Biochemical Pharmacology, Vol. 59, pp. 1365–1373, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00273-2

Protection of Erythrocytes against Oxidative Damage and Autologous Immunoglobulin G (IgG) Binding by Iron Chelator Fluor-benzoil-pyridoxal Hydrazone

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ABSTRACT. Iron is released in a free desferrioxamine-chelatable form when erythrocytes are challenged by an oxidative stress. The release of iron is believed to play an important role in inducing destructive damage (lipid peroxidation and hemolysis) or in producing membrane protein oxidation and generation of senescent cell antigens (SCA). In this report, we further tested the hypothesis that intracellular chelation of iron released under conditions of oxidative stress prevents erythrocyte damage or SCA formation. Fluor-benzoil-pyridoxal hydrazone (FBPH), an iron-chelating molecule of the family of aromatic hydrazones, was prepared by synthesis and used for the above purpose after the capacity of the product to enter cells had been ascertained. GSH-depleted mouse erythrocytes were incubated with the oxidant drug phenylhydrazine in order to produce iron release, lipid peroxidation, and hemolysis. FBPH at a concentration of 200 μM prevented lipid peroxidation and hemolysis in spite of equal values of iron release. FBPH was active even at a lower concentration (100 μM) when the erythrocytes were preincubated with it for 15 min. No preventive effect was seen when FBPH saturated with iron was used. Prolonged aerobic incubation (60 hr) of erythrocytes produced iron release and formation of SCA as determined by autologous immunoglobulin G (IgG) binding. The IgG binding was detected by using an anti-IgG antibody labeled with fluorescein and by examining the cells for fluorescence by confocal microscopy. FBPH prevented SCA formation in a dose-related manner. These results lend further support to the hypothesis that iron release is a key factor in erythrocyte ageing. BIOCHEM PHARMACOL **59**;11:1365–1373, 2000.

KEY WORDS. erythrocytes; oxidative stress; iron release; senescent cell antigen; fluor-benzoil-pyridoxal hydrazone; iron chelation

It is well known that redox cycling of iron promotes the Fenton reaction in which the potent oxidant hydroxyl radical is produced [1–4]. It is also known that iron, to be redox-cycling-active, has to be released from its complexes (transport or store proteins) [5]. Our previous studies have shown [6–8] that iron is released in a free (DFO†-chelatable) form when mouse erythrocytes are incubated with a number of oxidizing agents, such as phenylhydrazine, divicine, isouramil, acrolein, phenylhydroxylamine, dapsone hydroxylamine, and others. Iron is released from hemoglobin or heme [9], and the release is accompanied by Met-Hb formation [7]. When cells are depleted of GSH, the release is also accompanied by lipid peroxidation and hemolysis [6, 7, 10]. Iron acts from the inside of the cell; in fact, in experiments [7] in which mouse erythrocytes were preloaded with DFO (preincubated with a large excess of DFO), washed, depleted of GSH, and then incubated with phenylhydrazine, the released iron was chelated at the intracellular level, as shown by the fact that iron could be extracted from the erythrocytes as ferrioxamine, i.e. the DFO plus iron complex. Lipid peroxidation and hemolysis were completely prevented in these samples, which did not occur when the amount of intracellular DFO was not sufficient to chelate all the released iron. As is known [11, 12], DFO penetrates cells in minimal amounts only (it cannot penetrate human or bovine erythrocytes). Therefore, further studies were carried out with substances capable of penetrating cells and chelating iron to investigate whether intracellular chelation of the released iron could prevent the oxidative alterations of membrane components which are induced by the oxidizing agents mentioned above and which appear to be mediated by the release of iron.

With human or bovine erythrocytes, phenylhydrazine, divicine, and isouramil induced iron release, but not lipid peroxidation or hemolysis, even if the cells were depleted of GSH [13]. They did, however, induce the formation of SCA, as measured by the binding of autologous IgG [13]. The latter seems to result from oxidative degradation of

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† *Abbreviations:* DFO, desferrioxamine; Met-Hb, methemoglobin; SCA, senescent cell antigen; IgG, immunoglobulin G; PIH, pyridoxal isonicotinoyl hydrazone; FBPH, fluor-benzoil-pyridoxal hydrazone; DEM, diethylmaleate; and MDA, malonaldehyde.

Received 3 August 1999; accepted 29 November 1999.

some membrane proteins, particularly band 3, and acts as a specific signal for termination of old cells [14, 15]. We have shown [16] that the aerobic incubation of human or bovine erythrocytes in buffer for 24–60 hr (a model of rapid *in vitro* ageing) induces a progressive and marked iron release and Met-Hb formation. The release is accompanied by membrane protein oxidation as well as by the appearance of SCA. Addition, during the aerobic incubation, of the iron chelator ferrozine, which freely enters the cells, prevents both protein alterations and senescent antigen formation [16], suggesting that the release of iron is a relevant factor in generation of SCA. Similar results were obtained [13] by using the flavonoid quercetin, which can cross the cell membrane and is capable of chelating the iron released during the aerobic incubation of human erythrocytes and of preventing SCA formation. In view of the well-proven genotoxicity of quercetin [17, 18] and the fact that quercetin itself can react with iron to produce oxygen species [19], we searched for other iron-chelating drugs provided with no toxicity and capable of entering cells.

PIH is a molecule of the family of aromatic hydrazones synthesized by condensation of an aromatic aldehyde with an acid hydrazide [20–23]. PIH readily enters and chelates iron from cells such as fibroblasts, peritoneal macrophages, hepatocytes, and reticulocytes [24–26]. The mechanism of PIH action was also studied [26, 27] and involves passive diffusion of PIH into cells, chelation of iron derived mainly from a ferrous iron source, and active extrusion of the Fe–PIH complex via an energy-dependent process. Clinical trials showed that PIH can safely produce a level of iron excretion that would be clinically useful in the treatment of non-transfusion-dependent patients with iron-loading anemias [28]. These results suggest that a more bioavailable formulation of PIH should be evaluated as a means of increasing iron excretion before increasing the dose of the chelator [28].

Ponka *et al.* [27] examined the capacity of several aromatic hydrazones with structures similar to PIH (and equally synthesized by the condensation of aromatic aldehydes such as pyridoxal with hydrazides) to release iron from macrophages, reticulocytes, and hepatocytes. It appeared that one of the most efficient compounds was FBPH. Therefore, in the present study, we examined FBPH for its capacity to prevent the effects produced by the release of iron in erythrocytes, particularly the formation of SCA.

MATERIALS AND METHODS

Chemicals

DFO (Desferal) was supplied by Ciba-Geigy. Phenylhydrazine hydrochloryde was from Carlo Erba, hydrazine hydrate was from Fluka, and pyridoxal hydrochloride and 3-fluorobenzoic acid were from Aldrich. The secondary antibody was goat anti-human IgG (Fc-specific) fluorescein isothiocyanate (FITC) conjugate from Sigma Immunochemicals. The solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade.

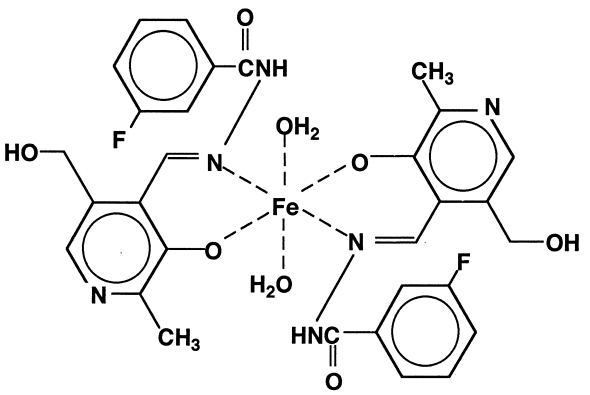

FIG. 1. Structure of the FBPH–iron complex.

Synthesis of FBPH

FBPH (which is not commercially available) was synthesized in our laboratory essentially according to Sah [29] and Edward *et al.* [30]. Briefly, the method implies the use of 3-fluorobenzoic acid and the preparation, in sequence, of its ethyl ester, hydrazide, and finally hydrazone. The ester was obtained by refluxing an ethanolic solution of fluorobenzoic acid with concentrated H_2SO_4 and removing the excess alcohol by distillation. The fluorobenzoate ethyl ester so obtained was repeatedly washed and then hydrazine was added. The mixture was refluxed at 135–140° for 2 hr, and the resulting hydrazide was obtained in crystallized form after evaporation of solvent. Pyridoxal hydrochloride was added to an aqueous solution of the hydrazide containing anhydrous sodium acetate (0.9 g%). The mixture was refluxed for 40 min at 110–115° and then filtered. The hydrazone remaining on the filter was washed and dessicated. The formation of FBPH was ascertained by mass spectrometry using a VG 70-250 S instrument (Interdepartmental Center for Analysis and Structural Determinations of Siena University). The formation of FBPH–Fe (the structure is given in Fig. 1) was equally ascertained by mass spectrometry.

Saturation of FBPH with Iron

FBPH (0.1 mmol) was dissolved in 50 mL of 28 mM sodium phosphate/potassium phosphate buffer, pH 7.4, at 50°. $FeSO_4$ (0.1 mmol) was added and allowed to react with FBPH for 2 hr at room temperature. The FBPH–Fe complex was extracted with ethyl acetate (in which $FeSO_4$ is not soluble). The ethyl acetate extract was washed with H_2O to remove possible iron sulphate traces. The solvent was evaporated and an aliquot of the residue was dissolved in the buffer and analyzed spectrophotometrically (Fig. 2). Another aliquot was dissolved in ethyl acetate and analyzed by mass spectrometry as mentioned above.

Erythrocyte Incubation

Mouse erythrocytes were used in experiments in which oxidative damage by phenylhydrazine was studied. Male

Swiss albino mice (Nossan, Correzzana, Milan) weighing 25–35 g and maintained on a pellet diet (Nossan) were used. Blood was withdrawn from the abdominal aorta under anesthesia and heparinized. After centrifugation, 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 [6]. The hemoglobin concentration, measured as in [31] and expressed per heme, was 7700 ± 220 nmol/mL. When GSH-depleted erythrocytes were to be used, GSH depletion was accomplished by a short (15-min) preincubation with 1.5 mM DEM. DEM was dissolved in a small volume of DMSO, which 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 was added to the incubation mixture at a concentration of 1 mM. FBPH was added at 100- and 200-μM concentrations. The incubation was carried out aerobically at 37° for 1 hr. At the end of the incubation, samples were withdrawn for the determination of "free" iron (DFO-chelatable), Met-Hb [32], GSH [33], MDA [34], and hemolysis [6]. "Free" iron was determined as a DFO–iron complex (ferrioxamine) as previously reported [6].

Attempts made to reveal by HPLC the FBPH–iron complex likely formed in erythrocytes incubated under conditions in which iron is released were unsuccessful. In fact, both FBPH and its iron complex, when injected into a silica column (LiChrosorb™ Si 60, 5 μm, Merck) and eluted with a mixture of 40% acetonitrile and 60% phosphate citrate buffer (0.2 M Na_2HPO_4 and 1 M citric acid, pH 4.2), showed identical retention times. Changing the proportions of acetonitrile: buffer gave the same results.

In the experiments in which the ability of FBPH to protect against erythrocyte ageing was tested, human erythrocytes were prepared from heparinized blood of volunteers. The cells were washed and resuspended in the NaCl–sodium phosphate/potassium phosphate buffer mentioned above as a 50% (v/v) suspension. The incubation was carried out aerobically for 60 hr at 37° in the presence of antibiotics (20 units penicillin and 20 μg streptomycin/mL of buffer). Determinations of "free" iron, Met-Hb, GSH, MDA, and hemolysis were performed as above. Erythrocyte samples were centrifuged and the packed cells were resuspended in Dulbecco–PBS prepared without $CaCl_2$ and $MgCl_2$ containing, in addition, 0.1 mM EGTA, pH 7.4, for determination of autologous IgG binding. All animal experimentation followed guidelines prescribed by Italian D.L. 27 of January 1992, no. 116, with approval of Siena University Ethic-Deontological Committee of Siena University.

Autologous IgG Binding to Erythrocytes

The binding of autologous IgG to human erythrocytes was detected by an anti-IgG antibody labeled with fluorescein and by examining the cells for fluorescence with confocal microscopy. IgG were prepared from the serum of the erythrocyte donor by precipitation in 40% ammonium sulphate according to Good *et al.* [35]. After dialysis, IgG in Dulbecco–PBS without $CaCl_2$ and $MgCl_2$, pH 7.4, were partially purified on diethylamino-ethylcellulose (DEAE) column, equilibrated with the same buffer, as reported by Levy *et al.* [36]. The recovered IgG were measured spectrophotometrically at 280 nm using $E^{1\%} = 14.0$. Aliquots of erythrocyte suspensions in Dulbecco–PBS containing 0.1 mM EGTA were layered (approx. 100,000 cells/well) on multiwell glass slides, previously washed and coated with polylysine (10 μg/mL, 30 min at room temperature). Erythrocytes were left to adhere for 60 min; excess buffer was then removed and slides were fixed by immersion (2 min) in acetone at −20°. Specimens were subsequently washed (10 min × 3) with the buffer above, plus 2% BSA for blockade of aspecific binding sites. Fixed erythrocytes were then exposed to autologous IgG solution (0.75 mg/mL in Dulbecco–PBS, containing 0.1 mM EGTA and 0.7% BSA) and incubated for 60 min at room temperature. After thorough washing in Dulbecco–PBS with 0.1 mM EGTA, fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (dilution, 1:64) were added, and slides were incubated for an additional 60 min. Finally, specimens were washed, mounted in glycerol (90%, v/v, in 1.0 M Tris, pH 9.0), and analyzed by confocal microscopy.

Confocal Laser-Scanning Fluorescence Microscopy

A Bio-Rad MRC-500 confocal imaging system equipped with a 20 mW argon ion laser was used. The system included a Nikon Optiphot fluorescence microscope and a Nimbus 80286 computer. Confocal images were obtained, processed, and stored employing the Bio-Rad COMOS™ software. A Nikon Plan-Apo 60 (NA 1.4) oil immersion objective was used. Optical sections approximately 1.0 μm in thickness were obtained at the largest apparent cell diameter. In each experiment, exciting light intensity, black level, and photomultiplier gain were adjusted on specimens reacted with the secondary antibody only; the same setting was then employed for scanning of experimental specimens. The instrument was set to 488 nm exciting light, with a filter barrier of 515 nm on the emission pathway (BHS filter block). To avoid fluorescence bleaching, cells to be analyzed were framed under bright-field observation, and confocal scanning was then performed. All fields were scanned only once and then discarded. Since FBPH is a fluorescent compound, it was verified that its presence in the samples incubated aerobically did not interfere with the fluorescence due to the fluorescein conjugated to the secondary antibody. In effect, a sample of erythrocytes incubated with 200 μM FBPH and reacted with the autologous IgG only did not show any fluorescence.

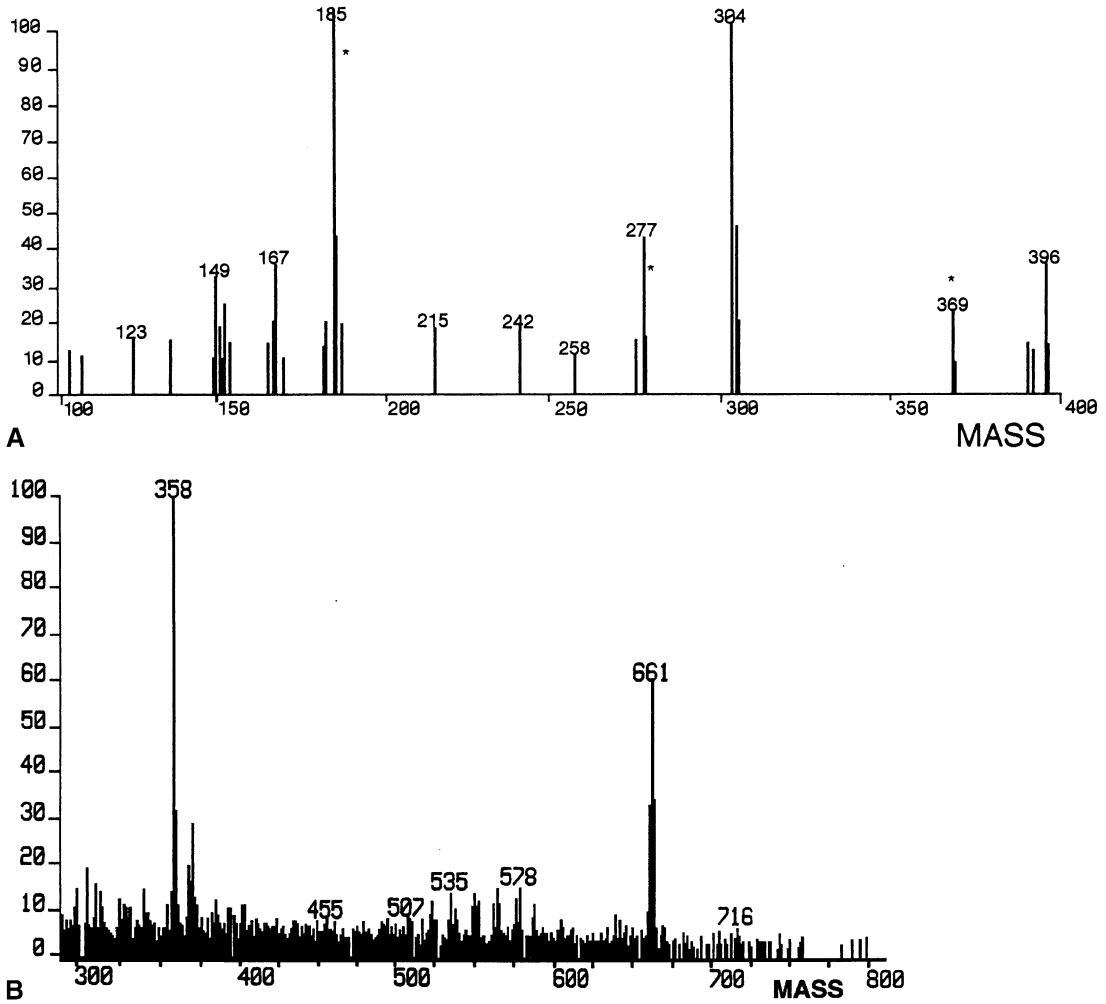

FIG. 2. (A) Liquid secondary ion mass spectrum of FBPH. The ions indicated with * are relevant to the matrix (glycerol); (B) Liquid secondary ion mass spectrum of the FBPH–iron complex.

RESULTS

The mass spectrometry analysis of FBPH as obtained by the synthetic procedure reported in Materials and Methods is shown in Fig. 2A. The peak at m/z 304 $[(C_{15}H_{14}N_3O_3F) + H]^+$ is relevant to its protonated molecular ions. Figure 2B shows the mass spectrometry analysis of the FBPH–iron complex. Ions at m/z 661 show that two molecules of FBPH are complexed with one iron atom, with elimination of two hydrogen atoms yielding protonated molecular ions with stoichiometry $[Fe(FBPH\text{-}H)_2 + H]^+$. The spectrophotometric analysis of the products (FBPH and FBPH–iron complex) further confirmed the formation of $(FBPH)_2$–Fe complex (Fig. 3, A and B). In fact, the addition of increasing amounts (5, 10, and 25 μM) of $FeSO_4$ to FBPH (50 μM) in sodium phosphate buffer, pH 7.4, resulted in a progressive shift of the absorption maximum from 385 to 365 nm (Fig. 3A). Further addition of $FeSO_4$ (50 μM) did not modify the 365-nm peak (Fig. 3A). This means that at an iron ion concentration half that of FBPH, a complex is formed in which the molar ratio FBPH:iron is 2:1. The addition of DFO (50 μM) to the complex (Fig. 3B) extracted iron from the iron–FBPH complex, as shown by the restoration of the FBPH spectrum. In view of the structure of this class of acylhydrazones, the spectrofluorophotometric analysis of the FBPH was also performed. Figure 4 shows the excitation and emission spectra of FBPH (50 μM) in sodium phosphate buffer. As can be seen, the excitation and emission maxima are at 395 and 480 nm, respectively. It can also be seen that the addition of iron sulphate (25 μM) quenches the fluorescence. The fluorescence characteristics of FBPH were used to detect the product in biological samples.

To confirm the capacity of FBPH to enter the erythrocytes used in the experiments performed, washed mouse erythrocytes were incubated in phosphate buffer with various concentrations of FBPH for 15 and 60 min. At the end of the incubation times, the cells were recovered by centrifugation and lysed by the addition of one volume of H_2O. The lysate was extracted with ethyl-acetate, and the extract was analyzed fluorimetrically by setting the excitation at 375 nm (the presence of residual amounts of Hb shifts the excitation maximum from 395 to 375 nm) and by

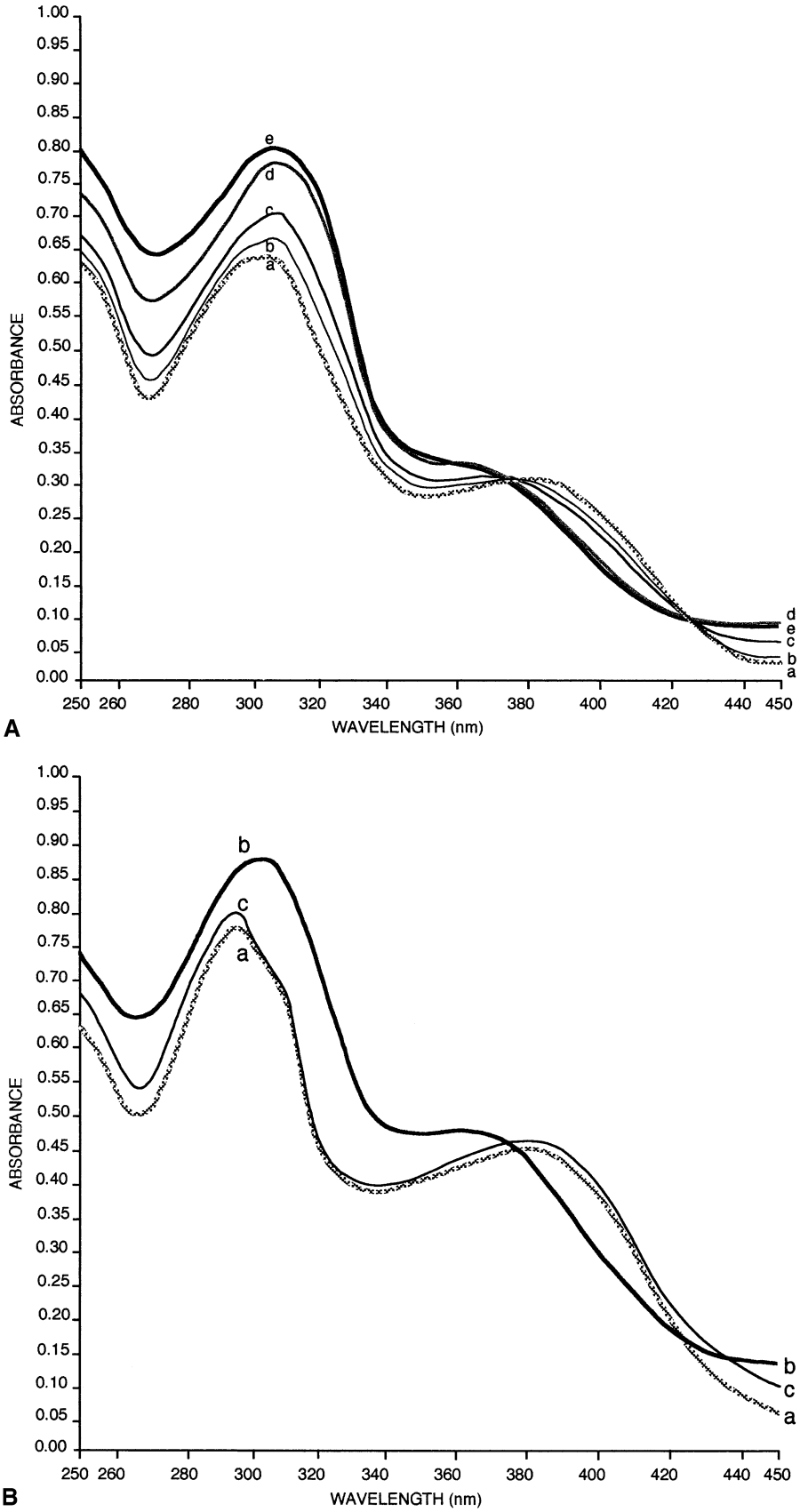

FIG. 3. (A) Absorption spectra of FBPH (50 μM) before (a) and after the addition of increasing concentrations of iron sulfate [(5 μM (b), 10 μM (c), 25 μM (d), and 50 μM (e)]; (B) Absorption spectra of FBPH [50 μM (a)] and of the FBPH–iron complex before (b) and after (c) DFO (50 μM) addition.

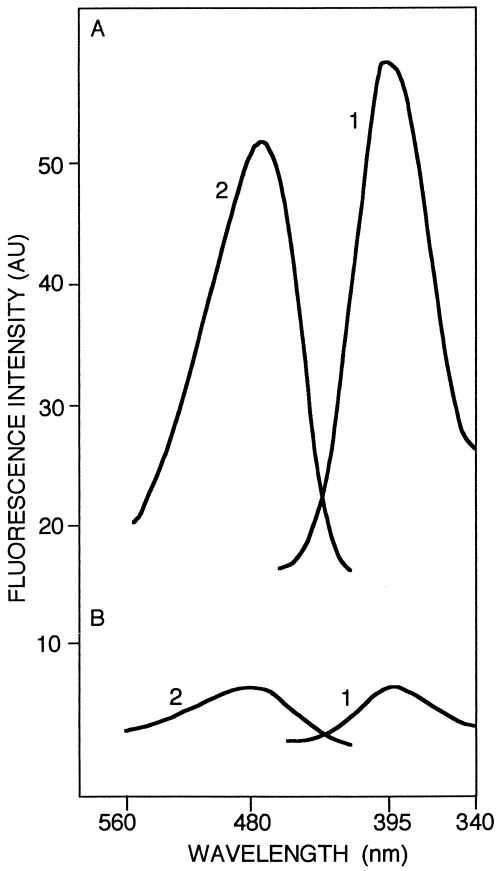

FIG. 4. Excitation (1) and emission (2) spectra of FBPH (50 μM) before (A) and after (B) the addition of iron sulfate (25 μM). AU, arbitrary units.

recording the emission maximum at 482 nm. The fluorescence (arbitrary units) was compared to that of a standard curve obtained with various concentrations of FBPH in buffer. Accordingly, the partition of FBPH between the medium and the erythrocytes could be obtained. This partition coefficient was 1.34 and 0.92 at 15 and 60 min of incubation, respectively, when 100 μM FBPH was added to the medium; it was 1.03 and 0.69 at 15 and 60 min, respectively, when 200 μM FBPH was used. It seems, therefore, that FBPH enters the erythrocytes and that, at both concentrations, the equilibrium between the medium and the cells is reached within less than 60 min.

In view of its capacity to chelate iron and to penetrate cells, FBPH was tested for its ability to prevent the effects induced by the oxidizing agent phenylhydrazine and allegedly related to the release of iron in the erythrocytes. Table 1 shows that incubation of native mouse erythrocytes with phenylhydrazine induced iron release and Met-Hb formation, but not lipid peroxidation or hemolysis, whereas with erythrocytes previously depleted of GSH (by a short preincubation with DEM), iron release and Met-Hb formation were accompanied by lipid peroxidation and hemolysis. This confirms our previous results [7]. The addition of 100 μM FBPH to GSH-depleted erythrocytes did not prevent lipid peroxidation and hemolysis. On the other hand, the addition to the same erythrocytes of 200 μM FBPH completely prevented both lipid peroxidation and hemolysis. It is likely that the lack of effect seen with 100 μM FBPH is due to the fact that the hydrazone has not entered erythrocytes in sufficient amounts at the moment when lipid peroxidation and hemolysis begin; however, when a higher concentration (200 μM) of the hydrazone is used, the intracellular concentration of the drug is now sufficient to chelate the released iron and to prevent lipid peroxidation. Indeed, preincubation of the erythrocytes with 100 μM FBPH for 15 min prevented lipid peroxidation and hemolysis upon subsequent exposure to phenylhydrazine. Moreover, the same concentration (100 μM) of FBPH was able to prevent lipid peroxidation (Table 2) when the experiment was repeated using an erythrocyte lysate (from GSH-depleted erythrocytes) rather than whole cells, so that the chelating agent (FBPH) could now be equally

TABLE 1. Release of iron (DFO-chelatable iron), Met-Hb formation, GSH decrease, lipid peroxidation (MDA formation) and hemolysis in mouse erythrocytes

Line		Free iron (nmol/mL)	Met-Hb (nmol/mL)	GSH (nmol/mL)	MDA (nmol/mL)	Hemolysis (%)
1	Control (non-incubated cells, 0 time)	1.5 ± 0.1	115 ± 8	970 ± 60	—	3.8 ± 0.7
2	Control (incubated cells)	2.4 ± 0.1	107 ± 4	913 ± 39	1.2 ± 0.2	4.1 ± 0.7
3	Phz	18.3 ± 0.5	555 ± 10	691 ± 25	2.8 ± 0.4	6.1 ± 0.6
4	DEM + Phz	23.0 ± 3.4	677 ± 63	159 ± 26	80.4 ± 2.7	88.5 ± 3.0
5	DEM + Phz + FBPH (100 μM)	21.1 ± 0.1	679 ± 109	101 ± 66	80.7 ± 8.3	86.6 ± 7.5
6	DEM + Phz + FBPH (200 μM)	20.7 ± 1.3	314 ± 19	74 ± 12	0.2 ± 0.2	7.2 ± 1.6
7	DEM + FBPH (100 μM), preincubation, + Phz	26.6 ± 3.2	346 ± 90	180 ± 41	5.2 ± 0.8	7.8 ± 1.0
8	DEM + Phz + FBPH (200 μM, saturated with iron)	—	768 ± 26	52 ± 8	86.2 ± 7.1	98.0 ± 7.3

Mouse erythrocytes were incubated with phenylhydrazine (Phz) (line 3) or preincubated with DEM and then incubated with Phz in the presence (lines 5–8) or absence (line 4) of FBPH. Lines 5 and 6, Phz and FBPH were added at the start of incubation. Line 7, erythrocytes were preincubated for 15 min with FBPH (100 μM) and Phz was added at the start of incubation. Line 8, erythrocytes were incubated with Phz and with FBPH (200 μM, saturated with iron). The value for free iron, which was 34.3 ± 4.0 nmol/mL, was not reported in the table, since it is likely that some DFO-chelatable iron derived from the complex rather than from the erythrocytes. Phz was added to erythrocytes at 1-mM concentration. The incubation was carried out aerobically at 37° for 60 min. 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 Phz did not result in any iron release, lipid peroxidation, or hemolysis. FBPH was added to erythrocytes at 100- and 200-μM concentrations. Met-Hb is expressed per heme. Zero time values for MDA were subtracted from those of the incubated samples. Results are the means ± SEM of three experiments.

TABLE 2. Met-Hb formation and lipid peroxidation (MDA formation) in a lysate of mouse erythrocytes depleted of GSH and incubated with phenylhydrazine (Phz) in the presence or absence of FBPH

	Met-Hb (nmol/mL)	MDA (nmol/mL)
Control (non-incubated cells, 0 time)	122	—
Control (incubated cells)	140	1.5
Lysate + Phz	589	43.5
Lysate + Phz + FBPH (100 μM)	461	3.5
Lysate + Phz + FBPH (200 μM)	384	3.3

Erythrocytes were depleted of GSH by preincubation with 1.5 mM DEM for 15 min. The erythrocyte lysate was prepared by freeze (at −196°)–thawing. Phz was added to erythrocyte lysate at 1-mM concentration. The incubation was carried out aerobically at 37° for 60 min. Zero time values for MDA were subtracted from those of the incubated samples. Results are the means of two experiments. The error spread was in the range of 10–15%. Met-Hb is expressed per heme.

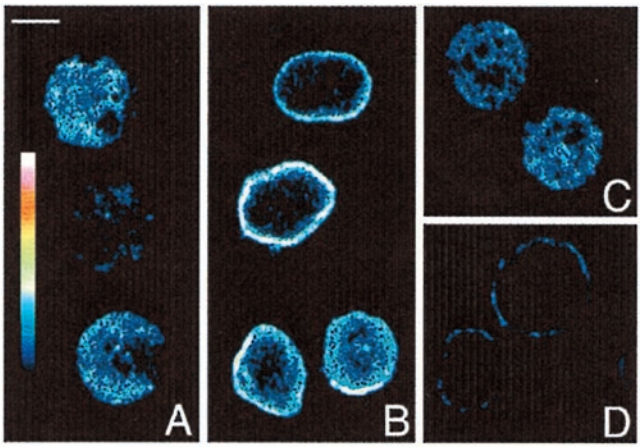

FIG. 6. Confocal imaging of aerobically incubated erythrocytes. A, B, C, D: Magnifications of selected fields, as from legend to Fig. 5. Bar in panel A corresponds to 5 μm.

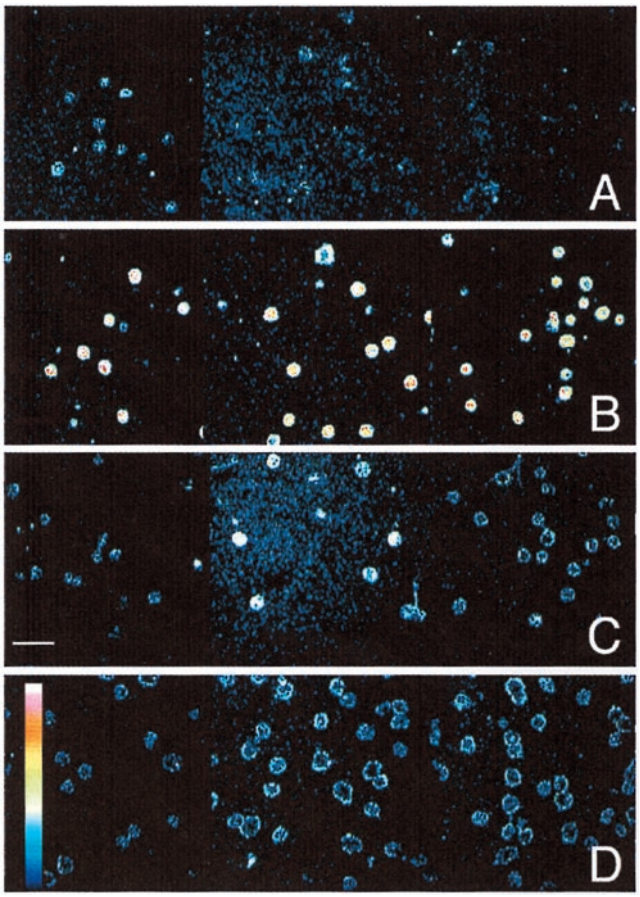

FIG. 5. Visualization by confocal laser-scanning fluorescence microscopy of autologous IgG binding to erythrocytes incubated aerobically for 60 hr. False color, confocal imaging of fluorescence intensities. See Materials and Methods for incubation and staining analytical procedures. (A) Non-incubated cells (0 time), showing little or no fluorescence at all. (B) Aerobically incubated cells (60 hr), presenting with intense fluorescence, indicating the binding of autologous IgG. (C) Cells incubated as in (B), but in the presence of 100 μM FBPH, showing less fluorescence. (D) Cells incubated as in (B), but in the presence of 200 μM FBPH; mean fluorescence is further decreased. Bar in panel C corresponds to 10 μm.

distributed in the incubation mixture. Interestingly, when 200 μM FBPH was presaturated with iron (see Materials and Methods) prior to addition to the preincubation mixture, its protective effect against phenylhydrazine-induced lipid peroxidation and hemolysis was completely abolished (Table 1). This indicates that the protective effect of FBPH is indeed due to its iron-chelating activity and not to other possible antioxidant effects of a different nature. Incidentally, 200 μM FBPH further decreased GSH (Table 1). A tentative explanation could be that chelation of iron by FBPH prevents its reaction with hydrogen peroxide (H_2O_2) (Fenton reaction), which would likely result in increased levels of H_2O_2 and consequent increased consumption of GSH through GSH–peroxidase activity. The decrease in Met-Hb with 200 μM FBPH seems to point to a possible involvement of iron-dependent oxidative mechanisms.

In view of the protective effect of FBPH on the oxidative damage induced by phenylhydrazine, experiments were performed to test the ability of the chelating agent FBPH to prevent erythrocyte ageing, i.e. oxidative alterations of membrane components resulting in senescent cell antigen formation, as measured by the binding of autologous IgG. To this end, human erythrocytes were incubated aerobically in buffer for 48 or 60 hr (a model of rapid *in vitro* ageing) and then exposed to autologous IgG (see Materials and Methods). The IgG binding was detected by using an anti-human IgG antibody labeled with fluorescein and by examining the cells for fluorescence by confocal microscopy. A typical experiment is reported in Fig. 5. As can be seen, the non-incubated cells (0 time) showed no fluorescence (Fig. 5A), while the aerobically incubated cells showed extensive fluorescence (Fig. 5B), i.e. extensive IgG binding (SCA formation). It can also be seen that cells incubated in the presence of FBPH demonstrated much less fluorescence (Fig. 5, C and D). The concentrations of FBPH tested (100 and 200 μM, Fig. 5, C and D, respectively) showed proportional responses. Figure 6 shows the

TABLE 3. Release of iron (DFO-chelatable iron), Met-Hb formation, GSH decrease, and hemolysis in human erythrocytes incubated aerobically in the presence or absence of FBPH

	Free iron (nmol/mL)	Met-Hb (nmol/mL)	GSH (nmol/mL)	Hemolysis (%)
0 time	1.9 ± 0.5	77 ± 6	834 ± 45	2.6 ± 0.3
Aerobic incubation	23.5 ± 2.9	1664 ± 178	122 ± 14	8.7 ± 2.3
Aerobic incubation + FBPH (100 μM)	25.7 ± 2.9	2048 ± 166	123 ± 21	8.5 ± 2.0
Aerobic incubation + FBPH (200 μM)	29.5 ± 2.9	2316 ± 300	137 ± 4	11.9 ± 1.5

The erythrocytes were incubated aerobically for 60 hr at 37°. Results are the means ± SEM of six or more experiments. Met-Hb is expressed per heme.

same experiment at higher magnification (0 time, Fig. 6A; aerobic incubation, Fig. 6B; protection by FBPH, Fig. 6C). Table 3 shows iron release, Met-Hb formation, GSH decrease, and hemolysis in all the experiments carried out under these conditions of prolonged aerobic incubation. No lipid peroxidation (MDA formation) was observed in these incubates, and the moderate hemolysis occasionally seen in some experiments could not be attributed to this process. In addition, there was no relation between hemolysis and the detectable fluorescence (SCA formation).

DISCUSSION

The present results show that the iron chelator FBPH is able to prevent oxidative damage (lipid peroxidation and hemolysis) induced in the erythrocyte membrane by the oxidizing agent phenylhydrazine as well as the formation of SCA, as determined by the binding of autologous IgG, during the prolonged aerobic incubation of human erythrocytes. Under both experimental conditions, iron is released in a free, redox-active form. The release of iron appears to be responsible for lipid peroxidation and subsequent hemolysis when GSH-depleted mouse erythrocytes are challenged with a number of oxidizing agents, as shown by the present study and previous reports from our laboratory [6–9]. It was also shown that the membrane protein alterations resulting in the formation of SCA appear to be related to the release of iron [16]. The results of the present report seem to indicate, in agreement with previous reports [7, 13, 16], that when an effective iron chelator is present in the cell in sufficient concentration, the iron released under various conditions is chelated and thus rendered inactive for redox-cycling reactions allegedly involving several membrane components.

Recently, we have shown [13] that the flavonoid quercetin which, like FBPH, can enter cells and chelate iron, affords a similar protection against erythrocyte damage (lipid peroxidation and hemolysis) induced by oxidants (phenylhydrazine and acrolein) and erythrocyte senescent antigen formation induced by prolonged aerobic incubation. As in the case of quercetin [13], attempts made to reveal the FBPH–iron complex which is likely formed in the erythrocytes incubated under conditions in which iron is released were unsuccessful. Since the iron-chelating capacity of FBPH is well known and since FBPH enters erythrocytes (see Results section), we believe that the data reported here enable us to conclude that the protection seen with FBPH is due to intracellular chelation of free iron.

It was also considered whether the effects of FBPH were due to its possible antioxidant activity, independent of metal chelation. However, in a simple test where lipid peroxidation was induced in erythrocytes or in phosphatidylcholine liposomes by the azo compound [2,2′-azobis(2-amidinopropane)dihydrochloride, APPH], as reported by Miki *et al.* [37], FBPH did not show any antioxidant activity (data not shown). Moreover, iron-presaturated FBPH was devoid of any protective effect (see Table 1), which would exclude the possibility that FBPH may exert antioxidant effects unrelated to its iron-chelating capacity. Therefore, the protective effect of FBPH on oxidant-induced erythrocyte damage (lipid peroxidation, hemolysis, SCA formation) really seems to be due to the chelation of the released iron. 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.

Since iron is released in the erythrocytes during blood storage [16], chelation of iron at the intracellular level could represent a means to delay the formation of SCA and to prolong the storage time of blood in blood stores. Furthermore, it has become clear that Fe chelators may be useful for the treatment of a wide variety of disease states, including cancer, malaria, and free radical-mediated tissue damage [38].

This work was supported by grants from the Italian Ministry of University and Scientific Research (Programs of National Relevance "Redox regulation of cellular processes" and "Cellular and molecular biology of hepatic fibrosis").

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