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Iron reverses impermeable chelator inhibition of DNA synthesis in CCl 39 cells

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ABSTRACT **Treatment of Chinese hamster lung fibroblasts (CCl 39 cells) with the impermeable iron(II) chelator bathophenanthroline disulfonate (BPS) inhibits DNA synthesis when cell growth is initiated with growth factors including epidermal growth factor plus insulin, thrombin, or ceruloplasmin, but not with 10% fetal calf serum. The BPS treatment inhibits transplasma membrane electron transport. The treatment leads to release of iron from the cells as determined by BPS iron(II) complex formation over 90 min. Growth factor stimulation of DNA synthesis and electron transport are restored by addition of di- or trivalent iron to the cells in the form of ferric ammonium citrate, ferrous ammonium sulfate, or diferric transferrin. The effect with BPS differs from the inhibition of growth by hydroxyurea, which acts on the ribonucleotide reductase, or diethylenetriaminepentaacetic acid, which is another impermeable chelating agent, in that these agents inhibit growth in 10% fetal calf serum. The BPS effect is consistent with removal of iron from a site on the cell surface that controls DNA synthesis.**

Iron is essential for many oxidoreductase activities in cells. With intact cells, the most important function to be identified is synthesis of deoxyribonucleotides through the ribonucleotide reductase (1, 2). Direct demonstration of this essential function is possible through the use of permeable iron(III)-specific chelators, which prevent iron-tyrosyl radical function in this enzyme by removal of the iron (3–5). Permeable iron(II) chelators also inhibit cell growth, but their site of action is less defined (6).

Inhibition of DNA synthesis has also been observed with impermeable chelators such as diethylenetriaminepentaacetic acid (DTPA) or EDTA. These chelators are not very specific for transition elements such as iron, so the basis of the inhibition is not known. S. Nyholm, A. Johansson, G. J. Mann, R. J. Bergeron, A. Gräslund, and L. Thelander (personal communication) showed that DTPA does not inhibit ribonucleotide reductase activity in whole cells, so this impermeable chelator acts at another site.

A transplasma membrane electron transport system that functions to oxidize cytosolic NADH has also been related to stimulation of cell proliferation (7–9). Since this enzyme system is located at the cell surface and is inhibited by the impermeable chelator bathophenanthroline disulfonate (BPS), it can be a site for chelator inhibition (10).

In this paper, we describe the inhibition of DNA synthesis in CCl 39 cells by BPS in comparison to inhibition by hydroxyurea and DTPA to provide evidence that reversible removal of externally exposed iron from cells is a basis for inhibition of growth factor-initiated DNA synthesis regardless of the kinase system activated by the growth factors.

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MATERIALS AND METHODS

Materials. Human ceruloplasmin and α-thrombin were from Sigma, epidermal growth factor (EGF) was from Collaborative Research, and insulin was from Novo Industri (Bagsvaerd, Denmark). Diferric transferrin (Fe_2Tf) was prepared according to Löw *et al.* (25). [^{3}H]Thymidine and *myo*-[2-^{3}H]inositol were from Amersham. One unit of ceruloplasmin per ml is 76 nM.

Cell Culture Conditions. CCl 39 cells are an established cell line of Chinese hamster lung fibroblast. Cells were maintained in minimum Eagle's medium (MEM; Flow Laboratories) supplemented with 10% fetal calf serum (FCS), 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 25 mM sodium bicarbonate at 37°C in a 95% air/5% CO_2 atmosphere. Confluent cultures in 24-well plates were rendered quiescent by 24-h incubation in MEM after washing in MEM (serum starvation). Mitogens were added in serum-free medium after starvation to initiate growth. Chelators or hydroxyurea present during the 24-h starvation were removed before initiation of growth.

Measurement of DNA Synthesis Reinitiation. Quiescent cultures were exposed for 24 h with the indicated growth factors in the presence of [^{3}H]thymidine (0.25 μCi/ml; 1 Ci = 37 GBq) and 0.5 μM unlabeled thymidine. After incubation, the cells were washed twice with ice-cold 0.9% NaCl and fixed for 15 min in 5% cold trichloroacetic acid. The trichloroacetic acid-precipitated material was extracted from each well for 2 hr at room temperature with 0.1 M NaOH, and the radioactivity incorporated was counted.

Ferricyanide Reduction Assay. Reduction of ferricyanide was measured in Hepes buffer (130 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/20 mM Hepes, pH 7.4) with 0.2 mM potassium ferricyanide and 5×10^5 cells per ml. Absorbance at 500 nm was subtracted from absorbance at 420 nm in the dual-beam mode of the Shimadzu spectrophotometer. The extinction coefficient used was 1 $mM^{-1} \cdot cm^{-1}$.

RESULTS

Impermeable iron chelators inhibit DNA synthesis in CCl 39 cells if they are present during the starvation period before growth is initiated with mitogens or if they are present during growth. In contrast, hydroxyurea, which inhibits iron tyrosine radical function in the ribonucleotide reductase (1), inhibits DNA synthesis only when present during mitogen-initiated growth (Table 1). Both BPS and DTPA are effective inhibitors when ceruloplasmin or EGF plus insulin is used to stimulate growth after the preincubation with chelator. When growth is stimulated with 10% FCS, BPS and DTPA do not

Abbreviations: BPS, bathophenanthroline disulfonate; EGF, epidermal growth factor; DTPA, diethylenetriaminepentaacetic acid; Fe_2Tf, diferric transferrin.

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Table 1. Effect of BPS, DTPA, and hydroxyurea (HU) on DNA synthesis by CCl 39 cells stimulated with FCS, ceruloplasmin (CP), or EGF plus insulin

		DNA synthesis, % of maximum		
	Time of exposure	With 10% FCS	With CP (1 unit/ml)	With EGF (10 μg/ml) and 1 μM insulin
BPS (0.3 mM)	During growth	98	17	20
BPS (0.3 mM)	Starvation	93	22	26
DTPA (1 mM)	During growth	15	20	14
DTPA (1 mM)	Starvation	95	20	67
HU (1 mM)	During growth	17	10	2
HU (1 mM)	Starvation	87	96	101

Percentage of maximum [^{3}H]thymidine incorporation with FCS, CP, or EGF plus insulin. Incorporation was as follows: with 10% FCS, 26,260 ± 1109 cpm; with CP (1 unit/ml), 17,911 ± 320 cpm; with EGF plus insulin, 161,183 ± 13,085 cpm. In another experiment, an 80-min starvation treatment with DTPA inhibited incorporation to 43% of maximum on EGF plus insulin after removal of DTPA. Serum starvation was for 24 h in MEM.

Table 2. Effect of Fe_2Tf on growth factor-induced DNA synthesis in CCl 39 cells after iron removal by BPS

Transferrin, μM	Ceruloplasmin		EGF + Ins		Thrombin	
	Fe_2Tf	Apo	Fe_2Tf	Apo	Fe_2Tf	Apo
0.00	23	25	35	32	29	29
0.001	39	25	39	34		
0.01	69	25	72	36		
0.1	114	35	72	38	87	29
1.0	100	43	134	54	102	34
3.0	86	46	72	65	78	36

Cells were treated with 0.3 mM BPS during the 24-h starvation period in MEM and washed to remove BPS. Results indicate percentage thymidine incorporation; 100% represents maximum thymidine incorporation into cells with ceruloplasmin without BPS treatment, which is 170,000 cpm, for EGF plus insulin (ins) it is 165,000, and for thrombin it is 150,000. DNA synthesis was measured as [^{3}H]thymidine incorporation into quiescent CCl 39 cells. Apo, apotransferrin.

give inhibition when present only during starvation. DTPA inhibits like hydroxyurea when present during growth on FCS (Table 1).

Treatment with BPS during starvation inhibits growth initiated with EGF plus insulin or thrombin or ceruloplasmin (Tables 1 and 2). BPS at the same concentration also inhibits DNA synthesis on EGF and insulin and thrombin when present during growth (data not shown).

The BPS treatment does not inhibit uptake of [^{3}H]thymidine. Cells treated with BPS up to 24 h show the same uptake of [^{3}H]thymidine in the cytosol over 2 h (≈4000 cpm) as cells not treated with BPS independent of the mitogen present.

When 5×10^6 cells are incubated at 37°C with 1 mM BPS for 90 min and centrifuged to remove cells, the supernatant shows an absorption increase in the region from 520 to 570 nm with a peak at 540 nm (Fig. 1). This spectrum is consistent with the spectrum of ferrous BPS. During a 90-min incubation period, up to 0.128 nmol of Fe per 10^6 cells can be observed in the supernatant after removal of cells. In contrast, only 0.016 nmol of Fe per 10^6 cells is found when BPS is added to the supernatant after removal of nontreated cells. If cells are lysed at pH 7.4 with 0.025% Triton X-100, the total iron(II) available for chelation with BPS is 0.057 nmol per 10^6 cells. This concentration was calculated by subtraction of absorbance from lysed cells without BPS from absorption of lysed cells with BPS. After exposure of intact cells to BPS for 90 min, the total iron(II) released by detergent treatment is 0.037 nmol per 10^6 cells.

Assay of detergent extract from 0.3 mM BPS-treated cells by adding ferrous iron shows no free BPS present in the cells as measured by an absorbance increase at 535 nm (Table 3). If BPS remained in the treated cells after removal of the medium containing BPS, the internal BPS would be released by the Triton X-100 treatment to react with the added ferrous iron. No increase in absorbance is observed, so no detectable BPS is in the cells.

Ferricyanide reduction can be used to measure transplasma membrane electron transport (11). CCl 39 cells, after 24 h in Hepes buffer (pH 7.5), show reduction with 0.1 mM potassium ferricyanide. After treatment of quiescent cells with 300 μM BPS for 24 h, the ferricyanide reduction rate decreased (Table 4). Incubation of BPS-treated cells for another 24 h with 10 μM ferric ammonium citrate restores ferricyanide reduction to the original rate.

DNA synthesis is restored when low concentrations of ferric or ferrous iron are added to the BPS-treated cells (Figs. 2 and 3). Other trivalent or divalent ions such as aluminum or an anion such as ferricyanide or divalent ions are ineffective for reversal of inhibition. Calcium (1.8 mM) is present in the growth medium throughout, so its removal is not a basis for inhibition. Ferric ammonium citrate (0.3 mM) restores thymidine incorporation into CCl 39 cells after 80 min of exposure to 1 mM DTPA during starvation before growth factor

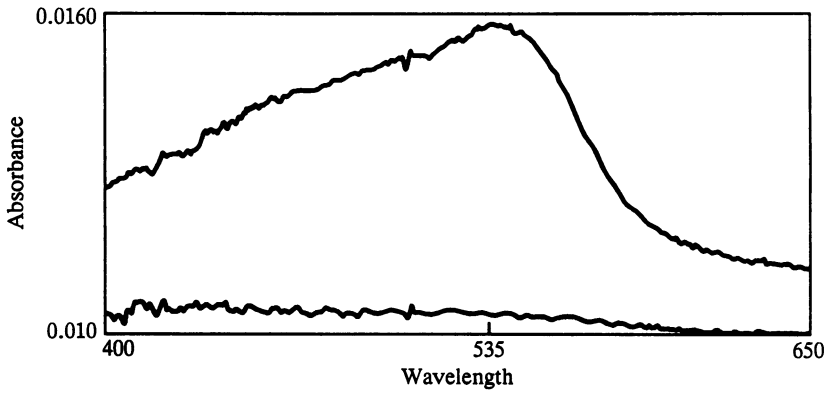

FIG. 1. Absorption spectrum of the supernatant obtained after incubation of CCl 39 cells in 2 mM BPS in phosphate-buffered saline (PBS) (pH 7.4). A confluent culture of CCl 39 cells in a 175-cm^2 flask (≈10^7 cells) was washed twice with PBS and extracted with 2 mM BPS in 6 ml of PBS under slow shaking at 37°C for 60 min in the dark. The medium was decanted and spun for 90 sec at high speed to remove any cells that might have loosened from the flask. Inspection of the flask after extraction showed that the cell layer remained intact and viable. The decanted extract was scanned in a Beckman DK 7500 spectrophotometer for the absorbance between 400 and 650 nm. Baseline was obtained by scanning 2 mM BPS in PBS between the same wavelengths.

Table 3. Assay of cells for the presence of internal BPS after BPS treatment

Treatment	Addition	A_{535} of broken cells	Absorbance increase
Control	None	0.543	
Control	40 μM Fe^{2+}	0.754	0.211
BPS extracted	None	0.561	
BPS extracted	40 μM Fe^{2+}	0.727	0.166

Cells in Hepes buffer (pH 7.5) were starved 24 h without or with 1 mM BPS. Cells were collected and resuspended in buffer with 0.025% Triton X-100 to release cell contents. Absorbance of suspension was measured at 535 nm to determine formation of the ferrous BPS complex.

addition when growth is on thrombin or EGF plus insulin after DTPA removal. Simple removal of DTPA does not restore synthesis. DNA synthesis is not inhibited by DTPA pretreatment when cells are grown on FCS (data not shown).

Fe_2Tf is effective in reversal of DNA synthesis inhibition after BPS treatment regardless of the mitogens used to activate synthesis in the treated cells. The concentration of Fe_2Tf required for complete restoration of DNA synthesis is between 0.1 and 1.0 μM, which is consistent with the binding affinity of the transferrin receptor (12). Similar concentrations of apotransferrin show only slight effects on growth, which may be attributed to adventitious iron (Table 2). Since FCS contains Fe_2Tf, the growth of cells on serum is not inhibited by BPS.

The inhibition of DNA synthesis by impermeable chelators is specific for iron(II) since treatment with Tiron, an iron(III)-specific chelator, at up to 0.3 mM does not inhibit [^{3}H]thymidine incorporation with EGF plus insulin or with 0.01 unit of thrombin when present during starvation and removed during growth. If Tiron (0.3 mM) is present during thymidine incorporation, it inhibits only 20% or less (data not shown).

DISCUSSION

Permeable lipophilic chelators of ferric iron including desferrioxamine, parabactin, and picolinic acid (3–5) are well-recognized inhibitors of DNA synthesis in cells. The effect of these agents, as well as of hydroxyurea, is based on destruction of the iron tyrosine radical in ribonucleotide reductase. Ribonucleotide reductase has two chelator-sensitive iron atoms per molecule (2). 1,10-Phenanthroline, a lipophilic iron(II) chelator, inhibits growth of carcinoma cells on 10% FCS (6). This effect and α,α-dipyridyl inhibition have been related to inhibition of iron uptake by lipophilic iron(II) chelators (12–14), whereas impermeable EDTA and BPS show little inhibition of uptake (15, 16).

DTPA is an impermeable metal ion chelator, which Thelander and his group (personal communication) have shown to inhibit proliferation of TA3H2 cells without inhibition of ribonucleotide reductase. DTPA inhibits growth of Chang cells at 6 mM but does not affect DNA synthesis or dATP or dTTP pools, indicating lack of penetration into the cells (17). Both hydroxyurea and DTPA inhibit CCl 39 cell DNA

Table 4. Inhibition of ferricyanide reduction by BPS treatment of CCl 39 cells and restoration of activity with iron addition

Treatment	Ferricyanide reduction rate, nmol per min per 10^6 cells
Control	1.92
After BPS treatment	0.74
After BPS plus ferric salt	1.97

Control, cells in Hepes buffer (pH 7.5) with no BPS for 24 h. BPS treatment was for 24 h with 300 μM BPS. Restoration was with 10 μM ferric ammonium citrate after removal of BPS.

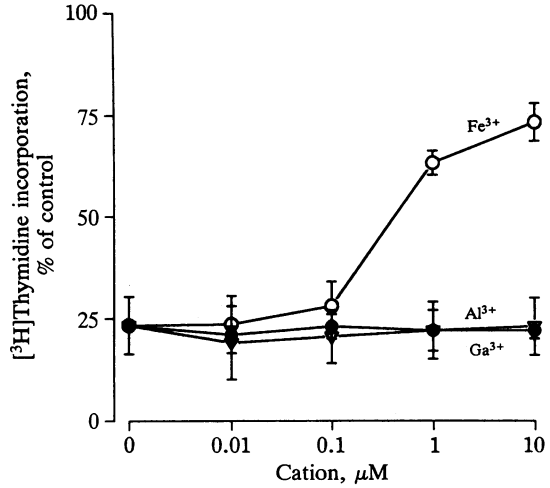

FIG. 2. Effect of trivalent cations on the restoration of EGF plus insulin stimulation of thymidine incorporation after treatment of CCl 39 cells with 0.3 mM BPS during 24-h serum starvation. BPS was removed and cells were washed with MEM before stimulation with EGF (10 ng/ml) plus 1 μM insulin in MEM without serum. The salts used were ferric ammonium citrate, gallium nitrate, and aluminum sulfate. Control ^{3}H incorporation is 210,000 cpm per well.

synthesis if present during growth on FCS or the mitogen ceruloplasmin. Pretreatment during starvation with DTPA also gives inhibition of DNA synthesis with mitogen, in contrast to hydroxyurea, which is not inhibitory after pretreatment. BPS inhibits growth only on mitogen regardless of exposure time, and FCS reverses inhibition under all conditions. The presence of Fe_2Tf in the FCS would supply iron to reverse the inhibition by the BPS during growth or in pretreatment. The continued inhibition with DTPA or hydroxyurea in growth with FCS indicates a stronger effect than simple surface iron removal. The effectiveness of either ferric or ferrous iron in reversal of inhibition after BPS treatment during starvation is consistent with the spectral evidence that iron is removed when cells are exposed to the chelator. The lack of inhibition by the iron(III) chelator Tiron shows that iron(II) is the form available for removal. Iron uptake into cells would be favored by ferrous iron, so the reversal by ferric iron is consistent with action at a surface site (18). A

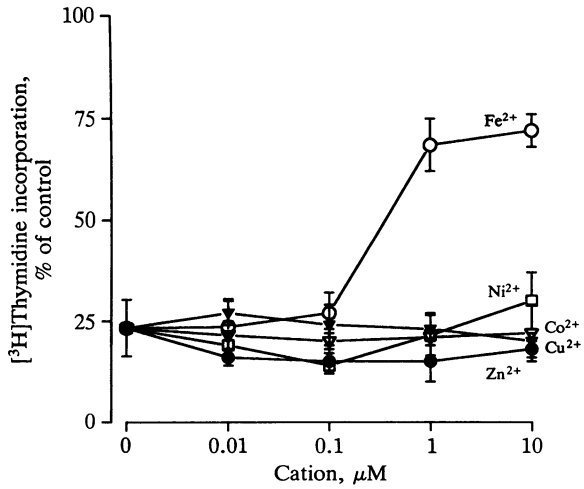

FIG. 3. Effect of divalent cations on restoration of thymidine incorporation after BPS treatment of CCl 39 cells as described for Fig. 2. BPS was removed before addition of the salts ferrous ammonium sulfate, cobalt sulfate, zinc chloride, copper sulfate, and nickel sulfate. Control ^{3}H incorporation was 210,000 cpm per well. Incorporation stimulated with EGF plus insulin is as in Fig. 2.

stoichiometric concentration of iron(II) protects against BPS inhibition when present in growth medium, so the preformed $Fe(BPS)_3$ chelate is not inhibitory (data not shown), and BPS is not taken up into cells to chelate internal iron.

A possible site for the BPS inhibition is in the NADH oxidase in the plasma membrane. BPS inhibits NADH ferricyanide reductase (19) and NADH oxidase (20) in isolated plasma membranes in the same concentration range.

The site in the cell from which iron is removed remains to be clearly established. Maximum extraction of iron from hepatocytes with 1 mM BPS has been shown at 90 min (21). The amount of iron freely available to BPS in lysed CCl 39 cells at neutral pH is limited and is less than the amount extracted during treatment. Short-term washing of cells with BPS removes a small amount of iron, as measured by a decrease in free iron. This iron could be removed either from the surface or from the interior. Some cells, such as BeWo, slowly release 50% of accumulated iron in 30 h to the medium, whereas others, such as HeLa, do not (22). In any event, the iron removal by DTPA and BPS does not give results consistent with those observed with inhibition of ribonucleotide reductase. The release of iron from the plasma membrane could be a basis for the inhibition of electron transport in the plasma membrane, which has been related to cell growth control (7–9). Strongly attached ^{59}Fe is found on the plasma membrane of reticulocytes after incubation with ferric transferrin, consistent with a strong iron-binding site on the plasma membrane (23). A chelator-sensitive iron site on the cell surface can also explain why ferricyanide can stimulate growth of untreated L1210 cells (11) but gives only a small stimulation compared to ferric iron after chelator extraction (24).

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