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REDOX CHANGES IN NORMAL AND NEOPLASTIC CELLS DURING THE CELL CYCLE I. BIOREDUCTION OF NITROXIDES BY CHO CELLS WITH DIFFERENT MITOTIC ACTIVITY

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Chinese Hamster Ovary (CHO) cells cultured *in vitro* and isolated in logarithmic phase of growth reduced spin probes at a lower rates than cells isolated during plateau phase of growth. This phenomenon was observed for nitroxides located in cell membranes and those penetrating into the cells. Blocking of electron transport in mitochondria with inhibitors slowed down the bioreduction, whereas uncoupling of mitochondrial phosphorylation increased the rate of this process.

INTRODUCTION

In the first quarter of this century some scientists (Hammett, 1929a, b; Hopkins, 1921; Hopkins & Dixon, 1922; Rapkine, 1931; Warburg, 1966) indicated the ties between cell division and redox properties of cells. This idea was developed later, among others, by Otto Warburg and Albert Szent-Gyorgyi - both Nobel laureates (Szent-Gyorgyi, 1957, 1960, 1968, 1972, 1976; Warburg 1966). The progress in this field connected with such vital biological problems as cell division and cancerogenesis was strongly dependent on the availability of suitable tools. It seems that the method of electron paramagnetic resonance combined with the use of nitroxide spin labels (NSL) may provide interesting information about the redox state of cell membranes and cell interior (Pająk, Subczyński, Panz & Łukiewicz, 1980; Sentjurc, Morse II & Swartz, 1986; Swartz, Sentjurc & Morse II, 1986; Swartz & Glockner, 1989; Swartz, 1987).

MATERIALS AND METHODS

Chemicals

Minimal Essential Medium (MEM), Phosphate Buffered Saline (PBS) and bovine serum were purchased in WSiS Lublin, Poland; antibiotics: penicillin and streptomycin were from Polfa Tarchomin, Poland; spin probes were from Molecular Probes, USA; methocel cellulose ether was obtained from DOW Chemicals, USA; 5-D-thioglucose and 1,1'azobis-N,N-dimethylformamide were from Aldrich, USA; L-buthionine sulfoximine was obtained from Chemical Dynamics Corp., USA; potassium cyanide was obtained from POCh, Poland and rotenone was purchased in Sigma, USA. All chemicals were of reagent class purity.

Cell culture

CHO cells were cultured in closed glass vessels in 25 ml of MEM supplemented with 10% of bovine serum and antibiotics. The cultures were maintained at 37°C. The passages were done every 3-4 days.

Sample preparation

The cells were isolated using 0.25% trypsin, washed twice in PBS and suspended in PBS. Just before experiments cells were diluted with the isotonic solution of methocell cellulose ether (methylcellulose). The final concentration of methylcellulose was 1% which was sufficient to reduce sedimentation of cells during measurements. Final concentration of cells was about 10^7/ml. Hydrophilic spin labels were added directly to cell suspensions up to a final concentration of 10^{-4}M. Measurements were started after about 5 min. period of preincubation. Control experiments indicated that oxygen concentration was close to zero after preincubation. In the case of hydrophilic spin labels, a thin film of probe was first obtained by drying the chloroform solution in test tubes, then cell suspension was added and shaken vigorously with "Vortex" for 30 seconds. Finally cell suspen-

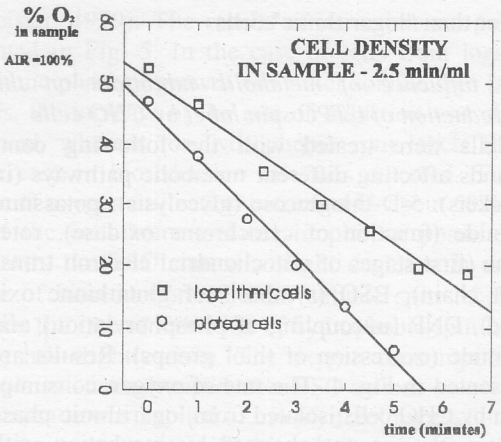

Fig. 1 Oxygen consumption by CHO cells in logarithmic and plateau phases of growth

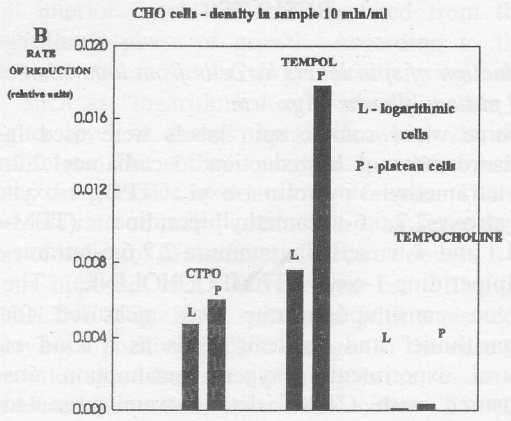

Fig. 2 Reduction rates of CTPO, TEMPOL AND TEMPOCHOLINE by CHO cells in logarithmic and plateau phases of growth

sion was transferred to Pasteur pipettes and placed in the resonator of ESR spectrometer.

ESR measurements

All ESR data were obtained with the use of Varian E3 spectrometer equipped with temperature control unit. Cells suspended in PBS with methyl cellulose were placed in Pasteur pipettes the bubbles of air were removed by vigorous shaking. The temperature of cell suspension was kept at 37°C. In the case of hydrophilic spin labels the conditions of recording spectra were as follows:

microwave power = 1 mW
frequency = 9.2 GHz
center magnetic field = 3230 Gs
magnetic field sweep range = 50 Gs
amplitude of modulation = 1 Gs

The low field line was recorded every 2-3 minutes and the height of that line served as an indicator of spin label concentration. In the case of hydrophobic spin labels the microwave power was 10 mW and central line of spectrum was recorded. The other conditions were the same as for hydrophilic spin probes.

Blocking the metabolic pathways

In the present work the following metabolic pathways were selectively inhibited:

a. glycolysis — affected by thioglucose (Chen & Whistler, 1975). Cells were incubated in standard medium with the addition of thioglucose at a concentration of 10-20 mM for 24 hours before experiments. After isolation of cells measurements were performed in PBS supplemented with thioglucose in concentration of 20 mM.

b. mitochondrial electron transport chain — affected by rotenone, dinitrophenole (DNP) and potassium cyanide. Rotenone, potassium cyanide and DPN were used at final concentrations of 3 mM, 30 μM and 0.05 mM respectively. These compounds were added to samples just before ESR measurements. The control experiments have shown that selected concentrations of inhibitors caused only a few percent decrease in cell viability as measured with trypan blue test.

c. synthesis of glutathione — affected by L-buthionine sulfoximine (BSO) (Clark, Epp, Biaglow, Morse-Gaudio & Zagcho, 1984; Griffith & Meister, 1979). BSO was added to the culture vessels 6 or 12 hours before measurements up to final concentrations of 10 or 20 mM. After isolation of cells measurements were done in PBS without BSO.

d. expression of thiol groups affected by 1,1'-azobis-N,N-dimethylformamide (diamide) (Kosower, Kosower, Wertheim & Correa 1969). Isolated cells were incubated for 5 minutes with diamide at a concentration of 0.8 mM, temperature of 4°C, and resuspended in PBS for measurements.

Data processing

It was assumed that bioreduction followed the equation of an exponential decay:

$$y(t) = A \cdot \exp(-Bt) \qquad (1)$$

where: y — amplitude of ESR signal, t — time (in minutes), A — numeric parameter connected with the initial amplitude of ESR signal, B — numeric parameter connected with the rate of bioreduction

Equation (1) corresponds to the chemical reactions of the first order. The reactions studied can sometimes be of higher order, but in most cases the amount of cells was high enough to fit the kinetics of bioreduction to the quasi-first order reactions.

RESULTS

Reduction of spin labels by cells from logarithmic and plateau phases of growth

Three water soluble spin labels were used in measurements of bioreduction: 3-carbamoyl-2,2,5,5-tetramethyl-3 pyrrolin-1-oxyl (CTPO), 1-oxyl-4-hydroxy-2,2,6,6-tetramethylpiperidine (TEMPOL) and 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOCHOLINE). The oxygen consumption rate was measured for "logarithmic" and "plateau" cells as a kind of control experiment. Oxygen consumption was measured with CTPO. details were presented elsewhere (Backer, Budker, Eremenko & Molin, 1977; Pająk *et al.*, 1980). CHO cells isolated in plateau phase of growth used oxygen 58% faster than cells isolated in logarithmic phase of growth (see Fig. 1). In Fig. 2 the numeric values of parameter connected with the rate of reduction of CTPO by CHO cells are presented. The cells isolated during plateau phase of growth reduced CTPO about 20% faster than cells isolated during logarithmic phase of growth. For water soluble spin label - TEMPOL, the plateau cells reduced spin label faster by 250% than cells isolated in logarithmic phase of growth. In the case of another hydrophilic spin label, TEMPOCHOLINE, the "plateau" cells reduced spin label 47% slower than "logarithmic" cells.

The bioreduction of spin labels locating in cell membranes was also investigated. Two spin probes were used, the analogs of stearic acid: 5-SASL and 16-SASL. Results are presented in Fig. 3. Spin label 5-SASL was reduced about 70% faster by "plateau" cells in comparison with cells isolated in phase of logarithmic growth. The "plateau" cells reduced spin label 16-SASL 86% faster than "logarithmic" cells.

The influence of metabolic inhibitors on the bioreduction of CTPO spin label by CHO cells

Cells were treated with the following compounds affecting different metabolic pathways (in brackets): 5-D-thioglucose (glycolysis), potassium cyanide (function of cytochrome oxidase), rotenone (first stages of mitochondrial electron transport chain), BSO (synthesis of glutathione oxidase), DNP (uncoupling of phosphorylation) and diamide (expression of thiol groups). Results are presented in Fig. 4. The rate of oxygen consumption by CHO cells isolated from logarithmic phase of growth was not changed by incubation with thioglucose. The bioreduction of thioglucose-treated CHO cells, isolated from logarithmic phase of growth, was slower by 27% in comparison with untreated cells. The thioglucose treatment applied to "plateau" CHO cells caused no statistically significant difference in the rate of bioreduction of CTPO.

It is visible that potassium cyanide caused slight acceleration (by 6%) of CTPO bioreduction in comparison with control. The treatment of cells with rotenone brought about a decrease in the rate of reduction by 33%. The effects of the uncoupler (DNP) are also shown in Fig. 4. One can see that the rate of bioreduction of CTPO by CHO cells is twice as high in the presence of DNP. It is interesting to note that at the same time the oxygen consumption has been found to increase by about 40% (Panz, 1992).

Another group of inhibitors checked for their ability to affect the bioreduction of spin labels by CHO cells were blockers of thiol metabolism and expression. A well known inhibitor of glutathione synthase is BSO (Clark *et al.*, 1984; Griffith &

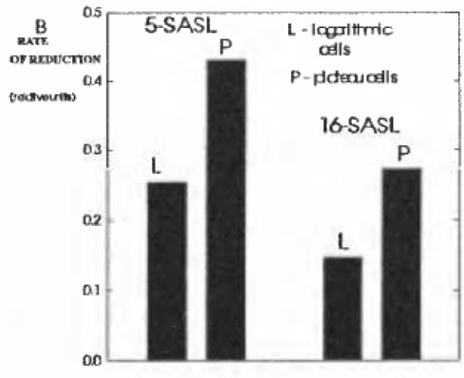

Fig. 3 Reduction rates of 5-SASL and 16-SASL by CHO cells in logarithmic and plateau phases of growth

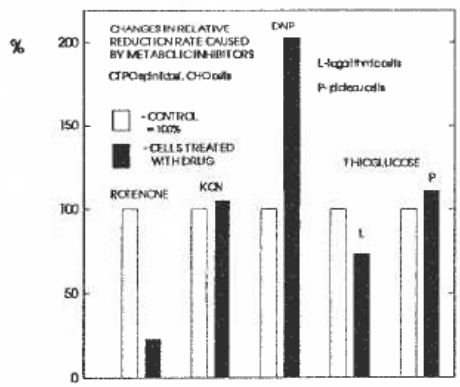

Fig. 4 Effect of rotenone, potassium cyanide, DNP and thioglucose on bioreduction rate of CTPO by CHO cells

Meister, 1979). The results of its action are presented in Fig. 5. In the case of cells from logarithmic phase of growth and water soluble spin labels, the increase of the rate of reduction was observed, whereas for hydrophobic spin labels the decrease in bioreduction rate was detected. The rate of oxygen utilization increased by about 50%.

If the cells were isolated in plateau phase of growth, the BSO caused a slight decrease in the rate of bioreduction of water soluble spin labels while the rate of reduction of hydrophobic spin labels has slightly risen. The oxygen consumption by plateau cells dropped after BSO action.

Diamide was applied to modify thiol expression. For the experiments with this substance CHO cells from the logarithmic phase were used (see Fig. 5). The data clearly indicate that diamide is able to cause a decrease in bioreduction rate of all spin labels examined except for a hydrophobic - 16-SASL. The oxygen consumption remained unchanged.

DISCUSSION

Dependence of bioreduction of spin labels on the phase of cell culture

The data presented in the preceding section demonstrate that CHO cells from the plateau phase of growth reduce spin labels penetrating the cell interior faster than cells isolated from the logarithmic phase of growth. According to the views of Albert Szent-Gyorgyi, proliferating cells — such as "logarithmic" cells should posses a higher level of donors than cells which do not proliferate. The ability to reduce spin label indicates the presence of donors of electrons. Therefore the rate of such bioreduction may serve as a sort of indicator of the level of electron donors.

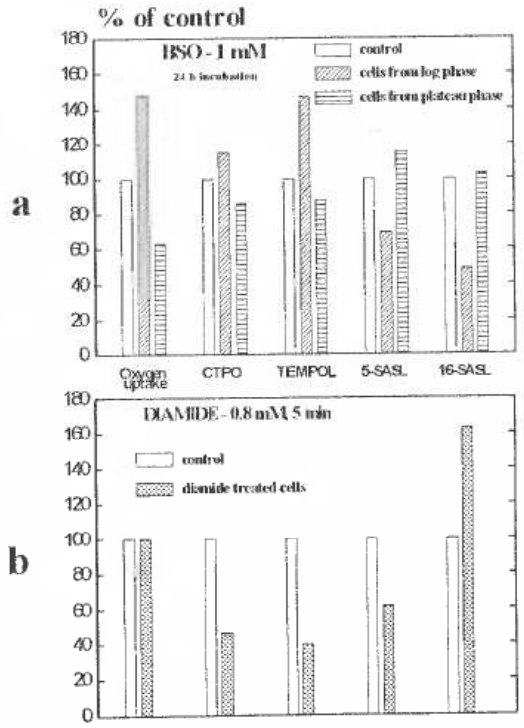

Fig. 5 Effect of BSO and diamide on bioreduction rates of spin labels by CHO cells

An opposite effect is observed for TEMPO-CHOLINE (Fig. 2), a spin label with molecules carrying a positive electric charge. In the case of the latter spin label, the plateau cells are slower in its bioreduction than logarithmic cells. TEMPO-CHOLINE is positively charged. On the other hand the surface of proliferating and transformed cells is more electro-negative than the surface of resting and normal cells (Doi, Tokuda, Itano, Matsui, Ohmura & Hatase, 1984). As a result, the electrostatic forces may tend to enhance the attraction between the cell surface and spin label, and to accelerate the bioreduction. This might account for the different rate of bioreduction of charged spin labels versus spin labels bearing no electric charge.

The observed oxygen consumption (see Fig. 1) is higher for "plateau" cells. This fact indicates that these cells rely on oxygen metabolism to a higher extent than "logarithmic" cells. This observation is consistent with Albert Szent-Gyorgyi's theory, which assumed that dividing cells turn to the primitive, archaic way of acquiring energy (so called "alpha state"), namely to glycolysis.

Interpretation of experiments on modification of metabolic pathways

The inhibition of glycolysis in fast proliferating cells causes decrease in bioreduction of CTPO (see Fig. 4). The possibilities of switching the mitochondrial mechanisms of that process are apparently limited for cells relying mostly on glycolysis. The opposite effect, the increase in bioreduction rate was observed for plateau cells. These cells are able to compensate the lower intensity of glycolysis by enhancing mitochondrial activity, which is reflected by a higher level of substances responsible for CTPO bioreduction. The blockade of glycolysis significantly increased the rate of oxygen consumption by "plateau" CHO cells.

Low concentrations of KCN, which do not kill cells, block cytochrome oxidase, so that electrons from the initial parts of the respiratory chain cannot reach oxygen, but reduce instead CTPO at a higher rate, whereas oxygen is "saved", its uptake decreasing at the same time. This means that the site of generation of electrons responsible for the reduction of CTPO must be located prior to the

cytochrome oxidase. In contrast, the site of inhibitory action of rotenone, known to affect initial stages of respiratory chain, must precede the place where electrons reducing CTPO are generated. Therefore, rotenone brings about a significant decrease in the rate of bioreduction of CTPO.

The increase in oxygen consumption and bioreduction, caused by the uncoupler DNP indicates that there are interactions between donors capable to reduce spin labels and respiratory electron transport chain, whereas reduction of thiols and oxidative phosphorylation are not directly connected.

CHO cells isolated in logarithmic phase of growth and treated with BSO (the inhibitor of glutathione synthase) exhibit a faster rate of bioreduction of hydrophilic spin labels. This could be taken to mean that a system of homeostatic equilibrium between donors and acceptors of electrons might be postulated. The "donor pool" deprived of glutathione supply trends to restore its reduction potential by using alternative pathways, e.g. mitochondrial activity.

The cells in "plateau" phase of growth are in a different situation, because the glutathione synthesis naturally drops down as the cells stop to divide, so that a hypothetic "reduction capacity" of a cellular pool remains the same. The results of diamide action (see Fig. 5) suggest a serious impairment of the "reduction pool" resulting from blockade of thiol groups. It seems that a decrease in the bioreduction rate of all investigated spin labels, except for those located in the deepest region of cell membranes, indicates the existence of a source of electron donors inside biological membranes.

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