Covance Studies

The attached report summarizes the results from the assessment of mitochondrial bioenergetics of hepatic mitochondria isolated from rats treated for 3 weeks with one of 4 doses (plus control) of dietary FC10. What is obvious from the data is the ability to detect dramatic differences among the 13 animals tested. However, attempts to group animals according to degree of effect failed to correlate with the exposure dose. Further inquiry revealed that all animals were euthanized with ca., 50 mg/kg pentobarbital, which we subsequently demonstrated to have profound effects on inhibiting pyruvate oxidation at complex I of the mitochondrial electron transport chain. We attribute our inability to establish a dose-response correlation to this effect of pentobarbital. Subsequent attempts to eliminate this confounding effect from the analyses were unsuccessful. However, we remain convinced of the importance of collecting data describing mitochondrial deficits in tissues from exposed animals and in our ability to detect such effects with sufficient sensitivity. Rather than relying on the isolation of high integrity, intact mitochondria for measuring coupled bioenergetic properties, we suggest measuring glycolytic and tricarboxylic metabolites in frozen tissues in order to derive a profile of intermediary metabolism for each exposed animal. From the relative concentrations of the individual metabolites (e.g., the oxoglutarate-to-pyruvate or succinate-to-fumarate ratios), we can estimate relative degrees of metabolic dysfunction in exposed animals[4]. Rather than requiring one of us to be present on-site at the time of sacrifice, we can perform such analyses on tissues that are collected in liquid nitrogen and kept frozen for shipping to our laboratory. This is considerably more convenient for all parties involved and we are anxious to discuss potential opportunities to continue to contribute to the *in vivo* monitoring studies.

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[4] Colson, C. and Klapper, M.H. (1979). Sequential measurements of glycolytic and tricarboxylic acid cycle metabolites in single sample aliquots. Anal. Biochem. 97, 394399.

PROBE DESCRIPTIONS

The introduction.

The rationale of the following experiments was to determine as many "independent" characteristics of energetics of mitochondria as it is possible from a single probe and having time limitations. Due to this, the specific experimental procedure and conditions were designed. The pyruvate –supported respiration and the membrane potential were measured in a simple slightly hypotonic incubation media. Other compounds, such as ADP, Ca^{2+}, and succinate, were added to mitochondria at saturating concentrations and at different times but at least 2 min after the mitochondria were introduced to the pyruvate –containing medium. No inhibitors of oxidative phosphorylation, the respiratory chain, Ca^{2+} transport, or uncouplers were used. Also, no attempts were taken to reduce the concentration of free fatty acids co-isolated with mitochondria or generated during incubation.

The logic of the experimental design was to measure in a single probe the most vulnerable characteristics of mitochondria, such as pyruvate-supported respiration and oxidative phosphorylation, and the most "insensitive", easily reproducible parameters such as succinate-supported respiration and Ca^{2+} transport. Under such limitations, there is no possibility to obtain the precise characteristics for any of the parameters. However, the combinatory analysis of all the measured parameters allows to estimate, at first approximation, the energetics of mitochondria, and make some conclusions about the *in vivo* conditions, in a tissue, where the mitochondria were isolated from.

The interpretation of any in *vitro* data, and especially the backward deduction of *in vivo* conditions in a tissue from the data obtained with isolated mitochondria, is always a kind of "might be" guessing. It is rather tempting to thing in terms like "bad mitochondria", say, if they can not oxidize a substrate fast enough, or "good mitochondria", if they exerted an RCI more then 20. We would prefer another mode of thinking, namely that mitochondria are good when the energetics characteristics they exert fit the cellular demands. And the particular values for mitochondrial energetics usually depend on the particular experimental conditions. Due to this, it seems to be necessary to remind shortly the peculiarities of mitochondrial biochemistry underlying the experimental conditions we have chosen.

The most vulnerable functions of mitochondria are pyruvate – supported respiration and oxidative phosphorylation. The oxidation of pyruvate by liver mitochondria depends on the availability of CoASH in the mitochondrial matrix, on the overall activity of the TCA cycle, and on the activity of respiratory chain (especially, on the Complex I activity). In addition to these, maximal rates of pyruvate oxidation usually require the presence of catalytic amounts of malate in the incubation medium (to stimulate the complete turnover of TCA cycle). Also, it is under allosteric control by of naturally occurring endogenous compounds, such as free fatty acids, acetyl-CoA, and ATP inhibiting the pyruvate dehydrogenase activity. The respiration rate of pyruvate-supported mitochondria phosphorylating ADP depends on all the mentioned parameters plus the activities of oxidative phosphorylation system (Pi transporter, ADP/ATP-translocase, and ATP-synthase). These latter are also can be negatively regulated by free long chain fatty acids and long-chain acyl-CoA.

The oxidation of succinate is not so vulnerable. The sensitivity of succinate dehydrogenase and Complex III to free fatty acids is much less then that of pyruvate dehydrogenase and Complex I. Practically, under any "regular" experimental conditions the rate of succinate-supported respiration is limited only by transmembrane electrochemical proton gradient. The only know strong endogenous inhibitor of succinate dehydrogenase is oxaloacetic acid

However, in the absence of malate and the presence of sufficient amounts of pyruvate in the incubation medium, the accumulation of oxaloacetate could not be [2]expected.

The respiration rate that can be observed during Ca2+ uptake is practically insensitive to any endogenous compounds, which may be co-isolated with mitochondria. Providing that phosphate concentration is sufficient, the respiration rate during Ca2+ transport can be considered as the maximal possible rate of oxidation of a respiratory substrate by the respiratory chain. It is limited mainly by the activity of a Ca2+ transporting system, which is kinetically faster, then any other transport system in mitochondria, including the transport of the majority of respiratory substrates, and much faster then ADP transport. However, when a NAD – dependent substrate is used, the respiration may become inhibited due to fast opening of a Ca2+ -induced pore in the inner mitochondrial membrane, and subsequent release and dilution of mitochondrial NAD. For succinate-supported respiration, the same event would provide the fastest respiration rate because the succinate dehydrogenase is a FAD-dependent enzyme, and pore opening completely discharge the transmembrane proton gradient.

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[2] due to kinetic limitation to TCA cycle arising from overreduction of NAD and fast removal of oxaloacetyc acid stimulated by pyruvate-derived acetyl-CoA.

MATERIALS AND METHODS

The isolation of mitochondria.

Mitochondria were isolated from liver pieces (~1.5 g wet weight) of adult male and female rats by a conventional differential centrifugation procedure. Liver pieces were cooled in 20 ml of isolation medium (210 mM mannitol, 10 mM sucrose, 5mM HEPES-KOH (pH 7.4), 1 mM EGTA). Cooled tissue was minced with scissors and washed once with 20 ml of isolation medium, then diluted with the same medium and homogenized for ~45 sec with a motor-driven Potter homogenizer (Teflon pestle, glass beaker) and placed on ice. The tissue to medium ratio was approximately 1:20 (g:ml). This procedure was applied sequentially to 8 liver specimens. The time interval between homogenizing of the first and the last liver pieces did not exceed([1]) 9 min. The homogenates were simultaneously centrifuged for 10 min x 700 g, $t^{\circ} = 4^{\circ}$ C, and mitochondrial pellets were recovered by centrifugation at 10,000 g x 10 min. The pellets were resuspended in 2 ml of washing medium (210 mM mannitol, 10 mM sucrose, 5mM HEPES-KOH, pH 7.4). The suspensions of mitochondria were diluted to 35 ml with the same medium, and centrifuged at 10,000 g x 10 min. The final mitochondrial pellets were resuspended in washing medium to a protein concentration of 80-120 mg x ml^{-1} and stored on ice. The isolation procedure took exactly 1 hour.

This isolation procedure was repeated for the second set of 8 liver specimens. The processing of the first set (samples 1 – 8) was completed at ~ 10:35 AM and the processing of the second set (samples 9 – 16) was started at ~12:35 AM and completed within 1 hour. The measurements were started at ~2:00 PM.

Measurements.

Mitochondrial membrane potential ($\Delta\Psi$) was estimated from TPP^+ ions distribution measured with a TPP^+-selective electrode. Mitochondrial membrane potential was calculated as described elsewhere.

The rate of oxygen consumption by mitochondria was measured with a hand-made Clark –type oxygen electrode.

Both the mitochondrial membrane potential and the respiration rate were recorded simultaneously using a multichannel incubation chamber equipped with a magnetic stirrer. The volume of the chamber was 1.8 ml. All experiments were performed at room temperature (22° C). The TPP^+ -sensitive electrode was calibrated by sequential additions of known amounts of TPP^+Cl^- before the addition of mitochondria (see Fig.1). The respiration rates were calculated assuming the initial oxygen concentration to be equal to 240 μM.

The incubation medium containing 210 mM mannitol, 10 mM sucrose, 5mM HEPES-KOH, pH 7.4, 4 mM KH_2PO_4, and 10 mM pyruvate was used in all experiments. The same volumes of mitochondrial suspension (30 μl) were added, regardless of the protein concentrations.

Protein concentrations were determined after the experiment by the Bradford assay. Bovine serum albumin was used as a standard.

Additions. Where mentioned, the additions were as following: 5 mM succinate, 200 μM ADP, and 264 μM $CaCl_2$.

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(1) – this modification of the conventional isolation procedure can be expected to decrease the quality of mitochondria. According to our experience, 10 min of storing of homogenized tissue on ice can produce approximately 20 - 30% decrease in RCI, depending on the perfectness of cooling.

The results

Table 1 shows the relation of sample labeling used in the following text to the labels of liver specimens.

Table 1. The sample labels :

1	2	3	4	5	6	7
C90753 m	C90719 m	C91230 f	C90935 m	C91024 m	C91350 f	C90890 m

8	11	12	13	14	15
C91165 f	C91205 f	C91430 f	C90814 m	C91135 f	C91169 f

The samples were measured in the following time sequence: **3 - 4 – 5 – 6 – 8 – 12 – 13 – 11 – 14 – 2 – 1 – 7 - 15.**

An average time spent for each probe was about 12 min, with approximately 5 min interval between probes. All measurements were completed within 4 hours. Table 2 shows the values of the respiration rate obtained in the presence of various additions.

Table 2. The respiration parameters measured.

For the incubation medium and other conditions, see Materials & Methods. For the sequence of additions, see Probe Descriptions chapter. Data are expressed in nmol O_2 /min/mg of protein. V_{pyr}, the rate of pyruvate – supported respiration; V_{adp}, the rate of respiration produced by ADP addition (St. 3); V_{ca}, the rate of respiration during Ca^{2+} uptake; V_{succ}, succinate- supported respiration in the presence of pyruvate and in the absence of other additions (unless stated otherwise); Ca/O, the ratio of Ca^{2+} transported (nmol) to the amount of oxygen consumed (nmol); ADP/O, the ratio of ADP phosphorylated (nmol) to the amount of oxygen consumed (nmol).

	1_t	2_t	3	4	5	6	7	8_t	11	12	13	14_t	15_t
V_{pyr}	3.7	3.1	2	6.8	5.5	4	5.7	0	4.6	2.1	1.8	2.7	2.7
V_{ADP}	0	^{P}5.7	30.5	50.5	^{P}10.2	^{P}5.1	0	ND	^{P}8	19	^{P}7.7	42.7	34.1
V_{Ca}	44.5	94.3	73.2	ND	^{P}6.4	96	36.8	ND	ND	78.9	^{P}14.2	ND	46.9
V_{succ}	10.4	14.7	13.6	33.4	*119	28.8	15.9	24	*69	10.6	*87	16.7	12.3
Ca/O	7.6	7.3	4.3	ND	^{P}7.2	4.9	5.5	ND	ND	4.4	^{P}12	ND	4.7
ADP/O	—	N.R.	N.R	N.R.	3.6	N.R.	—	ND	3.1	N.R.	3.5	0.7	1

ND – not determined

N.R. – not resolved (see Probe Description chapter)

^{P} – pyruvate –supported respiration

* - the respiration rate was measured in the presence of the excess of Ca2+, which induced the opening of pore, so it should be considered as the maximal rate of succinate oxidation.

Probe 1.

The sequence of additions was ADP, succinate, Ca2+, Ca2+. Mitochondria were able to maintain the membrane potential of 144 mV by pyruvate oxidation. However, ADP did not stimulate the respiration, although induced the $\Delta\Psi$ decrease to 130 mV. The subsequent addition of succinate stimulated the respiration and increased the $\Delta\Psi$ to 170 mV. The increase in respiration and in the $\Delta\Psi$ was biphasic, indicating incomplete phosphorylation of ADP to the moment of the addition of succinate. Only steady-state values for $\Delta\Psi$ and respiration are shown. RCI for phosphorylation was about 1.2. Subsequent addition of Ca2+ induced ~4 fold transient stimulation of respiration and decrease in $\Delta\Psi$ to 149 mV. These mitochondria consumed 360 nmol Ca2+/mg of protein, the pore was not observed.

Interpretation: the pyruvate transport system or pyruvate oxidation is inhibited; the activity of respiratory chain with succinate during Ca2+ uptake is ~2 fold less then usual (or the Ca2+ transporting system is inhibited); the resistance of these mitochondria to pore opening is in the upper top of normal range (or even slightly higher) for a well – coupled rat liver mitochondria.

Probe 2.

The sequence of additions was as in Probe 1. The responses on ADP, succinate, and Ca2+ were as in Probe 1, too. The $\Delta\Psi$ value for pyruvate oxidation was 138 mV, the ADP –induced $\Delta\Psi$ decrease was to 127 mV, the succinate – supported $\Delta\Psi$ was 166 mV, and Ca2+ induced the decrease in $\Delta\Psi$ to 127 mV.

Interpretations: The same as for Probe 1 for except that apparent Ca2+ capacity (the resistance to pore opening, determined by the characteristic $\Delta\Psi$ decrease) was less then for Probe 1 (between 200 and 360 nmol Ca2+/mg of protein).

Probe 3.

The sequence of additions was succinate, ADP, Ca2+. These mitochondria were unable to maintain the $\Delta\Psi$ with pyruvate. The $\Delta\Psi$ with succinate was also low, 144 mV. The addition of ADP induced the decrease in $\Delta\Psi$ to 137 mV and irreversible stimulated the respiration (Table 2). The addition of Ca2+ further stimulated the respiration, but in this case the stimulation was transient (normal response). The pore was opened by 164 nmol Ca2+/mg of protein.

Interpretations: These mitochondria a)uncoupled, b)the pyruvate oxidation is inhibited at the level of transport, pyruvate dehydrogenase, or Complex I. The rest of respiratory chain and/or succinate dehydrogenase activity are in the normal range. The sensitivity to Ca2+ is too high. It may be due to accumulation of a pore – inducing compound like free long-chain fatty acids. The decrease in Ca/O (Table 1) is probably due to uncoupling and/or incomplete phosphorylation of ADP to the moment of Ca2+ addition.

Probe 4.

The sequence of additions was as in Probe 3. These mitochondria were not able to maintain the $\Delta\Psi$ by pyruvate oxidation. The ADP irreversibly slightly stimulated the succinate –supported respiration, the addition of Ca2+ was without further effect. The resistance to pore opening was higher then 135 nmol Ca2+/mg of protein.

Interpretations: Mitochondria were uncoupled and inhibited with both substrates. However, pyruvate oxidation was less inhibited then in the probes described above (compare the Vpyr values in Table 1). The amount of Ca2+ required for pore opening bear no information in this case (the protein concentration, which was measured after the experiment, was too high, 1.97 mg/ml, so the added amount of Ca2+ was insufficient).

Probe 5.

The sequence of additions was ADP, Ca2+, Ca2+, and succinate. These mitochondria were able to maintain ΔΨ of 143 mV by pyruvate oxidation. The addition of ADP transiently stimulated the respiration and decreased the ΔΨ to 115 mV, the RCI was 2.7. The measurement of the ΔΨ revealed an unusual pattern, after the completion of ADP phosphorylation the ΔΨ increased to the higher level, than that before the ADP addition (a hyperpolarisation). The response of respiration on the Ca2+ addition was unusually slower then the response on ADP (see Table 2). The pore was opened by 243 nmol Ca2+/ mg of protein. Succinate-supported respiration was recorded after the pore opening, so it reflects the maximal activity of succinate dehydrogenase + the rest of respiratory chain, and it is in the normal range.

Interpretations: These mitochondria were coupled, the respiration with both substrates was not significantly inhibited. However, the response on Ca2+ was unusual, it might indicate some alteration in the Ca2+ - transporting system. Actually, the "good" pyruvate oxidation may also reflect the availability of free CoASH in the mitochondrial matrix, that can results from the inhibition of fatty acids β-oxidation.

Probe **6**.

The sequence of additions was ADP, succinate, Ca2+. These mitochondria maintained the ΔΨ of 122 mV by pyruvate oxidation. ADP slightly stimulated the respiration. The response on the succinate, Ca2+ additions were like these in Probe 3, for except that 155 nmol Ca2+/mg of protein did not open the pore (normal response).

Interpretations: These mitochondria were uncoupled, and the pyruvate oxidation inhibited. The data provide no information on the sensitivity to pore by the same reasons as in Probe 4 (insufficient Ca2+ loading).

Probe **7**.

The sequence of additions was ADP, succinate, Ca2+, Ca2+, Ca2+. The responses were essentially as in Probes 1, 2. The ΔΨ for pyruvate oxidation was 143 mV, and that for succinate-supported respiration was 167 mV. Ca2+ opened the pore in the range 306 –465 nmol/ mg of protein (see Probe 1).

Interpretations: See Probe 1, Probe 2.

Probe **8**.

The only succinate was added because these mitochondria appeared to be unable to maintain the ΔΨ both with pyruvate and with succinate. Succinate-supported respiration was partially inhibited. It should be noted also, that the yield of mitochondria from this liver specimen was the lowest, the initial concentration of mitochondria in suspension was 75 mg/ml and that in probe was 1.25 mg/ml

Interpretations: for a regular rat liver mitochondria, 1.25 mg/ml concentration in probe is more then sufficient to observe all the mitochondrial functions. These mitochondria were inhibited and completely uncoupled.

Probe **11**.

The sequence of additions was ADP, Ca2+, and succinate. These mitochondria were able to maintain the ΔΨ of 136 mV by pyruvate oxidation. The addition of ADP slightly but reversible stimulated the respiration, but that of Ca2+ was without effect. However, the addition of Ca2+ induced complete deenergization, and the subsequent succinate addition did not increase the ΔΨ whereas stimulated the respiration.

Interpretations: The absence of the stimulation of pyruvate oxidation by the Ca2+ addition is probably due to the opening of pore (see Introduction).The sensitivity to Ca2+ is unusually high, less then 150 nmol Ca2+/ mg of protein, as in Probe 3.