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Communication

Sadly, Professor Jerzy Popinigis passed away on September 6th, 2003. This work is dedicated to his memory.

Oxidation of glycerol-3-phosphate in porcine and bovine adrenal cortex mitochondria★✪

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The capabilities of porcine adrenal cortex mitochondria to oxidize glycerol-3-phosphate (GP) were studied. In comparison with bovine adrenal cortex mitochondria, porcine mitochondria oxidized GP about three times more actively (18.9 *vs* 6.1 nmol O_2/min per mg protein in the presence of ADP) and the activity of mitochondrial glycerol-3-phosphate dehydrogenase was about four times higher (33.4 *vs* 8.2 nmol/min per mg protein). In porcine adrenal cortex mitochondria we found similar values for succinate and GP oxidation both in the absence and presence of ADP or

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Abbreviations: BSA, bovine serum albumin; DOC, deoxycorticosterone; GP, glycerol-3-phosphate; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase.

deoxycorticosterone (DOC). Rotenone sensitivity of DOC stimulation of GP oxidation indicated that porcine adrenal cortex mitochondria are able to oxidize GP and thus to generate NADPH from GP, presumably *via* reverse electron transport followed by energy-dependent NADH-NADP transhydrogenation.

The major function of mitochondria in aerobic tissues is to catalyze the terminal reactions of metabolic substrates utilization and to produce sufficient high-energy bond of ATP that meets the energy demands of the cells and drives their activities. However, mitochondria from adrenal cortex (similarly as from other steroidogenic cells, including several cell types in the ovary and placenta or the Leydig cells of the testis) represent an important source of cholesterol. Cholesterol serves as the initial substrate for all steroid hormones synthesized in the body regardless of the tissue or the final steroid products. Therefore, mitochondria from steroidogenic cells also contain enzymes that catalyze reactions involved in the synthesis of steroid hormones (Orme-Johnson, 1990; Stocco, 2000; Jefcoate, 2002).

As concerns the energy metabolism in adrenal cortex mitochondria, the most active substrates in supporting the respiratory functions are intermediates of the Krebs cycle (see, Brownie & Grant, 1954; Grant & Mongkolkul, 1959; Wakabayashi *et al.,* 1976; Mandrik *et al.,* 1982). For a long time glutamate (a NADH-dependent substrate) was thought to be metabolically inert as a substrate for bovine adrenal cortex mitochondria (Launay *et al.,* 1974; Wakabayashi *et al.,* 1976). In our previous studies we found differences in glutamate oxidation between bovine and porcine adrenal cortex mitochondria (Litwińska *et al.*, 1984; Popinigis *et al.,* 1990). Bovine mitochondria oxidize glutamate only if oxaloacetate or malate in trace amounts is present in the incubation medium and the respiration supports both oxidative phosphorylation and 11β-hydroxylation of deoxycorticosterone (DOC) to corticosterone (Litwińska *et al.*, 1984). Porcine mitochondria oxidize glutamate at about half the rate observed with succinate (Popinigis *et al.,* 1990).

In our preliminary report we showed that mitochondria from porcine adrenal cortex actively oxidized glycerol-3-phosphate (Popinigis *et al.,* 2003). Oxidation of the FAD-dependent substrate glycerol-3-phosphate (GP) varies in mitochondrial preparations from different tissues. High mitochondrial GP oxidation indicates high expression of mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), which (together with its cytosolic counterpart) enables activity of the glycerol-3-phosphate shuttle. The shuttle provides an additional source of hydrogens for the respiratory chain and can also ensure optimal conditions for glycolysis because cytosolic NADH is reoxidized without lactate production. The aim of this study was to compare the oxidation of two FAD-dependent substrates, succinate and GP, in porcine and bovine adrenal cortex mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from porcine or bovine adrenal cortex by the same procedure as described earlier (Litwińska *et al.*, 1984; Popinigis *et al.*, 1990). In short, adrenal glands from freshly killed animals were collected, rapidly freed of all connective tissue and fat and the central medulla was scraped away and discarded. The cortex placed in ice-cold 330 mM sucrose, 20 mM Tris/HCl, 2 mM EDTA and 0.2% bovine serum albumin (BSA) medium (pH 7.4) was finely minced and gently homogenized in a glass-Teflon homogenizer in the above medium. All procedures were carried out at 0–4°C. The mitochondrial fraction was isolated by differential centrifugation and the mitochondrial pellet was washed three times in incubation medium containing 330 mM sucrose, 20 mM Tris/HCl and 0.5 mM EDTA (pH 7.4). The

analysis of protein content was according to Lowry *et al.* (1951) using BSA as a standard.

Oxygen uptake was measured with a Clark oxygen electrode in a Gilson polarograph (U.S.A.). All measurements were performed in 2 ml of incubation medium containing 200 mM sucrose, 50 mM Tris/HCl, 15 mM KCl, 5 mM K-phosphate buffer, 2 mM EDTA and 0.2% BSA (pH 7.2) at 22°C. State 3 was estimated with 0.2 mM ADP, and 11β-hydroxylase activity after introduction of 0.02 mM DOC if not indicated differently. The protein content of measured samples was approximately 3 mg. Oxygen consumption was expressed as nmol O_2 per min per mg protein. The ADP/O ratio was calculated from oxygen electrode traces according to Estabrook (1967). Activity of 11β-hydroxylase was measured as the transient increase in oxygen uptake above the resting value after addition of DOC.

Mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH; EC 1.1.99.5) activity was measured spectrophotometrically using a Pye Unicam SP-8 VIS spectrophotometer at 600 nm in 50 mM K-phosphate buffer and 1 mM KCN (pH 7.4) with combined artificial electron acceptors 1 mM phenazine methosulfate (PMS) and 0.05 mM 2,6-dichlorophenol-indophenol (DCIP) as the terminal acceptor, at 30°C. The amount of mitochondrial protein in the 2-ml cuvette was about 0.5 mg. The reaction was started by the addition of 20 mM GP. The enzyme activity was expressed as nmol DCIP reduced per min per mg protein using a molar extinction coefficient of 21 $\times 10^3$ M^{-1} cm^{-1}.

Glycerol-3-phosphate, bovine serum albumin, tris(hydroxymethyl)aminomethane and rotenone were products of Sigma Chemical Co. (U.S.A.). All other chemicals were of the highest purity available commercially from POCh, Gliwice (Poland).

RESULTS

As was shown in our preliminary report, mitochondria from porcine adrenal cortex actively oxidized GP (Popinigis *et al.,* 2003). Table 1 shows that the GP oxidation was stimulated in a reversible manner by the addition of ADP and was coupled to ATP synthesis. After returning to the resting value a subsequent addition of DOC supported 11β-hydroxylation of DOC to corticosterone (Table 2). In contrast, mitochondria from bovine adrenal cortex oxidized GP very slowly (Table 1). The stimulation of respiration by ADP was by only 30% in these mitochondria (*vs* 42% in porcine mitochondria) and O_2 uptake did not return to the resting values. Subsequent addi-

Table 1. Oxidation of glycerol-3-phosphate by porcine and bovine adrenal cortex mitochondria and activity of mitochondrial glycerol-3-phosphate dehydrogenase

	Oxygen uptake (nmol O_2/min per mg protein)	
Additions	Porcine mitochondria	Bovine mitochondria
State 4 (–ADP)	13.2 ± 0.8 (n = 8)	4.7 ± 0.9 (n = 3)
State 3 (+ADP)	18.9 ± 1.3 (n = 8)	6.1 ± 1.2 (n = 3)
+DOC	15.4 ± 0.9 (n = 8)	6.1 ± 1.2 (n = 3)
mGPDH (nmol/min per mg protein)	33.4 ± 4.3 (n = 9)	8.2 ± 2.5 (n = 3)

Data indicate values of oxygen uptake with glycerol-3-phosphate (15 mM) in the absence of ADP, State 4 (–ADP). After the addition of ADP the increase in oxygen uptake indicates State 3 (+ADP). When the respiration returns to resting values (not shown) DOC is added (+DOC). Experimental conditions are given in Materials and Methods. The data are expressed as the mean ± S.E.M. The number of experiments is shown in parentheses.

Table 2. Oxidation of succinate and glycerol-3-phosphate by porcine adrenal cortex mitochondria

	Oxygen uptake (nmol O_2/min per mg protein)	
Additions	Succinate	Glycerol-3-phosphate
State 4 (–ADP)	11.2 ± 0.7	13.2 ± 0.8
State 3 (+ADP)	20.9 ± 1.5	18.9 ± 1.3
State 4′ (–ADP)	9.8 ± 0.8	10.9 ± 0.7
+DOC	16.3 ± 0.8	15.4 ± 0.9
–DOC	10.8 ± 0.5	10.9 ± 0.5
ADP:O	1.1 ± 0.1	0.8 ± 0.1

Data indicate values of oxygen uptake with succinate (5 mM) or glycerol-3-phosphate (15 mM) in the absence of ADP State 4 (–ADP). After the addition of ADP the increase in oxygen uptake indicates State 3 (+ADP). When the respiration returns to resting values, State 4′ (–ADP), DOC is added (+DOC). Values (–DOC) indicate returning to the State 4 values. Experimental conditions are given in Materials and Methods. The data are expressed as the mean ± S.E.M. The number of experiments is 8.

tion of DOC did not change the oxygen uptake. Table 1 also shows mGPDH activities in mitochondria of these two species. The activity from porcine mitochondria reached four times higher values.

Succinate is known as the substrate that is oxidized by adrenal cortex mitochondria at the highest rate (Mandrik *et al.,* 1982; Popinigis *et al.,* 1990). We compared the oxidation of two FAD-dependent substrates, succinate and GP, on isolated porcine adrenal cortex mitochondria (Table 2). As can be seen, there is no remarkable difference between the two substrates. The ADP/O ratio is lower (1.1 for succinate and 0.8 for GP) than the theoretical values probably because of the problem of coupling in adrenal cortex mitochondria (Shears & Boyd, 1982).

The most interesting difference in the oxidation of the two substrates (succinate and GP) was found after inhibition by rotenone (a potent inhibitor of complex I in the respiratory chain). The presence of rotenone (5 μM) in the incubation medium completely abolished DOC stimulation of GP oxidation (Fig. 1). However, the ADP and DOC stimulation of succinate oxidation and ADP stimulation of GP oxidation were not affected by rotenone (Fig. 1).

DISCUSSION

Mitochondria from adrenal cortex cells represent an interesting system because they carry out the reactions of oxidative phosphorylation coupled to ATP synthesis and

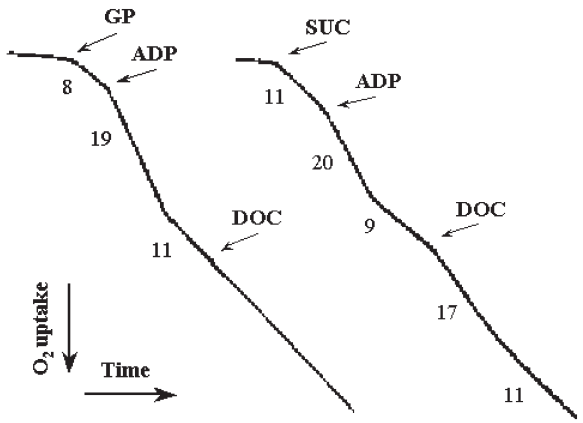

Figure 1. Oxidation of glycerol-3-phosphate and of succinate in the presence of rotenone by porcine adrenal cortex mitochondria.

Experimental conditions are identical as in Materials and Methods. Where indicated, glycerol-3-phosphate (GP) 15 mM, succinate (SUC) 5 mM, ADP 0.15 mM and deoxycorticosterone (DOC) 0.02 mM were added. Oxygen uptake is expressed as nmol O_2/min per mg protein. Similar results were obtained using three preparations of porcine adrenal cortex mitochondria.

also have unique mechanisms for regulating the steroid hydroxylation reactions. Both these oxidative pathways compete for oxygen and reducing equivalents.

Our data showed that the GP:succinate oxidation ratio (18.9:20.9) in porcine adrenal cortex mitochondria was 0.9, indicating an almost equal capacity of both substrates to support the respiratory functions of mitochondria. On the other hand, bovine adrenal cortex mitochondria oxidized succinate at the high rate of 49.8 ± 6.2 nmol O_2/min per mg protein in the presence of ADP and 28.6 ± 2.6 nmol O_2/min per mg protein in the presence of DOC (Popinigis *et al.,* 1990), but oxidation of GP was low (6.1 ± 1.2 nmol O_2/min per mg protein). Accordingly, the GP:succinate oxidation ratio (6.1:49.8) is 0.12.

The finding of active GP oxidation in adrenal cortex mitochondria allowed Simpson and Frenkel (1969) to postulate the possibility of GP cycle and the access to the cytosolic pool of NADH. GP could act as an electron donor for both chains (respiratory and steroidogenic) of adrenal cortex mitochondria. The reducing equivalents derived from GP reach the mitochondrial respiratory chain at the level of the coenzyme Q pool (Rauchová *et al.*, 1992). Their first pathway is the reduction of oxygen *via* complexes III and IV of the respiratory chain leading to ATP synthesis. The second pathway can be the passage to the steroidogenic electron transport chain through the coenzyme Q pool to NAD *via* reversed electron transport and then from NADH to NADPH by NADH:NADP transhydrogenation. Our results in Fig. 1 show relative insensitivity of the succinate-supported 11β-hydroxylation reaction to rotenone. However, as succinate can be converted to malate *via* part of the Krebs cycle (providing NADPH by the action of the malic enzyme), the reverse electron transport is essential in the case of GP-supported 11β-hydroxylation. The rotenone sensitivity of DOC stimulation of GP oxidation in porcine adrenal cortex mitochondria shows the role of the reversed electron transport in GP-supported hydroxylations.

A possible explanation of our findings is that the amount of mGPDH in the adrenal cortex could be related to the nutritional habit of the animals. It is possible to speculate that the adrenal cortex mitochondria of an omnivore (e.g., pig), in addition to acting in the Krebs cycle, could be active in oxidizing a "spare substrate", such as GP. This has been described in the insect flight muscle (Bücher & Klingenberg, 1958), brown adipose tissue (Houštěk *et al.*, 1975) or β-pancreatic isle cells (MacDonald, 1981) where an active GP shuttle was postulated previously.

In conclusion, our results showed a three times more active glycerol-3-phosphate oxidation in porcine adrenal cortex mitochondria in comparison with the bovine ones. The rotenone sensitivity of deoxycorticosterone stimulation of GP oxidation indicates a role of the reversed electron transport in glycerol-3-phosphate-supported hydroxylation in porcine adrenal cortex mitochondria.

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