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# LACTOFERRIN ACTIVATES PLASMA MEMBRANE OXIDASE AND $Na^+/H^+$ ANTIPORT ACTIVITY

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Lactoferrin is a growth stimulant. The basis for this effect is not clear since it is not thought to be involved in iron uptake through endocytosis. Ferric lactoferrin supports external ferrous chelate formation by K562 and HeLa cells, and ferric lactoferrin stimulates the reduction of external ferric iron by cells. Ferric lactoferrin also stimulates NADH oxidase activity in isolated rat liver plasma membranes and stimulates amiloride sensitive proton release from K562 cells. The evidence that ferric lactoferrin can participate in oxidoreduction reactions at the plasma membrane leading to activation of $Na^+/H^+$ exchange provides an alternative explanation for the proliferative effect. 

The requirement for ferric transferrin for cell growth has been attributed to satisfaction of cellular requirements for iron as a constituent of essential catalytic proteins (1-4). The partial replacement of ferric transferrin by an impermeable oxidant, ferricyanide, which does not supply iron for cell growth, introduced the possibility that a second role for ferric transferrin was based on reduction at the cell surface (5,6). A role for ferric transferrin as an external oxidant at the plasma membrane has been supported by evidence that iron in ferric transferrin can be reduced at the level of the plasma membrane (7) and that isolated plasma membranes contain a system which acts as an NADH ferric transferrin reductase or as a ferric transferrin stimulated NADH:oxygen oxidoreductase (4,8,9). Since impermeable oxidants which do not contain iron can replace ferric transferrin in stimulation of serum-free growth of cells (10,11), the oxidoreductase activity in the plasma membrane may be responsible for part of the growth stimulation by diferric transferrin (12,13).

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Ferric lactoferrin also can stimulate cell proliferation even though it is not taken up by the cell through endocytosis which is the normal mechanism for entry of ferric transferrin (14,15). As noted by Roiron-Lagroux and Figarella (16), the basis for the growth stimulation by lactoferrin is unclear. They consider the possible reduction of ferric transferrin at the cell surface as an alternative basis for growth stimulation.

We have examined the reduction of ferric lactoferrin at the plasma membrane of K562 and HeLa cells to see if it can act as an electron acceptor or if it can stimulate NADH oxidase activity in liver plasma membranes. We also have tested ferric lactoferrin for stimulation of proton release by cells analogous to the effects of ferric transferrin.

## Materials and Methods

HeLa cells were grown in α MEM media under 5% $CO_2$ at 37° with 10% fetal calf serum, 100 U penicillin and 170 µg streptomycin per ml. Monolayer cultures were trypsinized and centrifuged at 1500 x g. The cells were taken up in TD-Tris buffer (NaCl 0.14 M, KCl 5 mM, $Na_2HPO_4$ 0.7 mM, Tris base 25 mM, final pH 7.4) to a final cell concentration of 0.1 g cell wet weight per ml (19). K562 cells were grown under 5% $CO_2$ in suspension culture in RPMI-1640 media with 25 mM Hepes, pH 7.0, and 10% fetal calf serum, 11 U $ml^{-1}$ penicillin, 11 mg $ml^{-1}$ streptomycin and 9.4 mg $ml^{-1}$ L-glutamine. Iron saturated ferric lactoferrin (human) and ferric transferrin (human) were obtained from Sigma, St. Louis, MO. The presence of loosely bound iron in the lactoferrin and transferrin was measured by change in absorbance at 535 nm when 100 µmoles of neutral ascorbate were added to 10 µM lactoferrin or transferrin and 10 µM BPS in TD buffer, pH 7.4 (20). Reduction of ferric transferrin and ferric ammonium sulfate was done as described previously (7,20,21). Stimulation of proton release was done as previously described for diferric transferrin (22). The pH of a cell suspension in isoosmolar salts with 0.25 mM Tris chloride was continuously measured with an Orion pH meter and a Corning combination electrode in a jacketed cuvette at 23° bubbled with air to equilibrate $CO_2$. After equilibration, ferric lactoferrin (3 to 17 µM) was added to change the rate of acidification of the media. Calibration of proton release was by addition of 100 nmoles HCl at the beginning and end of each assay.

## Results and Discussion

K562 cells show formation of ferrous bathophenanthroline disulfonate (BPS) when incubated with ferric lactoferrin (Table I). The rate of iron reduction with ferric lactoferrin is more rapid than the rate of iron reduction observed with K562 cells and ferric transferrin. The concentration of ferric lactoferrin required for maximum reduction is over 10 µM. The same relatively high concentration of diferric transferrin has been shown to be required for maximum rate of diferric transferrin reduction by other cells (12,21,25).

In addition to the direct formation of ferrous BPS chelate when cells are incubated with ferric transferrin or ferric lactoferrin, ferric ammonium citrate (FAC) can be reduced by cells and the reduction is stimulated by

TABLE I
Formation of ferrous bathophenanthroline disulfonate chelate by K562 cells

| Addition | $Fe(BPS)_3$ formation rate nmole $min^{-1}$gww $cells^{-1}$ |
|---|---|
| None (cells only) | 0 |
| Ferric lactoferrin 3 μM | 1.8 (2) |
| Ferric lactoferrin 10 μM | 3.5 (2) |
| Ferric lactoferrin 10 μM + anti-lactoferrin (1:100) | 0 |
| Ferric transferrin 10 μM | 1.6 (2) |

Assay in 2.8 ml TD buffer with 10 μM BPS and 0.15 gww cells at 37° with absorbance at 600 nm subtracted from absorbance at 535 nm using the Aminco DW2a spectrophotometer in dual beam phase. The chelate remains in the supernatant if the cells are removed after the reaction. The antibody to lactoferrin was produced in rabbits to human lactoferrin and was purchased from Dakopatts (Santa Barbara, CA).

ferric lactoferrin or ferric transferrin (Fig. 1). The stimulation of FAC reduction has been observed with both K562 and HeLa cells.

Isolated rat liver plasma membranes have a cyanide insensitive, hormone stimulated NADH oxidase which is stimulated by diferric transferrin (7-9).

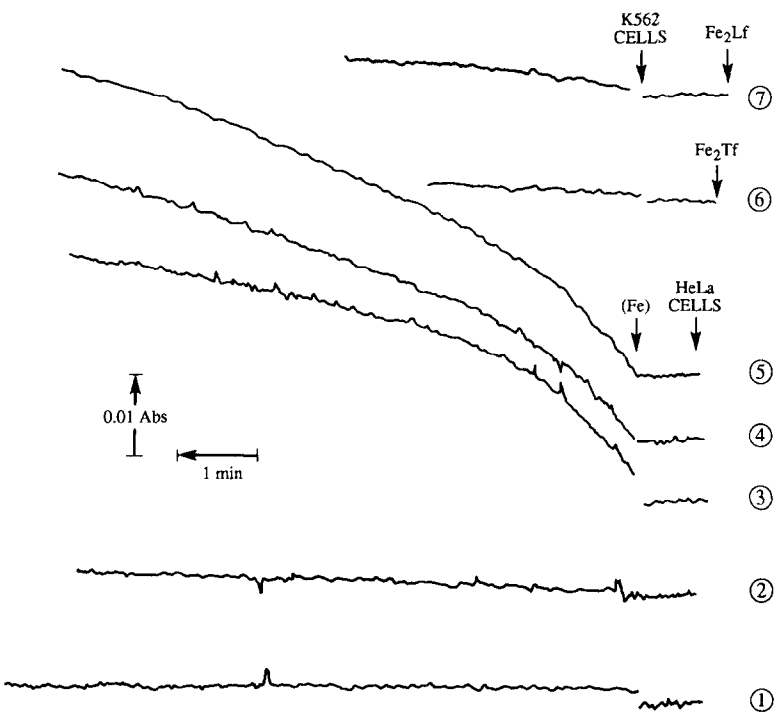


Fig. 1. Spectrophotometric tracing of ferrous bathophenanthroline disulfonate formation by HeLa and K562 cells with diferric lactoferrin and diferric transferrin and the effects on ferric ammonium citrate reduction. (1-5) HeLa cells added first with iron compound added later. (6-7) Ferric lacto or transferrin added first with K562 cell later. Assay in Na (140), K (5), Mg (1), Ca (1) chloride (mM as in parenthesis) with 10 mM Hepes buffer, pH 7.4. Absorbance at 600 nm subtracted from 535 nm in the Shimadzu spectrophotometer dual beam mode. 0.75 x $10^6$ cells for each assay. Ferric lactoferrin ($Fe_2Lf$) and ferric transferrin ($Fe_2Tf$) added to make 14 μM. (1) $Fe_2Tf$, (2) $Fe_2Lf$, (3) FAC, 7.5 μM, (4) FAC + $Fe_2Tf$, (5) FAC + $Fe_2Lf$.

TABLE II

Effect of ferric lactoferrin on NADH oxidase activity of rat liver plasma membrane

| Addition | NADH oxidase nmole $min^{-1}$ mg $protein^{-1}$ |
|---|---|
| Membrane only | 1.5 ± 0.14 (4) |
| Membrane + $Fe_2Lf$ | 2.7 (2) |
| Membrane + $Fe_2Lf$ + Lf antibody (1:20) | 1.2 (2) |
| Membrane + $Fe_2Tf$ | 2.6 |
| Membrane + $Fe_2Tf$ + Tf antibody (1:20) | 0.8 |
| No membrane + $Fe_2Lf$ | 0.0 |

Assay in 2.8 ml Tris chloride, pH 7.4, 50 mM, 1 mM KCN, 25 µM NADH with 0.35 mg membrane protein with absorption at 430 nm subtracted from absorption at 340 nm in the Aminco DW2a dual beam mode. $Fe_2Tf$, holotransferrin, $Fe_2Lf$, holotransferrin. Lactoferrin and transferrin added to 17 µM final concentration. Antilactoferrin antibody as in Table I, 25 µl.

The NADH oxidase in rat liver plasma membranes also is stimulated by ferric lactoferrin at concentrations and extents similar to ferric transferrin (Table II).

Ferric lactoferrin stimulates proton release from K562 cells. This effect has been observed with ferric transferrin with other cells, but with K562 cells the proton release with ferric transferrin is less than with ferric lactoferrin. The proton release is partially inhibited by 0.2 mM amiloride and by antibody to lactoferrin (Table III).

The formation of ferrous BPS in the media by cells is consistent with a transmembrane reduction of ferric lactoferrin. Because of the presence of BPS, which alters the apparent redox potential of the bound iron, the reaction does not measure ferric lactoferrin reduction at the natural redox potential (-450 mV) of free lactoferrin (9). Despite the effect of redox potential

TABLE III

Effect of amiloride and antilactoferrin antibody on $H^+$ release from K562 cells stimulated by ferric lactoferrin

| | Addition | Rate of $H^+$ release nequiv $H^+$ $min^{-1}$ $gww^{-1}$ |
|---|---|---|
| 1. | Ferric lactoferrin 3.4 µM | 2550 (2) |
| | (1) plus amiloride 0.1 mM | 1710 |
| | (1) plus amiloride 0.2 mM | 1250 |
| | (1) plus amiloride 0.3 mM | 330 |
| | (1) plus antibody to lactoferrin (1:1000) | 790 |
| | (1) plus antibody to lactoferrin (1:500) | 0 |
| 2. | Ferric transferrin 10 µM | 501 |

Assay in 3 ml sucrose salts with 0.25 mM Tris chloride, pH 7.4 for stabilization, and 0.01 gww cells. Lactoferrin and transferrin added after equilibration of cells in buffer. Calibration with 100 nmoles HCl before and after transferrin additions. Antibody and amiloride added with cells during equilibration. Antibody as in Table I, 25 µl.

shift, the reduction of iron in lactoferrin demonstrates transplasma membrane electron transport, since ferrous chelate is recovered outside the cell (7,12). The activity is not inhibited by superoxide dismutase, so is not based on superoxide production (23). Inhibition of the reduction by antibody to lactoferrin shows that the ferrous iron does not derive from iron loosely bound to lactoferrin (20). In addition to its action as an electron acceptor, hololactoferrin stimulates the reduction of ferric ammonium citrate (FAC) by HeLa cells (Fig. 1). This stimulation resembles the stimulation of FAC reduction previously observed with holotransferrin (7,12).

The affinity of ferric lactoferrin for the redox system is less than expected for the high affinity binding site on the lactoferrin receptor (18). The tight binding is saturated below 1 μM whereas the redox system requires greater than 10 μM to reach a maximum rate. This suggests that the low affinity binding reported for lactoferrin has a role in the redox activity (16). A similar low affinity site for the ferric transferrin redox has been suggested because of a similar concentration dependence (12). The transferrin response also is not inhibited by monoclonal antibodies which bind at the high affinity site on transferrin receptors, but is inhibited by monoclonal antibodies to epitopes not at the high affinity site (12).

The cyanide-insensitive NADH oxidase in plasma membranes is unique in that it is stimulated directly by hormones or growth factors (24,26) and is inhibited by low concentrations of antitumor drugs (8). The stimulation of NADH oxidase activity by ferric transferrin can be part of the reason that transferrin stimulates growth, since oxygen is required for mammalian cell growth, even in the absence of functional mitochondria (27). Ferric lactoferrin also stimulates NADH oxidase activity in liver cell membranes, and this could be the basis for the reported stimulation of growth by lactoferrin (15,16).

The mechanism by which plasma membrane oxidoreductase stimulates cell proliferation is unknown. Redox activity in the plasma membrane has been shown to affect other cell functions known to be involved in growth control. These effects include induction of c-fos and c-myc protooncogene expression (27,28), activation of specific tyrosine kinase (29), activation of calcium transport (30) and stimulation of the $Na^+/H^+$ antiport (12,22,29). It is clear that ferric lactoferrin activates proton release from K562 cells (Table III). The amiloride inhibition of proton release is consistent with activation of the $Na^+/H^+$ antiport. This indicates that part of the lactoferrin growth stimulation can be based on increase in internal pH (31).

Cell proliferation induced by lactoferrin, which is not taken up by cells (15,16), can be based on activation of an NADH oxidase in the plasma membrane in response to ferric lactoferrin binding at a low affinity receptor. Lactoferrin has been reported not to stimulate growth of non-lymphocyte

derived cells such as HeLa cells, but the concentration used was 0.44 μM, which would give a low electron transport rate with HeLa cells (17). In lymphoid cells, growth stimulation is observed as low as 0.02 μM, which indicates either a higher affinity for stimulation of NADH oxidase or a different site of action than the redox system (18).

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