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Diferric Transferrin Reduction Stimulates the Na^+/H^+ Antiport of HeLa Cells

I. L. Sun[1], R. Garcia-Cañero[3], W. Liu[1], W. Toole-Simms[1], F. L. Crane[1], D. J. Morré[2], and H. Löw[4]

[1]Departments of Biological Sciences and [2]Medicinal Chemistry, Purdue University, West Lafayette, Indiana, 47907
[3]Servicio de Bioquimica Experimental, Clinica Puerta de Hierro, 28035 Madrid, Spain
[4]Endocrinology Department, Karolinska Institute, S104 01 Stockholm, Sweden

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Summary: Proton release from HeLa cells is stimulated by external oxidants for the transplasmalemma electron transport enzymes. These oxidants, such as ferricyanide and diferric transferrin, also stimulate cell growth. We now present evidence that proton release associated with the reduction of ferricyanide and diferric transferrin is through the Na^+/H^+ antiport. The stoichiometry of H^+/e^- release with diferric transferrin is over 50 to 1, which is greater than expected for oxidation of a protonated transmembrane electron carrier. Diferric transferrin induced proton release depends on external sodium and is inhibited by amiloride. Proton release is also inhibited when diferric transferrin reduction is inhibited by apotransferrin. A tightly coupled association between the redox system and the antiport is shown by sodium dependence and amiloride inhibition of diferric transferrin reduction. The results indicate a new role for ferric transferrin in growth stimulation by activation of the sodium-proton antiport.

External oxidants such as ferricyanide and diferric transferrin, which can be reduced by the transplasma membrane electron transport system, stimulate the growth of HeLa and other cells in serum deficient media (1-6). Reduction of ferricyanide by HeLa and other cells is accompanied by proton release from the cells (7-9). This proton release by HeLa cells under the conditions used (10 mM NaCl) was in the range of two to three protons released per ferricyanide reduced or an H^+/e^- ratio less than 3 (8). Recent studies by Garcia-Cañero and coworkers (10,11) have shown that in young HeLa and liver cells the rate of ferricyanide reduction is stimulated by

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high sodium concentration (>50 mM), and is accompanied by sodium uptake and is inhibited by amiloride, indicating that the ferricyanide reduction system is connected to the Na^+/H^+ antiport. Diferric transferrin is a natural electron acceptor in serum which is reduced by the transplasmalemma electron transport system in conjunction with the transferrin receptor (4,12,13). We now show that the reduction of diferric transferrin by HeLa cells is accompanied by a remarkably rapid proton release from the cells with an H^+/e^- ratio over 50. Sodium dependency and amiloride inhibition indicate that this redox induced proton flow is mediated through the Na^+/H^+ antiport (14). Activation of proton release by the Na^+/H^+ antiport and consequent alkalinization of the cytoplasm has been related to the mitogenic action of growth factors (15-17). Growth stimulation by the transplasmalemma NADH diferric transferrin reductase (12,18) can therefore be related to activation of the Na^+/H^+ antiport.

Materials and Methods: HeLa cells, S3 strain, were grown in roller bottles on Joklik minimal essential media in liquid culture at pH 7.4 at 37° with 10% serum, 100 μ penicillin and 100 μg/ml streptomycin. Cells were harvested by centrifugation and taken up in TD-tris buffer (150 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4 and 3.0 g/l Trizma base, pH 7.4) to a final cell concentration of 0.2 g wet weight (gww) per ml. Cell survival was determined by eosin Y exclusion.

Reduction of iron in diferric transferrin was determined by the formation of ferrous bathophenanthroline disulfonate based on the procedure of Avron and Shavit (19). 10-20 mgww cells were suspended in appropriate salt solutions with 3.4 μM bathophenanthroline sulfonate (BPS) and inhibitors as required. The reaction was started with addition of diferric transferrin to 17 μM concentration. Absorbance change was measured with the dual beam of the Aminco DW2a spectrophotometer at 535 minus 600 nm. At these wavelengths the extinction coefficient difference for ferrous bathophenanthroline sulfonate is 17.1 $mM^{-1}cm^{-1}$. The reaction can be started either by addition of cells or diferric transferrin. Ferricyanide reduction was measured with the dual beam at 420 minus 500 nm in appropriate salt solutions with 0.1 mM ferricyanide and 10-20 mgww of cells. Extinction coefficient is 1 $mM^{-1}cm^{-1}$. All reactions were at 37°. Proton release was measured in a water jacketed 2 ml cuvette with an Orion 701A pH meter and a Corning combination glass electrode with 10 mgww cells in appropriate salt solutions buffered with 1.5 mM tris chloride at starting pH 7.4. Air was bubbled continuously to remove CO_2. The reaction was started with diferric transferrin (17 μM) or ferricyanide (0.1 mM). Cells were incubated with inhibitors for 3 min before assay.

NADH diferric transferrin reductase was determined by disappearance of NADH, 20 μM, at 340 minus 430 nm, in presence of 0.05 mg rat liver plasma membrane in 2.5 ml salt solutions with 25 mM tris Cl, pH 7.4. 1.0 mM KCN is present to inhibit any mitochondrial oxidase. Diferric transferrin was prepared according to Karin and Mintz (20) or obtained from Miles Laboratories. Apotransferrin and other reagents were from Sigma. The B3/25 monoclonal antibody to the transferrin receptor was from Hybritech. Goat antihuman transferrin antibody was from Cooper Biomedical Co.

Plasma membranes were prepared from rat liver by centrifugation on sucrose gradients and characterized by marker enzymes and morphometric analysis (21).

Results and Discussion: Both ferricyanide reduction and diferric transferrin reduction by HeLa cells is faster in 150 mM sodium chloride than in 150 mM choline chloride. The rate in sucrose is also slower, but with ferricyanide as electron acceptor this may partly reflect the repulsion of the ferricyanide by the negative charge on the cell surface, as discussed previously (Ref. 9; Table I). The sodium chloride stimulation of diferric transferrin reduction increases up to 100 mM NaCl (Fig. 1 or Table I). Ferricyanide reduction is also stimulated.

Proton release induced by both ferricyanide and diferric transferrin is greater in sodium chloride than in choline chloride (Table I). The ratio of nanoequivalents H^+ released to nanomols acceptor reduced is increased in sodium chloride for both acceptors, but the H^+/e^- for transferrin is much higher than for ferricyanide.

Amiloride, which is an inhibitor of the sodium dependent proton antiport, inhibits proton release induced by both ferricyanide and diferric transferrin. The amiloride also inhibits electron transport across the plasma membrane to both acceptors (Table II).

Specific inhibitors for diferric transferrin reduction by HeLa cells

Table I. Effect of sodium ions on the rate of ferricyanide and diferric transferrin reduction and associated proton release by HeLa cells (2 day culture)

	Acceptor ferricyanide		
	Reduction rate neq Fe $min^{-1}gww^{-1}$	H^+ release rate neq H^+ $min^{-1}gww^{-1}$	H^+/e^-
250 mM Sucrose	131	133	1
150 mM NaCl	261	273	1.1
150 mM Choline Chloride	160	132	0.8
	Acceptor diferric transferrin		
	Reduction rate	H^+ release rate	H^+/e^-
250 mM sucrose	4.9	200	41
150 mM NaCl	13.0	833	64
150 mM Choline Cl	5.6	0	0

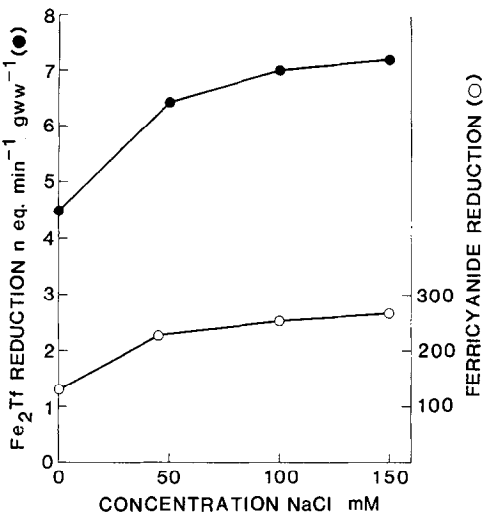

Fig. 1. Effect of Na^+ concentration on the rate of diferric transferrin and ferricyanide reduction by HeLa cells. Cells grown 48 hr. Assay in sodium chloride at indicated concentration with sucrose to make the final concentration equiosmolar to 250 mM sucrose. Diferric transferrin and ferricyanide reduction determined as described under methods.

such as apotransferrin, goat antihuman transferrin antibody and the B3/25 monoclonal antibody for the transferrin receptor (12) also inhibit the H^+ release induced by diferric transferrin (Table III). When ferricyanide is added to HeLa cells in media containing sodium ions in presence of ouabain there is a rapid uptake of sodium ions by the cells (10).

To test if the inhibition of diferric transferrin reduction through the plasma membrane by amiloride was a direct effect on the dehydrogenase, the effect of amiloride on the NADH diferric transferrin reductase activity of rat liver plasma membrane was tested (Table IV). It is clear that the NADH diferric transferrin reduction by rat liver plasma membrane is strongly inhibited by amiloride.

Table II. Amiloride inhibition of ferricyanide and diferric transferrin reduction and stimulated proton release by HeLa cells

	Reduction n eq $min^{-1}gww^{-1}$		H^+ release n eq $min^{-1}gww^{-1}$	
	$Fe(CN)_6$	Fe_2Tf	$Fe(CN)_6$	Fe_2Tf
Control	160	28	190	880
Amiloride 0.2 mM	105	9.3	102	456

Table III. Effect of apotransferrin and transferrin antibody on the rate of H^+ release stimulated by diferric transferrin with HeLa cells

	Fe_2Tf induced H^+ release n eq $min^{-1}gww^{-1}$
Control	1923
Apotransferrin (17 μM)	800
Transferrin antibody (75 μg/ml)	0
B3/25 antibody to Tf receptor (54 μg/ml)	625
Apotransferrin only, no Fe_2Tf	0

All activity induced with 17 μM diferric transferrin, in 100 mM NaCl, 83 mM sucrose buffer.

The rate of diferric transferrin reduction by HeLa cells is much slower than the rate of ferricyanide reduction, but the proton release induced by diferric transferrin is much greater than with ferricyanide. The H^+/e^- ratio with diferric transferrin as external electron acceptor is much higher than with ferricyanide and is so high that it suggests opening of a channel for protons rather than anisotropic oxidation-reduction of a protonated electron carrier. Garcia-Cañero and coworkers (10,11) have shown that ferricyanide reduction by HeLa and liver cells is stimulated by sodium ions and is amiloride sensitive at early stages of growth. They also show that ferricyanide stimulates sodium uptake, which indicates a connection between the redox system and the Na^+/H^+ antiport. Since the proton release induced by ferricyanide and diferric transferrin is also strongly dependent on high sodium ion concentration and is inhibited by amiloride, our evidence is consistent with the view that the transplasmalemma electron transport system can activate the Na^+/H^+ antiport. The major part of the proton release accompanying reduction of external oxidants would be through this antiport and would account for the high H^+/e^- ratio.

Table IV. Inhibition of NADH diferric transferrin reductase activity of rat liver plasma membrane by amiloride

Addition	NADH diferric transferrin reduction nmol NADH min^{-1} mg $protein^{-1}$
Control	17.5
0.2 mM amiloride	4.8
17 μM apotransferrin	8.1

Assay with 17 μM diferric transferrin in 50 mM $NaPO_4$ buffer, pH 7.0.

The antiport has been shown to be activated by a proton binding site on the cytoplasmic side of the membrane (14). Since the transmembrane dehydrogenase will release protons during the oxidation of cytosolic NADH, it could act as a source of protons for activation of this allosteric activation site. If the dehydrogenase is a flavoprotein or a pteridino protein then proton release may come at a site deeper in the membrane (22), which would be protected from rapid equilibration with the cytoplasm and would permit activation when the cytoplasmic bulk pH is alkaline.

Activation of the Na^+/H^+ antiport by diferric transferrin would provide a further basis for diferric transferrin stimulation of growth other than a role only in iron transport to the cell. It remains to be seen if this activation is direct or through activation of other signals such as phospholipase c activation (15). If the action is direct, then hormone activation of the antiport may be mediated through the electron transport system and it would be expected that hormones such as PDGF which activate the antiport in appropriate cells would increase the rate of diferric transferrin reduction (15). A tight coupling between electron transport and the antiport is evidenced by the activation of electron transport by high sodium and inhibition of electron transport by amiloride. If the redox system was distant from the antiport, a control of electron transport through the external sodium site would be unlikely (23). The effect of insulin in growth stimulation at the IGF site may be based on increased exposure of transferrin receptors (24) to increase electron transport across the membrane (8).

The inhibition of diferric transferrin reduction by amiloride in the whole cell might be accounted for on the basis of tight coupling between the redox system and the antiport. The inhibition of the transferrin reduction by isolated membranes in the absence of sodium ions indicates that the amiloride effect may be by direct interaction with the dehydrogenase. This amiloride inhibition of the electron transport is consistent with previous

inhibition observed with liver cells using ferricyanide as electron acceptor (10).

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