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IRON AT THE CELL SURFACE CONTROLS DNA SYNTHESIS IN CCl 39 CELLS

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SUMMARY: Treatment of CCl 39 cells with the impermeable iron II chelator bathophenanthroline disulfonate (BPS) inhibits both DNA synthesis and transplasma membrane electron transport. The inhibition persists when the BPS is removed, and the extract from 10^6 cells contains up to 1.28 nmoles iron II chelated to BPS. The BPS iron II chelate itself is not inhibitory. Both DNA synthesis and electron transport are restored by addition of μM iron II or iron III compounds to extracted cells. Other impermeable chelators for iron II give similar inhibition, whereas the iron III-specific Tiron or copper-specific bathocuproine sulfonate do not inhibit. The inhibition differs from the permeable iron III chelator inhibition of ribonucleotide reductase, because inhibition of DNA synthesis by the permeable chelators is reversed when chelator is removed. The response to growth factors also differs, with no impermeable chelator inhibition on 10% fetal calf serum contrasting to inhibition by permeable chelators. DNA synthesis with both activation of tyrosine kinase with EGF plus insulin or by thrombin or ceruloplasmin led to protein kinase C activation as inhibited by the impermeable chelators. It is proposed that an iron available on the cell surface is required for DNA synthesis and plasma membrane electron transport.

Transplasma membrane NADH oxidase has been related to the control of cell growth (1). This association is based on (a) stimulation of cell proliferation and DNA synthesis by impermeable oxidants, (b) stimulation of plasma membrane electron transport by growth factors (2) and oncogenes (3,4), and (c) inhibition of growth and plasma membrane electron transport by antitumor drugs (5). Responses within the cell to activation of transplasma membrane electron transport include transient expression of c *myc* and c *fos* protooncogenes (6), increase in protein

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tyrosine phosphorylation (7), increase in intracellular calcium (8), and activation of the Na^+/H^+ antiport to increase cytosolic pH (9).

A number of prosthetic groups have been recognized in plasma membranes which could participate in electron transport, including flavin, b type cytochromes, coenzyme Q, thiols, copper, and iron (10,11). Evidence for coenzyme Q function has been presented (12). This report describes evidence for a chelate sensitive iron at the plasma membrane which is necessary for electron transport and DNA synthesis in certain cells.

METHODS: Chinese hamster lung fibroblasts (CCl 39) cells were maintained in minimal Eagle's medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 units/ml penicillin, 50 µg/ml streptomycin, and 25 mM sodium bicarbonate at 37° C with 95% air/5% CO_2. Confluent cultures in 24-well plates were rendered quiescent by 24 hr incubation in MEM after washing in MEM. For measurement of DNA synthesis, quiescent cultures were exposed for 24 hr to the indicated growth factor plus 0.25 µCi/ml [^{3}H]thymidine in 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 material precipitated by trichloroacetic acid was extracted from each well with 0.1 N NaOH for 2 hr at room temperature. MEM and FCS were from Flow Labs, ceruloplasmin and transferrin from Sigma, epidermal growth factor (EGF) from Collaborative Research, insulin from Nova Industri, and [^{3}H]thymidine from Amersham Corporation. Diferric transferrin was prepared according to (13).

Ferricyanide reduction was in Hepes salts buffer (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2 mM $CaCl_2$ with 20 mM Hepes pH 7.4 at 37° C and 0.2 mM $K_3Fe(CN)_6$ and 10^6 cells added to start the reaction in 3.0 ml. Absorbance change at 420 minus 500 nm was measured on the Shimadzu dual beam spectrophotometer. The extinction coefficient for ferricyanide is 1 $mM^{-1}cm^{-1}$.

RESULTS

Addition of BPS to media containing EGF plus insulin, thrombin, or ceruloplasmin as growth factors inhibits the DNA synthesis of CCl 39 cells (Fig. 1). Growth on FCS is not inhibited by BPS. The inhibitory effect of BPS reaches a maximum at 0.3 mM. EDTA, another impermeable chelator, inhibits DNA synthesis with ceruloplasmin 62% at 0.3 mM. Other impermeable chelators of iron II inhibit DNA synthesis at the same concentrations as BPS. These include DTPA and ferrozine. The iron III-specific 2,3-dihydroxybenzene sulfonate (Tiron) and copper-specific bathocuproine sulfonate (BCS) inhibit DNA synthesis less than 10% (not shown).

The inhibition by BPS is reversed by inclusion of stoichiometric iron II in the growth media such that 0.1 mM ferrous ammonium sulfate gives 90% reversal with 0.3 mM BPS. Other metals with less affinity for BPS, such as copper II, cobalt II, or aluminum III, give less or no reversal (Table 1).

If quiescent CCl 39 cells are incubated with BPS during a 24 hour serum starvation and BPS is removed before growth factors or FCS are added to initiate growth, the DNA synthesis by the BPS-treated cells is inhibited in the presence of the growth factors EGF plus insulin or ceruloplasmin, but synthesis is not inhibited with FCS. Incubation with and removal of DTPA also inhibits DNA synthesis with ceruloplasmin but not with FCS. On the other hand, hydroxyurea, which inhibits DNA synthesis while present, does not inhibit after removal. For example, when ceruloplasmin gives 17.9 x 10^3 cpm/well [^{3}H]thymidine incorporation, it gives only 9.0 x 10^3 cpm/well after 1 mM DTPA treatment and 8.0 x 10^3 cpm/well after 0.3 mM BPS

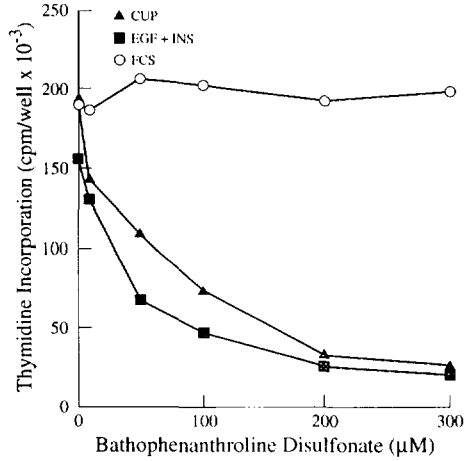

Fig. 1. Effect of BPS on [^{3}H]thymidine incorporation into DNA when DNA synthesis is stimulated by growth factors in serum-free media contrasted to synthesis in FCS. Open circles are 10% fetal calf serum, triangles are 1 U/ml ceruloplasmin, and squares are 10 ng/ml EGF and 1 μM insulin.

treatment and 17.2 x 10^3 cpm/well after 1 mM hydroxyurea. DNA synthesis in the cells treated with chelator for 24 hr can be restored by μM concentrations of iron compounds such as ferric or ferrous ammonium citrate. Diferric transferrin restores activity at concentrations which react with the transferrin receptor (Fig. 2). Apotransferrin is not effective.

Impermeable chelators also inhibit transplasma membrane electron transport under the same conditions and concentrations which produce inhibition of DNA synthesis. Reduction of copper ions is 75% inhibited at 300 μM BPS (14). Ferricyanide reduction by BPS-treated cells after removal of BPS decreases from 1.9 to 0.7 nmoles min^{-1} 10^{-6} $cells^{-1}$ and is completely restored by incubation of the treated cells with 10 μM ferric ammonium citrate.

When CCl 39 cells are exposed to BPS for 90 min under the extraction conditions for inhibition of DNA synthesis and electron transport, an average of 0.9 nmoles per 10^6 cells is measured as the pink iron II BPS chelate in the supernatant after removal of the cells. When cells are centrifuged after incubation without BPS and BPS is added to the supernatant, only 0.16 nmoles per 10^6 cells of iron is found.

Similar inhibition of DNA synthesis by BPS treatment was found in swiss 3T3 cells but not with HeLa cells. In contrast, permeable desferrioxamine is reported to inhibit DNA synthesis by HeLa cells 100% (15) because of inhibition of ribonucleotide reductase.

DISCUSSION

Impermeable chelators with high affinity for iron II inhibit DNA synthesis as measured by [^{3}H]thymidine incorporation in CCl 39 and swiss 3T3 cells. The inhibitory chelators include iron II-specific BPS, ferrozine, DTPA, and EDTA, whereas BCS and iron III-specific Tiron

Table 1. Restoration of the DNA synthesis in the presence of 0.1 mM of different cations. Cells were stimulated with 1 U/ml ceruloplasmin for 24 h in the presence of 0.3 mM BPS. Values are expressed as the percentage of the DNA synthesis found in cells stimulated with ceruloplasmin in absence of BPS and cations. Data are the average of two different experiments made in triplicate.

Cations	DNA Synthesis % Control
None	17
Fe^{2+}	87
Co^{2+}	40
Cu^{2+}	18
Al^{3+}	17

(1,2-dihydroxybenzene sulfonate) have little effect. Inhibition is observed regardless of the pathway involved in activation of DNA synthesis (16). Stimulation of [^{3}H]thymidine incorporation by EGF plus insulin through a tyrosine kinase or by α-thrombin or ceruloplasmin, which act through PKC, are equally inhibited. These growth factors all give over 80% of the growth stimulation of FCS. In contrast, stimulation of DNA synthesis by 10% FCS is not inhibited by the impermeable chelators.

Permeable iron III chelators inhibit DNA synthesis primarily by removal of iron from ribonucleotide reductase (17,18,19). Hydroxyurea inhibits the same enzyme by destruction of the iron tyrosyl radical (20). The inhibition by the permeable chelators and hydroxyurea differs from the impermeable chelators in that the former inhibit when cells are grown in 10% FCS. A further

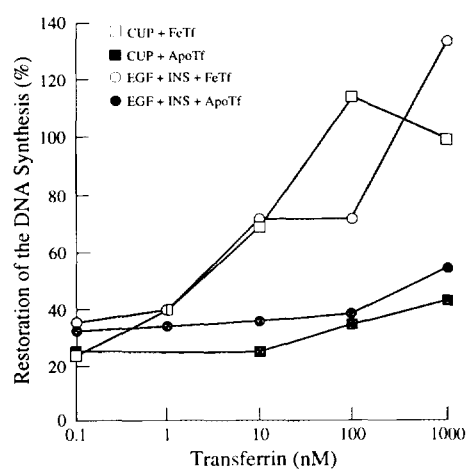

Fig. 2. Effect of diferric transferrin and apotransferrin on growth factor induced DNA synthesis in CCl 39 cells after iron removal by BPS. Filled squares are 1 U/ml ceruloplasmin plus apotransferrin, open squares are ceruloplasmin plus diferric transferrin, open circles are 10 ng/ml EGF plus insulin (1 μM) plus diferric transferrin, and filled circles are EGF plus insulin plus apotransferrin.

difference is seen in that the inhibition by the permeable iron III chelators and hydroxyurea does not persist after removal of the chelator from the cells, whereas the inhibition by impermeable iron II chelators persists after removal of the chelator. A further difference has been found by Thelander and coworkers in that DTPA inhibits TA3H2 cell growth without an effect on the ESR signal of the ribonucleotide reductase (personal communications). DTPA also inhibits Chang cell growth without effect on DNA synthesis or deoxynucleotide pools (21). Formation of an iron II BPS complex occurs when liver cells are exposed to 300 μM BPS (22), as also observed here for CCl 39 cells. EDTA does not inhibit iron uptake (23,24). Iron has been found in plasma membranes (25,26) and is extracted by impermeable chelators (27,28). Since acid treatment is not required for the iron extraction, the iron on the plasma membrane is most likely in a chelate form similar to the iron bound at cys-α-α-cys sites which control activity of tyrosinase (29).

The NADH ferricyanide reductase of erythrocyte membranes (30) and the NADH oxidase of liver plasma membranes are inhibited by treatment with 300 μM BPS, and the inhibition persists after the BPS is removed. Use of ferricyanide and copper as electron acceptors avoids reactivation of the enzyme by compounds which restore iron. Addition of small amounts of iron II or iron III compounds restores the plasma membrane electron transport and DNA synthesis after BPS has been removed. When BPS is still present, the interpretation is more difficult since iron II is needed for reversal of inhibition at sufficiently high concentration to chelate away all the BPS. The reversal of inhibition here is evidence that an iron II BPS chelate is not the inhibitor. The lack of inhibition by BPS in the presence of FCS indicates that low levels of transferrin or ferritin in the serum can restore the extracted iron. Since permeable chelator inhibition of DNA synthesis is released by removal of the chelator without extra added iron, then internal stores of iron must be available to reverse the ribonucleotide reductase inhibition. The requirement for added external iron to reverse the inhibition of DNA synthesis and electron transport by impermeable iron II chelators indicates that internal iron is not readily available to replenish the external site. An external iron site may also explain inhibition of CHO cell DNA synthesis by desferrioamine conjugated to 70 kDa starch (31).

Activation of plasma membrane electron transport stimulates DNA synthesis and cell proliferation (1,6,32,33). The reversible external iron II chelator inhibition of both DNA synthesis and electron transport is evidence for a unique iron component in the redox chain which has a controlling effect on response to agonists which activate both tyrosine kinase and PKC based pathways for proliferation.

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