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EFFECT OF RHEIN ON THE GLUCOSE METABOLISM OF EHRLICH ASCITES TUMOR CELLS

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Abstract—The effect of rhein, 4,5 dihydroxyanthraquinone-2-carboxylic acid, on oxygen consumption and the rate of aerobic and anaerobic lactate production by Ehrlich ascites tumor cells has been investigated. The rate of oxygen uptake decreases with the increase of rhein concentration. Rhein also inhibits aerobic and anaerobic glycolysis. The rate of aerobic lactate production decreases with the drug concentration and the maximal effect was observed at 0.100 mM. Anaerobic lactate production is also inhibited and the maximum effect is reached at 0.220 mM. The possibility that the lactate production decrease was secondary to an effect on mitochondrial ATPase was excluded on the basis of the data with DNP and oligomycin. Rhein reduces the intracellular level of lactate, pyruvate and glucose-6-phosphate. Glucose utilization and 2-deoxy-D-glucose uptake are decreased to the same extent as the inhibition of aerobic lactate production, whereas glucose phosphorylation is unaffected. It is, therefore, concluded that the inhibition of glycolysis of Ehrlich ascites tumor cells by rhein is caused by an impairment of glucose uptake.

It is well known that tumor cells possess an abnormal energy metabolism characterized by an elevated rate of glycolysis [1–3]. Although the high lactic acid-producing capacity is not a peculiarity of rapidly growing tumors [4–7], the increased glycolysis rate in tumor cells is a specific effect of the transformed process [8, 9]. The high rate of glucose utilization provides some selective advantages for tumor cell growth and survival in comparison to the normal surrounding cells. The elevated glycolysis of neoplastic cells increases the intracellular content of glucose-6-phosphate, a central division point for the energy metabolism, lipogenesis, the uronic acid pathway and nucleotide synthesis. An elevated glycolysis also provides an alternate source of ATP production and this effect is particularly important in low oxygen environments such as in poorly vascularized solid tumors.

The significance of glycolysis for neoplastic growth has been clearly established by Burk *et al.* [10] as well as the central importance of chemically inhibiting or modifying glycolysis for a rationale cancer chemotherapy.

In the recent years, a novel approach to the chemotherapeutic treatment of cancer has focused on the aberrant energy metabolism of neoplastic cells [11–17]. Both experimental and clinical evidence suggests that it may be tumor energy metabolism, growth rate and survival may be inhibited by targeting tumor-specific enzyme systems [18–22].

Furthermore, it has been demonstrated that rhein, 4,5 dihydroxyantraquinone-2-carboxylic acid, an anti-inflammatory drug [23–25], inhibits the oxidation of NAD- and FAD-linked substrates at dehydrogenase-coenzyme level [26–28] in such a way similar to lonidamine [14], an antitumor drug which selectively affects the energy metabolism of neoplastic cells [12, 13].

The effect of rhein on the energy metabolism of neoplastic cells was, therefore, investigated and the present report presents data on its action on the oxygen consumption, aerobic and anaerobic glycolysis of Ehrlich ascites tumor cells. The experiments were essentially designed to localize the site of action of rhein on the glycolytic pathway.

MATERIALS AND METHODS

Cells. Ehrlich ascites tumor cells were grown in 2-month-old male Swiss mice and harvested 7–11 days after inoculation. The cells were withdrawn from the killed animals and resuspended in a medium containing, in final concentration, NaCl 105 mM, KCl 5 mM, TES† 50 mM, pH 7.4 (NKT). The cells were centrifuged at 300 *g* for 5 min at room temperature and washed three times with NKT medium. The packed cells were resuspended in NKT medium at a concentration of 2×10^8 cells/mL. Contamination with other cells, such as leukocytes, did not exceed 0.6%, according to the differential counting of smears stained by the May–Grunwald method. The viability of the cells was about 95–98%, as indicated by phase-contrast microscopy in the presence of trypan blue.

Assay of respiration and glycolysis. Respiration was measured with a Clark oxygen electrode (Yellow Spring Instruments) at 30°; the concentration of dissolved oxygen was, in air-saturated medium, 444 ngatoms O/mL [29]. Reaction rates were determined by adding 0.2 mL of cell suspension (4×10^7 cells) to 2.8 mL of air-saturated NKT medium in a

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† Abbreviations: DNP, 2,4 dinitrophenol; TES, *N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid; NKT, NaCl, KCl, TES medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

closed glass chamber. The measurements of aerobic glycolysis were carried out in 25-mL Erlenmeyer flasks at 30° in a Dubnoff metabolic shaker. The complete reaction medium contained NKT medium, pH 7.4, and 0.2 mL of cellular suspension (4×10^7 cells). The other components are indicated in figures and tables. The final volume was always 3.0 mL. After 10 min of preincubation with or without the drug, the reaction was started by the addition of glucose, at a final concentration of 10 mM, and the incubation was allowed to proceed for 1 hr. At the end of the incubation, an aliquot (0.2 mL) was withdrawn to evaluate cell viability which, both in aerobiosis and anaerobiosis, was routinely greater than 85%. The flasks were then immersed in an ice-bath for 3 min and the cells were sedimented by centrifugation at 3000 *g* at 4°. L(−)-Lactate concentration in the supernatants was evaluated enzymatically according to Hohorst [30]. Anaerobic glycolysis was measured under the same conditions except that the solution and the vessels, 10 min before the addition of the cells and throughout the incubation, were gassed with ultrapure argon (Caracciolo Ossigeno, Rome, Italy). The cells and glucose were added by means of a syringe through the serum stopper.

Assay of intracellular metabolites. For the determination of intracellular metabolites, the cells (4×10^7) were incubated as described for the aerobic glycolysis. At the end of incubation (1 hr), aliquots (2 mL) were withdrawn with a Gilson pipette and added to 1 mL of ice-cold 2.54 M perchloric acid and immediately shaken by a vortex mixer. The acidified samples were allowed to stand at room temperature for 30 min and then centrifuged at 20,000 *g* for 10 min at 4°. The supernatants were neutralized with 0.5 mL of a buffered solution of potassium carbonate (K_2CO_3 3 M, triethanolamine 0.5 M, pH 9.0). The precipitate was removed by centrifugation and the metabolite content was evaluated on the supernatants essentially according to Maitra and Estabrook [31]. The only difference was the use of absorbance measurements rather than fluorescence. Fluorescence is usually considered to provide greater sensitivity, but with the Aminco DW-2a spectrophotometer sensitivity has not been a problem.

Incubation with [U-^{14}C]2-deoxy-D-glucose. The cells (4×10^7) were incubated in NKT buffer as described for the aerobic glycolysis. After 10 min of preincubation, glucose, at final concentration of 10 mM, and 2 μCi of [U-^{14}C]2-deoxy-D-glucose were added and the incubation was allowed to proceed for 30 min at 30°. At the end of the incubation, the cells were spotted onto GF/A filters (Whatman) and extensively washed with NKT buffer (30 mL). After drying, the disks were transferred into vials, containing 5 mL of Aquassure scintillation liquid and assayed for radioactivity in an LS 1800 Beckman liquid scintillation spectrometer.

Assay of hexokinase activity. In order to evaluate the effect of rhein on hexokinase activity, the cells (4×10^7) were incubated in triplicate for 1 hr at 30° with 0.220 mM rhein. At the end of incubation, the cells were collected by centrifugation at 1000 *g* at 4°, resuspended in 3 mL of H medium (220 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4), disrupted with digitonin and homogenized according to Parry and Pedersen [32]. The homogenate was then centrifuged at 1100 *g* for 5 min at 4° in a J21 Beckman centrifuge to sediment nuclei and cell debris. The post-nuclear supernatant was centrifuged at 48,500 *g* in J21 Beckman centrifuge for 30 min at 4° and the hexokinase (HK) activity evaluated both in the sediment ("bound" HK) and in the supernatant ("free" HK). Hexokinase (EC 2.7.1.1) was determined according to Parry and Pedersen [32] except that Lubrol was omitted from the incubation mixture. After 5 min at 30° the reaction was started by adding ATP. The protein content was evaluated by means of biuret reaction [33].

Chemicals. The following chemicals were purchased from the indicated sources: TES from the Sigma Chemical Co. (St Louis, MO); ATP, ADP, HEPES, oligomycin, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, hexokinase, phosphoenolpyruvate, pyruvate kinase. NAD, NADP, triethanolamine, HCl from Boehringer-Mannheim (Mannheim, F.R.G.); [U-^{14}C]2-deoxy-D-glucose and Aquassure from New England Nuclear (Boston, MA). All other reagents were analytical grade and were purchased from BDH Italia (Milan, Italy). Rhein was a gift of Dr Vittorio Behar, Proter Laboratories, Opera (Milan, Italy).

RESULTS

Effect of rhein concentration on respiration and glycolysis

Figure 1A shows the rate of oxygen consumption of Ehrlich ascites tumor cells as a function of rhein concentration. After the addition of the cells to the oxygraph chamber, the basal rate of oxygen consumption was recorded for 3 min. Rhein, at indicated concentration, was then added and the subsequent oxygen consumption was determined for 5 min and compared to the rate in the absence of the drug. The rate of oxygen uptake decreases with the increase of rhein concentration. The lower rate of oxygen utilization does not depend on the cell death because their viability, evaluated at the end of the experiment (8 min) by trypan blue exclusion test, was unmodified even at highest rhein concentrations.

The effect of rhein on the aerobic glycolysis is reported in Fig. 1B. The aerobic lactate production decreases with drug concentration up to 0.100 mM. Further increases, up to 0.4 mM, do not induce significant changes in the lactate production rate. The drug concentration required to obtain half-maximal inhibition is 0.035 mM.

Figure 1C shows the effect of rhein on anaerobic glycolysis. The lactate production is higher than under aerobic conditions and decreases with the drug concentration. Maximal inhibition is reached at 0.220 mM rhein. Half-maximal inhibition took place at a concentration of 0.040 mM.

Effect of rhein, DNP, oligomycin on aerobic and anaerobic glycolysis

In view of the decrease of lactate production brought about by rhein, experiments were carried out to localize its site of action and the effect of

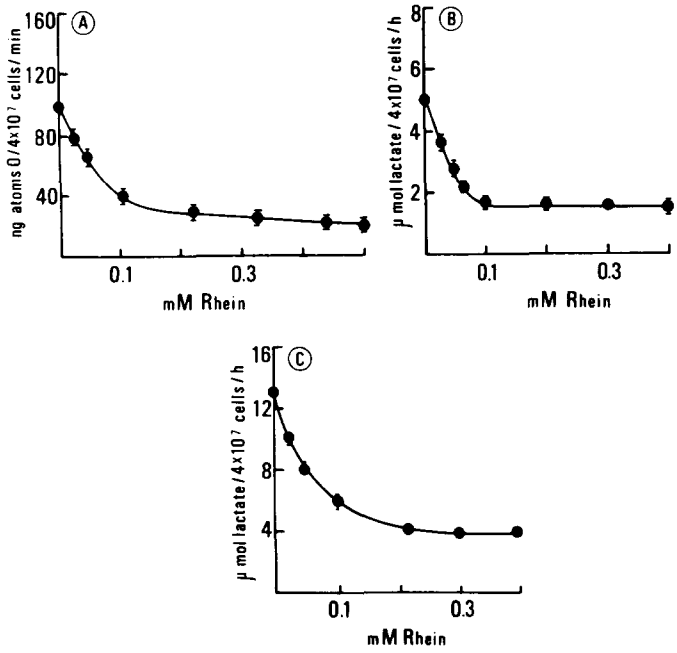

Fig. 1. Dependence of oxygen consumption (A), aerobic (B) and anaerobic (C) lactate production of Ehrlich ascites tumor cells on rhein concentration. Cells were incubated as described in Materials and Methods. Each point ± SD was averaged from 10 different experiments performed in duplicate.

Table 1. Effect of rhein, oligomycin and DNP on the lactate production by Ehrlich ascites tumor cells

Additions	μmoles lactate/(4 × 10^7 cells × hr) Anaerobiosis	Aerobiosis
None	14.4 ± 0.2	5.1 ± 0.4
Rhein	6.4 ± 0.1	1.8 ± 0.3
DNP	18.1 ± 0.7	13.2 ± 0.6
DNP + rhein	7.3 ± 0.7	1.9 ± 0.9
Oligomycin	15.0 ± 0.2	6.4 ± 0.6
Oligomycin + DNP	14.8 ± 0.3	6.6 ± 0.8
Oligomycin + rhein	7.0 ± 0.8	1.7 ± 0.5

Each value ± SD was averaged from eight different experiments performed in duplicate. The final concentrations of rhein, DNP, oligomycin and glucose were 0.220 mM, 0.100 mM, 0.33 μg/mL and 10 mM, respectively.

rhein, DNP and oligomycin on aerobic and anaerobic glycolysis is reported in Table 1.

DNP stimulates anaerobic glycolysis by an activation of mitochondrial ATPase; rhein abolishes this stimulation and lowers the lactate production to a value quite similar to that observed with rhein alone. A similar behaviour was found under aerobic conditions.

These results indicate that the inhibition of glycolysis brought about by rhein cannot be ascribed to an effect on mitochondrial ATPase, as further confirmed by the data with oligomycin which specifically inhibits this enzyme. Oligomycin abolishes DNP stimulation, does not inhibit lactate production, but slightly increases the aerobic lactate production. In the presence of oligomycin, the drug lowers the anaerobic and aerobic lactate production to the values obtained with rhein alone.

Effect of rhein on the level of lactate, pyruvate, glucose-6-phosphate, glucose utilization, hexokinase activity and [U-^{14}C]2-deoxy-D-glucose uptake

It has been previously reported [28, 34] that rhein inhibits lactate dehydrogenase as well as other dehydrogenases. Experiments were, therefore, carried out to ascertain whether the decrease in the lactate production brought about by the drug might be ascribed to an inhibition of this enzyme or other glycolytic ones and/or to lactate outward transport.

Table 2 reports data on the intra- and extracellular lactate concentration and the level of pyruvate and

Table 2. Effect of rhein on lactate, pyruvate and glucose-6-phosphate (G-6-P) content in Ehrlich ascites tumor cells

	Control	Rhein	Δ%
Total lactate	7.24 ± 0.90	1.77 ± 0.49	−76
Extracellular lactate	6.19 ± 0.80	1.70 ± 0.45	−73
Intracellular lactate	1.14 ± 0.03	0.09 ± 0.02	−92
Pyruvate	0.16 ± 0.02	0.07 ± 0.01	−54
G-6-P	45.0 ± 8.0	14.0 ± 4.0	−69

Each value ± SD was averaged from seven different experiments performed in duplicate. The values are expressed as μmoles/4 × 10^7 cells for lactate and pyruvate and as nmoles/4 × 10^7 cells for G-6-P. The final concentration of glucose and rhein was 10 and 0.220 mM, respectively. The gas phase was air.

Table 4. Effect of rhein on the activity of "free" and "bound" hexokinase (HK) of Ehrlich ascites tumor cells

	ΔA/min/mg	
	"Free" HK	"Bound" HK
Control	0.87 ± 0.07	1.20 ± 0.05
Rhein	0.85 ± 0.03	1.92 ± 0.08

Each value ± SD was averaged from four different experiments performed in triplicate. The final concentration of rhein was 0.220 mM.

activity of "free" enzyme and stimulates that of the "bound" form. This activating effect, most likely, may reflect an action of the drug on the mitochondrial

Table 3. Effect of rhein on aerobic glucose utilization by Ehrlich ascites tumor cells

	Glucose			
	Initial μmoles	Final μmoles	Utilized μmoles	Δ%
Control	25.2 ± 0.3	21.8 ± 0.6	3.4	−73
Rhein	25.2 ± 0.3	24.3 ± 0.8	0.9	

Each value ± SD was averaged from seven different experiments performed in duplicate. The values are expressed as μmoles/4 × 10^7 cells/hr. The final concentration of rhein was 0.220 mM.

glucose-6-phosphate in the control and in rhein-treated cells after 1 hr of incubation with 10 mM glucose.

Rhein strongly affects the total lactate production. To establish whether this decrease might be ascribed to an effect on the lactate outward transport, the concentration of extra- and intracellular lactate was evaluated. When the cells were incubated with rhein, the amount of lactate in the medium is decreased by 71%, i.e. to about the same extent as the total lactate. Since the intracellular level of lactate is also lowered in rhein-treated cell, the inhibition of aerobic glycolyis clearly cannot depend on a reduced lactate transport.

Rhein decreases the concentration of pyruvate and glucose-6-phosphate. The reduction of glucose-6-phosphate content might be caused by an inhibition of glucose inward transport or, alternatively, to an inhibition of glucose phosphorylation. To discriminate between these two possibilities, the effect of rhein on glucose utilization, hexokinase activity and on 2-deoxy-D-glucose uptake has been investigated. Table 3 reports data on the glucose utilization by Ehrlich ascites tumor cells in the presence and in the absence of 0.220 mM rhein. Rhein lowers the glucose utilization to a similar extent (−73%) as the glucose-6-phosphate level (−69%) and lactate production (−76%).

Table 4 shows the HK activity in control and rhein-treated cells. Because in tumor cells a certain amount of the enzyme is bound to the outer mitochondrial membrane [11], the activity of hexokinase was assayed both in supernatant ("free" HK) and in the sediment ("bound" HK). Rhein does not affect the

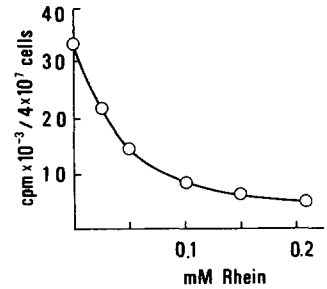

Fig. 2. Effect of rhein concentration on [U-^{14}C]2-deoxy-D-glucose (sp. act. 282 mCi/mmol) uptake by Ehrlich ascites tumor cells. Cells were incubated as described in Materials and Methods. Each point was averaged from five different experiments performed in triplicate.

membranes. Therefore, these experiments clearly demonstrate that the reduced glucose utilization by rhein-treated cells is not secondary to an inhibition of glucose phosphorylation. This conclusion is further supported by the fact that 0.220 mM rhein inhibits the 2-deoxy-D-glucose uptake (Fig. 2) by about 75%, i.e. to an extent quite similar to that found for aerobic glycolysis.

DISCUSSION

The results reported above demonstrate that rhein affects the glucose metabolism of Ehrlich ascites tumor cells by decreasing both the oxygen consumption and the aerobic and anaerobic glycolysis. The decrease of oxygen uptake is related to the

inhibition of electron transport at the dehydrogenase-coenzyme level [26].

As far as the inhibition of glycolysis by rhein is concerned, it can clearly not be ascribed to an effect on mitochondrial ATPase, as shown by the results obtained with DNP and oligomycin. DNP uncouples oxidative phosphorylation, activates mitochondrial ATPase, raising intracellular P_i concentration, and accelerates both the aerobic and anaerobic glycolysis. The increase in anaerobic glycolysis indicates that the activation of mitochondrial ATPase must be a major component. If rhein were to inhibit only mitochondrial ATPase, the lactate production should be lowered to values similar to or slightly higher than those observed in the absence of DNP. In fact, the specific inhibitor oligomycin did not decrease aerobic and anaerobic glycolysis, but slightly stimulates the former. This stimulation is probably caused by an inhibition of oxidative phosphorylation resulting in lowering of the intracellular ATP, thus releasing the inhibition of phosphofructokinase by ATP.

Yet, the inhibition of lactate production by rhein does not depend on an effect on lactate outward transport. Lactate is excreted in Ehrlich ascites tumor cells together with a proton [35]. The block of the lactate excretion lowers the intracellular pH, which, in turn, causes an inhibition of glycolysis [36, 37]. An inhibition of the lactate outward transport should increase the internal lactate concentration. The lower intracellular content of lactate found in rhein-treated cells demonstrates that the drug does not affect the glycolysis by means of an action on the lactate transport and, consequently, by a lowering of the intracellular pH. The nearly complete inhibition of lactate transport was required before an increase of internal lactate levels, a lowering of intracellular pH and an inhibition of glycolysis were observed [35, 36].

Although it has been reported that rhein inhibits purified lactate dehydrogenase [34], the low lactate production rate in Ehrlich ascites tumor cells achieved by the drug, under these experimental conditions, cannot be ascribed to an inhibition of this enzyme. A decrease in the lactate dehydrogenase activity increases the intracellular pyruvate concentration, whereas pyruvate content is remarkably lower in rhein-treated than in control cells. The lower extent of inhibition (−54%), as compared to that of lactate (−76%), may be explained considering that other compounds, such as alanine, may contribute to determine the pyruvate level.

A low intracellular pyruvate concentration in rhein-treated cells secondary to an effect on the phosphofructokinase must also be excluded. An inhibition of its activity raises the glucose-6-phosphate concentration, whereas a lower amount of this metabolite was found when the cells were incubated with rhein. Thus, the inhibition of glycolysis by rhein in Ehrlich ascites tumor cells must be ascribed to an effect on glucose uptake. Rhein inhibits the glucose utilization and 2-deoxy-D-glucose uptake to an extent compatible with the inhibition of aerobic lactate production. Neither can this reduced utilization be ascribed to an inhibition of glucose phosphorylation because the hexokinase activity is not affected (Table 4).

These data clearly demonstrate that the inhibition of glycolysis of Ehrlich ascites tumor cells by rhein depends on an effect on glucose utilization. Nevertheless, whereas it is clear that the effect of rhein is exerted at the level of the cell membrane, it still remains to be established whether the drug affects the glucose carrier or if it alters the functional properties of the cell membrane so as to inhibit the glucose transport.

The capacity of the rhein to inhibit both respiration and glycolysis of neoplastic cells makes this drug worthy of further interest. In fact, any attempt to inhibit tumor cell growth and survival by interfering with tumor energy production must take into account the ability of tumor cells to utilize equally well both oxidative phosphorylation and glycolysis to support cell growth [1, 38–41].

The inhibition of energy metabolism by rhein lowers intracellular concentration of ATP [42] so that, considering also the low general toxicity, it might be used as biochemical modulator [43] to reduce or to reverse the multidrug resistance. In fact, resistant cells are characterized by a decrease in net drug accumulation, but, when these cells are depleted of ATP energy, the steady-state level of drug increases to values similar to those of sensitive cells [44–46].

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