FEBS Letters 352 (1994) 71–75

FEBS 14495

# Differentiation potentiates oxidant injury to mitochondria by hydrogen peroxide in Friend's erythroleukemia cells

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Received 10 June 1994; revised version received 25 July 1994

**Abstract** Oxidative damage to mitochondrial functions was investigated upon non-lethal treatment with $H_2O_2$ of Friend's erythroleukemia cells induced to differentiate, in comparison with the parental cell line. Both respiration and maximal ATP synthase capacity were more severely diminished by $H_2O_2$ in induced cells. The effects were mediated by intracellular redox-active iron and $OH^{\cdot}$ radicals. Specifically, the mechanisms of the selective oxidant injury to $F_0F_1$ ATP synthase observed in differentiating cells likely involved impairment of $F_0$–$F_1$ coupling sensitive to oligomycin. We suggest a Fenton-like reaction of $H_2O_2$ with iron ions, more available in the differentiating cells, as occurring at the surface and/or in the lipid bulk phase of the inner mitochondrial membrane, thus injuring subunits responsible for the coupling of $F_0F_1$ ATP synthase through generation in situ of the actual damaging species. Besides, we propose heme iron as the most likely candidate for such reaction in induced cells actively synthesizing heme. In accordance, pretreatment of uninduced cells with hemin made $H_2O_2$-damage qualitatively identical.

*Key words:* Hydrogen peroxide; Oxidative phosphorylation; Heme; Iron; $F_0F_1$ ATPsynthase; Friend's erythroleukemia cells

## 1. Introduction

The exposure of Friend's virus-infected murine erythroleukemia cells (FELC) to HMBA initiates a coordinated erythroid differentiation program during which FELC synthesize characteristic proteins of mature erythrocytes [1], notably hemoglobin [2,3]. HMBA-induced FELC undergo substantial modifications in iron metabolism concomitant with the increase of heme biosynthesis [4–7]. We recently demonstrated that heme-synthesizing FELC were more susceptible to the oxidative insult inflicted by the anthracycline antibiotic daunomycin via $H_2O_2$ generation in a way closely related to their increased cellular iron levels [8]. Accordingly, iron is known to participate in a Fenton-type reaction with $H_2O_2$ producing highly reactive hydroxyl radical ($OH^{\cdot}$). As mitochondria were shown to require iron [9], as well as to be major intracellular targets in the mechanisms of oxidant-mediated injury to some tumor cell lines [10], we expected that alterations in the redox-active iron pool of mitochondria could be associated to FELC differentiation and could be critical to enhance the injury by $H_2O_2$. Interestingly, we recently documented $H_2O_2$ to determine inactivation with a strict requirement for iron of purified mitochondrial $F_1$ ATPase [11] and $F_0F_1$ ATP synthase [12]. The aim of the present investigation was to evaluate whether early mitochondrial dysfunctions were caused by $H_2O_2$ to FELC and whether differentiation could influence $H_2O_2$ effects on mitochondrial components, particularly on ATP synthase. Then, mitochondrial functions of both parental and induced FELC were assayed after incubation with $H_2O_2$ determining no more than 10% of cell mortality. The results showed that the maximal ATP synthase capacity was more severely impaired by $H_2O_2$ in HMBA-induced FELC. Differential effects were also observed on basal and FCCP-uncoupled respiration rates, as well as, and more notably, on the oligomycin-sensitive respiration rate, investigated as a measure of mitochondrial ATP synthesis flux. Evidence was reported that such effects were due to greater availability of redox-active iron, likely heme iron, at the level of mitochondrial membranes of heme-synthesizing FELC.

## 2. Materials and methods

FELC, clone 3CL8 *vitro*, kindly provided by Dr. Belardelli, Istituto Superiore di Sanità, Rome, Italy, were grown in RPMI 1640 medium (ICN Biomedicals, Inc. Costa Mesa, CA) supplemented with 10% heat-inactivated fetal calf serum (Seromed, Biochrom KG, Berlin, Germany) and maintained in logarithmic phase with appropriate dilutions every 3–4 days. To induce differentiation, cells from confluent cultures were seeded in complete medium added with 5 mM HMBA (Sigma, St. Louis, MO) and grown for 120 h, with a refeeding (including fresh inducer) after 96 h. Parallel cultures grown under identical conditions, except for the absence of HMBA, served as controls in all experiments. The erythroid differentiation was assessed by evaluating the increase (ca. 20-fold) of hemoglobin content of cells on the basis of the intensity of the Soret band in 17,000 × *g* supernatants from sonicated homogenates [8]. Cell viability, evaluated by the Trypan blue dye exclusion test, never was lower than 95%. The oxidant treatment was performed by exposing cells at confluence, suspended at $2 \times 10^6$ cells/ml in PBS (20 mM sodium phosphate pH 7.4, 140 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$) containing 5 mM glucose, to 0.25 mM $H_2O_2$ for 30 min; cell mortality never exceeded 10%. In some experiments 1 h preincubation was carried out in the presence of 20 mM DFO (Ciba Geigy, Basle, Switzerland), or 20 mM FO prepared by addition of ferric chloride to yield 95% saturation of DFO, or 100 μM DMSO (Merck, Darmstad, Germany), or 100 μM BHT (Sigma). The effects of exogenous hemin on $H_2O_2$-susceptibility of uninduced FELC were investigated by incubating cell suspensions ($2 \times 10^6$ cells/ml) with 25 μM hemin (Sigma) for 30 min; the drained pellets were then resuspended at the initial density and exposed to $H_2O_2$. For the measurements of mitochondrial oxygen uptake by intact cells [10], FELC were suspended at $5–10 \times 10^6$ cells/ml in respiration buffer (0.25 M sucrose, 10 mM HEPES, 5 mM phosphate, 1 mM $MgCl_2$, 2 mM EGTA, 5 mM glucose, pH 7.4) at 37°C and transferred into the polarographic cell (1 ml) of a Y.S.I. oxygraph

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*Abbreviations:* FELC, Friend's erythroleukemia cells; HMBA, *N,N'*-hexamethylene bisacetamide; PBS, phospate-buffered saline; HEPES, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); EGTA, ethylene glycol-bis (β-aminoethyl ether)*N,N,N,N'*-tetraacetic acid; RCR, respiratory control ratio; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; BHT, butylated hydroxytoluene; DMSO, dimethyl sulfoxide; DFO, desferrioxamine methanesulphonate; FO, ferrioxamine.


*SSDI* 0014-5793(94)00882-5

equipped with a Clark electrode (model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Oxygen consumption was completely suppressed by 1 mM KCN and was taken to be a direct measure of mitochondrial respiration. After 6–8 min recording of basal respiration rate, 10 μM oligomycin (Sigma), or 50 μM FCCP (Sigma), or 75 μM atractyloside (Sigma) were added. RCR was calculated as ratio of the rate of FCCP-stimulated respiration to that of basal respiration. The maximal efficiency of ATP hydrolysis by mitocondria was assayed as Das and Harris [13], by measuring NADH oxidation with a continuous spectrophotometric method at 340 nm, in the presence of a coupled lactate dehydrogenase-pyruvate kynase ATP regenerating system at 37° C and pH 7.4, on homogenates prepared as follows. Cell suspensions ($5 \times 10^7$ cells/ml) in 20 mM HEPES, 1 mM $MgCl_2$, 2 mM EGTA, pH 7.0, were subjected to brief controlled sonication with a 301 Sonic Dismembrator (Artek System Co.): disruption of mitochondrial membranes and formation of submitochondrial vesicles were monitored during sonication by evaluating the increase in ATPase activity and the decline in the effect of 5 μM FCCP or 50 μM atractyloside on such activity. Possible interferences of $Ca^{2+}$- and $Na^+/K^+$-ATPases were minimized by using an assay buffer containing 2 mM EGTA and less than 5 mM $Na^+$ [13]. Very low sensitivity to 10 μM sodium orthovanadate or to 2 mM ouabain (Sigma) were found (ca. 9% or 6%) in both parental and induced FELC, indicating that over 85% of the measured ATPase activity was mitochondrial in origin. Oligomycin-sensitive ATPase activity was assayed in the presence of 4 μM oligomycin and considered as maximal ATP synthesis capacity.

## 3. Results and discussion

### 3.1. Impairment of mitochondrial respiration of FELC by $H_2O_2$

The rate of mitochondrial oxygen consumption by intact FELC with glucose as the sole exogenous substrate was measured in the absence or in the presence of the uncoupler FCCP, as well as of specific inhibitors of adenine nucleotide traslocase and $F_0F_1$ ATP synthase, i.e. atractyloside and oligomycin, to investigate the effects of non-lethal treatment with $H_2O_2$ on the basal respiration, on the respiration uncoupled from the proton electrochemical gradient, on the resting respiration detectable under non-phosphorylating conditions and on the respiration coupled to ATP synthesis. The results are shown in Table 1A).

Basal respiration was largely inhibited, as for other glucose-fed intact cells [14,15], by oligomycin (ca. 70%) or by atractyloside (ca. 60%) in both parental and HMBA-induced FELC. This indicated that electron transfer was similarly coupled to ATP synthesis and transport in both cell types, despite the lower values of the respiration rates observed in induced cells when expressed per cell ratio, reflecting the reduction of cell volume and protein content of such cells [1,8]. Upon $H_2O_2$- exposure of the parental FELC a 40% decrease was observed in the basal respiration rate, which still showed full sensitivity to oligomycin or to atractyloside. Moreover, a 39% decline in FCCP-uncoupled respiration rate occurred, indicating an impairment inflicted by the oxidant to the respiratory chain. On the other hand, the FCCP stimulating effect on the basal respiration (see RCR values) showed that the proton-motive force of the mitochondrial membrane was not diminished. The resting respiration measured in the presence of oligomycin (state 4), or of atractyloside (not shown), containing components of oxygen consumption coupled to ion transport [14], was inhibited by 34%, suggesting $H_2O_2$ may have caused a decrease in membrane conductance [16]. Oligomycin- and atractyloside-sensitive respiration rates, reflecting ADP phosphorylation flux, were also declined by 43% and 41%, respectively. In the case of differentiating FELC, $H_2O_2$ caused significantly larger ($P < 0.001$) decrease in the basal respiration rate (54% decrease). Even the decrease in FCCP-uncoupled respiration rate (56%) was significantly greater ($P < 0.001$) than that observed in the parental cells, indicating a significant additional damage to the electron transport chain. Nevertheless, similarly to control cells, the resting respiration declined only by 40%, and the effect of the uncoupler on $H_2O_2$-inhibited basal oxygen consumption (see RCR) suggested that the proton electrochemical gradient still affected the respiration. Furthermore, the most notable differential effect observed in heme-synthesizing FELC upon $H_2O_2$-exposure was on ADP phosphorylation, as oligomycin- and atractyloside-sensitive respiration rates declined by 65%. Oli-

Table 1
Effects of HMBA-induced differentiation of FELC on dysfunctions caused by $H_2O_2$ to mitochondrial respiration and maximal ATP synthesis capacity

| | Parental cells | | HMBA-induced cells | |
|---|---|---|---|---|
| | No treatment | $H_2O_2$ | No treatment | $H_2O_2$ |
| (A) | | | | |
| Basal respiration rate | 1.92 ± 0.09 | 1.16 ± 0.14* | 1.22 ± 0.09 | 0.56 ± 0.08* |
| FCCP-uncoupled respiration rate | 2.93 ± 0.09 | 1.78 ± 0.03* | 1.88 ± 0.07 | 0.82 ± 0.07* |
| State 4 respiration rate | 0.56 ± 0.06 | 0.37 ± 0.09* | 0.38 ± 0.06 | 0.27 ± 0.05* |
| Oligomycin-sensitive respiration rate | 1.36 ± 0.06 (71%) | 0.79 ± 0.09* (68%) | 0.84 ± 0.07 (69%) | 0.29 ± 0.04* (52%)* |
| Atractyloside-sensitive respiration rate | 1.19 ± 0.05 (62%) | 0.70 ± 0.08* (60%) | 0.75 ± 0.06 (61%) | 0.26 ± 0.04* (47%)* |
| RCR | 1.53 ± 0.09 | 1.53 ± 0.11 | 1.54 ± 0.12 | 1.46 ± 0.08 |
| (B) | | | | |
| ATPase activity | 18.9 ± 1.9 | 12.1 ± 1.2* | 9.2 ± 1.6 | 5.9 ± 1.0* |
| Oligomycin-sensitive ATPase activity | 13.8 ± 1.5 (73%) | 8.2 ± 1.0* (67%) | 6.4 ± 0.9 (70%) | 2.9 ± 0.9* (50%)* |

FELC were cultured in the absence or in the presence of 5 mM HMBA. Then, cells at confluence were incubated in PBS for 30 min with or without 0.25 mM $H_2O_2$. The rate of mitochondrial oxygen consumption by intact cells (A) and maximal ATPase activity of briefly sonicated cell suspensions (B) were assayed. Data of respiration rates are expressed as nmol $O_2$/min/$10^6$ cells. Oligomycin- and atractyloside-sensitive respiration rates were calculated as difference between the basal respiration rate and that detected in the presence of 10 μM oligomycin (state 4) and 75 μM atractyloside, respectively. The values in parentheses are % of oligomycin-sensitive or atractyloside-sensitive oxygen consumption rate taking the basal respiration as 100%. RCR was calculated as the ratio of FCCP-uncoupled respiration rate, divided by the rate of the basal respiration. The values of both total and oligomycin-sensitive maximal ATPase activity are expressed as nmol ATP/min/$10^6$cells. The values in parentheses are% of oligomycin-sensitive activity with respect to the total ATPase activity taken as 100%. All data represent means ± S.D. of 10 different experiments in which the assays were at least in duplicate. *Significantly different from the appropriate controls in the absence of $H_2O_2$, $P < 0.001$, Student's $t$-test.

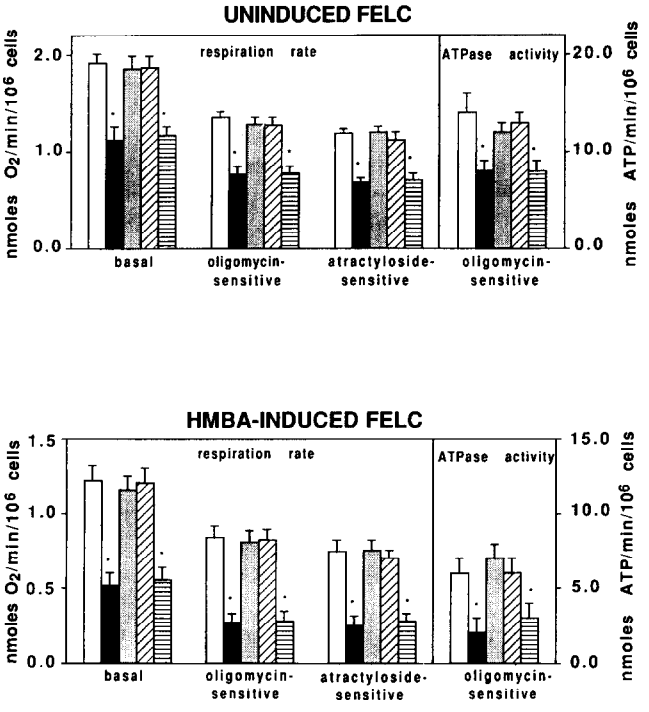


Fig. 1. Preventive effect of DFO or DMSO and ineffectiveness of BHT on $H_2O_2$-dependent damage to respiration rate and oligomycin-sensitive ATPase activity in HMBA-induced and uninduced FELC. Cells from confluent cultures were treated with 0.25 mM $H_2O_2$ for 30 min after 1 h preincubation in PBS + 5 mM glucose in the absence (full columns) or in the presence of 20 mM DFO (dotted columns), 100 $\mu$M DMSO (diagonal columns), 100 $\mu$M BHT (dashed columns). Control experiments without $H_2O_2$ either in the absence or in the presence of DFO, or DMSO, or BHT were also performed and reported as empty columns. The measurements of rates of basal, oligomycin- and atractyloside-sensitive respiration and of oligomycin-sensitive maximal ATPase activity were carried out as specified in section 2. Data are mean values ± S.D. of at least 3 different experiments in which the assays were performed in duplicate. *$P < 0.001$, Student's $t$-test, with respect to the appropriate controls in the absence of $H_2O_2$ (empty columns).

gomycin- and atractyloside-sensitivity of $H_2O_2$-inhibited basal respiration was then diminished to about 50%, suggesting that the coupling of the ATP synthesis to the respiration was impaired by $H_2O_2$ in this case, but not in undifferentiated cells, where, as a matter of fact, the full sensitivity was maintained. Assuming a high degree of control of 'phosphorylation flux' over basal respiration and a non-ohmic proton conductance of the mitochondrial inner membrane, that were suggested as general phenomena in intact cells [14,16], taking also into account that FCCP- uncoupled respiration rates were greater than the basal rates of respiration, the results indicated that the impairment of ADP phosphorylation caused by $H_2O_2$, mainly in differentiating FELC, appeared to be related to damage to the ATP synthesis/transport system rather than to electron transport.

### 3.2. Damage by $H_2O_2$ to maximal mitochondrial ATP synthase capacity of FELC

We next investigated the susceptibility to $H_2O_2$ of the maximal ATP synthase capacity of the mitochondria, which we assayed by measuring the ATP hydrolysis rate at saturating ATP concentration sustained by homogenates prepared by brief controlled sonication of FELC, either induced to differentiate or not. In both cell types, treated or not with $H_2O_2$, the addition of FCCP or atractyloside had no effect on the ATPase activity measured, indicating that the sonication of cell suspensions provided a correct and complete exposure of the enzyme complex through the formation of submitochondrial vesicles. The results are reported in Table IB), where the oligomycin-sensitive ATPase activity represents the maximal ATP synthase capacity. 70% sensitivity to oligomycin was observed (see values in parentheses) in both parental and HMBA-induced cells, pointing to a correct coupling of $F_0$ and $F_1$ moieties of $F_0F_1$ ATP synthase complex in both cases, despite the lower value of ATPase activity observed in induced cells. The exposure to $H_2O_2$ apparently caused a comparable significant decrease in ATPase activity of both cell types. Conversely, % oligomycin-sensitivity of ATPase activity was diminished only in the differentiating FELC from 70% to 50%, so that the maximal ATP synthesizing capacity more markedly declined in this case (55% decrease with respect to 41% in the parental cells). These results indicated that $H_2O_2$ was able to affect $F_0F_1$ ATP synthase complex in intact FELC, in a way apparently stronger in heme-synthesizing cells and presumably different from a qualitatively point of view. In fact, in this case subunits responsible for the proper assembling of $F_0$ and $F_1$ may have been damaged, whereas in control cells the full oligomycin-sensitivity of ATPase activity excluded this possibility.

### 3.3. Protection experiments with cell-permeable 'anti- oxy-radical' agents

Fig. 1 shows that 1 h preincubation of intact cells with the iron chelator DFO, as well as with the OH$^\bullet$ radical scavenger DMSO, completely prevented the damage caused by $H_2O_2$ to both control and differentiating FELC, indicating that intracellular redox-active chelatable iron and the OH$^\bullet$ radical were involved in both cases. In accordance with this hypothesis was the observed ineffectiveness of FO, 95% iron-saturated DFO derivative, in preventing $H_2O_2$-damage (not shown), which ruled out direct reactions of DFO with $H_2O_2$, OH$^\bullet$, or other oxy-radicals, as responsible for the protective effect under our conditions. On the other hand, the lipid antioxidant BHT never showed any protection, suggesting that lipid peroxidation did not occur to such extent as to mediate the impairment of mitochondrial functions. Then, it is tempting to argue that, upon the non- lethal treatment of FELC with $H_2O_2$ which we carried out, the mitochondrial dysfunctions observed in both parental and differentiating cells were due to iron-catalyzed peroxidative attack to proteins, rather than to lipids, of the inner membrane. The results of Davies and coworkers [17], showing oxidative inactivation of electron transport chain components and ATPase of submitochondrial particles as independent of lipid peroxidation, support our hypothesis.

### 3.4. The selective injury caused by $H_2O_2$ to mitochondrial functions of heme-synthesizing FELC was mimicked by pre-exposure of uninduced FELC to hemin

We have recently shown that the iron content of HMBA-induced FELC was apparently increased with respect to the uninduced cells by about 3-fold, with non- hemoglobin-bound iron being only slightly augmented, while hemoglobin-bound iron was more than 20-fold greater [8]. In accordance, it was reasonable that non- heme chelatable iron affected in both cell types $H_2O_2$- dependent damage to mitochondrial functions, as

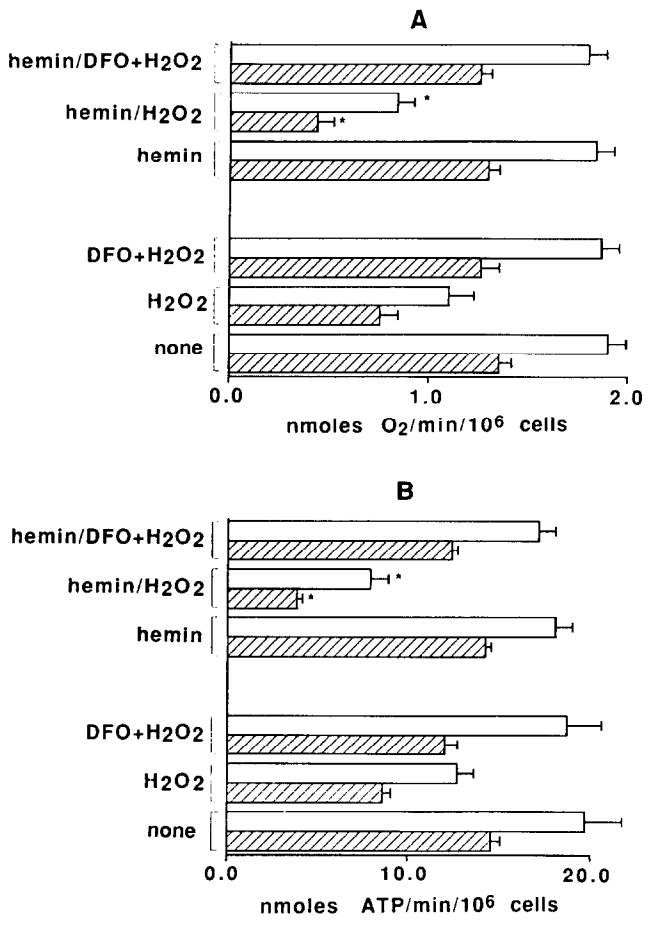


Fig. 2. Pre-exposure to exogenous hemin potentiates the mitochondrial dysfunctions caused by $H_2O_2$ to uninduced FELC. Uniduced FELC ($2 \times 10^6$cells/ml) were preincubated in PBS + 5 mM glucose for 30 min in the presence of 25 $\mu$M hemin (hemin). Cell mortality never exceeded 5%. Then, the drained pellet was resuspended at the initial density and exposed to 0.25 mM $H_2O_2$ for 30 min after 1 hour incubation with 20 mM DFO (hemin/DFO + $H_2O_2$) or without DFO (hemin/$H_2O_2$). Control experiments were performed in the absence of hemin and indicated as: none, $H_2O_2$, DFO + $H_2O_2$. Panel A shows the basal respiration rate (empty columns) and the oligomycin-sensitive respiration rate (diagonal columns) of intact FELC. Panel B reports the maximal ATPase activity of sonicated cell suspensions: total activity (empty columns) and oligomycin-sensitive activity (diagonal columns). Data are mean values ± S.D. of 3 different experiments in which the assays were performed at least in duplicate. *Significantly different from the appropriate controls in the absence of hemin, $P < 0.001$, Student's $t$-test.

suggested by the results of the protection experiments above reported, indicating that the oxidative insult occurred in the parental cells as mediated, as a matter of fact, by chelatable iron. Conversely, we suggest, considering the augmented heme synthesis, that an increase in heme levels of the inner mitochondrial membrane could be a significant factor in determining the selective injury caused by $H_2O_2$ to differentating FELC, whereby the metalloporphyrin promoted OH$^\cdot$ radical formation at the membrane surface and/or in the lipid bulk phase. This hypothesis was supported by the results shown in Fig. 2, demonstrating that by treating uninduced FELC with hemin before the exposure to $H_2O_2$ (hemin/$H_2O_2$) the effects of the oxidant, both on respiration rate (panel A) and on maximal ATPase activity (panel B), became qualitatively identical to those before described for the heme-synthesizing cells. Specifically, as for the damage to $F_0F_1$ ATP synthase, it appeared to result in a decline in the % oligomycin-sensitivity of ATPase activity (from 68% to 49%) and then to alter some oligomycin-sensitivity responsible subunits of the complex. In this respect, it is noteworthy to emphasize that we recently demonstrated that $H_2O_2$ was not able to cause any damage affecting the correct oligomycin-sensitive coupling of the components $F_0$ and $F_1$ assembled in the purified native enzyme complex, when it was exposed to the oxidant in the presence of redox-active adventitious iron ions [12]. Taking into account such results, hemin, as a lipid-soluble iron chelate, could be likely supposed to provide redox-active iron ions in a more appropriate form accessible to ATP synthase subunits responsible for the coupling. Such subunits should thus be damaged directly, and/or through oxidative injury to adenine nucleotide translocase, whose conformational rearrangements may be transmitted to $F_0$ moiety of the complex [18]. Finally, it should be considered that heme has been long documented to efficiently react with $H_2O_2$ leading to the formation of hyper-valent iron species and/or OH$^\cdot$ radicals [19], as well as of protein-derived radicals of hemoproteins [20], or of proteins like albumin present together hemin in the bulk solution [21]. The reaction was reported to eventually release iron from heme (22). In this regard, because DFO does not chelate iron from hemin in vitro [23], the preventive effect of DFO shown in Fig. 2 (hemin/DFO+$H_2O_2$) indicates that liberation of iron from hemin at the level of the inner mitochondrial membranes was required for $H_2O_2$-damage to occur, rather than direct interaction of hemin iron with $H_2O_2$ and target protein simultaneously. This may be considered a possible mechanism for the protective effect exhibited by DFO on the selective damage to HMBA-induced FELC, supporting our hypothesis that it was mediated by heme.

### 3.5. Conclusions

The results reported show that, due to the modifications in their iron metabolism associated to the augmentation of heme synthesis, FELC committed to the erythroid differentiation provided us with a good model to investigate the role of intracellular iron in the mechanisms of oxidative damage to cells and specifically to mitochondria. The potentiating effects by heme iron evidenced in intact FELC toward the injury caused by $H_2O_2$ to proteins of inner mitochondrial membranes, and particularly to the $F_0F_1$ ATP synthase complex, suggest that the selective damage inflicted by $H_2O_2$ to HMBA-induced FELC may be explained by the presence of a suitable amount of heme at the level of the mitochondrial membranes of these cells. The occurrence of such condition can be reasonably expected on the basis of the modifications of heme metabolism described as concomitant with the differentiation [24,25].

*Acknowledgements:* Supported by 'Consiglio Nazionale delle Ricerche' C.N.R., 'Ministero Università e Ricerca Scientifica e Tecnologica' M.U.R.S.T. (40%, 60%), and C.N.R. Targeted Project 'Applicazioni Cliniche Ricerca Oncologica: A.C.R.O.'. The expert photographic work of Dr. Giancarlo Cro 'Studio Fotografico Controluce' is gratefully acknowledged.

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