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# ION TRANSPORT ATPases AS TARGETS FOR FREE RADICAL DAMAGE

## PROTECTION BY AN AMINOSTEROID OF THE $Ca^{2+}$ PUMP ATPase AND $Na^+/K^+$ PUMP ATPase OF HUMAN RED BLOOD CELL MEMBRANES

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**Abstract**—Preincubation of red blood cell membranes in the presence of ferrous sulfate and EDTA resulted in both a concentration- and time-dependent inhibition of the $Na^+/K^+$ pump ATPase, basal $Ca^{2+}$ pump ATPase, and the calmodulin- (CaM) activated $Ca^{2+}$ pump ATPase. The $IC_{50}$ for all three ATPases was approximately $2.5 \times 10^{-5}$ M iron. The addition to membranes of ferrous iron and EDTA in an approximately 1:1 ratio resulted in conversion to the ferric iron form in several minutes. However, inhibition of the ion pump ATPases and cross-linking of membrane proteins occurred over the course of several hours. The time course of formation of thiobarbituric acid-reactive substances (TBARS) closely paralleled inhibition of the ion pump ATPases. Inhibition of the ion pump ATPases was prevented by the addition of deferoxamine or superoxide dismutase but not by mannitol, ethanol, or catalase. Both butylated hydroxytoluene and tirilazad mesylate (U74006F) prevented the formation of TBARS, limited the inhibition of the ion pump ATPases, and reduced cross-linking of membrane proteins. These data may be interpreted to suggest that inhibition of ion pump ATPases in plasma membranes may occur as a result of iron-promoted formation of superoxide and subsequent lipid peroxidation, which can be prevented by free-radical scavengers including butylated hydroxytoluene and U74006F.

Current evidence suggests that during and/or following stroke, ischemia, or CNS trauma, changes occur which favor the production of oxygen radicals [1, 2]. These changes include consumption of ATP and concomitant accumulation of AMP [3], which is degraded to hypoxanthine. After ischemia, xanthine oxidase may catalyze the formation of superoxide ion ($O_1^{-\cdot}$), an oxygen free radical, in the presence of hypoxanthine [4]. In addition, ischemia or stroke may lead to increases in lactate levels as cells convert to anaerobic glycolysis following the reduction in oxygen delivery. These changes lead to tissue acidosis in regions of the CNS with a fall in local pH to values as low as 6.0 [5]. Both ferritin and transferrin can release iron at a pH of 6.0 or less [1], and evidence points to the involvement of iron in the initiation and propagation of lipid peroxidation in CNS trauma [1]. Iron facilitates the production of oxygen free radicals [6] via the so-called Fenton reaction. Ion gradients are disrupted by conditions that promote the formation of oxygen radicals. In particular, $Ca^{2+}$ accumulates in damaged cells [7], and increased cytosolic $Ca^{2+}$ may be a final common pathway in cell death [8–10]. We hypothesize that inhibition of both the $Na^+/K^+$ pump ATPase and the $Ca^{2+}$ pump ATPase as caused by oxygen free radicals leads to the accumulation of intracellular $Ca^{2+}$ to toxic levels. Increased intracellular $Ca^{2+}$ causes or contributes to the cell injury and death seen in a variety of pathological states. Intracellular $Ca^{2+}$ is normally maintained at extremely low levels in the cytoplasm by one or more processes, including a calmodulin- (CaM†) activated $Ca^{2+}$ pump [11–13], $Na^+/K^+$ exchange [14], which is energized indirectly by the $Na^+/K^+$ pump, and the endoplasmic reticulum $Ca^{2+}$ pump ATPase [15].

Tirilazad mesylate (U74006F) is a non-glucocorticoid, 21-aminosteroid [16] that is undergoing clinical trials for treatment of head injury, spinal cord injury and subarachnoid hemorrhage. U74006F is an antioxidant and an inhibitor of iron-dependent lipid peroxidation [16], which has shown promising activity in experimental models of CNS ischemia [17–20]. Using chelated iron and red blood cell (RBC) membranes, we examined the potential of U74006F to protect the $Ca^{2+}$ pump ATPase and the $Na^+/K^+$ ATPase from inhibition that may occur as a result of oxygen free radicals and/or lipid peroxidation.

### MATERIALS AND METHODS

Vanadium-free ATP was purchased from Boehringer Mannheim (Indianapolis, IN). Butylated hydroxytoluene (BHT), 1,10-phenanthroline, bovine serum albumin (BSA), deferoxamine (DFO), ferrous sulfate, mannitol, catalase, copper/zinc, superoxide

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† Abbreviations: CaM, calmodulin; RBC, red blood cell; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; DFO, deferoxamine; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; and MDA, malondialdehyde.

dismutase (SOD), thiobarbituric acid, 1,1,3,3-tetraethoxypropane, iron stock standard, *p*-chloromercuriphenylsulfonic acid, and EDTA were all purchased from the Sigma Chemical Co. (St. Louis, MO). U74006F was provided by Edward Hall of the Upjohn Co. (Kalamazoo, MI). All chemicals were of the highest purity grade available.

*Membrane preparation.* Human RBC membranes were prepared as previously described [21]. Briefly, outdated RBCs from the blood bank were washed three times in cold isotonic saline and spun at 5000 *g* for 5 min in a Sorvall RC2B centrifuge. Membranes were prepared by hemolysis of 2.5 mL of washed, packed RBCs in 35 mL of imidazole (20 mM, pH 7.4) buffer containing 0.57 mM ethylene glycol-bis (β-aminoethyl-ether)*N,N,N',N'*-tetraacetic acid (EGTA) with rapid mixing in capped tubes. The hemolysate was centrifuged for 10 min at 17,000 *g*, and the resultant supernate was removed by aspiration. This step was repeated once. The membranes were then washed with about 35 mL of 20 mM imidazole buffer, pH 7.4, two more times. The membranes were finally washed with 9 mL (per original 2.5 mL of packed RBCs) of 40 mM/40 mM histidine/imidazole buffer, pH 7.1 (H/I). At each centrifugation step, care was taken to remove the brown-colored button that formed on the bottom of the membrane fraction. After removal of the supernate, an approximately equal volume of H/I was added to the white and apparently hemoglobin-free pellet. Membrane protein content was determined by the bicinchoninic acid method [22] using BSA as a standard. The membranes were stored on ice in the refrigerator until used.

*Preincubation.* To minimize free radical scavenging by H/I, membranes (0.5 to 1 mL) were first spun at 5000 *g* for 25 min in a Fisher 235B microcentrifuge, and the supernate was removed. Membranes were then resuspended in an equal volume of distilled water and immediately diluted in the preincubation medium. Specific activities of membranes suspended in water were comparable to those obtained using membranes suspended in buffer (data not shown). Others have used a saline solution to preincubate RBC membranes and have not reported any adverse effects from such treatment [23]. Unbuffered membranes were then added to reaction vessels with the following reagents: 500 μL of 1.8% NaCl, with or without ferrous sulfate/EDTA in nominally equimolar amounts, and other reagents as specified. Distilled water was added to a final volume of 1 mL. Protein concentration was 1 mg/mL and pH was 6.8 to 7.0. Incubations were carried out in a Dubnoff shaking incubator at 37°. At the conclusion of the preincubation period, 4 mM DFO was added to stop the iron-mediated reactions.

*Determination of nonheme iron.* Ferrous and ferric iron content was determined by the method of Brumby and Massey [24]. In 96-well microtiter plates, 100 μL trichloroacetic acid (20%, w/v) with or without 0.005 M *p*-chloromercuriphenylsulfonic acid, 12.5 μL of 0.06 N acetic or ascorbic acid, 37.5 μL of 0.1% 1,10-phenanthroline, 10 μL saturated ammonium acetate, and 90 μL of $H_2O$ were added for a final volume of 250 μL. Covered plates were incubated for 30 min at 37° in a Dubnoff shaking incubator with developed color read using a Dynatech MR650 plate reader with a 570 nm filter. A standard curve was prepared with known amounts of iron.

*TBARS measurement.* Thiobarbituric acid-reactive substances (TBARS) were assayed as previously described by Buege and Aust [25] with some modification. The reaction of 100 μL of preincubation solution containing 1 mg/mL protein was stopped by the addition of 670 μL of a solution containing 12.5% trichloroacetic acid, 0.67% thiobarbituric acid, and 0.8 N HCl. Ten microliters of 0.5% BHT was then added to prevent further iron-catalyzed TBARS formation during the boiling step. $H_2O$ (220 μL) was added to a final volume of 1.0 mL. Samples were boiled for 15 min in capped microcentrifuge tubes, cooled on ice, and centrifuged at 1500 *g* for 5 min. The absorbance of the supernate was read at 535 nm. Quantification was based upon a molar extinction coefficient of $1.21 \times 10^5$/mol·cm obtained from standard curves using 1,1,3,3-tetraethoxypropane. This molar extinction coefficient is in close agreement with $1.56 \times 10^5$/mol·cm described by Buege and Aust [25].

*ATPase activities.* Membrane ATPase activities were measured by modification of the method of Raess and Vincenzi [26]. ATPase activities were assayed by incubations carried out in triplicate in covered 96-well microtiter plates at 37° in a Dubnoff shaking incubator. Buffer, membranes, and other reagents were placed in wells as outlined in Table 1. The $Na^+/K^+,Mg^{2+}$ buffer (Table 1) represents 18 mM histidine, 18 mM imidazole, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.1. When added, CaM was 30 nM, $CaCl_2$ was 0.2 mM, and ouabain was 0.1 mM. All wells contained isolated human RBC membranes equivalent to approximately 0.75 μg of membrane protein. The reaction mixture (90 μL) was preincubated for 10 min, and following this preincubation the enzymatic ATPase reaction was initiated by the addition of 10 μL ATP (final concentration of 3.0 mM). The final reaction volume was 100 μL. The reaction was terminated after 60 min by the addition of 25 μL of 5% sodium dodecyl sulfate (SDS). The blank (well 1) was stopped at time zero with SDS. ATPase activity was determined by inorganic phosphate using the colorimetric method of Fiske and Subbarow [27] with developed color read using a plate reader with an 810 nm filter.

*SDS–polyacrylamide gel electrophoresis (SDS–PAGE).* Sodium dodecyl sulfate–polyacrylamide slab gels were prepared according to the method of Laemmli [28] using an SE 250 min-gel unit. A 10% polyacrylamide separating gel and a 4% polyacrylamide stacking gel were used for resolving proteins denatured with SDS. Gels were stained with Coomassie brilliant blue.

## RESULTS

Incubation of ferrous sulfate and EDTA with RBC membranes resulted in conversion of ferrous iron to ferric iron (Fig. 1). The conversion of ferrous iron to ferric iron was rapid, with essentially complete conversion to ferric iron occurring within 5 min. Following preincubation in 200 μM ferrous sulfate

Table 1. Operational definitions of ATPase activities in human RBC membranes

| Additions to: | $Na^+,K^+,Mg^{2+}$ buffer | $Ca^{2+}$ | Ouabain | CaM |
|---|---|---|---|---|
| Well 1 (blank) | + | + or − | + or − | + or − |
| Well 2 | + | − | + | − |
| Well 3 | + | − | − | − |
| Well 4 | + | + | + | − |
| Well 5 | + | + | + | + |

| | Typical specific activities (nmol/min/mg) |
|---|---|
| CaM-activated $Ca^{2+}$ pump ATPase = Well 5 − Well 2 | 50 |
| Basal $Ca^{2+}$ pump ATPase = Well 4 − Well 2 | 12 |
| $Na^+/K^+$ pump ATPase = Well 3 − Well 2 | 8 |
| $Mg^{2+}$-ATPase = Well 2 − Well 1 | 3 |

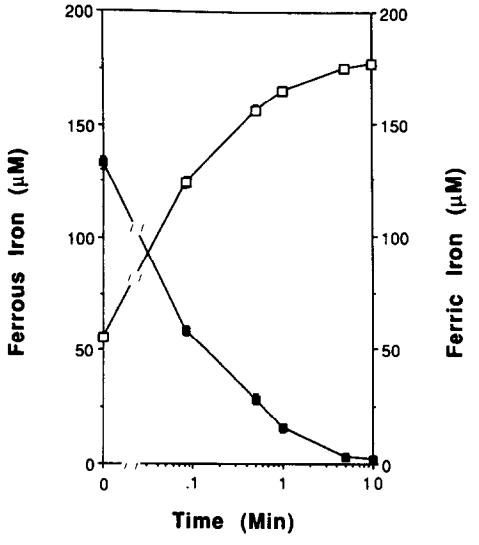


Fig. 1. Oxidation of iron by RBC membranes. Red cell membranes (1 mg protein/mL) were preincubated in the presence of 200 μM $Fe^{2+}$/EDTA for: 0, 0.083, 0.5, 1.0, 5.0, and 10 min. Following preincubation, ferrous (■) and ferric (□) iron were determined. Data represent the mean of three different experiments (±SEM). For most points, the standard error bars were smaller than the symbols.

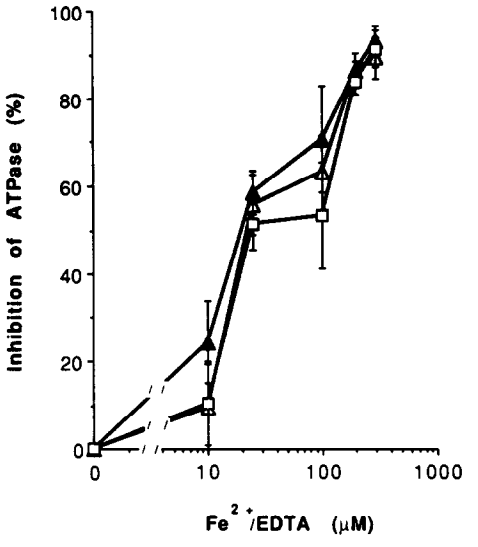


Fig. 2. Effect of iron on ATPase activities. Data (±SEM) are expressed as per cent inhibition of control activities plotted as a function of the log concentration of $Fe^{2+}$/EDTA. RBC membranes (1 mg protein/mL) were preincubated with $Fe^{2+}$/EDTA for 4 hr. Following preincubation, ATPase activities for the CaM-activated $Ca^{2+}$ pump ATPase (▲), basal $Ca^{2+}$ pump ATPase (△), and the $Na^+/K^+$ pump ATPase (□) were determined. Specific activities (nmol $P_i$/min/mg protein) for controls (no $Fe^{2+}$/EDTA) were: 8.84 ± 0.85, 15.9 ± 1.48, and 51.6 ± 4.19 for the $Na^+/K^+$, basal $Ca^{2+}$, and CaM-activated $Ca^{2+}$ pump ATPases (N = 6), respectively, and 0.83 ± 0.34, 2.00 ± 0.90, and 3.75 ± 1.80, respectively, in the presence of 300 μM $Fe^{2+}$/EDTA (N = 3).

and 200 μM EDTA, the $Na^+/K^+$ pump ATPase, basal $Ca^{2+}$ pump ATPase, and the CaM-activated $Ca^{2+}$ pump ATPase were inhibited in a concentration- and time-dependent manner (Figs. 2 and 3), with an apparent $IC_{50}$ for all three ATPases of approximately $2.5 \times 10^{-5}$ M iron.

The formation of TBARS increased over time and resembled the time-dependent inhibition of the ion pump ATPases (compare Figs. 3 and 4). However, there were observable differences: TBARS production and inhibition of enzyme activities exhibited different time courses, with a lag phase in the case of TBARS and a hyperbolic curve for enzyme inhibition. These results suggest that the formation of TBARS is not the rate-limiting step in this process and that the production of a small amount of TBARS that occurs during the first hour of preincubation represents lipid peroxidation that is enough to produce substantial inhibition of the ion pump ATPases.

It is noteworthy that while the initial conversion of ferrous iron to ferric iron occurred in minutes, the formation of TBARS and inhibition of ATPase

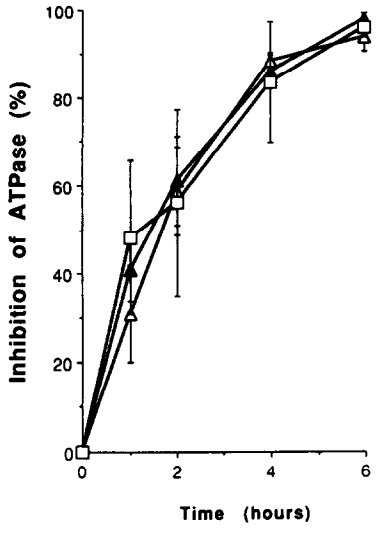


Fig. 3. Time course of iron-induced inhibition of ATPase activities. RBC membranes were preincubated with 200 $\mu$M $Fe^{2+}$/EDTA for various times and assayed as in Fig. 2. Key: CaM-activated $Ca^{2+}$ pump ATPase (▲), basal $Ca^{2+}$ pump ATPase (△), and the $Na^+/K^+$ pump ATPase (□). Data (±SEM) are expressed as per cent inhibition compared to control activities plotted as a function of time (N = 3). Specific activities (nmol $P_i$/min/mg protein) at $t$ = 0 were: 6.27 ± 0.89, 16.4 ± 4.25, and 47.3 ± 6.39 for the $Na^+/K^+$, basal $Ca^{2+}$, and CaM-activated $Ca^{2+}$ pump ATPases (N = 3), respectively, and 0.30 ± 0.16, 0.82 ± 0.28, and 1.04 ± 0.58, respectively, at $t$ = 6 hr (N = 3).

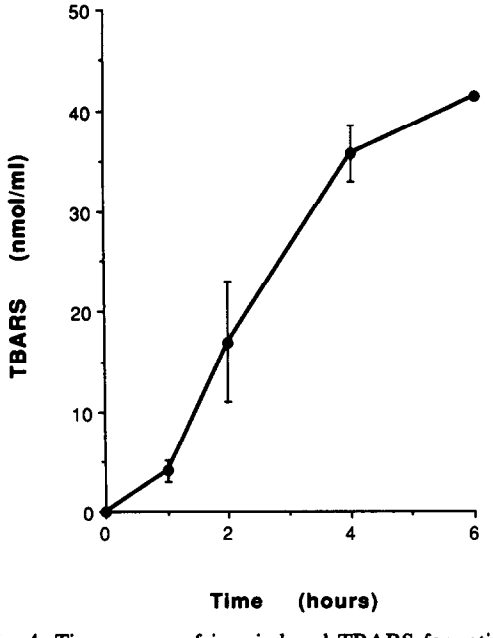


Fig. 4. Time course of iron-induced TBARS formation. TBARS were determined following preincubation of red cell membranes with 200 $\mu$M $Fe^{2+}$/EDTA for various time periods. Data represent the mean of three different experiments (±SEM).

activities occurred over the course of some hours. Because of this striking difference between the kinetics of oxidation of $Fe^{2+}$/EDTA and inactivation of the ion pump ATPases, we decided to explore the possibility that this was due either to a redox cycling of the $Fe^{2+}$/EDTA, or to initiation of a process that continued after oxidation of the $Fe^{2+}$/EDTA. Thus, we compared the effects of adding DFO at time zero with adding it after 5 min of incubation with $Fe^{2+}$/EDTA. In either case, once DFO was added, incubation was then allowed to continue for 4 hr, after which ATPase activities were determined. With DFO added at the onset of preincubation, specific activities for the ion pump ATPases were comparable with control values in the absence of $Fe^{2+}$/EDTA (see below). DFO added 5 min after incubation with $Fe^{2+}$/EDTA gave similar results: specific activities (expressed as % of controls) for the ion pump ATPases were roughly 94.9 ± 3.58 for DFO added after 5 min versus 19.1 ± 10.3 in the presence of $Fe^{2+}$/EDTA alone (N = 3).

SOD (120 units) prevented iron-mediated inhibition of the ion pump ATPases. Ion pump ATPase activities were 2- to 3-fold higher in the presence of SOD and $Fe^{2+}$/EDTA than in the presence of $Fe^{2+}$/EDTA alone (Table 2). By contrast, denatured SOD provided minimal protection of the ion pump ATPases from iron-induced inhibition (Table 2). In addition, SOD limited the formation of TBARS; TBARS were much lower (near control values) following preincubation in the presence of both SOD and $Fe^{2+}$/EDTA than in the presence of $Fe^{2+}$/EDTA alone (see Fig. 6). As stated above, DFO prevented iron-induced inhibition of the iron pump ATPase activities. Mean activities of the $Na^+/K^+$ pump ATPase, basal $Ca^{2+}$ pump ATPase, and CaM-activated $Ca^{2+}$ pump ATPase were 100, 107 and 94.5%, respectively, following preincubation in the presence of 4 mM DFO and $Fe^{2+}$/EDTA, as compared with those reactions incubated in the control ($Fe^{2+}$/EDTA alone) (Table 2). Catalase, mannitol, and ethanol did not protect the ion pump ATPases (Table 2), and had a minimal effect on TBARS formation as well (see Fig. 6).

We examined the abilities of two antioxidants, BHT and U74006F, to prevent iron-induced inhibition of the ion pump ATPases. Figure 5 shows that U74006F inhibited the ion pump ATPases with an apparent $IC_{50}$ of 60 $\mu$M for the $Na^+/K^+$ pump ATPase and 85 $\mu$M for both the basal $Ca^{2+}$ pump ATPase and the CaM-activated $Ca^{2+}$ pump ATPase, respectively. The reason for the inhibition of the ion pump ATPases by U74006F is unknown to us. The solvent, ethanol, had no effect on the ion pump ATPases up to 2%, which corresponded to the highest concentration of U74006F (data not shown). Because only minor inhibition of ATPase activities was caused by 10 $\mu$M U74006F, this concentration was chosen for further experiments.

U74006F (10 $\mu$M) protected ion transport ATPases from iron-induced inhibition. Specific activities were approximately 3- to 5-fold higher following preincubation in the presence of 10 $\mu$M U74006F and 200 $\mu$M $Fe^{2+}$/EDTA than following preincubation in the presence of $Fe^{2+}$/EDTA alone (Table 2). BHT was also very effective in preventing iron-induced

Table 2. Effect of various reagents on $Fe^{2+}$/EDTA-induced inhibition of the ion pump ATPases in red blood cell membranes

| Preincubation conditions | ATPase specific activities (nmol $P_i$/min/mg) | | |
|---|---|---|---|
| | $Na^+/K^+$ ATPase | Basal $Ca^{2+}$ ATPase | CaM-act. $Ca^{2+}$ ATPase |
| Control | 7.15 ± 0.40 | 13.5 ± 1.63 | 50.3 ± 5.16 |
| $Fe^{2+}$ | 2.04 ± 0.80 | 3.52 ± 0.57 | 7.98 ± 2.00 |
| $Fe^{2+}$ and SOD* (120 units) | 1.96 ± 0.98 | 3.90 ± 1.96 | 10.1 ± 4.84 |
| $Fe^{2+}$ and SOD (120 units) | 5.44 ± 0.50 | 9.74 ± 1.55 | 33.0 ± 4.79 |
| Control | 7.14 ± 0.21 | 16.2 ± 1.94 | 30.5 ± 2.19 |
| $Fe^{2+}$ | 0.057 ± 0.057 | 0.25 ± 0.25 | 0.16 ± 0.16 |
| $Fe^{2+}$ and catalase* (132 units) | 0.20 ± 0.16 | 0.62 ± 0.23 | 0.34 ± 0.19 |
| $Fe^{2+}$ and catalase (132 units) | 0.53 ± 0.42 | 0.25 ± 0.13 | 0.55 ± 0.27 |
| Control | 5.90 ± 1.25 | 12.0 ± 1.53 | 47.7 ± 4.83 |
| $Fe^{2+}$ | 2.15 ± 0.87 | 3.51 ± 0.89 | 9.87 ± 3.03 |
| $Fe^{2+}$ and mannitol (15 mM) | 2.82 ± 0.85 | 4.96 ± 0.96 | 12.6 ± 4.30 |
| Control | 6.72 ± 0.13 | 16.9 ± 2.09 | 60.5 ± 1.45 |
| $Fe^{2+}$ | 0.85 ± 0.15 | 2.05 ± 0.35 | 3.38 ± 1.26 |
| $Fe^{2+}$ and ethanol (82.7 mM) | 0.64 ± 0.32 | 2.69 ± 0.66 | 4.75 ± 1.41 |
| Control | 9.54 ± 0.68 | 9.33 ± 0.16 | 32.6 ± 1.95 |
| $Fe^{2+}$ | 1.65 ± 0.44 | 1.40 ± 0.65 | 2.75 ± 1.86 |
| $Fe^{2+}$ and BHT (10 μM) | 8.45 ± 0.69 | 8.64 ± 0.16 | 27.6 ± 2.04 |
| $Fe^{2+}$ and U74006F (10 μM) | 5.19 ± 0.39 | 3.72 ± 1.08 | 13.1 ± 2.62 |
| Control | 11.1 ± 0.20 | 15.2 ± 1.20 | 46.7 ± 2.54 |
| $Fe^{2+}$ | 1.36 ± 0.22 | 2.31 ± 0.11 | 3.39 ± 0.20 |
| $Fe^{2+}$ and DFO (4 mM) | 11.2 ± 1.32 | 16.3 ± 1.83 | 44.3 ± 5.40 |

For each condition, RBC membranes (1 mg protein/mL) were preincubated for 4 hr with an approximately equimolar concentration of 200 μM ferrous sulfate and EDTA ($Fe^{2+}$/EDTA) or in the presence of $Fe^{2+}$/EDTA and each reagent as specified. Control experiments were run without $Fe^{2+}$/EDTA. Following preincubation, ATPase activities for the CaM-activated $Ca^{2+}$ pump ATPase, basal $Ca^{2+}$ pump ATPase, and the $Na^+/K^+$ pump ATPase were determined. Data (±SEM) are expressed as specific activity (nmol $P_i$/min/mg membrane protein). For all conditions N = 3, except for SOD control and SOD where N = 6.

* Denatured by boiling for 10 min.

inhibition of the ion pump ATPases. Following preincubation in the presence of 200 μM $Fe^{2+}$/EDTA, specific activities (expressed as a % of controls) for the $Na^+/K^+$ pump ATPase, basal $Ca^{2+}$ pump ATPase, and the CaM-activated $Ca^{2+}$ pump ATPase were 17.1, 15.0 and 8.46%, respectively. Following preincubation in the presence of both $Fe^{2+}$/EDTA and 10 μM BHT, specific activities were 88.5% for the $Na^+/K^+$ pump ATPase, 92.6% for the basal $Ca^{2+}$ pump ATPase, and 84.7% for the CaM-activated $Ca^{2+}$ pump ATPase. By itself, BHT had no effect on ion pump ATPase activities using up to 1 mM concentrations (data not shown). Under the same conditions, BHT and U74006F inhibited the formation of TBARS (Fig. 6). Again, BHT appeared to be the more potent of the two antioxidants. Ethanol, the solvent used for both BHT and U74006F, had no effect on ion pump ATPase activities or TBARS formation.

$Fe^{2+}$/EDTA promoted cross-linking of membrane proteins, as shown in Figs. 7 and 8. SDS–PAGE revealed the presence of a high molecular weight, proteinaceous material that formed in a time-dependent manner when RBC membranes were preincubated with $Fe^{2+}$/EDTA (Fig. 7). Compared with control membranes, membranes exposed to $Fe^{2+}$/EDTA demonstrated a general loss of the well-known major membrane proteins of the RBC membrane including spectrin, actin and glyceraldehyde-3-phosphate dehydrogenase [29]. The appearance of proteinaceous material with molecular weight sufficiently high that it barely entered the stacking gel (Figs. 7 and 8) was associated with the general loss of known proteins. In the additional presence of U74006F or BHT, by contrast, there was less generalized loss of protein bands, with little or no proteinaceous polymer in the stacking gel (Fig. 8). However, iron-induced protein cross-linking was not completely antagonized by these agents, as there was some protein polymer at the interface of the stacking and running gel.

## DISCUSSION

Trauma or ischemia in the CNS produces a fertile environment for the production of reactive oxygen species, including free radicals. The brain is an abundant source of iron [30], and its release by hemorrhage or reductive mobilization from ferritin by $O_2^{-\cdot}$ [31] may contribute to lipid peroxidation. Using RBC membranes and iron we examined a model system for iron-induced lipid peroxidation.

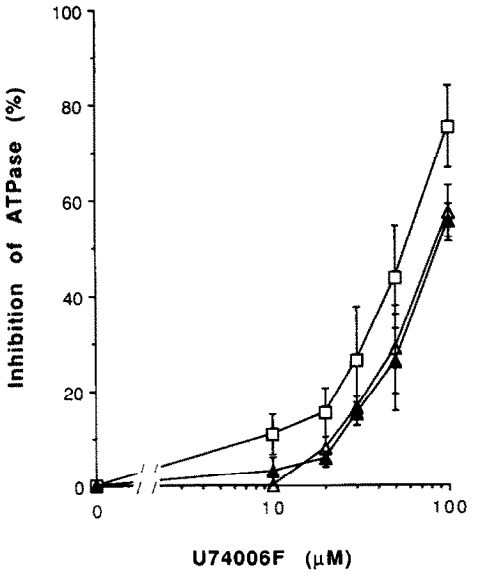


Fig. 5. Effect of U74006F on ATPase activities. RBC membranes were preincubated for 4 hr without $Fe^{2+}$/EDTA in the presence of various concentrations of U74006F. Following preincubation, ATPase activities were determined as in Fig. 2. Key: CaM-activated $Ca^{2+}$ pump ATPase (▲), basal $Ca^{2+}$ pump ATPase (△), and the $Na^+/K^+$ pump ATPase (□). Data (±SEM) represent three experiments. Specific activities (nmol $P_i$/min/mg protein) for controls (no U74006F) were: 9.14 ± 0.65, 10.8 ± 0.86, and 35.9 ± 1.50 for the $Na^+/K^+$, basal $Ca^{2+}$, and CaM-activated $Ca^{2+}$ pump ATPases, respectively, and 2.21 ± 0.79, 4.64 ± 0.87, and 16.0 ± 1.5, respectively, at 100 μM U74006F.

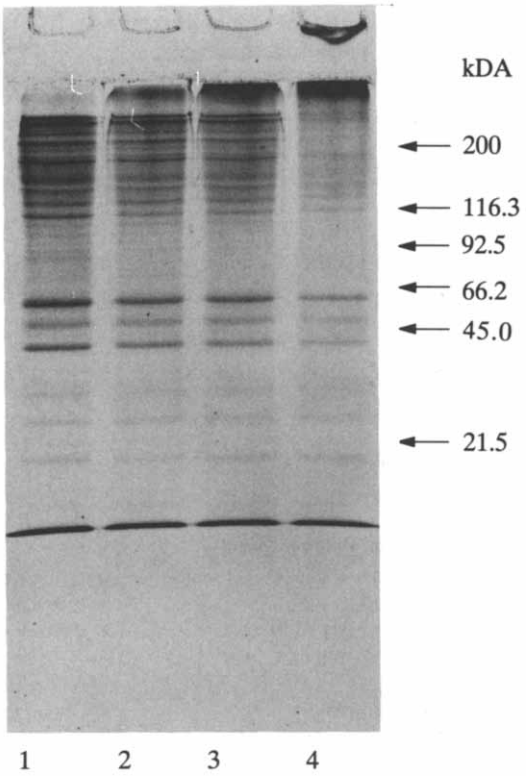


Fig. 7. SDS-PAGE of RBC membranes incubated for various times with $Fe^{2+}$/EDTA. Preincubation reactions were assayed as in Fig. 3 and 13 μg of RBC membrane proteins from each reaction was used for an SDS-gel prepared as described under Materials and Methods. Samples are: lane 1, control (no $Fe^{2+}$/EDTA); lane 2, $Fe^{2+}$/EDTA, 1 hr; lane 3, $Fe^{2+}$/EDTA, 2 hr; and lane 4, $Fe^{2+}$/EDTA, 4 hr.

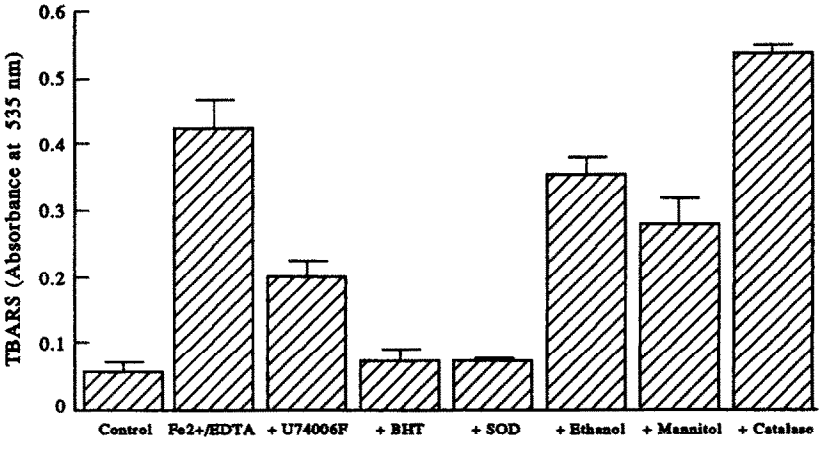


Fig. 6. Effect of various antioxidants on TBARS formation. RBC membranes were preincubated for 4 hr in the absence (control) or the presence of 200 μM $Fe^{2+}$/EDTA ($Fe^{2+}$/EDTA), as in Fig. 2, without or with various antioxidants as labeled. The concentration used for each antioxidant was identical to that listed in Table 2. Following preincubation, TBARS were assayed as in Fig. 4. Data (±SEM) represent three experiments, except for catalase where N = 2 (mean ± range) and $Fe^{2+}$/EDTA where N = 8.

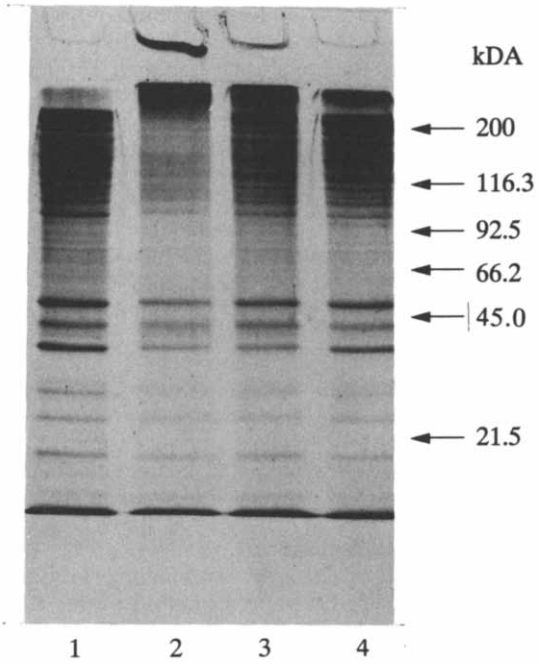


Fig. 8. SDS–PAGE of RBC membranes incubated in the presence of $Fe^{2+}$/EDTA alone or in the presence of $Fe^{2+}$/EDTA and 10 $\mu$M BHT or U74006F. Preincubation reactions were assayed as in Fig. 6 and 13 $\mu$g of RBC membrane proteins from each reaction was analyzed on an SDS-gel. Samples are: lane 1, control; lane 2, $Fe^{2+}$/EDTA; lane 3, $Fe^{2+}$/EDTA and U74006F; and lane 4, $Fe^{2+}$/EDTA and BHT.

EDTA has been shown to enhance iron-dependent lipid peroxidation when iron and EDTA are present in a 1:1 ratio [32]. Iron is poorly soluble in aqueous solution at physiological pH, forming hydrated iron complexes that precipitate as $Fe(OH)_3$ [33]. Chelation of iron by EDTA may keep iron in a form that remains in solution and that is catalytically active [34]. It has also been demonstrated that when the concentration of EDTA exceeds that of the ferrous iron, lipid peroxidation is substantially inhibited [35].

Iron salts do not exist "free" in biological systems. They are essentially always bound to proteins, membranes, nucleic acids, or low-molecular-weight chelating agents [36]. Examples of biological chelators of iron include ATP, ADP, and citrate, all of which may act similarly to EDTA in that they keep iron in solution at physiological pH values. EDTA was used in our preincubation reactions because these other biological complexing agents are only about 10% as effective as EDTA in generating free radicals under reaction conditions normally used *in vitro* [36].

We used a nominal concentration of 200 $\mu$M ferrous sulfate in our reactions. However, when actually measured in the reaction mixtures, the ferrous content was lower, typically between 185 and 190 $\mu$M with a ferric content of 10–15 $\mu$M. For this reason, and because lipid peroxidation is substantially inhibited when [EDTA] exceeds [$Fe^{2+}$], a concentration of 180 $\mu$M EDTA was used together with a nominal ferrous sulfate concentration of 200 $\mu$M. For simplicity, figure legends and tables specify equimolar $Fe^{2+}$/EDTA. It should be noted this is an approximate value. In actuality, the [$Fe^{2+}$] was slightly greater than [EDTA].

$Fe^{2+}$/EDTA resulted in both a concentration- and time-dependent inhibition of the ion pump ATPases. While the conversion of ferrous to ferric iron occurred in minutes, the formation of TBARS and inhibition of ATPase activities occurred over the course of some hours. These facts may be interpreted to suggest that inhibition of the ion pump ATPases occurred secondarily to the initial conversion of ferrous to ferric iron. It must be qualified that $Fe^{2+}$/EDTA can spontaneously oxidize in the presence of dioxygen. However, in control experiments in the absence of RBC membranes (data not shown), there was no detectable conversion of ferrous to ferric iron in a 10-min time period.Therefore, it appears that RBC membranes accelerated oxidation of $Fe^{2+}$/EDTA. In any event, the results of adding DFO 5 min after incubation with $Fe^{2+}$/EDTA would seem to suggest that iron continued to "cycle" from ferrous to ferric and ferric to ferrous in spite of the fact that no exogenous reducing agent was added in these experiments. There may have been small amounts of endogenous reducing agents such as ascorbate and glutathione present in our system.

The ability of DFO to prevent inhibition of the ion pump ATPases presumably was related to its ability to chelate iron, and in a sense, "steal" iron away from the $Fe^{2+}$/EDTA chelate. We noticed that upon addition of DFO, the color of the reaction changed to yellow/orange, which is an indication that the chromophore, ferrioxamine, had formed. In addition, it is known that DFO tightly binds $Fe^{3+}$ and renders it inactive as a redox-catalyst [37]. However, because DFO is also a "weak" scavenger of superoxide [36], it is possible, though unlikely, that DFO prevented iron-induced inhibition of the ion pump ATPases by acting as a free radical scavenger. We used an excessive amount of DFO (4 mM) to ensure that all available iron (attached to membranes or EDTA) was chelated and rendered inactive as a free radical catalyst. Leclerc *et al.* [23] also used 4 mM DFO to prevent inhibition of the $Ca^{2+}$ pump ATPase by ammonium iron citrate, and did not report any damaging effects of DFO on their model system.

Iron is known to initiate and propagate lipid peroxidation via a series of reactions (Equations 1–3) that produce oxygen free radicals [38, 39]. The addition of SOD in our system prevented iron-mediated inhibition of the ion pump ATPases. Thus, it may be suggested that superoxide is a critical oxygen free radical produced in our model system. Previous work has demonstrated that superoxide inactivates the endoplasmic reticulum (ER) $Ca^{2+}$ pump ATPase of the coronary artery and the sarcoplasmic reticulum (SR) $Ca^{2+}$ pump ATPase of vascular smooth muscle and this inhibition can be prevented by SOD [40, 41].

As previously stated, DFO added after 5 min of incubation with $Fe^{2+}$/EDTA protected the ion pump

ATPases from inhibition. This would seem to suggest that iron continued to "cycle" from ferrous to ferric and ferric to ferrous in spite of the fact that no exogenous reducing agent was added in these experiments. It could be that as iron cycled between these two states, superoxide was continually produced throughout the entire incubation process. An additional explanation would be that the superoxide formed immediately after addition of $Fe^{2+}$/EDTA (in the presence of oxygen) initiated lipid peroxidation and then lipid peroxidation proceeded as a radical chain reaction, no longer requiring the presence of a primordial free radical such as superoxide. In future experiments, we plan to measure any superoxide that might be generated during long-term incubations to address these questions.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -} \quad (1)$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+} \quad (3)$$

Formation of hydrogen peroxide ($H_2O_2$) and the subsequent Fenton reaction (Eqn. 3) may not be occurring in our system because catalase, which would destroy $H_2O_2$, did not prevent iron-induced inhibition of the ion pump ATPases. Our data support the work of Suzuki and Ford [41] who demonstrated that inhibition of the SR $Ca^{2+}$ pump ATPase by superoxide is blocked by SOD but not by catalase. We have no evidence that hydroxyl radical was responsible for inhibition of ion pump ATPases in our system because neither mannitol nor ethanol, both of which are known $OH^{\cdot}$ scavengers [32, 42], prevented iron-induced inhibition in our system. Likewise, neither mannitol nor ethanol substantially limited the formation of TBARS. However, the inability of $OH^{\cdot}$ scavengers to prevent iron-stimulated peroxidation does not necessarily mean that $OH^{\cdot}$ did not participate in initiation of lipid peroxidation [36]. The high reactivity of $OH^{\cdot}$ may prevent $OH^{\cdot}$ scavengers from interception before $OH^{\cdot}$ reacts with the lipid bilayer. Thus, it is possible that the initial burst of superoxide production ultimately led to the production of hydroxyl radical by way of Equations 2 and 3. This would explain why SOD prevented iron-induced inhibition of the ion pump ATPases.

It is possible that iron-induced inhibition of the ion pump ATPases occurred as a result of oxidation of sulfhydryl groups and subsequent disulfide bond formation. Results from this laboratory [43] and others [44] suggest that thiol oxidation inhibits $Ca^{2+}$ pump ATPase activity. Moore *et al.* [45] found that inhibition of the $Ca^{2+}$ pump ATPase might result from oxidation of sulfhydryl groups. The results of Moore *et al.* [45] are similar to those of Hebbel *et al.* [46] who found evidence for inhibition of the $Ca^{2+}$ pump ATPase, as caused by *t*-butylhydroperoxide, based on both thiol- and lipid-dependent mechanisms. In addition, proteins containing the amino acids tryptophan, tyrosine, phenylalanine, and histidine can undergo free radical-mediated amino acid modification [47], which may potentially inhibit ion pump ATPase activity. Thus, we cannot rule out the possibility that iron-induced inhibition of the ion pump ATPases occurred, at least in part, due to direct effects on the membrane transport enzymes of iron and/or iron-generated free radicals.

As noted, TBARS were produced in a time-dependent fashion consistent with the interpretation that iron promoted lipid peroxidation in RBC membranes. TBARS were assayed by the reaction of malonaldehyde [48], also called malondialdehyde (MDA), with thiobarbituric acid. The reaction creates a chromophore with a maximum absorption at 532 nm. MDA is a secondary component of lipid peroxidation capable of cross-linking membrane components containing amino groups [49]. However, cross-linking of membrane proteins can also occur by direct interaction of superoxide or other free radicals independent of lipid peroxidation [50]. Tappel [49] has shown that a fluorescent compound is formed when MDA reacts with the amino groups of proteins and phospholipids. Thus, one potential mechanism for iron-mediated inhibition of the ion pump ATPases is through cross-linking [51] or scission of proteins by MDA produced during the lipid peroxidation process. Results of SDS–PAGE support this as one possible mechanism. The cross-linking we observed appears to have been non-specific, as might be expected. Bands of well-known proteins that decreased in association with the appearance of high molecular weight proteinaceous material included both extrinsic proteins such as spectrin and actin and intrinsic membrane proteins such as glycophorin and, presumably, ion pump ATPases [52]. The effects of $Fe^{2+}$/EDTA were widespread, suggesting a lack of selectivity of this treatment. Because we were using protein cross-linking as a marker for lipid peroxidation, the concentration of $Fe^{2+}$/EDTA used was high and the time of preincubation was long. We would not expect such extensive damage to occur as a result of events related to hypoxia in whole cells *in vivo*. In general, there was reasonable correlation between the degree of enzyme inhibition and TBARS formation and protein polymerization. However, there is probably not a simple causal relationship between these measures.

Evidence is accumulating that suggests that iron, or iron containing molecules like heme, can inhibit ion pump ATPases. Leclerc *et al.* [53] have shown that hemin inhibits the activity of the CaM-activated $Ca^{2+}$ pump ATPase of RBC membranes via oxidative damage caused by free radicals. More recently, the same authors showed that nonheme iron inhibits and BHT protects the $Ca^{2+}$ pump ATPase of RBC membranes [23]. Our results are in agreement with those of Leclerc *et al.* and support the broader interpretation that under some conditions iron may promote relatively widespread and non-specific inhibition of ion pump ATPases.

In our hands, the classical antioxidant, BHT, was effective in protecting ATPase activities. Both U74006F and BHT inhibited the formation of TBARS under the same conditions. BHT was more potent *in vitro*. This result is compatible with those of Braughler *et al.* [16], who have also shown that BHT is more potent than U74006F in preventing the formation of TBARS. It may be that *in vivo*,

U74006F is more accessible to and effective at CNS target sites than BHT. Another possible explanation is that, *in vivo*, U74006F may be modified to a more active compound. Thus, the model system we used may not have provided an optimal environment to display effectiveness of U74006F.

In a number of studies, emphasis has been on the influx of $Ca^{2+}$ into the cells as a critical prelude to cell death in the CNS [8–10]. We propose that failure to extrude $Ca^{2+}$ is the more critical issue. For example, from the work of Hall *et al.* [7], it appears that influx of $Ca^{2+}$ occurs early in the ischemic phase of the gerbil model of stroke. However, during reperfusion, failure of $Ca^{2+}$ extrusion is apparent in untreated animals, whereas $Ca^{2+}$ is extruded from the cells in animals treated with U74006F. The current results are consistent with the interpretation that U74006F protects ion transport ATPases from inhibition caused by conditions associated with lipid peroxidation of cell membranes. Protection of ion transport ATPases may be a critical factor in the ability of cells to recover from ischemic insult and the burst of free radicals associated with reperfusion.

Efflux of $Ca^{2+}$ from neurons is dependent not only on the plasma membrane $Ca^{2+}$ pump, but on the $Na^+/Ca^{2+}$ exchanger [14]. Thus, the exchanger is another potential site for altered ionic regulation. The exchanger might be inhibited directly by lipid peroxidation and/or by protein cross-linking or scission. The present results shed no light on that question because human RBCs do not express the exchanger [54]. However, indirect inhibition is clearly predicted by the present results. Inhibition of the $Na^+/K^+$ pump would lead to a decreased $Na^+$ gradient and less driving force for extrusion of $Ca^{2+}$. Obviously, inhibition of the $Ca^{2+}$ pump ATPase would also lead to increased $Ca^{2+}$ in the cell. Increased $Ca^{2+}$ has perilous results that contribute to the pathophysiology seen in CNS trauma, stroke, or ischemia. An increase of intracellular $Ca^{2+}$ from 0.1 to 0.5 $\mu$M is sufficient to activate calpain, a proteinase capable of degrading the $Ca^{2+}$ pump ATPase [55], the cytoskeleton, and ion channels [56–58]. In addition, high intracellular $Ca^{2+}$ levels may also activate transglutaminases that are capable of cross-linking and thus inactivating a variety of intracellular proteins [59–61]. Finally, $Ca^{2+}$-activated phospholipases, such as phospholipase $A_2$, may be important in contributing to the degradation of the plasma membrane by hydrolysis of membrane phospholipids [62].

In conclusion, both U74006F and BHT protected ion pump ATPases from iron-induced inhibition, presumably by limiting the degree of lipid peroxidation and levels of lipid peroxidation by-products such as MDA. Lipophilic antioxidants may spare membrane proteins including the $Na^+/K^+$ and $Ca^{2+}$ pump ATPases from inhibition. The demonstrated effectiveness of U74006F in preventing CNS injury in a variety of animal models may be related to its effectiveness in protecting plasma membrane ion transport ATPases from inhibition. This protection, it is suggested, helps ensure that extrusion from $Ca^{2+}$-loaded cells occurs following restoration of circulation in compromised tissues.

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