Journal of Bioenergetics and Biomembranes, Vol. 14, Nos. 5/6, 1982

Insulin Control of a Transplasma Membrane NADH Dehydrogenase in Erythrocyte Membranes

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Received April 2, 1982; revised May 18, 1982

Abstract

A study of NADH ferricyanide reductase activity in oriented vesicles or open ghosts of human and porcine erythrocytes shows that the dehydrogenase activity can have three types of orientation in the membrane. There is activity which responds only to acceptors and NADH exclusively on the inside face, or exclusively on the outer surface. There is also activity which requires exposure of both sides of the membrane and thus is transmembranous. The transmembrane activity is inhibited by insulin, whereas the internal and external enzymes do not respond to insulin. The transmembrane dehydrogenase can be a basis for proton transport in the plasma membrane.

KEY WORDS: Plasma membrane; NADH dehydroganase; erythrocyte membrane; insulin; transplasma membrane dehydrogenase; human erythrocyte; porcine erythrocyte; hormone action.

Introduction

The mechanism by which insulin transmits its message through the plasma membrane is unknown. Several effects of insulin have been observed which have been suggested to be related to its mode of action. These include release of a low-molecular-weight compound from the plasma membrane (Czech, 1981; Seals and Jarett, 1980; Kiechle *et al.,* 1981), production of hydrogen peroxide (May and DeHaën, 1979), and changes in membrane thiols (Mukherjee and Mukherjee, 1981) or stimulation of phosphodiesterase and inhibition of adenylate cyclase (Hepp and Renner, 1972; Illiano and Cuatrecasas, 1972; Torres *et al.,* 1978; Zinman and Hollenberg 1974).

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Inhibition of a calcium-stimulated ATPase has also been described (Pershardsingh and MacDonald, 1979). We have previously shown insulin inhibition of NADH dehydrogenase activity in liver and fat cell membranes (Goldenberg *et al.*, 1978; Löw *et al.*, 1978). Subsequently it has been found that the NADH dehydrogenase actitity in plasma membrane has three different orientations in the membrane (Löw *et al.*, 1979; Cherry *et al.*, 1981; Clark *et al.*, 1981). The study of enzyme orientation is favored in mammalian erythrocyte plasma membranes because procedures have been developed for preparation of open ghosts (sheets), sealed inside-out vesicles, and resealed right-side-out ghosts (Steck and Kant, 1974). With these three orientations of the membrane, one can study the activity of dehydrogenases which are exclusively internal, exclusively external, and tranmembranous. The lack of other membrane types in the erythrocyte eliminates problems of enzyme activity on contaminating membranes.

In this paper we describe the effect of insulin on the transmembranous NADH dehydrogenase of human and porcine erythrocyte plasma membrane.

Methods

Human erythrocytes were obtained from volunteers and pig erythrocytes from a local abattoir. Washed open ghosts and sealed vesicles with inside-out and right-side-out (resealed) orientation were prepared according to Steck and Kant (1974). Acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase, assayed with and without 0.2% Triton X100, were used as markers for the exposure of the outer and inner surface, respectively, of the erythrocyte membranes. Assay of NADH ferricyanide reductase, NADH cytochrome *c* reductase, and NADH indophenol reductase activities were carried out as previously described (Crane *et al.*, 1956; Crane and Löw, 1976). Human erythrocyte membranes were assayed in 0.1 M sodium phosphate, pH 7.0. In some ferricyanide reduction experiments more dilute buffer was used with no significant effect on insulin inhibition. Potassium ferricyanide (0.25 mM) and NADH (20 μM) were used. A split-beam spectrophotometer was used for all human membrane assays. The extinction coefficients used were: ferricyanide, 1.0 mM^{-1} cm^{-1} at 420 nm; cytochrome *c*, 19.5 mM^{-1} cm^{-1}; and 2,6-dichloroindophenol, 20 mM^{-1} at 600 nm. Porcine membrane assays were carried out in a dual mode using the difference between absorption at 420 and 500 nm for ferricyanide reduction at 37°C. For porcine membrane all assays were in 0.05 M sodium phosphate, pH 7.0.

Assay of ferricyanide reduction in intact pig erythrocytes was in "standard medium" as described by Orringer and Roer (1979) with 1 mM

ferricyanide. Ferrous chelate was measured at 535 nm. Acetylcholinesterase and glyceraldehyde-3-phosphate dehydrogenase were assayed by the method of Steck and Kant (1974). Pork insulin was obtained from Novo and Eli Lilly Co.

Results

Both human and porcine erythrocyte membranes show NADH dehydrogenase actitivy. The best activity is found using ferricyanide as an acceptor, whereas activity with cytochrome *c* or 2,6-dichloroindophenol as acceptor is relatively low (Table I).

With oriented preparations of human erythrocyte membranes, it is possible to determine the membrane surface with which the dehydrogenase activity is associated. As shown previously by Kant and Steck (1972), the NADH cytochrome *c* reductase activity is seen only with open ghosts or sealed wrong-side-out vesicles, and the activity is not changed by detergent treatment, which indicates a position exclusively on the cytoplasmic surface. The NADH cytochrome *c* reductase is revealed by detergent treatment of right-side-out vesicles. Since ferricyanide is impermeable to erythrocyte membranes (Mishra and Passow, 1969), the orientation of the NADH ferricyanide reductase can also be determined. Contrary to the pattern shown by NADH cytochrome *c* reductase, the ferricyanide reductase is seen with all membrane orientations (Table II). There is a very low activity on the external surface. The activity seen with sealed wrong-side-out vesicles is increased by Triton X100 treatment to approach the activity seen with open ghosts. The exposure of new ferricyanide reductase activity by detergent treatment of wrong-side-out vesicles may partly be accounted for by exposure of an external enzyme and partly by the presence of an NADH ferricyanide reductase which requires exposure of both surfaces in order to react with the NADH and ferricyanide, respectively. In other words, the presence of a transmembranous dehydrogenase is indicated, which would be consistent with ferricyanide

Table I. NADH Dehydrogenase Activity of Washed Erythrocyte Ghost Preparations

	Dehydrogenase activity	
Reaction	Human erythrocyte (nmol/mg/min)	Pig erythrocyte (nmol/mg/min)
NADH ferricyanide reductase	455 ± 32 (5)	250
NADH cytochrome *c* reductase	13 ± 3 (5)	4
NADH indophenol reductase	37 ± 8 (4)	7
NADPH ferricyanide reductase	0	0
Succinate cytochrome *c* reductase	0	0

Table II. Effect of Erythrocyte Membrane Orientations on NADH Ferricyanide Reductase Activity

	Enzyme activity (nmol/min/mg)		
Activity	Open membrane	Right-side-out vesicle	Wrong-side-out vesicle
NADH → ferricyanide	420	80	220
NADH → ferricyanide plus Triton X100	400	350	360
NADH → cytochrome *c*	7.5	0	4.2
NADH → cytochrome *c* plus saponin	8.8	6.7	4.2
Acetylcholinesterase[a]	1.2	0.9	0.25
Acetylcholinesterase plus Triton X100	1.2	0.9	1.5
Glyceraldehyde-3-phosphate dehydrogenase[b]	1.8	0.2	1.8
Glyceraldehyde-3-phosphate dehydrogenase plus Triton X100	1.8	1.4	2.2

[a] Marker for outer surface of erythrocyte membrane.
[b] Marker for inner surface of erythrocyte membrane. For opening vesicles 0.2% Triton X100 was added to the assay mixture. For cytochrome *c* reductase activity 0.01% saponin was used in place of Triton X100 to avoid inhibition of the enzyme. Ferricyanide and cytochrome *c* reductase rates are for one-electron transfer.

reduction by intact erythrocytes (Mishra and Passow, 1969). Diazobenzene sulfonate inhibition of part of the NADH ferricyanide reductase after exposure of intact cells to the reagent (MacKellar *et al.*, 1979) also indicates a transmembrane enzyme.

Insulin inhibits the NADH dehydrogenase activity of both human and porcine erythrocyte ghosts (Table III). Maximum inhibition is seen at 30–70 μU insulin per milliliter for both NADH ferricyanide and NADH indophelol reductase activity. There is no inhibition of the NADH cytochrome *c* reductase (Figs. 1 and 2). The inhibition produced by insulin has not been seen on addition of other proteins. For example, beef serum albumin shows no inhibition when added at concentrations from 0.03 up to 0.2 mg/ml. As

Table III. Effect of Insulin on NADH Dehydrogenase Activity of Erythrocyte Ghosts[a]

Erythrocyte membrane	NADH dehydrogenase acceptor	Maximum percent inhibition at 30–70 μU/ml insulin, mean and standard deviation
Human	Ferricyanide	23.8 ± 8.5 (18)
Human	2,6-Dichloroindophenol	19.3 ± 11.3 (8)
Human	Cytochrome *c*	2.3 ± 2.9 (4)
Porcine	Ferricyanide	20.5

[a] Assays as described in Methods.

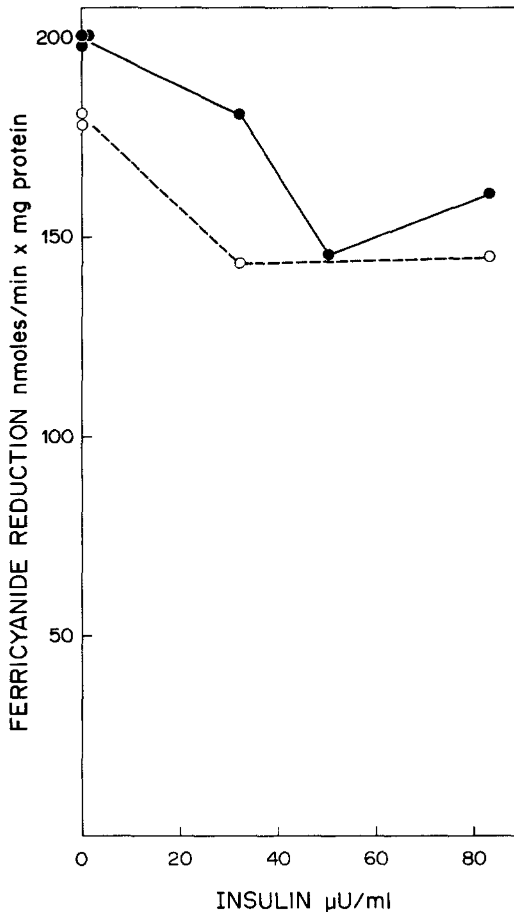

Fig. 1. Insulin inhibition of ferricyanide reduction by human erythrocyte ghosts. Assay as described in Methods except 1 mM sodium phosphate buffer (●) or 15 μM adenosine (O) added. Insulin incubated with membranes 2 min before addition of ferricyanide.

observed with other plasma membranes (Löw *et al.*, 1978; Goldenberg *et al.*, 1978), the insulin inhibition reaches a maximum at 30–70 μU/ml, and at higher concentrations the inhibition is decreased. The effect of adenosine was tested since it has been suggested that adenosine may increase sensitivity of adipocytes to insulin (Green *et al.*, 1981).

Insulin inhibition is most apparent with erythrocyte ghost membranes in which both sides of the membrane are exposed (Table IV). Inside-out vesicles show almost no effect of insulin. No effect of insulin has been seen on the very

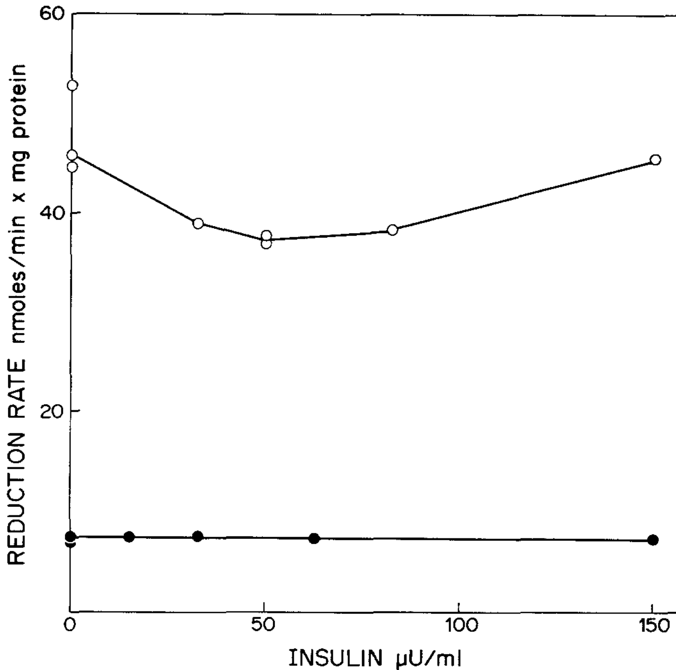

Fig. 2. Insulin inhibition of NADH indophenol reductase of human erythrocyte ghosts (○), and lack of effect on NADH cytochrome *c* reductase (●). Assays as described in Methods.

low NADH ferricyanide reductase activity of right-side-out vesicles. Transmembrane ferricyanide reduction can also be studied by using intact erythrocytes to reduce external ferricyanide. A very good insulin inhibition of the ferricyanide reduction by intact pig erythrocytes of up to 38% can be shown (Fig. 3).

Table IV. Insulin Inhibition of NADH Ferricyanide Reductase with Oriented Porcine Erythrocyte Membranes[a]

	Percent inhibition	
Insulin (porcine) concentration (μU/ml)	Open ghosts	Inside-out sealed vesicles
10	16	0
20	20	0
25	25	0
40	18	4
50	12	4

[a]Assay in 50 mM $NaPO_4$, pH 7.0. Open ghosts, NADH ferricyanide reductase 208 nmol/min/mg. Inside-out vesicles, ferricyanide reductase 111 nmol/min/mg.

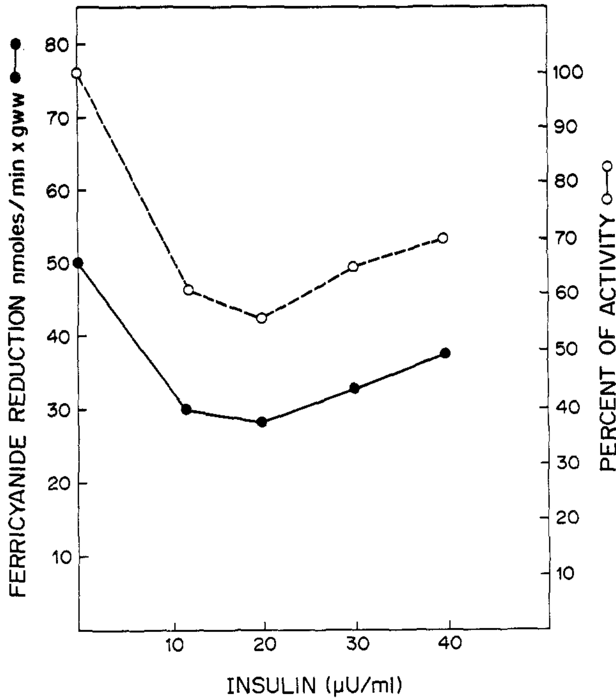

Fig. 3. Insulin inhibition of ferricyanide reduction by pig erythrocytes. Assay as described in Methods by formation of ferrous bathophenanthroline sulfonate by reduction of ferric iron by ferrocyanide formed in the reaction.

Discussion

Dormandy and Zardy (1965) presented evidence that insulin could prevent acidification of the external media by erythrocytes exposed to ferricyanide. The effects were seen at high levels of insulin (500 μU/ml) but would be consistent with an effect of insulin on a transmembrane ferricyanide reductase in which the reduction of external ferricyanide would be accompanied by an outward movement of protons. This movement of protons could be analogous to the redox-driven transfer of protons across the mitochondrial membrane (Reynafarje *et al.*, 1976; Boyer *et al.*, 1977).

The data we present are consistent with insulin inhibition of a transmembranous NADH ferricyanide reductase. No inhibition of the external NADH ferricyanide reductase is seen. There is also no significant inhibition of the ferricyanide reductase on inside-out vesicles, but if reaction with an external insulin receptor (Grambhir *et al.*, 1978) is necessary for inhibition, then

inhibition would not be expected since the receptor would be sequestered. Since the internal ferricyanide reductase presumably represents the dehydrogenase activity of the NADH cytochrome b_5 reductase on the inside of the membrane (Strittmatter and Velick 1956; Schafer and Hultquist, 1981), then the fact that NADH cytochrome *c* reductase in open ghosts is not inhibited by insulin is better evidence that insulin cannot inhibit the internal enzyme since insulin can react with receptor in open membranes.

Insulin inhibition of ferricyanide reduction by whole erythrocytes is also consistent with insulin inhibition of a transmembrane dehydrogenase. The problem here is that it is difficult to know the substrate on the inside of the cell in whole cell studies. It remains to be conclusively demonstrated that all or part of the ferricyanide reduction by whole cells is based on oxidation of NADH since internal glutathione has also been proposed as a substrate for this reaction (Arese *et al.*, 1972). It is doubtful that the activity we observed with pig erythrocytes can be based on transmembrane migration of an ascorbate–dehydroascorbate couple (Orringer and Roer (1979) since addition of dehydroascorbate to whole pig erythrocytes only increases ferricyanide reduction by 10% (MacKellar, 1981).

The evidence is quite clear that with isolated plasma membranes the transmembrane NADH dehydrogenase is inhibited by physiological levels of insulin. It is also clear that the reduction of ferricyanide by the intact erythrocytes is inhibited by the same low concentrations of insulin. A method for definition of the internal electron donor in whole cells is needed to show that the effect in whole cells is the same as that seen on the NADH dehydrogenase in the isolated membranes.

There are diverse proposals for the mechanism of insulin action. The transmembrane dehydrogenase provides a suggestion for the mechanism of the immediate postreceptor action of insulin constituting a framework in which all other mechanisms can be placed. The redox-mediated proton migration through the membrane would be a basis for insulin-induced membrane potential changes, calcium transport modifications, or activation of proteolysis. The redox function itself may be related to disulfide reduction or formation of hydrogen peroxide. Thus the actions of insulin discussed in the Introduction can be related to the insulin-sensitive dehydrogenase which we describe here.

Acknowledgments

This research was supported by grants AM25235 from the National Institute of Arthritis, Metabolic, Digestive, and Kidney Diseases and GMk621839 (F. L. C.). Research in Stockholm was supported by the Swedish Medical Research Council and Nordisk Insulin Fond.

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