Multiple Effects of Amobarbital on Ehrlich Ascites Tumor Cells. Inhibition of Pyruvate Dehydrogenase

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The inhibition of the proliferation of hyperdiploid Ehrlich ascites tumor cells in suspension cultures by amobarbital is coupled to an increased glycolytic activity as shown by lactic acid production and glucose consumption; higher concentrations of amobarbital than 1 mM enhance the ATP/ADP ratio of the total cell. The actual activity of pyruvate dehydrogenase of intact cells is completely inhibited in the presence of 2 mM amobarbital as was shown by the $^{14}CO_2$ evolution from [1-^{14}C]pyruvate or the incorporation of $^{14}CO_2$ into the total lipid fraction of the cells from [U-^{14}C]pyruvate or from [U-^{14}C]lactate. The pyruvate dehydrogenase complex from Ehrlich ascites tumor cells is completely inhibited by 1 mM amobarbital *in vitro*. The activity of α-oxoglutarate dehydrogenase is inhibited by amobarbital, too, as was shown by measuring the $^{14}CO_2$ evolution from [1-^{14}C]glutamate with intact cells.

It is suggested that the inhibition of pyruvate dehydrogenase in the presence of amobarbital is the result of a direct action on the enzyme as well as the consequence of a change in the cellular redox state or its energy charge.

Barbiturates are known to inhibit the growth of mammalian cells as has been demonstrated for monolayer culture of mouse heteroploid cells [1], for rat hepatoma cells *in vitro* [2], for mice fibroblast cultures and for hyperdiploid Ehrlich ascites tumor cells in suspension cultures [4]. Extensive investigations of the mechanism of this effect indicate that barbiturates affect cell proliferation and cell metabolism by inhibition of the respiratory chain [5] as well as biosynthetic processes such as DNA synthesis [4, 6–8] and RNA synthesis [4, 7, 8] or protein synthesis [4, 7, 8]. This impairment of biosynthetic activities might in turn be the result of the influence of amobarbital on enzymes such as flavoenzymes [9, 10] and ATPase [11] or of barbiturate-membrane-interactions leading to perturbations of membrane functions such as transport processes [12].

In the present communication we report our experiments on the effect of amobarbital on the pyruvate dehydrogenase of EAT cells *in vivo* and *in vitro*. These experiments were performed in the course of our studies on the regulation of the pyruvate dehydrogenase *in vivo* which were started following the observation of a correlation between cell proliferation and pyruvate dehydrogenase activity of EAT cells [13].

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Abbreviations: PDH, Pyruvate dehydrogenase (E.C. 1.2.4.1); HEPES, 2-(N-2-hydroxyethyl-piperazin-N'yl) ethane sulfonic acid.

Materials

Amobarbital and HEPES [2-(N-2-hydroxyethyl-piperazin-N′-yl) ethane sulfonic acid] buffer were purchased from Serva, Heidelberg; NAD, LDH, and glucose kits from Boehringer, Mannheim, and scintillation liquid 'Rotiszint 22' from Roth, Karlsruhe. Sodium salts of [U-^{14}C]acetate, [U-^{14}C]pyruvate, [U-^{14}C]lactate, [1-^{14}C]pyruvate and [1-^{14}C]glutamate were from Amersham Buchler, Braunschweig. All other chemicals were from Merck, Darmstadt, or Riedel de Haën, Hannover.

Methods

All experiments were performed with hyperdiploid Ehrlich ascites tumor cells which were cultured in the peritoneal space of female NMRI mice (20 – 25 g) from Ivanovas Co., Kisslegg. The cells were harvested 7 – 10 days after inoculation of about 2×10^7 cells and washed once with medium [14]. Cell concentrations were routinely determined by nephelometry [15]; the viability of cells was tested with nigrosin [16]. All incubations were carried out in a medium [14] modified by the addition of 10 – 20 mM HEPES-buffer pH 7.5 at 37 °C for 30 min. Under these conditions the cells retain a viability of 95 – 97%. Lactate was determined by the UV-method [17], glucose by the test combination GOD-Perid from Boehringer, Mannheim, and protein by microbiuret [18]. ATP was assayed by the luciferin-luciferase method [19] using an Amino Chem-Glow photometer. Standardization was performed with equivalents of 10 pmol of ATP. For ATP determination 250 µl of cell suspensions were injected into

1 ml of redistilled water of 95 °C and cooled rapidly after 3 min to 0 °C. After centrifugation ATP was assayed in the supernatant. Recovery of ATP was never observed to be below 95%. ADP was determined in the same supernatant after transformation to ATP with creatinkinase and creatinphosphate [20]. Pyruvate dehydrogenase activity was measured by the following three assays.

a) By $^{14}CO_2$ evolution from [1-^{14}C]pyruvate in Warburg vessels under 'Carbogen' (95% O_2 + 5% CO_2) at 37 °C for 30 min. $^{14}CO_2$ evolved was trapped in 0.2 ml of 3 M KOH during at least 90 min at 0 °C, after stop of incubations by addition of 0.4 ml of 5 N H_2SO_4. The KOH was dissolved in 10 ml of 'Rotiszint 22' and counted in a Packard Tricarb scintillation counter. All values were corrected by $^{14}CO_2$ evolved in the daily medium blank.

b) By ^{14}C incorporation into total lipids [21] from [U-^{14}C]pyruvate under the same conditions as in a), except with 15 min of nonradioactive preincubation under air instead of 'Carbogen'. After extraction of the total lipids [22] aliquots of air-dried chloroform extracts were counted in 10 ml of 'Rotiszint 22' in a Packard Tricarb scintillation counter. Isolation of triglycerides [23] prior to counting delivered the same results.

c) The activity of isolated pyruvate dehydrogenase from EAT cells or from pig heart was measured by NAD^+ reduction [24].

The pyruvate dehydrogenase complex was isolated (for more sophisticated preparation see [25]) from mitochondria of EAT cells [26] or from pig heart [27]. The mitochondria were frozen, thawed and disrupted for 2 min by ultrasonic treatment. The supernatant from the sedimentation of 48000 × *g* was chromatographed on Sepharose 4 B (4 × 13 cm) and the active fractions of PDH were collected by centrifugation at 150000 × *g*. A suspension of the pellet in 20 mM phosphate-buffer pH 7.0 and 10 mM mercaptoethanol, was used for the experiments. No activation with pyruvate dehydrogenase phosphatase was carried out before the assay. Freezing is not suitable for preserving the enzyme preparation, however addition of 50% glycerol stabilized the enzyme.

Results and Discussion

The effect of amobarbital on the glycolytic activity of EAT cells

Addition of 2 mM amobarbital to EAT cells, incubated in a complete culture medium, stimulated the lactate production to about 180% of the controls (Fig. 1). In contrast, glucose consumption was stimulated only to about 120%. These data clearly demonstrate an enhanced glycolytic activity of EAT cells upon addition of amobarbital. Since the output of pyruvate is not significantly changed in the presence of amobarbital, the ratio of lactate to pyruvate increases, indicating a higher reduced redox state of the cells. This in turn might be caused by an inhibition of respiration by amobarbital [5]. However, a change in lactate production might also be related to a change of the actual pyruvate dehydrogenase activity. Previously we reported namely a decrease in lactate production in the presence of increasing amounts of trichloro acetic acid or dichloro acetic [13] known as inhibitors of pyruvate dehydrogenase kinase [28]. Moreover, in several cases of human lactic acidosis a pyruvate dehydrogenase deficiency could be demonstrated [29]. For further elucidation of the question whether there is a correlation between lactate production and actual pyruvate dehydrogenase activity, the latter was measured *in vivo* by two different assays.

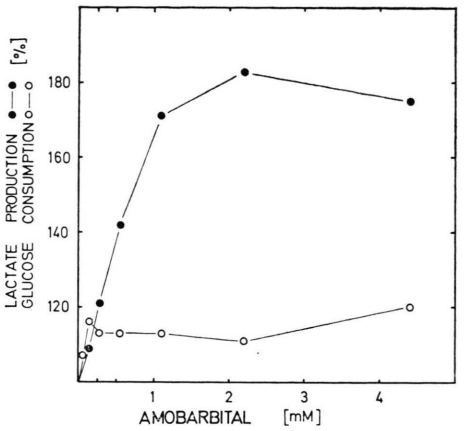

Fig. 1. Stimulation of lactate production and glucose consumption of EAT cells by amobarbital. 1.1 × 10^7 cells were incubated as described under "Methods". The rates of stimulation were identical after 15 or 30 min of incubation time. 100% lactate = 0.95 μmol lactate/10^6 cell × hour and 100% glucose = 0.79 μmol glucose/10^6 cells × hour.

The effect of amobarbital on the pyruvate dehydrogenase activity of intact cells

a) $^{14}CO_2$ evolution from [1-^{14}C]pyruvate

In the presence of 2 mM amobarbital a strong inhibition of the pyruvate dehydrogenase activity of intact EAT cells is observed (Fig. 2 a); it is evident that maximum inhibition is obtained in the same concentration range where the amobarbital induced

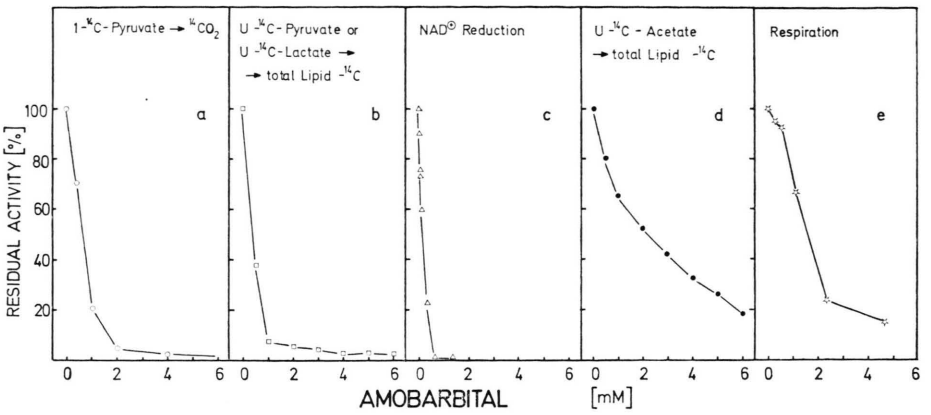

Fig. 2. Effect of amobarbital on pyruvate dehydrogenase.

a. Inhibition of $^{14}CO_2$ evolution from [1-^{14}C]pyruvate by amobarbital with intact EAT cells. 2.2×10^7 cells were incubated in 2 ml of modified medium as described under "Methods". PDH activity was measured in the presence of 0.5 μCi [1-^{14}C] pyruvate and 1 mM pyruvate. 100% = 913 CPM = 8.3 nmol CO_2/min from pyruvate.

b and d. Inhibition of ^{14}C incorporation from [U-^{14}C]pyruvate, [U-^{14}C]lactate or from [U-^{14}C]acetate into total lipids of EAT cells by amobarbital. 100% in presence of 2 μCi [U-^{14}C]pyruvate = 4000 CPM, [U-^{14}C]lactate = 5600 CPM and [U-^{14}C] acetate = 12000 CPM. Fig. 2d as control for Fig. 2b demonstrates the specifity of the effect of amobarbital within the used assay system.

c. Inhibition of isolated PDH from EAT cells by amobarbital. The activity of the PDH was measured by NAD^+ reduction at 340 nm at 37 °C. The PDH was not activated by PDH phosphatase treatment before assay. 100% represents a reduction rate of 0.15 nmol NAD^+/mg protein × minute.

e. Inhibition of respiration of intact EAT-cells by amobarbital under growth conditions. The oxygen consumption of 0.5×10^6 cells in 0.5 ml complete medium was measured with an oxygen electrode of the Clark type at 37 °C. The delayed inhibition of respiration by amobarbital supports the observation that the strong inhibition of PDH activity (Fig. 2a–c) by 1 mM amobarbital might be related rather to a direct effect than a metabolic one.

Table I. Effect of amobarbital on α-oxoglutarate dehydrogenase of intact EAT cells. 2.2×10^7 cells were incubated in 2 ml of modified medium as described under 'Methods'. $^{14}CO_2$ was evolved from 1 μCi [1-^{14}C]glutamate in presence of 2 mM glutamine. An activity of 1147 cpm corresponds to 10.4 nmol CO_2/min from α-oxoglutarate.

Inhibitor	[mM]	Activity of α-oxoglutarate dehydrogenase [cpm]	% of control
none		1147	100
amobarbital	0.5	883	77
	1.0	436	38
	2.0	333	29
	4.0	206	18

lactate production is highest. Corresponding experiments on α-oxoglutarate dehydrogenase of intact EAT cells (Table I) revealed that this enzyme is less sensitive against 1 – 4 mM amobarbital than pyruvate dehydrogenase. With other tissues such as liver slices or brain homogenates from female NMRI mice it was found that the actual pyruvate dehydrogenase activity was inhibited by 70 – 80% in the presence of 2 mM amobarbital. Even after addition of 0.125 mM dinitrophenol, where pyruvate dehydrogenase activity is normally stimulated up to five-fold [30], the presence of 2 mM amobarbital resulted in an almost total inhibition of the pyruvate dehydrogenase activity of intact EAT cells. The amobarbital-induced inhibition can be reversed by washing the cells with amobarbital-free medium; after washing for 15 min no remaining inhibition of pyruvate dehydrogenase was observed. This confirms other investigations with monolayer cultures of mouse heteroploid cells [1] demonstrating a reversible arrest of cell growth and stimulation of glycolytic activity by amobarbital.

b) ^{14}C incorporation into total lipid fraction from [U-^{14}C]pyruvate

To confirm the data obtained by method a) we employed a further assay system to evaluate the actual pyruvate dehydrogenase activity *in vivo* [21]. In parallel experiments ^{14}C incorporation from [U-^{14}C]acetate into total lipids was measured to get a measure of the influence of amobarbital on other reactions in this assay system besides pyruvate dehydrogenase. Whereas the ^{14}C incorporation from [U-^{14}C]pyruvate or from [U-^{14}C]lactate (Fig. 2 b) follows the same inhibition pattern as in the case

of $^{14}CO_2$ evolution, the ^{14}C incorporation from [U-^{14}C]acetate differs quite strikingly (Fig. 2 d), as significant incorporation still occurs even in the presence of 6 mM amobarbital.

The presented data demonstrate the metabolic effects of amobarbital on pyruvate dehydrogenase activity in intact EAT cells. However, the mechanism of the resulting inhibition is not yet clear. On the basis of the present knowledge at least three mechanisms must be discussed with regard to the regulation of the *in vivo* activity of pyruvate dehydrogenase. First the $NADH + H^+/NAD^+$ ratio, as reflected by the lactate/pyruvate ratio [30–34]; secondly the acetyl CoA/CoASH ratio [31–33, 35–37], and finally the ATP/ADP ratio or the energy charge [38–40]. Since the addition of amobarbital induces a rise in lactate production [1, 4], and an inhibition of respiration [5] (Fig. 2 e) an increase of $NADH + H^+$ level within the cell has to be assumed, $NADH + H^+$ being a competitive inhibitor of pyruvate dehydrogenase [32]. On the basis of the data from (Table II) an inhibition of the *in vivo* activity of pyruvate dehydrogenase by enhanced ATP/ADP ratio after addition of amobarbital has to be taken into account [41, 42].

Effect of amobarbital on the activity of the isolated pyruvate dehydrogenase complex

Studying the isolated pyruvate dehydrogenase complex from Ehrlich ascites tumor cells or from pig heart, it was observed that amobarbital inhibited the pyruvate dehydrogenase activity *in vitro* much stronger than *in vivo*. As is shown in Fig. 2 c, 1 mM amobarbital completely inhibits the enzyme. This shows that amobarbital has – besides its indirect ('metabolic') effects on PDH – additionally a 'direct' one. This becomes quite intelligible on the basis of results reporting a number of flavoenzymes to be inhibited by barbiturates [9]. Since one pyruvate dehydrogenase complex contains 10 – 12 molecules of dihydrolipoamide dehydrogenase [43] it seems reasonable that the total reaction catalyzed by pyruvate dehydrogenase is inhibited by amobarbital. However, it has to be pointed out that the observed inhibition of pyruvate dehydrogenase by amobarbital *in vitro* is only observed in the presence of membrane fractions which are associated with pyruvate dehydrogenase under physiological conditions.

Table II. Influence of amobarbital on the levels of ATP and ADP and the ATP/ADP ratio of intact EAT cells. Values are means ± S.E.M.with the number of experiments in parentheses.

Inhibitor	[mM]	ATP [nmol/10^6 cells]	ADP [nmol/10^6 cells]	ATP/ADP ratio	% of control
none		$1.98_{(6)} \pm 0.24$	$1.49_{(4)} \pm 0.06$	1.33 *	100
amobarbital	0.07	$1.96_{(3)} \pm 0.10$	$1.34_{(1)}$	1.46	110
	0.14	$1.99_{(3)} \pm 0.04$	$1.36_{(2)} \pm 0.06$	1.46	110
	0.58	$2.15_{(3)} \pm 0.11$	$1.64_{(2)} \pm 0.16$	1.31	99
	1.17	$2.20_{(3)} \pm 0.06$	$0.99_{(2)} \pm 0.10$	2.22	167
	2.34	$2.28_{(3)} \pm 0.11$	$1.32_{(2)} \pm 0.03$	1.73	130

* 1.33 represents the value after 30 min of incubation. This value increases with incubation time and approaches a maximum of 2.6 after 4 hours.

There are several contradictory reports concerning the effect of amobarbital or other barbiturates on the glycolytic activity of cells. Thus, an inhibition of lactic acid production of jejunum or brain tissue in the presence of 4 mM pentobarbital was described [44]. Recently reported experiments indicate that barbiturates inhibit glycolysis in the early steps [45]. These observations with EAT cells suggest a decreased rate of glycolysis caused by thiopental. However, other results [1, 4] and the present data demonstrate the opposite effect of amobarbital with EAT cells. We conclude that the observed stimulation of lactate production of EAT cells by amobarbital might be related to inhibition of pyruvate dehydrogenase activity.

Concerning the correlation between cell proliferation and actual pyruvate dehydrogenase activity [13] it has to be emphasized that amobarbital has several points of attack in the living cell; it is therefore difficult to decide what the main reason for the inhibition of cell proliferation might be. With regard to the narcotic effect of barbiturates we would like to propose that the 'metabolic effects' in addition to the 'direct effect' might be sufficient to reduce physiological activity of nerve or brain tissue, since an accumulation of several barbiturates within lipid-rich tissue or lipid-rich compartments such as membrane has to be assumed [46]. The significance of the described effects of amobarbital with respect to its narcotic action is further confirmed by the reversibility observed with cell cultures [1].

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