Effect of Phorbol Esters on Iron Uptake in Human Hematopoietic Cell Lines

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ABSTRACT

We have investigated the effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on iron uptake into human hematopoietic cell lines K562, U937, and HL-60. TPA inhibited both cell growth and iron uptake by these cell lines. This effect was rapid, which is typical of phorbol esters which are biologically active, and it occurred at very low concentrations of TPA. This effect of TPA was dependent upon an inhibition of the transferrin-binding capacity as estimated on intact cells. However, experiments with transferrin binding on cell samples dissolved in 1% Triton X-100 showed that TPA-treated cells exhibited a transferrin-binding capacity similar to that of control cells. On the basis of this result, it is suggested that TPA modified a part of transferrin receptors present in the cells; as a result of this modification, these receptors became unavailable for binding transferrin, but they remained physically present in the cell. Other compounds capable of inducing the differentiation of leukemic cells, such as dimethyl sulfoxide, butyrate, retinoic acid, and 1α,25-dihydroxyvitamin D₃, did not acutely inhibit iron uptake. We also investigated the effect of TPA on transferrin receptors in a cellular system in which phorbol esters stimulate cell proliferation. At 16x 10⁻⁹ M, TPA markedly stimulated the proliferation of T-lymphocytes. However, in spite of this marked stimulation of cell proliferation, TPA-stimulated lymphocytes exhibited a transferrin-binding capacity much inferior to cells stimulated by other mitogens, such as phytohemagglutinin.

INTRODUCTION

All living systems appear to need iron, and it has been suggested that "life in any form without iron is in all likelihood impossible" (6). Iron is required in substantial amounts by reticulocytes (for hemoglobin synthesis), by oxidative respiratory enzymes, and by the developing embryo. The major carrier of iron in the circulation of vertebrates is transferrin; hence, cells grown in a serum-free medium must be provided with transferrin (2). The richest source of transferrin receptors is in hemoglobin-synthesizing reticulocytes and in the placental trophoblast. However, transferrin receptors have been demonstrated on a variety of cell types including fibroblasts, lymphocytes, kidney tissue, epithelial cells, hematopoietic cell lines, and various tumor cell types (12, 15, 16, 36, 40, 42, 46). It is likely that virtually all vertebrate cells (excluding mature erythrocytes) have transferrin receptors, their density being greatly increased by cell activation and proliferation (20). TPA³ and other phorbol esters that are biologically active have profound effects on cellular differentiation and proliferation. In fact, TPA induces the differentiation in vitro of several human leukemic cell lines (11, 22); this process is usually associated with complete inhibition of cell proliferation (10, 19, 31, 34, 35, 41). We have examined the effect of TPA on iron uptake in several human hematopoietic cell lines. We report here that TPA acutely inhibits the uptake of iron by these hematopoietic cells.

MATERIALS AND METHODS

Cell Line Culture

K562 (23), HL-60 (5), and U937 (39) cells were grown in suspension culture in RPMI 1640 (Boehringer/Mannheim, Mannheim, Germany) containing 10% fetal calf serum (Boehringer/Mannheim) and antibiotics. The cells have a doubling time of about 24 hr. Cell viability was assessed by trypan blue exclusion test. Dimethyl sulfoxide, butyrate, retinoic acid, and hemin were obtained from Sigma Chemical Co. (St. Louis, MO). 1α,25-Dihydroxyvitamin D₃ was a generous gift of Roche Laboratories (Switzerland).

Lymphocyte Culture

Heparinized blood was obtained from healthy adults. Mononuclear cells were separated on Ficoll-Isoaque gradients, washed 3 times with Hank's balanced salt solution, and then suspended in RPMI 1640 to 1 x 10⁶ cells/ml. The cells were then cultured in tissue culture plates (Corning) in the presence of phytohemagglutinin (2.5 μg/ml; Difco Laboratories, Detroit, MI). Fetal calf serum (from 2.5 to 10%) and human serum (1%) were used. Each well contained 2 ml of tissue culture medium with 2 x 10⁶ cells. These suspensions were cultured at 37°C in an atmosphere of 5% CO₂ and 95% air.

Iron Uptake

Cells (10 x 10⁶) were preincubated for 1 hr at 37°C with 16 nm TPA (Sigma; TPA was dissolved in acetone and stored at -20°C as a 160 μM stock solution), washed 3 times in serum-free RPMI 1640, and then incubated in the presence of [⁵⁹Fe]transferrin (125 μg/ml) in RPMI 1640. To measure "nonspecific" iron uptake, cells were incubated with [⁵⁹Fe]transferrin (125 μg/ml) in the presence of unlabeled transferrin (10 mg/ml) at 37°C. At the end of the incubation period, the cells (1 x 10⁶) were layered over a cushion of phthalate oil (density, 1.02) and centrifuged for 2 min at 13,000 x g to remove unbound [⁵⁹Fe]transferrin. The ⁵⁹Fe content of the cell pellet was measured in a gamma counter. All data were averages of duplicate determinations (which were usually within 10% of each other) and were corrected for nonspecific binding (which did not exceed 5%).

Iron-free human transferrin was obtained from Sigma and was radio-labeled with ⁵⁷Fe following a method described previously (25). ⁵⁷Fe as ferric chloride, 30 Ci/g of iron, was obtained from Amersham.

¹²⁵I-Transferrin-binding Assay

Purified human transferrin (>99% pure) was conjugated with ¹²⁵I by the solid-phase lactoperoxidase system (New England Nuclear radioiodination system), as reported previously (42).
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Transferrin-binding capacity was investigated both on intact cells and on cell samples dissolved in Triton X-100 (1%).

Transferrin Receptor Assay on Intact Cells. The binding reactions were performed in 12- x 75-mm polypropylene tubes in RPMI 1640 containing 0.1% human serum albumin (Sigma Fraction V). Cell concentrations were 5 x 10⁶ cells/ml, labeled transferrin was 500 ng/ml, and unlabeled transferrin was 0 to 1 mg/ml. Unbound ligand was removed by passage of cells through a density cushion, as described previously (42). After incubation, 200-μl aliquots of the cell suspension were layered over 150 μl of a mixture of dibutyl phthalate and dinonyl phthalate (Merck) to a final density of 1.025 in 400-μl plastic microfuge tubes and centrifuged in a Hettich microfuge (10,000 x g for 5 min). At the end of centrifugation, the supernatant and most of the dibutyl phthalate cushion were aspirated. The tips of the vials containing the cell pellet were then severed with a scalpel and transferred to plastic vials, and the radioactivity was measured in a gamma counter. Total binding corresponded to the radioactivity in the cell pellet. "Nonspecific" binding was represented by the radioactivity bound to the cells in the presence of cold transferrin (1 mg/ml) and was less than 5% of the total radioactivity bound per 10⁶ cells. "Specific" binding was the difference between total and nonspecific.

Solubilized Transferrin Receptor Assay. A simple assay was devised to measure solubilized transferrin receptor activity. It is based on a difference in solubility of free and transferrin-bound receptors in polyethylene glycol. Dissolved receptors (1% Triton X-100) were incubated in a total volume of 0.2 ml for 30 min at 37° in a 0.1 M citrate-Tris buffer solution (pH 5.0) containing 0.1% bovine serum albumin, 0.1% Triton X-100, and 200 ng of 125I-transferrin. The receptor-transferrin complex was precipitated with 0.8 ml of polyethylene glycol (12% w/v) in 0.1 M citrate-Tris buffer (pH 5.0) containing the carrier human γ-globulin (0.1%). The tubes were placed in an ice bath for 30 min and then centrifuged at 13,000 x g for 15 min at 4°. The supernatant and the precipitate were tested for radioactivity. Coprecipitation of free transferrin was measured by omitting the receptor from the tubes while nonspecific binding of transferrin was determined by preincubating the samples with 1 mg of nonradioactive transferrin before adding the radioactive transferrin.

Results

Effect of TPA on Iron Uptake and Transferrin Binding in Human Leukemic Cell Lines. Incubation of U937, K562, or HL-60 cells in the presence of TPA (16 nm) induced a marked reduction of iron uptake (Chart 1). This phenomenon appeared 15 min after the addition of TPA to the cells (Chart 1). The degree of inhibition of iron uptake by TPA was comparable when measured in cells made adherent to plastic bottles by TPA or in cells in suspension in polypropylene tubes. The treatment of the cells with TPA did not modify cellular viability as assessed by the trypan blue exclusion test.

The inhibition of iron uptake induced by TPA seems to be dependent upon a reduction of the number of iron molecules transported into the cells by transferrin (Chart 2). Incubation of the cells in the presence of increasing amounts of [59Fe]transferrin showed that TPA induced a marked reduction of the maximal amount of 59Fe taken up by the cells, without affecting the Kₘ of the process of iron uptake by the cells (control cell Kₘ, 0.45 x 10⁻⁶ M; TPA-treated cells Kₘ, 0.5 x 10⁻⁶ M).

The effect of various concentrations of TPA and other phorbol esters on their ability to inhibit iron uptake by K562 cells was...
Table 1

<table>
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<th>Cell line</th>
<th>Time (hr)</th>
<th>125I-Transferrin binding (%) of control</th>
<th>[55Fe]Transferrin uptake (%) of control</th>
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Effect of Other Inducers of Cellular Differentiation on Iron Uptake in Human Leukemic Cell Lines. We tested also whether other compounds capable of inducing the differentiation of human leukemic cell lines could acutely modify the rate of iron uptake (Chart 4, A and C). On the basis of this result, it can be suggested that TPA modified a part of transferrin receptor present in the cells; as a result of this modification, these receptors were not available for binding transferrin, but they remained physically present into the cell.

Investigation of transferrin binding to TPA-treated cells showed a contemporaneous and parallel inhibition of both transferrin binding and iron uptake (Table 1). Scatchard analysis of 125I-transferrin binding to the cells showed that TPA modified both the number of transferrin-binding sites and the affinity of transferrin receptors for transferrin (Chart 4, B and D). Thus, TPA induced a marked reduction of transferrin-binding sites both in K562 cells (control, $5.4 \times 10^5$ sites/cell; TPA-treated cells, $3.4 \times 10^5$ sites/cell) and in U937 cells (control, $2.16 \times 10^5$ sites/cell; TPA-treated cells, $1.63 \times 10^5$ sites/cell). In contrast, TPA reduced the affinity of transferrin receptors for transferrin in U937 (control, $1.22 \times 10^{-8}$ M; TPA-treated cells, $2.4 \times 10^{-8}$ M) but not in K562 cells (control, $2.4 \times 10^{-8}$ M; TPA-treated cells, $2.5 \times 10^{-8}$ M).

These results were constantly observed in 3 independent experiments. This result strongly suggested that the effect of TPA on iron uptake may be dependent upon an inhibition of the transferrin binding to the cells. However, when transferrin-binding capacity was investigated in cell samples dissolved in Triton X-100 (1%), it appeared clear that no significant difference existed in TPA-treated cells with respect to control untreated cells (Chart 4, A and C). On the basis of this result, it can be suggested that TPA modified a part of transferrin receptor present in the cells; as a result of this modification, these receptors were not available for binding transferrin, but they remained physically present into the cell.
uptake in leukemic cell lines. Our results showed that retinoic acid, dimethyl sulfoxide, sodium butyrate, and vitamin D₃ did not significantly inhibit iron uptake by K562 and U937 cells (Chart 5). However, hemin induced a significant reduction of iron uptake in both K562 and U937 cells (Chart 5).

Effect of DNA Synthesis Inhibitors on Iron Uptake and Transferrin Binding. TPA completely inhibited cell growth of U937, HL-60, and K562 cells as shown in Chart 6. Interestingly, the addition in the culture medium of picolinic acid, an iron chelator, induced an inhibition of both iron uptake (comparable to that induced by TPA) and cell growth (Chart 6).

The effect of TPA on iron uptake and transferrin binding could be dependent upon the inhibition of cell proliferation induced by this compound or by another mechanism. To investigate this point, we incubated K562 cells in the presence of hydroxyurea. At 0.75 × 10⁻³ M, this drug completely inhibited cell growth. Cells incubated in the presence of hydroxyurea did not change their transferrin-binding capacity for a period of time of at least 96 hr, in spite a complete inhibition of cell growth (Table 2).

Effect of TPA on Transferrin-binding Capacity of Human T-Lymphocytes. We also investigated the effect of TPA on the expression of transferrin receptors in a cellular system in which phorbol esters stimulate cell proliferation. In fact, TPA may replace lectins or other mitogens in the stimulation of the proliferation of human T-lymphocytes (1, 17, 43). At 16 × 10⁻⁹ M, TPA stimulated T-lymphocyte proliferation, as estimated by [³H]thymidine incorporation, almost as well as phytohemagglutinin (Chart 7). However, in spite this marked stimulation of cell proliferation, TPA-stimulated lymphocytes exhibited a transferrin-binding capacity much inferior with respect to cells stimulated by phytohemagglutinin.

DISCUSSION

Our results afford evidence that several human hematopoietic cell lines exhibit a rapid reduction in iron uptake following phorbol ester treatment. Specifically, TPA induced a reduction of the maximal amount of ⁵⁹Fe taken by the cells, without affecting the Kₘ of the process of iron uptake by the cells. This effect occurs 15 min after the addition of TPA to the cells. The inhibition of iron uptake induced by TPA exhibits a biphasic pattern, a loss of about 50% of the iron uptake capacity during the first 3 hr of incubation, followed by a slower decrease. After 24 hr of incubation in the presence of TPA, the cells retained no more than 25 to 30% of the initial iron uptake capacity. The reduction in iron uptake is proportional to both the concentration and the tumor-promoting activity of the phorbol derivative.

The inhibition of iron uptake induced by TPA is associated to a parallel inhibition of transferrin-binding capacity. This last phenomenon appears evident when investigated on whole cells. However, experiments with transferrin binding in cell samples dissolved in 1% Triton X-100 showed that TPA-treated cells exhibited a transferrin-binding capacity similar to that of control
transferrin, but they remained physically present in the cells. The transferrin receptors present into the cells; as a result of this cells. These results strongly suggest that TPA modified a part of its receptor which appears to possess a Ca2+, phospholipid-dependent protein kinase (4). As a result, transferrin receptors are phosphorylated (26) and partially internalized (17). Similar to the regulation of insulin receptors, the effect of TPA on transferrin receptors could be explained by the phosphorylation of these receptors (10, 11, 12).

In conclusion, the observation that iron uptake is reduced by TPA in systems where proliferation is stimulated as well as those in which it is inhibited could be tentatively explained as follows.

Since TPA inhibits transferrin binding also in a system in which phorbol esters are a mitogenic stimulus, it seems difficult to directly correlate the effects of TPA on iron uptake with the effects on cellular proliferation. Thus, it appears quite clear that the effect of phorbol esters on transferrin receptors and iron uptake seems to be not correlated with the proliferative response of the cells to these compounds. Previous studies have shown that TPA may replace mitogens in the stimulation of the proliferation of human T-lymphocytes (1, 18, 44, 45). Our results confirmed this observation since lymphocytes grown in the presence of TPA exhibited [3H]thymidine incorporation comparable to that observed in lymphocytes stimulated by mitogenic lectins. However, in spite of a great stimulation of DNA synthesis, TPA-stimulated lymphocytes exhibited a much lower transferrin-binding capacity with respect to lymphocytes grown in the presence of phytohemagglutinin.

In order to verify whether the inhibition of iron uptake induced by TPA could be directly correlated with the inhibition of cellular proliferation, we investigated the effect of TPA on transferrin binding and iron uptake in lymphocytes. Previous studies have shown that TPA may replace mitogens in the stimulation of the proliferation of human T-lymphocytes (1, 18, 44, 45). Our results confirmed this observation since lymphocytes grown in the presence of TPA exhibited [3H]thymidine incorporation comparable to that observed in lymphocytes stimulated by mitogenic lectins. However, in spite of a great stimulation of DNA synthesis, TPA-stimulated lymphocytes exhibited a much lower transferrin-binding capacity with respect to lymphocytes grown in the presence of phytohemagglutinin.

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The common mechanism and direct effect of TPA could be to cause phosphorylation and internalization of the receptor, and any subsequent indirect effect on cell proliferation would depend on the cell system.

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