Biochimica et Biophysica Acta, 946 (1988) 1–4
Elsevier

BBA 74228

Transmembrane ferricyanide reductase activity in Ehrlich ascites tumor cells

Miguel A. Medina, Francisca Sánchez-Jiménez, Juan A. Segura and Ignacio Núñez de Castro

Laboratorio de Bioquímica, Facultad de Ciencias, Universidad de Málaga, Málaga (Spain)

(Received 4 July 1988)

Key words: Ferricyanide reductase; Antitumor drug; (Ehrlich tumor cell)

A transmembrane ferricyanide reductase activity was assayed in intact Ehrlich ascites tumor cells. Kinetic measurements gave a K_m of 0.14 mM and a V_{max} of 0.31 μmol/min per 10^6 cells. In short-term batch experiments, this activity was enhanced in the presence of 10 mM lactate, a source of cytosolic NADH. The transmembrane redox activity was accompanied by alkalinization of the cytosol. Both ferricyanide reduction and proton extrusion were diminished in the presence of 0.2 mM amiloride. Several cytotoxic drugs significantly inhibited the ferricyanide reductase activity at concentrations at which they show antineoplastic activity.

Introduction

Plasma membrane redox activities have been found in a number of eukaryotic cells, including yeasts [1], plant cells [2–5] and animal cells [6–8]. These activities can now be related to several vital functions [9]. One of these functions is the control of cell growth. As a matter of fact, stimulation of cell growth has been shown to occur when the transplasma membrane redox system is active.

Transplasma membrane electron-transport activity is usually depressed in tumor or transformed cells compared with nontransformed control cells [10] and is inhibited by antitumor drugs at concentrations which inhibit cell growth [9].

Recently, a NADH-ferricyanide reductase activity has been shown in plasma membrane vesicles from Ehrlich ascites tumor cells [11]. In this report, this ferricyanide reductase transmembrane activity is studied in intact cells, focusing the attention in the role of NADH-yielding substrates, the connection between ferricyanide reduction and proton release and the inhibitory effect of some antitumor drugs.

Material and Methods

Hyperdiploid Lettré Ehrlich ascites tumor cells were grown in the peritoneal cavity of 2-month-old Swiss female mice, and harvested as described elsewhere [12]. The harvested cells were suspended in a phosphate-buffered saline with 11 mM phosphate, 6 mM KCl and 154 mM NaCl (pH 7.4). Cellular density was adjusted to $120 \cdot 10^6$ cells/ml. The cells were preincubated at 37°C in a 50 ml flask under an atmosphere of 95% O_2 and 5% CO_2 for 15 min. The suspension was then diluted to $60 \cdot 10^6$ cells/ml in flasks containing the buffered saline, ferricyanide and metabolic substrates. The incubations were carried out at 37°C in a Grant metabolic incubator with continuous shaking (140 strokes/min). Aliquots of 1 ml were removed from the flasks at specific times, poured into

Correspondence: M.A. Medina, Laboratorio de Bioquímica, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain.

Eppendorf tubes and centrifuged at 12800 × *g* for 2 min in a Centaur micro-centrifuge. Ferricyanide reduction was followed by measuring the absorbance at 420 nm in the supernatants. The ferricyanide absorption coefficient is taken as 1 $cm^{-1} \cdot mM^{-1}$. Extracellular recovery of ferricyanide was tested by measuring the absorbance of the supernatants at 420 nm after oxidation by persulfate.

Kinetics measurements were carried out in a Beckman DU-8B spectrophotometer in the program mode dual wavelength analysis at 420 nm minus 500 nm.

Cytosolic and extracellular pH were monitored with neutral red as described by Bashford [13].

To determine the effects of amiloride on ferricyanide reduction and proton extrusion, cells were preincubated with 0.2 mM amiloride for 10 min; afterwards, ferricyanide was added and cells were incubated for 15 min.

Results and Discussion

The plasma membrane ferricyanide reductase activity of intact Ehrlich ascites tumor cells has been studied in pH and temperature conditions considered as optima for this activity in plasma membrane vesicles [11]. Data in Table I show the time course of ferricyanide reduction by intact Ehrlich cells incubated with no other substrates added, and with 5 mM glucose, 0.5 mM glutamine or 10 mM lactate. After the first few minutes, there was a decrease in the velocity of ferricyanide reduction, most probably due to a consumption of the intracellular electron donor, namely NADH. From minute 15 on, lactate, an immediate source of NADH, was able to maintain a higher ferricyanide reduction capacity when compared with data obtained with only 0.5 mM ferricyanide. Under these conditions, the oxidation of endogenous substrates would provide the required NADH for ferricyanide reduction.

In short-term incubations there was no significant difference in the ferricyanide reduction rate between cells incubated with no metabolic substrate and those incubated with glucose or glutamine, showing that these substrates are not immediate sources of available cytosolic NADH.

TABLE II

EFFECT OF GLUCOSE ON FERRICYANIDE REDUCTASE ACTIVITY IN INTACT EHRLICH ASCITES TUMOR CELLS DURING LONG-TERM INCUBATION

Data are given as pmol/10^6 cells and are means ± S.E. of three separate experiments. Values in brackets are the percentages of total ferricyanide reduced.

Time (min)	Ferricyanide reductase activity	
	ferricyanide (0.5 mM)	+ glucose (5 mM)
60	2350 ± 200 (26%)	3117 ± 17 * (35%)
90	2400 ± 200 (27%)	4017 ± 140 * (45%)
120	2500 ± 140 (28%)	4950 ± 212 * (55%)

* Significant as compared with cells incubated with only 0.5 mM ferricyanide ($P < 0.01$).

TABLE I

TRANSPLASMA MEMBRANE FERRICYANIDE REDUCTASE ACTIVITY IN INTACT EHRLICH ASCITES TUMOR CELLS

Data are given as pmol/10^6 cells and are means ± S.E. of four separate experiments. In all the cases, ferricyanide was present at 0.5 mM concentration. n.d., not determined.

Incubation time (min)	Ferricyanide reductase activity			
	control	+ glucose (5 mM)	+ glutamine (0.5 mM)	+ lactate (10 mM)
5	630 ± 75	542 ± 110	753 ± 240	722 ± 143
10	1050 ± 83	938 ± 43	1151 ± 197	1255 ± 170
15	1322 ± 118	1258 ± 63	1501 ± 212	1708 ± 193 *
20	1567 ± 118	1388 ± 65	1566 ± 105	2017 ± 192 *
25	1700 ± 92	1617 ± 97	n.d.	2388 ± 238 *
30	1883 ± 93	1805 ± 115	n.d.	2725 ± 193 *

* Significant as compared with cells incubated with only 0.5 mM ferricyanide ($P < 0.01$).

The differences between glucose and lactate incubations could be explained taking into account that Ehrlich ascites tumor cells maintain a very high aerobic glycolysis [14] and in short-term incubations they use a great percentage of the consumed glucose in lactate production. However, as Table II shows, glucose may be used as a source of NADH in long-term incubations. After 60 min of incubation, when almost all the glucose had been consumed, the lactate produced and ejected into the medium was used as the immediate source of NADH.

In all cases, the recovery of total extracellular ferricyanide after oxidation of the supernatant by persulfate was almost 100%, showing that the decrease in absorbance measured at 420 nm was not due to ferricyanide uptake by the cells, but to ferricyanide reduction by the transplasma membrane oxidoreductase activity.

Kinetics measurements of initial velocities showed that the reduction of ferricyanide is a saturable process. The double-reciprocal plot gave a K_m of 0.14 mM and a V_{max} of 312 pmol · min^{-1} · 10^6 cells.

Transmembrane redox activity has been shown to be coupled with an increase in extracellular protons [2–4,9,10,15]. Crane et al. [9] take these data as evidence for a coupling of proton release with transmembrane redox activity. However, whether this is a vectorial or scalar proton release still remains under discussion. Very recently, it has been shown that extracellular proton release during ferricyanide reduction is accompanied by a moderate acidification of the cytoplasm and no significant variation of the vacuolar pH in *Elodea densa* leaves, as measured by ^{31}P-NMR [16]. We have found a moderate alkalinization in the internal pH of intact Ehrlich ascites tumor cells incubated with 0.5 mM ferricyanide. After 15 min, there was an increase of 0.06 in the cytosolic pH and a proton release of 414 pmol/10^6 cells. These data support a vectorial proton release in Ehrlich cells during ferricyanide reduction. Alkalinization of the cytosol has been related to cell growth [17].

Amiloride, an inhibitor of Na^+/H^+ antiport [18,19], showed inhibitory effects both on ferricyanide reductase activity (38% inhibition with respect to control cells) and proton extrusion (88% inhibition). These results are in agreement with those reported for HeLa cells [15] and isolated hepatocytes [20], and seem to indicate a connection between the redox system and the Na^+/H^+ antiport. In fact, this evidence is consistent with the view that the transplasmalemma electron transport system can activate the Na^+/H^+ antiport. However, these data must be taken with care because amiloride is not a totally specific Na^+/H^+ antiport inhibitor and has been shown to exhibit a variety of nonspecific effects, including inhibition of Na^+/K^+-ATPase [21–24]. Further studies are required in order to test the connection between the redox system and Na^+/H^+ antiport.

TABLE III

EFFECT OF SOME ANTITUMOR DRUGS ON FERRICYANIDE REDUCTION BY EHRLICH ASCITES TUMOR CELLS

Cells were preincubated with the antitumor drugs tested for 10 min. Afterwards, ferricyanide was added to a final concentration 0.5 mM and cells were further incubated for 15 min. Data are given as percentage of inhibition with respect to control cells with no antitumor drug added and are means ± S.E. of three separate experiments, except for that of DON (6-diazo-5-oxo-L-norleucine), which is the value obtained in two determinations.

Antitumor drug	% Inhibition
Acivicin ($5 \cdot 10^{-6}$ M)	66 ± 3
Azaserine (10^{-5} M)	60 ± 5
Daunomicin (10^{-6} M)	48 ± 7
DON (10^{-3} M)	10
Methotrexate ($5 \cdot 10^{-5}$ M)	28 ± 5
Mitoxantrone ($2 \cdot 10^{-5}$ M)	48 ± 3

The effect of different antitumor drugs on transplasma membrane redox activity was studied (Table III). All the antitumor drugs tested induced a significant and high inhibition of ferricyanide oxidoreductase at concentrations at which they show antineoplastic activity. Only 6-diazo-5-oxo-L-norleucine (DON) at such a high concentration as 1 mM did not show any significant effect. These results enlarge the list of antineoplastic drugs inducing inhibition of the transmembrane redox activity [9]. Besides, they suggest that cytotoxicity may be somehow linked to the inhibition of transmembrane ferricyanide reductase activity. The mechanism of inhibition of this activity by antineoplastic drugs must be further investigated.

Acknowledgements

This work was supported by Grant 962/84 from the CAICYT and Grant 87/1518 from the FIS. Thanks are due to M. Meaney for correcting this manuscript. J.A. Segura is recipient of a Fundación Averroes Scholarship.

References

1 Crane, F.L., Roberts, H., Linnane, A.W. and Löw, H. (1982) J. Bioenerg. Biomembr. 14, 191–205.
2 Thom, M. and Maretzki, A. (1985) Plant Physiol. 77, 873–876.
3 Rubinstein, B. and Stern, A.I. (1986) Plant Physiol. 80, 805–811.
4 Sandelius, A.S., Barr, R., Crane, F.L. and Morré, D.J. (1986) Plant Sci. 48, 1–10.
5 Neufeld, E. and Bown, A.W. (1987) Plant Physiol. 83, 895–899.
6 Goldenberg, H., Crane, F.L. and Morré, D.J. (1979) J. Biol. Chem. 254, 2491–2498.
7 Goldenberg, H. (1982) Biochim. Biophys. Acta 694, 203–223.
8 Löw, H., Crane, F.L., Partick, E.J., Patten, G.S. and Clark, M.G. (1984) Biochim. Biophys. Acta 804, 253–260.
9 Crane, F.L., Sun, I.L., Clark, M.G., Grebing, C. and Löw, H. (1985) Biochim. Biophys. Acta 811, 233–264.
10 Sun, I.L., Crane, F.L. and Chou, J.Y. (1986) Biochim. Biophys. Acta 886, 327–336.
11 Kilberg, M.S. and Christensen, H.N. (1979) Biochemistry 18, 1525–1530.
12 Sánchez Olavarriia, J., Chico, E., Giménez-Gallego, G. and Núñez de Castro, I. (1981) Biochimie 63, 469–475.
13 Bashford, C.L. (1987) in Spectrophotometry and Spectrofluorimetry (Harris, D.A. and Bashford, C.L., eds.), pp. 115–135, IRL Press, Oxford.
14 Medina, M.A., Sánchez Jiménez, F., Márquez, F.J., Pérez Rodríguez, J., Quesada, A.R. and Núñez de Castro, I. (1988) Biochem. Int. 16, 339–347.
15 Sun, I.L., García-Cañero, R., Liu, W., Toole-Simms, W., Crane, F.L., Morré, D.J. and Löw, H. (1987) Biochem. Biophys. Res. Commun. 145, 467–473.
16 Guern, J., Mathieu, Y., Ulrich-Eberius, C.I., Marré, M.T. and Marré, E. (1988) in the Proceedings of the NATO Advance Research Workshop on Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth, Plenum Press, New York, in press.
17 Gerson, D.F., Kiefer, H. and Eufe, W. (1982) Science 216, 1009–1010.
18 Kinsella, J.L. and Aronson, P.S. (1980) Am. J. Physiol. 238, F461–F469.
19 Kinsella, J.L. and Aronson, P.S. (1981) Am. J. Physiol. 241, F374–F379.
20 García-Cañero, R., Díaz-Gil, J.J. and Guerra, M.A. (1987) in Redox Functions of the Eukaryotic Plasma Membrane (Ramírez, J.M., ed.), pp. 43–47, CSIC Publications Office, Madrid.
21 Zhuang, Y.X., Cragoe, E.J., Jr., Shaikewitz, T., Glaser, L. and Cassell, D. (1984) Biochemistry 23, 4481–4488.
22 Soltoff, S.P. and Mandel, L.J. (1983) Science 220, 957–959.
23 Soltoff, S.P., Cragoe, E.J., Jr. and Mandel, L.J. (1986) Am. J. Physiol. 250, C744–C747.
24 Renner, E.L., Lake, J.R., Cragoe, E.J., Jr. and Scharschmidt, B.F. (1988) Biochim. Biophys. Acta 938, 386–394.