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Dissociation of iron transport and heme biosynthesis from commitment to terminal maturation of murine erythroleukemia cells

(iron uptake/dimethyl sulfoxide/imidazole)

ASTERIOS S. TSIFTSOGLOU, MARCO T. NUNEZ, WILLIE WONG, AND STEPHEN H. ROBINSON

Charles A. Dana Research Institute, Department of Medicine and Harvard–Thorndike Laboratory, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215

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ABSTRACT **Treatment of murine erythroleukemia (MEL) cells with imidazole in the presence of dimethyl sulfoxide (Me_2SO) has been shown to dissociate hemoglobin accumulation from commitment to terminal maturation. To explore the mechanism(s) of this effect, we studied iron transport and heme and hemoglobin synthesis in Me_2SO-induced MEL cells that were then exposed to imidazole. Imidazole treatment (*i*) causes moderate inhibition of ^{125}I-labeled transferrin binding to both control and Me_2SO-treated MEL cells; (*ii*) markedly suppresses Me_2SO-induced activation of iron uptake into MEL cells; (*iii*) markedly decreases the incorporation of iron into ferritin; and (*iv*) abolishes heme biosynthesis from [2-^{14}C]glycine and hemoglobin accumulation in Me_2SO-treated cells. Imidazole treatment does not inhibit other aspects of cellular maturation; cells treated with Me_2SO in the presence or absence of imidazole exhibit similar changes in proliferative activity and protein synthesis and, as shown previously, in cell morphology. Inhibition of hemoglobin accumulation in MEL cells is reversible on withdrawal of imidazole but is not altered by exogenous hemin. These data indicate that commitment to terminal maturation is regulated independently from the systems for iron transport and heme biosynthesis during early phases of erythroid cell differentiation.**

Recently, it has been shown that general aspects of the commitment to maturation in murine erythroleukemia (MEL) cells induced by dimethyl sulfoxide (Me_2SO) can occur in the absence of hemoglobin synthesis; this occurs when Me_2SO-treated MEL cells are also exposed to imidazole (1). These cells fail to produce globin mRNA and to accumulate hemoglobin. However, they exhibit nuclear condensation, morphological maturation, an increase in the nuclear protein IP-25 [a protein associated with commitment to maturation (2, 3)], and loss of proliferative capacity (1, 4). The mechanism by which imidazole exerts this selective inhibitory effect on the hemoglobin accumulation program is not known. It might be mediated through an effect on the iron transport pathway that is activated in Me_2SO-treated MEL cells (5–7). Imidazole might also block the formation of heme by impairing the activity of enzymes (e.g., δ-aminolevulinic acid synthetase) involved in protoporphyrin synthesis; these enzymes are extensively induced during MEL cell differentiation (8). Alternatively, imidazole might alter mitochondrial membrane functions involved in iron transport and heme production. If imidazole indeed proves to interfere with one or more of these early processes, the fact that globin mRNA production fails to occur would suggest that the inhibited process is critical to a coordinated sequence of biochemical events leading to hemoglobin synthesis.

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The studies presented here analyze the effects of imidazole on (*i*) iron metabolism, (*ii*) heme biosynthesis and hemoglobin accumulation, and (*iii*) cell growth and protein synthesis during MEL cell differentiation. Additional experiments were carried out to determine whether exogenous hemin can overcome the imidazole-induced block in hemoglobin synthesis.

MATERIALS AND METHODS

Cell Culture and Chemicals. Cells were MEL (745-PC-4) derived from the MEL 745 cell line, originally isolated by Friend *et al.* (5). Cells were grown in α medium lacking nucleosides and supplemented with 13% fetal calf serum and penicillin and streptomycin in a humidified atmosphere with 5% CO_2. Continuous growth was ensured by maintaining the cells at densities of 3×10^4 to 3×10^5 cells per ml. Cell growth was monitored with a Coulter Counter (model ZBI). On certain occasions, as indicated in the text, cells were grown in α medium containing 10% fetal calf serum and 3% mouse serum as a source of mouse transferrin.

Me_2SO (Mallinckrodt) was added directly to cultures without previous sterilization. Imidazole (Sigma; Grade III) was dissolved to 50 mg/ml in distilled water, sterilized by filtration, and stored at −35°C prior to its use. The experimental protocol used in this study was similar to that published previously (1); cells were treated with Me_2SO for 24 hr and then exposed to imidazole at 500 μg/ml in the continuing presence of Me_2SO. Cells exposed to no drug, Me_2SO, or only imidazole served as controls.

Determination of Benzidine-Positive Cells and of Hemoglobin Content. The proportion of hemoglobin-containing cells was determined by scoring more than 250 cells on cytocentrifuge slides stained with benzidine/H_2O_2. Cellular hemoglobin levels were determined spectrophotometrically as described by Tsiftsoglou *et al.* (9).

Transferrin Binding and Iron Uptake. Transferrin binding to MEL cells was measured as described (7). ^{125}I-Labeled transferrin was incubated at various concentrations (0.5–5 μM) with MEL cells ($2–5 \times 10^6$ cells) in phosphate-buffered saline containing 5% bovine serum albumin at 37°C. The cells were then washed (three times) in cold phosphate-buffered saline/0.2% bovine serum albumin/5 M glucose (PBG), and radioactivity was determined in a Nuclear Chicago gamma counter. The number of transferrin receptors per cell was determined from the binding data plotted in a Scatchard plot (10). Iron uptake was measured by incubating cells for various times with 2 μM ^{59}Fe-labeled diferric transferrin. After 30 min of incubation,

Abbreviations: MEL, murine erythroleukemia; Me_2SO, dimethyl sulfoxide.

cells were removed and washed with PBG and radioactivity was measured.

^{59}Fe Incorporation into Ferritin. Cells were incubated with [^{59}Fe]transferrin for 30 min at 37°C as described for ^{59}Fe uptake (7), and the washed cells were lysed with 10 vol of 0.5% Triton X-100 in 10 mM Tris·HCl, pH 7.6/0.5 mM KCl/2 mM $MgCl_2$. After 10 min on ice, cells were centrifuged and the pellet was washed once with 10 vol of phosphate-buffered saline. Enough carrier mouse ferritin and purified rabbit anti-mouse ferritin were added to the pooled supernatants to ensure quantitative ferritin precipitation (7). After 2 hr on ice the mixture was centrifuged, the pellet was washed twice with cold phosphate-buffered saline, and the ^{59}Fe radioactivity was measured.

Rate of Total Protein and Ferritin Synthesis. Cells were labeled for 2 hr with [4,5-^{3}H]leucine (10 μCi/ml, 5 Ci/mmol; New England Nuclear; 1 Ci = 3.7×10^{10} Bq) and cell lysates were prepared at different times. One part of the radiolabeled lysates was used to measure the rate of total protein synthesis by precipitation with trichloroacetic acid and collection of the precipitates on glass fiber filters (GF/B, Whatman; 2.4-cm circles), while the rest was used to measure ferritin synthesis by precipitation of [^{3}H]leucine-labeled ferritin with rabbit anti-mouse ferritin as described above. The rate of ferritin synthesis was expressed as cpm/10^5 cells and as the percent of total protein synthesis.

Heme Synthesis. Cells were incubated with [2-^{14}C]glycine (2.5 μCi/ml, 40–60 mCi/mmol) in α medium for 4 hr, then washed (three times) with phosphate-buffered saline. Heme was extracted from the acidified cell lysate with methyl ethyl ketone (11) and its radioactivity was measured in a liquid scintillation counter.

Electrophoretic Analysis of Cell Lysates and Identification of Hemoglobin. Cell lysates were analyzed by a slab polyacrylamide gel electrophoresis technique as described (12). Identification of hemoglobin was carried out by staining the gel with a methanol solution of 3,3'-dimethoxybenzidine (1%) (Eastman).

RESULTS

Imidazole Inhibits Heme Biosynthesis and Hemoglobin Accumulation in Me_2SO-Treated MEL Cells. As shown in Fig. 1*A*, addition of imidazole (500 μg/ml) to cultures after 24 hr of incubation with Me_2SO abolished the accumulation of benzidine-positive cells. Cultures treated with Me_2SO alone for 96 hr contained about 80% benzidine-positive cells, as compared to 0–3% benzidine-positive cells in cultures to which imidazole had also been added. This was associated with a similar decrease in hemoglobin accumulation (Fig. 1*B*). Me_2SO/imidazole-treated cells contained less than 1% of the hemoglobin present in cells treated with Me_2SO only. Fig. 2 shows that imidazole treatment also markedly suppressed the incorporation of [2-^{14}C]glycine into heme in both Me_2SO-treated and control cells. Inhibition of heme biosynthesis was evident 24 hr after the addition of imidazole and progressed gradually up to 96–120 hr of incubation. By this time, Me_2SO/imidazole-treated cultures contained only 5–6% of the labeled heme present in Me_2SO-treated cells. Imidazole also decreased spontaneous heme synthesis in control cultures not exposed to Me_2SO.

Effects of Imidazole on Cell Growth and Protein Synthesis. Me_2SO- and Me_2SO/imidazole-treated cells exhibited similar decreases in both growth and protein synthesis. In contrast to the findings with cells treated with no drug or imidazole only, cell growth began to decrease within 24 hr of adding Me_2SO and continued to do so gradually up to 96 hr. By this time, the rates of cell growth in Me_2SO- and Me_2SO/imidazole-treated cells were 12% and 18% of the values in control and imidazole-treated cells, respectively. A similar decrease was observed in the rate of protein synthesis. The rates of protein synthesis in Me_2SO- and Me_2SO/imidazole-treated cells were 16% and 20% of the values observed in control and imidazole-treated cells after 96 hr.

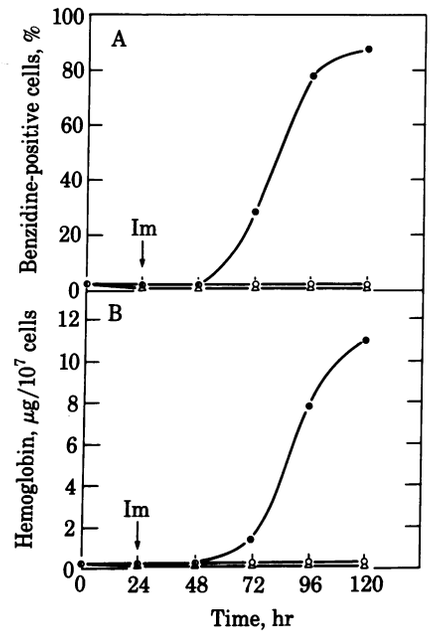

FIG. 1. Effects of imidazole treatment on hemoglobin accumulation. Exponentially growing MEL (745-PC-4) cells were incubated in culture (3×10^4 cells per ml) with and without Me_2SO (1.5%, vol/vol). After 24-hr incubation, imidazole (Im, 500 μg/ml) was added to one half of the control and one half of the Me_2SO-treated cultures, as indicated by the arrow, and incubation was continued at 37°C. The remaining cultures served as controls. At intervals thereafter, the cultures were scored for the proportion of benzidine-positive cells (*A*) and for hemoglobin content (*B*). ○, Control cultures; ●, Me_2SO-treated cultures; △, control/imidazole- or Me_2SO/imidazole-treated cultures.

Effects of Imidazole on Transferrin Receptors and Iron Uptake. Treatment of cells with Me_2SO alone led to a 30–33% increase in the number of transferrin receptors per cell (Fig. 3). The number of transferrin receptors then declined with longer incubation (96–120 hr), possibly reflecting late events in normal erythroid differentiation (13). Addition of imidazole to Me_2SO-treated cultures caused complete inhibition of the in-

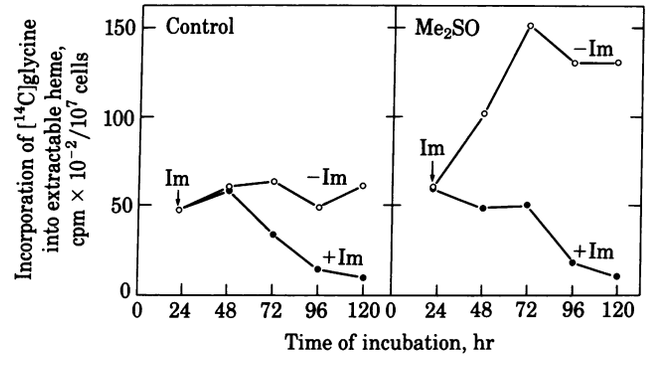

FIG. 2. Effects of imidazole on [^{14}C]glycine incorporation into heme. Cells were cultured with or without Me_2SO and imidazole (Im) as described for Fig. 1. At the times indicated, cells were pulsed with [2-^{14}C]glycine for 4 hr, heme was extracted, and radioactivity was determined.

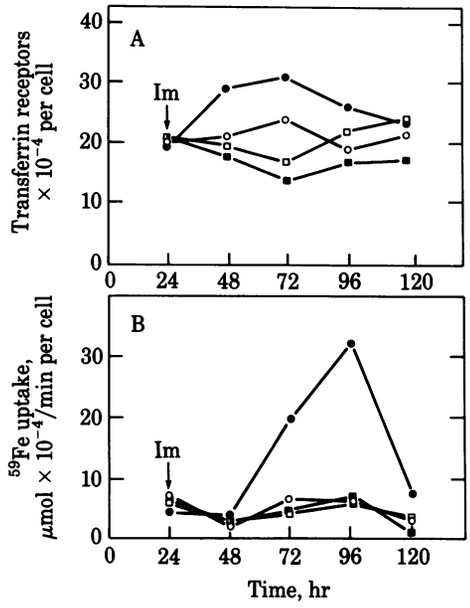

FIG. 3. Effects of imidazole on the number of transferrin receptors and iron uptake. Cells were cultured with and without Me_2SO and imidazole (Im) as described for Fig. 1. At the times indicated, cells were assayed for the number of transferrin receptors and for iron uptake. (*A*) Number of transferrin receptors; (*B*) rate of iron uptake. ○, Control cells; □, control/imidazole-treated cells; ●, Me_2SO-treated cells; ■, Me_2SO/imidazole-treated cells.

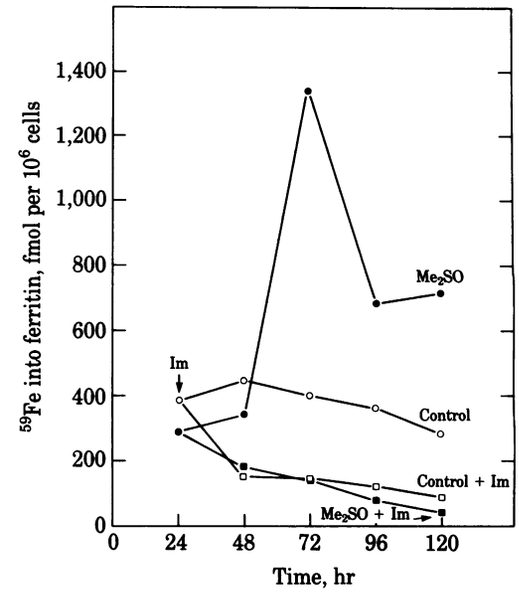

FIG. 4. Imidazole inhibits iron uptake into ferritin. Cells were cultured with or without Me_2SO and imidazole (Im) as described for Fig. 1. At the times indicated, cells were assayed for the amount of ^{59}Fe incorporated into ferritin, expressed per 30 min. ○, Control cells; □, control/imidazole-treated cells; ●, Me_2SO-treated cells; ■, Me_2SO/imidazole-treated cells.

crease in transferrin receptors. A much smaller and more equivocal effect of imidazole was observed in control cells not exposed to Me_2SO. Imidazole caused a dramatic inhibition of the increase of iron uptake induced by Me_2SO. Me_2SO-treated cells exposed to imidazole exhibited the low rate of iron uptake characterisitic of uninduced cells (7).

Ferritin synthesis remained constant in control MEL cells and declined in Me_2SO-treated cells but at a rate lower than that of total protein synthesis. Thus, as a percent of total newly synthesized cytoplasmic protein, ferritin synthesis increased in Me_2SO-treated cells by a factor of 2. Exposure of Me_2SO-treated cells to imidazole markedly suppressed this relative increase in ferritin synthesis (data not shown). In control non-Me_2SO-treated cells ferritin synthesis declined by 10–15% after imidazole treatment.

Imidazole effectively prevented the accumulation of ^{59}Fe in ferritin (Fig. 4), a step that is also activated during the differentiation of Me_2SO-treated cells (7). After 72-hr incubation, control and Me_2SO-treated cells exposed to imidazole contained less than 34% and 11% of the ferritin-bound ^{59}Fe found in control and Me_2SO-treated cells not exposed to imidazole, respectively. The different kinetics of activation of iron uptake and incorporation into ferritin, heme synthesis, and hemoglobin accumulation shown in Figs. 1–4 are probably due in part to experimental variation and in part to the sequential development of these processes during MEL cell differentiation (7). Imidazole interferes with all of these steps in hemoglobinization.

Reversibility of Imidazole Inhibition of Hemoglobin Accumulation. As shown in Fig. 5, Me_2SO/imidazole-treated cells washed free of imidazole and then incubated in medium containing Me_2SO alone accumulated virtually the same level of benzidine-positive cells as cultures exposed to Me_2SO alone from the beginning of incubation. The conclusion that the imidazole-induced block in hemoglobin accumulation is reversible was further supported by electrophoretic analysis of the cell lysates. Me_2SO/imidazole-treated cells failed to produce hemoglobin; in contrast, Me_2SO/imidazole-treated cells released from the imidazole block and then incubated with Me_2SO alone accumulated hemoglobin at levels comparable to those of cells exposed to Me_2SO alone from the beginning of incubation.

If imidazole-induced inhibition of hemoglobin accumulation in Me_2SO-treated cells is due to depletion of heme, then exogenously added hemin should overcome this block. As shown in Table 1, cells treated with Me_2SO alone or with both Me_2SO and hemin accumulated more than 95% benzidine-positive cells

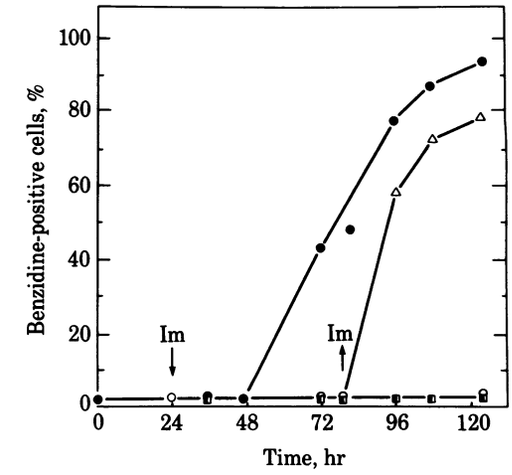

FIG. 5. Effects of imidazole removal on hemoglobin accumulation. Exponentially growing cells were incubated with or without Me_2SO for 24 hr. One half of the cultures were exposed to imidazole (Im) (↓) and incubation was continued at 37°C for up to 120 hr. Some Me_2SO/imidazole-treated cultures were washed free of imidazole 54 hr after the addition of imidazole (78 hr after addition of Me_2SO) and reincubated with Me_2SO alone (↑). The percentage of benzidine-positive cells in each culture was determined at different time intervals as indicated. ○, Control cells treated with no drug; □, imidazole-treated cells; ●, Me_2SO-treated cells; ■, Me_2SO/imidazole-treated cells; △, Me_2SO/imidazole-treated cells released from the imidazole block after 54 hr and then incubated with Me_2SO alone.

Table 1. Effect of hemin on imidazole inhibition of hemoglobin accumulation in Me_2SO-treated MEL cells

Treatment		Benzidine-positive cells, %
0–24 hr	24–120 hr	
None	None	<1
None	Imidazole	<1
None	Hemin	6.4
None	Hemin + imidazole	4.5
Me_2SO	Me_2SO	91.4
Me_2SO	Me_2SO + imidazole	0
Me_2SO	Me_2SO + hemin	94.3
Me_2SO	Me_2SO + imidazole + hemin	7.7

Exponentially growing MEL cells were incubated with and without Me_2SO (1.5%, vol/vol). After 24-hr incubation, some of the cultures were exposed to hemin (30 μM), imidazole (500 μg/ml), or both, and incubation was continued for 96 hr more. Cultures were then scored for the percentage of benzidine-positive cells.

after 120-hr incubation. In contrast, cultures exposed to both Me_2SO and imidazole in the presence of hemin did not accumulate a significant proportion of benzidine-positive cells. Additional evidence that hemin treatment fails to overcome the imidazole inhibition was obtained from the electrophoretic data. Me_2SO/imidazole and Me_2SO/imidazole/hemin-treated cells did not accumulate more than traces of hemoglobin.

DISCUSSION

Erythroid cell maturation is associated with hemoglobin biosynthesis, and it is widely assumed that these two events are highly interdependent. In studies with MEL cells, hemoglobin accumulation appears to be coordinated with commitment, the irreversible decision the cells make to differentiate when they are treated with an inducer. However, the observation that treatment with hemin stimulates globin synthesis but not commitment in MEL cells (3, 14), together with the finding that commitment in MEL cells treated with Me_2SO begins earlier than heme biosynthesis (3, 4, 7, 15), suggested that hemoglobin accumulation and commitment may be regulated independently. Direct evidence supporting this conclusion was obtained only recently by Gusella *et al.* (1), who showed that treatment with imidazole dissociates hemoglobin accumulation from other more general aspects of terminal maturation in inducer-treated MEL cells.

The present study confirms these observations and demonstrates that the inhibition of hemoglobin accumulation by imidazole is associated with suppression of the activation of iron transport and heme biosynthesis that normally accompanies MEL cell differentiation. Thus, these early steps in hemoglobinization can be regulated independently of the commitment to morphologic maturation and loss of proliferative capacity that proceeds normally in the presence of imidazole. Moreover, the processes regulating commitment must diverge very early from those involved in hemoglobin accumulation.

We do not yet understand precisely how imidazole interferes with activation of iron transport, heme biosynthesis, and hemoglobin accumulation. As outlined in the following discussion, the mechanism is probably complex.

Primary suppression of one or more of the steps involved in iron transport into the cell could explain the observed decrease in heme biosynthesis and ferritin synthesis as well, since both are regulated by iron availability. This would imply an increase in free protoporphyrin within the cells; cellular protoporphyrin levels were not measured in this study. It is unlikely that imidazole acts simply as an iron chelator, in the light of experiments (unpublished observations) that have shown that imidazole does not inactivate iron transport in murine reticulocytes as opposed to nucleated erythroid cells.

However, the imidazole block in hemoglobin accumulation in Me_2SO-treated cells is not reversed by hemin, an agent that stimulates globin mRNA synthesis (16) and β-minor globin accumulation (17) but does not induce commitment to terminal maturation (3, 14) in MEL cells. This suggests that the action of imidazole on hemoglobin accumulation is more complex than specific interference with iron transport, heme biosynthesis, or both. The lack of reversal of the imidazole block of hemoglobin production by hemin is surprising in light of the observation that hemin overcomes the imidazole block in globin mRNA accumulation in Me_2SO-treated MEL cells (V. Volloch, personal communication). These findings suggest that imidazole may block the translation of globin mRNA. If this is the primary effect of imidazole, the observed changes in iron transport and heme biosynthesis may be secondary feedback effects due to the decrease in globin production. Alternatively, imidazole may decrease the translation of several mRNA molecules coding for a variety of proteins involved in iron uptake, heme biosynthesis, and hemoglobin accumulation during erythroid cell maturation.

It has been reported that imidazole interacts with membranes and blocks sodium channels in neural cells (18). This information—taken together with earlier observations that MEL cell differentiation is associated both with changes in the metabolism of Ca^{2+} (19, 20), Fe^{2+} (5, 7, 13), and K^+ (21) and with mitochondrial membrane depolarization (22)—prompted us to investigate the effects of imidazole on the physical state of the mitochondrial membrane of MEL cells. In preliminary experiments with the use of rhodamine-123, a dye that accumulates specifically in mitochondria (23), we have found that imidazole causes extensive hyperpolarization of the mitochondrial membrane. Since the mitochondria are critical sites of iron incorporation into heme and of heme biosynthesis, impairment of mitochondrial function could explain the decreases in iron uptake and heme synthesis that were observed in Me_2SO/imidazole-treated cells. The diminution in globin mRNA accumulation and translation in these cells might then be attributed to the lack of heme, which has been shown to stimulate both the accumulation and translation of globin mRNA during MEL cell differentiation (3, 16, 17). The failure of exogenous hemin to correct a translational defect in globin synthesis might be due to imidazole-induced abnormalities in the mitochondrial membrane, which normally introduces endogenously produced heme into appropriate compartments of the cytosol where it can interact with the system for globin production.

Thus, although the observed decreases in iron transport and heme biosynthesis clearly play an important role in the suppression of hemoglobin accumulation in differentiating MEL cells treated with imidazole, it seems probable that these are not the specific targets of imidazole's action and that the latter works primarily by interfering with mitochondrial function, mRNA translation, or both. In either event, the findings make it clear that a series of critical early steps in hemoglobin synthesis are dissociable from other molecular events involved in the commitment of erythroid cells to terminal maturation.

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