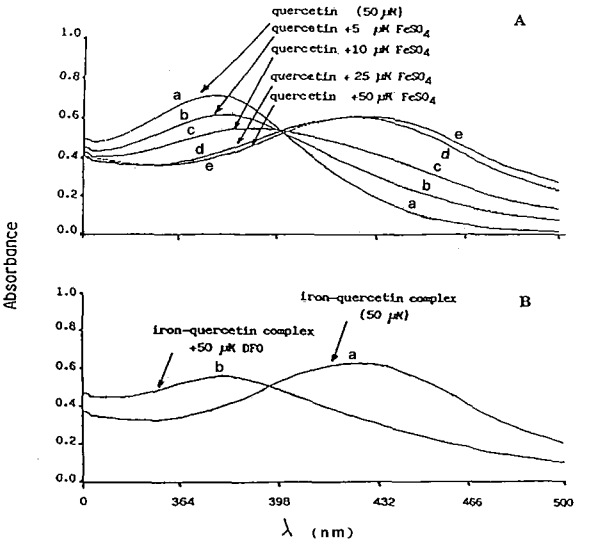

Fig. 1. A: Absorption spectra of quercetin before (a) and after (b,c,d,e) the addition of increasing concentrations of iron sulfate. B: Absorption spectra of iron-quercetin complex before (a) and after (b) DFO addition.

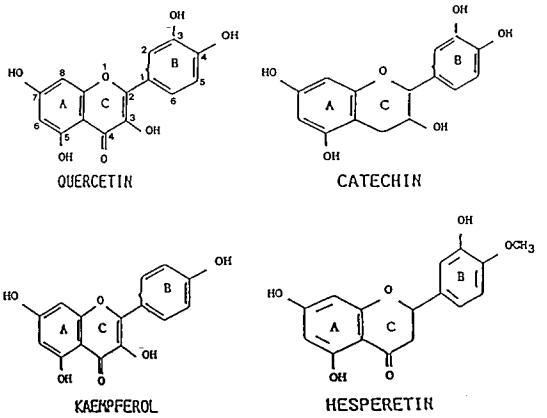

Fig. 2. Structure of quercetin and some related flavonoids.

lapachol and lawsone did not show similar spectral changes upon the addition of $FeSO_4$. From these data it could be deduced that both the hydroxyl (in C_3) and the carbonyl (in C_4) group of the C ring (see Fig. 2) are necessary to bind iron in a stoichiometric ratio of 2:1, at least in the flavonoids considered.

In order to confirm the ability of quercetin to penetrate mouse erythrocytes, we incubated 1 ml of packed cells with 1 ml of 100 μM quercetin in sodium phosphate buffer for 1 h. The cells were then removed by centrifugation and the absorption spectrum (375–380 nm) of the clear supernatant was recorded. The absorption was much less than half of that of the initial quercetin solution. On the other hand, when rutin was used in the same test, all the absorption of the initial rutin solution was recovered in the supernatant. Further indications of the ability of quercetin to penetrate erythrocytes came from the experiments in which the fluorescence of quercetin (several flavonoids are fluorescent substances) was recovered in erythrocyte ghosts. To this end the ghosts from 1 ml of packed cells were incubated (1 h) with 2 ml of sodium phosphate buffer containing 50 μM quercetin. After centrifugation, the ghosts and the supernatant were recovered. The ghosts were resuspended with 2 ml of the buffer and 2 ml of DMSO and examined by spectrofluorophotometry (Ex_{max}, 448 nm; Em_{max} 540 nm). About 90% of the fluorescence of the initial quercetin solution was recovered in the ghosts, whereas about 10% of the fluorescence was recovered in the supernatant to which 2 ml of DMSO had been added. These preliminary experiments indicated therefore, in agreement with previous reports [16,17], that quercetin, unlike rutin, is associated with erythrocytes to a great extent.

In order to investigate the intracellular distribution of quercetin, erythrocytes (50% suspension in buffer) were incubated (1 h) in the presence of 50 μM quercetin. After centrifugation the clear supernatant was extracted with ethyl acetate. The amount of quercetin was measured spectrophotometrically with the use of a standard quercetin solution in ethyl acetate and found to be 3.7 ± 0.2 μM. The erythrocytes were then lysed (freezing-thawing) and, after sedimentation of the ghosts, the ghost-free lysate was extracted with ethyl acetate. The amount of quercetin was measured spectrophotometrically in the extract and found to be, when referred to packed cells, 74.2 ± 2.3 μM. This indicates that the major part of quercetin was in the erythrocyte cytosol.

In view of the capacity to chelate iron and to penetrate cells, quercetin was tested for its ability to prevent the effects induced by the oxidizing agents mentioned above (see Section 1) and likely related to the release of iron into the erythrocytes. Table 1 shows that incubation of native mouse erythrocytes with phenylhydrazine induces iron release and methemoglobin formation, but not lipid peroxidation or hemolysis,

Table 1
Release of iron (DFO chelatable iron), methemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and hemolysis in mouse erythrocytes incubated with phenylhydrazine (Phz) or preincubated with diethylmaleate (DEM) and then incubated with phenylhydrazine in the presence or the absence of quercetin

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Hemolysis (%)
Control	0	1.6 ± 0.2	103 ± 18	876 ± 66	–	4.4 ± 0.8
	60	2.0 ± 0.4	99 ± 12	829 ± 93	2.0 ± 0.6	5.1 ± 0.9
Phz	60	19.3 ± 3.1	548 ± 24	642 ± 46	2.3 ± 0.5	6.6 ± 0.5
DEM+Phz	60	24.4 ± 1.8	1094 ± 272	236 ± 47	86.7 ± 4.2	89.2 ± 2.0
DEM+Phz+Quercetin (15 μM)	60	23.9 ± 2.6	819 ± 106	152 ± 34	25.2 ± 5.4	18.7 ± 1.4
DEM+Phz+Quercetin (50 μM)	60	18.3 ± 2.5	691 ± 155	192 ± 45	0	5.8 ± 0.7

Phz was added to erythrocytes at 1 mM concentration. When DEM pretreated cells were used, the erythrocytes were preincubated with 1.5 mM DEM for 15 min, recovered by centrifugation and then incubated with Phz. DEM pretreatment followed by incubation without phenylhydrazine did not result in any iron release, lipid peroxidation and hemolysis. Results are the means ± S.E.M. of three to six experiments. Met-Hb is expressed

Table 2
Release of iron (DFO chelatable iron), methemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and hemolysis in mouse erythrocytes incubated with acrolein in the presence or the absence of quercetin

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Hemolysis (%)
Control	0	2.1 ± 0.3	74 ± 7	921 ± 72	–	4.1 ± 0.7
	90	2.7 ± 0.3	96 ± 6	893 ± 57	2.3 ± 0.7	4.8 ± 0.5
Acrolein	90	11.3 ± 1.0	1295 ± 316	73 ± 11	43.5 ± 2.0	93.4 ± 2.1
Acrolein+Quercetin (5 μM)	90	6.5 ± 1.4	1339 ± 482	101 ± 0.3	39.9 ± 3.7	63.3 ± 14.6
Acrolein+Quercetin (15 μM)	90	10.9 ± 1.6	1383[a]	150 ± 38	19.2 ± 3.5	12.2 ± 3.2
Acrolein+Quercetin (25 μM)	90	7.0 ± 1.2	819[a]	62 ± 18	4.7 ± 1.7	8.4 ± 0.7

Acrolein was added at 6 mM concentration. Results are the means ± S.E.M. of three to six experiments. Met-Hb is expressed per heme.
[a]Mean of two experiments.

Table 3
Methemoglobin (Met-Hb) formation, lipid peroxidation (MDA formation) and hemolysis in mouse erythrocytes preincubated with diethylmaleate (DEM) and then incubated with phenylhydrazine (Phz) in the presence or the absence of kaempferol

	Incubation time	Met-Hb (nmol/ml)	MDA (nmol/ml)	Hemolysis (%)
Control	0	102	–	2.3
	60	119	1.9	2.9
DEM+Phz	60	512	72.2	89.0
DEM+Phz+Kaempferol (100 μM)	60	307	0.1	3.5

Phz was added to erythrocytes at 1 mM concentration. Preincubation with DEM was performed as reported in Table 1. Results are the means of two experiments. Met-Hb is expressed per heme. The GSH level in native erythrocytes and in erythrocytes preincubated with DEM was 1058 and 536 nmol/ml, respectively.

whereas with erythrocytes previously depleted of GSH (by a short preincubation with DEM) iron release and methemoglobin formation are accompanied by lipid peroxidation and hemolysis (Table 1). This confirms our previous results [11]. The addition of quercetin in an amount (15 μM) lower than that of the released iron (23.9 μM), even if it considerably decreases lipid peroxidation and hemolysis, does not suppress them (Table 1). On the other hand, the addition of quercetin in an amount (50 μM) higher than that of the released iron completely suppresses lipid peroxidation and hemolysis (Table 1). As stated above, the molar ratio of quercetin:iron is 2:1 in the complex and therefore 50 μM quercetin is apparently able to chelate all the iron (18.3 μM) released in this experiment. Indeed, the actual amounts of both free iron and quercetin inside the erythrocytes should be considered roughly double of those (18.3 and 50 μM, respectively) reported in Table 1, since, on the one hand, iron release was determined in the lysate from 1 ml of packed cells plus 1 ml of buffer (see Section 2), and, on the other hand, most quercetin added to the incubation mixture enters the erythrocytes.

The importance of the ratio between the amount of released iron and the amount of added quercetin for the prevention of lipid peroxidation and hemolysis is also shown by the experiments carried out with acrolein (Table 2). This aldehyde induces iron release in the erythrocytes [9,10,12], although to a lower extent than the other oxidants do. In addition it produces GSH depletion by directly binding GSH [33–35]. Therefore, no previous depletion with DEM is needed for lipid peroxidation and hemolysis to occur. When quercetin is added at a concentration (5 μM) lower than that theoretically equivalent to the amount (6.5 μM) of the released iron, lipid peroxidation and hemolysis still occur to a great extent (Table 2). When, on the other hand, quercetin is added in a higher amount (15 μM), but still slightly lower than that theoretically necessary to chelate all the released iron (10.9 μM), lipid peroxidation and hemolysis are markedly decreased but not completely suppressed. Finally, when quercetin is added in an amount (25 μM) definitely higher than that of the released iron (7 μM), lipid peroxidation and hemolysis are completely prevented (Table 2).

Further support for the hypothesis that the protective effect of quercetin depends on the chelation of the released iron is offered by the comparison of the data obtained with phenylhydrazine and with acrolein. The amount of iron released by phenylhydrazine is higher than that released by acrolein. Accordingly, the amount of quercetin necessary to completely prevent lipid peroxidation and hemolysis with phenylhydrazine is higher (50 μM) than that (25 μM) necessary with acrolein. Furthermore, the data obtained with acrolein at 60 min of incubation (not reported in Table 2) show that with 15 μM quercetin lipid peroxidation and hemolysis are completely prevented (MDA: 5.2 ± 1.6 nmol/ml; hemolysis: 7.6 ± 1.4%), while with phenylhydrazine the same amount of quercetin only decreases the two phenomena (see Table 1). Finally a remarkable protective effect on both lipid peroxidation and hemolysis induced by phenylhydrazine was also observed (Table 3) when using kaempferol, which seems able to chelate iron like quercetin. In contrast, no protection was seen with hesperitin, which like quercetin and kaempferol enters the cell, but seemingly does not chelate iron.

As stated in Section 2.3, in experiments in which GSH-depleted mouse erythrocytes were incubated with phenylhydrazine in the presence of 50 μM quercetin, the attempt to measure indirectly the quercetin-iron complex after dissociation of the complex and measurement of free quercetin resulted in a calculated value of total quercetin of 80 μM (referred to packed cells). If one considers that the amount of iron released in these conditions is 36.6 μM (referred to packed cells; see Table 1) an amount of 73.2 μM quercetin can be assumed to be involved in the complex. The amount of quercetin apparently remaining free after the incubation is 19.5 ± 4.6 μM (see Section 2.3). Therefore the amount of total

Table 4
Release of iron (DFO chelatable iron), methemoglobin (Met-Hb) formation, GSH decrease, lipid peroxidation (MDA formation) and hemolysis in mouse erythrocytes incubated with diethylmaleate (DEM) and then incubated with divicine or isouramil in the presence or the absence of quercetin

	Incubation time (min)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)	GSH (nmol/ml)	MDA (nmol/ml)	Hemolysis (%)
Control	0	2.0 ± 0.2	94 ± 8	862 ± 121	–	3.1 ± 0.5
	60	2.4 ± 0.3	119 ± 17	–	2.0 ± 0.4	4.9 ± 0.3
DEM+Divicine	60	24.1 ± 2.4	887 ± 151	131 ± 17	76.7 ± 4.0	88.6 ± 4.4
DEM+Divicine+Quercetin (100 μM)	60	23.0 ± 5.4	258 ± 2	210 ± 26	1.6 ± 1.3	6.7 ± 1.6
DEM+Isouramil	60	25.8 ± 1.7	1016 ± 248	198 ± 12	86.2 ± 5.1	81.5 ± 2.7
DEM+Isouramil+Quercetin (100 μM)	60	16.3 ± 1.8	162 ± 23	199 ± 34	1.2 ± 0.7	5.2 ± 1.0

Divicine and isouramil were added at 1 mM concentrations. Preincubation with DEM was performed as reported in Table 1. Results are the means ± S.E.M. of three experiments. Met-Hb is expressed per heme.

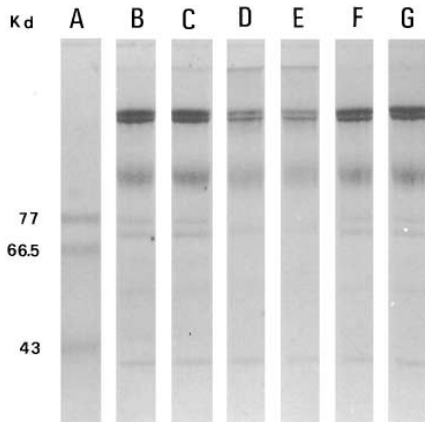

Fig. 3. Polyacrylamide gel electrophoresis of ghosts of GSH depleted mouse erythrocytes incubated with divicine (1 mM) or isouramil (1 mM) for 90 min, in the presence or the absence of quercetin (100 μM). Lanes: A, molecular weight markers (kDa); B, control, 0 time; C, control, incubated for 90 min; D, incubated with divicine; E, incubated with isouramil; F, incubated with divicine and quercetin; G, incubated with isouramil and quercetin. Ten percent polyacrylamide gel was used.

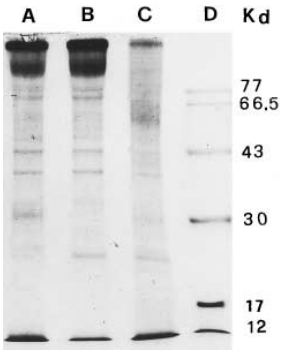

Fig. 4. Polyacrylamide gel electrophoresis of ghosts of GSH depleted human erythrocytes preincubated in the presence or the absence of quercetin (100 μM) and then incubated with divicine (0.5 mM) and isouramil (0.5 mM) for 48 h, as reported in Table 5. Lanes: A, control incubated for 48 h; B, preincubated with quercetin and incubated with divicine and isouramil; C, preincubated without quercetin and incubated with divicine and isouramil; D, molecular weight markers (kDa). Fifteen percent polyacrylamide gel was used.

quercetin calculated in the erythrocytes (80 μM) almost completely accounts for the sum of free quercetin (19.5 μM) plus the quercetin-iron complex (73.2 μM). Thus, according to these estimations, quercetin seems to have bound intracellularly the released iron.

The protective effect of quercetin was also seen with the pyrimidine aglycones, divicine and isouramil, derived from vicine and convicine respectively, the potentially toxic glucosides contained in fava beans (*Vicia faba*). Again in erythrocytes previously depleted of GSH divicine and isouramil induced, together with iron release and methemoglobin formation, lipid peroxidation and hemolysis (Table 4). Again quercetin (100 μM) was able to prevent lipid peroxidation and hemolysis (Table 4).

The electrophoretic analysis of the membrane proteins of the same GSH depleted erythrocytes incubated for 90 min with divicine and isouramil showed a remarkable decrease in α and β spectrin and in band 3 (Fig. 3). Also bands 4.1, 4.5, and 5 were markedly decreased. The addition of quercetin (100 μM) completely prevented all these membrane protein alterations, suggesting that they are related, at least in part, to the release of iron.

With human erythrocytes divicine and isouramil did not induce lipid peroxidation and hemolysis even when the cells were depleted of GSH. In these experiments NEM was used as GSH depleting agent, since DEM was found to induce very low GSH depletion in human erythrocytes. However, even after preincubation with NEM the GSH level was decreased by only 34% which is much less than the decrease (70–85%) obtained with DEM in mouse erythrocytes. Therefore the incubation with divicine and isouramil was prolonged to 24 and 48 h. Even at the latter time lipid peroxidation and hemolysis did not occur (Table 5), even if a substantial release of iron and a more severe GSH depletion (−70%) were seen. In spite of the inability to induce lipid peroxidation, divicine and isouramil produced, during the same incubation time (48 h), alterations of membrane proteins (Fig. 4) similar to those seen in mouse erythrocytes (see Fig. 3) and consisting in an almost complete disappearance of α and β spectrin and band 3, in a marked decrease of bands 4.1 and 4.2 and in the appearance of new bands around 66 kDa. Preincubation with quercetin (100 μM) completely prevented these membrane protein alterations, suggesting again that they are related to the release of iron.

It must be noted that in these experiments substantial iron release occurred even in control erythrocytes incubated for 48 h in the absence of divicine and isouramil (Table 5), which confirms our previous results [32] that prolonged aerobic incubation induces the release of iron. This release is not accompanied by membrane protein alterations (Fig. 4) probably because the GSH level is maintained to a certain extent.

We have recently suggested [32] the possibility that the release of iron in a redox active form is related to the generation of senescent antigen (the antigen appearing on aged erythro-

Table 5
Release of iron (DFO chelatable iron), methemoglobin (Met-Hb) formation, GSH decrease and hemolysis in human erythrocytes preincubated with quercetin and subsequently with *N*-ethylmaleimide (NEM), and then incubated with divicine and isouramil

	Incubation time (h)	'Free iron' (nmol/ml)	Met-Hb (nmol/ml)μ	GSH (nmol/ml)	Hemolysis (%)
No addition	0	1.0 ± 0.2	123 ± 11	521 ± 67	2.7 ± 0.3
	48	15.0 ± 2.1	322 ± 31	503 ± 72	9.2 ± 0.9
Divicine+Isouramil	48	21.0 ± 2.7	1177 ± 90	256 ± 30	11.7 ± 1.1
Divicine+Isouramil+Quercetin	48	20.8 ± 3.0	1240 ± 54	204 ± 54	7.5 ± 0.9

The erythrocytes were preincubated with 100 μM quercetin and subsequently with 1 mM NEM. The first preincubation step was in the only buffer when quercetin was not used. The cells were recovered by centrifugation and then incubated with 0.5 mM divicine and 0.5 mM isouramil. Results are the means ± S.E.M. of three experiments. Met-Hb is given per heme. The GSH level in native (not preincubated) erythrocytes was 885 ± 104 nmol/ml. Lipid peroxidation (MDA formation) was negligible (> 2 nmol/ml) in all the experiments. In some experiments severe hemolysis occurred at 24 or 48 h in all the samples (including controls, no addition). These experiments were discarded.

Table 6
Binding of autologous IgG to human erythrocytes preincubated with quercetin and subsequently with *N*-ethylmaleimide and then incubated with divicine and isouramil

	Incubation time (h)	GSH (nmol/ml	Bound IgG (No. of molecules per cell)
Control	0	323 ± 7	13.9 ± 2.3
	3	238 ± 19	17.5 ± 6.1
Divicine+Isouramil	3	213 ± 38	33.2 ± 9.4
Divicine+Isouramil+Quercetin	3	224 ± 65	17.1 ± 2.0

The erythrocytes were preincubated and incubated as reported in Table 5, with the only difference that the incubation period was 3 h.

cytes [36]), the link being represented by metal catalyzed oxidation of membrane proteins [32]. In fact, under some conditions in which a marked iron release occurs in the erythrocytes, senescent cell antigen is also formed. These conditions were prolonged (60 h) aerobic incubation (in vitro ageing) and incubation with phenylhydrazine [32]. Here we show (Table 6) that incubation (3 h) with divicine and isouramil also gives rise to the formation of senescent antigen (as measured by the binding of autologous IgG). Preincubation with quercetin (100 μM) almost completely prevents the autologous IgG binding that is the formation of senescent antigen.

4. Discussion

The present results clearly show that the flavonoid quercetin is able to prevent the oxidative damage induced in the erythrocyte membrane by a number of oxidizing agents, such as phenylhydrazine, acrolein, divicine and isouramil; damage to membrane lipids (lipid peroxidation and consequent hemolysis) or proteins (severe decrease in spectrin and band 3, and senescent cell antigen formation).

With all these agents iron is released in a free, redox active form. The release of iron appears to be responsible for lipid peroxidation and consequent hemolysis [11]. Also the membrane protein alterations resulting in the formation of senescent cell antigen appear to be related to the release of iron [32]. The results of the present report seem to indicate that the protection afforded by quercetin is due to intracellular chelation of iron. This possibility is supported by (i) the semi-stoichiometric ratio between the amount of released iron and the amount of quercetin necessary to prevent lipid peroxidation and hemolysis, as found in the experiments with phenylhydrazine and acrolein; (ii) the ability to prevent lipid peroxidation and hemolysis of kaempferol which binds iron and the inability of hesperitin which does not; (iii) the results of the experiments carried out in an attempt to extract the quercetin-iron complex from the erythrocytes incubated with phenylhydrazine. Unfortunately, the latter results proved the formation of the complex only indirectly, while in previous studies [11] with DFO preloaded erythrocytes (see Section 1) it was possible to extract the ferrioxamine complex.

The possibility must also be considered that the effect of quercetin is due to its antioxidant activity (which has been extensively documented, see [37–41]), independently of metal chelation. Probably both activities are involved in the protective effect and it is difficult to distinguish the respective roles played. We think, however, that, in our model system, the iron chelating capacity of quercetin is important, since in preliminary experiments we have observed that in GSH depleted mouse erythrocytes vitamin E (which does not chelate iron) is able to prevent the phenylhydrazine induced lipid peroxidation independently of the amount of released iron. In the present study the use of quercetin was aimed at blocking the redox cycling of free iron, which can act on both membrane lipids and proteins. Other iron chelators able to enter erythrocytes are currently being studied in our laboratory with the aim of preventing the damaging effect of iron release. For instance, a remarkably high free iron level and DNA damage have been observed in the hepatocytes [42] in phenylhydrazine induced iron overload. Furthermore, since iron is released in the erythrocytes during blood storage [32], chelation of iron at the intracellular level could represent a means to delay the formation of senescent antigen and to prolong the storage time of blood in blood stores.

Acknowledgements: The present study was supported by the Consiglio Nazionale delle Ricerche (C.N.R.), Grant 96.04985.ST74 and by M.U.R.S.T. 40% Funds (Progetto 'Cirrosi Epatica ed Epatite Virale').

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