Evaluation of Mitochondrial Function by Measuring the Heat Production in State 3 and State 4 Respiration

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Using a microcalorimetric method, we have measured the heat production in states 3 and 4 respiration of a mitochondrial preparation from rat heart ventricle. Adenosine triphosphate production in state 3 respiration was also determined for the same preparation after heat production was measured. In Tris-buffered solution with pyruvate and malate used as substrates, the total heat productions in states 3 and 4 respiration for a 10-min reaction period were about 709 and 207 mJ/mg of mitochondrial protein, respectively.

2,4-Dinitrophenol, an uncoupler of mitochondrial oxidative phosphorylation, increased the heat production in both states 3 and 4 respiration. On the other hand, antimycin A_1, an inhibitor of the electron transport system, decreased the heat production in both states.

When ventricle mitochondrial dysfunction was induced by the *in vivo* administration of a high dose of isoproterenol, heat production was increased in state 4 respiration.

These results indicate that the calorimetric method can be used to evaluate the mitochondrial function.

Keywords mitochondria; oxidative phosphorylation; microcalorimetry; heat production; rat ventricle; 2,4-dinitrophenol; antimycin A_1; isoproterenol

Mitochondrial dysfunction occurs in ischemia or hypoxia and causes a depression of adenosine triphosphate (ATP) synthesis followed by biomembrane damage. So it is important to evaluate the mitochondrial function.

This has been done by judging the morphological change or monitoring the oxygen consumption in mitochondrial respiration.[1)]

The reaction of mitochondrial oxidative phosphorylation is a very efficient and specialized body function that traps and stores biological energy as the high-energy phosphate bond of ATP produced from nutrients such as glucose. So, the heat production in this reaction should be closely related to mitochondrial function, and it should be very informative to measure the heat production in states 3 and 4 respiration.

Recently it has became possible to measure the heat production in cultured cells by microcalorimetry.[2)] We have adopted this method for measuring the heat production in mitochondrial oxidative phosphorylation, and succeeded in quantitative measurement.

In the present work, we measured the heat production in states 3 and 4 respiration, and clarified the relation between mitochondrial function and heat production. Furthermore, the data obtained by the calorimetric method were compared with the data by the polarographic method, measured separately.

Materials and Methods

Preparation of Mitochondria Rat heart ventricle mitochondria were prepared by the method of Chance and Hagihara[3)] with some modifications. Sprague-Dawley male rats aged 9—10 weeks purchased from CLEA Japan Inc. were used. The chest of an anesthetized rat was opened and the ventricle was immediately removed, minced and washed with chilled mannitol solution to remove the external blood. The mannitol solution, containing 0.21 M mannitol, 70 mM sucrose and 1 mM ethylene glycol bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetate (EGTA), was buffered with 10 mM Tris–HCl (pH 7.4). The minced ventricle was incubated with 4 ml of the mannitol solution containing 4 mg of Nagarse (an alkaline protease, from Nagase Co., Ltd., Osaka, Japan) for 10 min with continuous stirring in an ice-water bath. After the addition of 16 ml of the mannitol solution, the minced tissue was homogenized using a loosely fitting glass-Teflon homogenizer at about 400 rpm (5 strokes). The resulting homogenate was centrifuged at $400 \times g$ for 10 min at 4 °C. The supernatant obtained was then centrifuged at $10000 \times g$ for 10 min at 1 °C. The pellet obtained was rinsed with the mannitol solution, suspended in 10 ml of the mannitol solution and centrifuged again at $10000 \times g$ for 10 min at 1 °C. The pellet was suspended in 500 μl of ice-chilled reaction medium and used to measure heat production in oxidative phosphorylation reactions. The reaction medium, containing 0.23 M mannitol, 70 mM sucrose, 2 mM EGTA and 5 mM inorganic phosphorus, was buffered with 20 mM Tris–HCl (pH 7.4).

The mitochondrial protein was measured according to the method of Lowry *et al.*,[4)] using bovine serum albumin as a protein standard.

Measurement of Heat Production Fifty microliters of the mitochondrial suspension, 20 μl of 0.25 M sodium pyruvate–0.05 M sodium malate and 25 μl of 0.2 M adenosine diphosphate (ADP)–Na (pH 7.4) were added to 1 ml of oxygen-saturated reaction medium. Then 500 μl of the above mixture was immediately applied to an ESCO-3000 thermoactive cell analyzer (ESCO Co., Ltd., Tokyo, Japan), a flow-type microcalorimeter, to measure the heat production in state 3 respiration, using the method described in reference 2. To measure heat production in state 4 respiration, a similar procedure was followed but without adding the ADP. Heat production was measured for 10 min at 37 °C. After the measurement of heat production in state 3 respiration, the ATP concentration in the sample was also measured as follows.

Calculation of Heat Production Total heat production during a 10 min reaction period was calculated as shown in Fig. 1. Heat production of actual record is shown by the solid line. It is considered, however, that total heat production during the 10 min reaction period may be represented by the hatched area shown in Fig. 1 for the following reasons. 1) The onset of oxidative phosphorylation reaction is very fast. 2) When constant heat is supplied as current to the detector, a similar response delay occurs.

Measurement of ATP Production in State 3 Respiration To stop the reaction, immediately after measuring the heat production for 10 min, an equal volume of 0.9 M perchloric acid was added to the reaction mixture that came from the calorimeter outlet. It was then diluted with a sufficient amount of buffer to neutralize it, and the ATP was measured by the luciferin–luciferase method.[5)]

Preparation of Isoproterenol-Induced Myocardial Ischemia L-Isoproterenol (from Sigma, U.S.A.) was administered to the rat subcutaneously in 0.9% sodium chloride solution at a dose level of 600 mg/kg of body weight. Twenty-four hours after administration of the isoproterenol, the heart ventricle was removed and its mitochondrial function was evaluated.

Statistical Analysis The significance of differences between mean

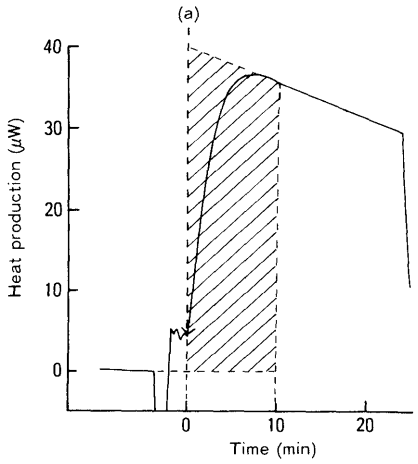

Fig. 1. A Chart of Heat Production in State 3 Respiration

The reaction mixture (see Materials and Methods) was pumped through thin Teflon tubing into the water bath at 37 °C. After 90 s, the sample flowed into the detector. Then the flow was stopped and the measurement of heat production was started (a).

Heat production of actual record is shown by the solid line. But it is considered that total heat production during 10 min of reaction period may be represented by the hatched area shown in the figure as mentioned in Materials and Methods.

The pattern of the chart in state 4 respiration was similar to that in state 3.

values was estimated by using Student's t-test. Differences with p values of 0.05 or lower were considered significant.

Results and Discussion

The mitochondrial preparations used in the present work showed a respiratory control ratio (*RCR*) of about 10 and a *P/O* ratio of about 3 with the pyruvate/malate mixture. This indicates that we consistently obtained "intact" mitochondria.

Heat Production in States 3 and 4 Respiration The precision of this method was determined by separate measurements in one mitochondria preparation. The coefficients of variation of this method in states 3 and 4 respiration were 6.3% and 4.4% ($n=10$ each), respectively. These values were smaller than with the polarographic method (8.6% in state 3 and 13.5% in state 4 respiration ($n=10$ each) in our experiment). This indicates that the heat measurement is superior to polarography in evaluating the mitochondrial function.

There was a linear relationship between the concentration of mitochondria and the heat production in both states 3 and 4 respiration (Fig. 2). In state 3, about 709 mJ/mg of mitochondrial protein was produced, while only about 207 mJ/mg was produced in state 4. The ratio of the heat of state 3 to state 4 was about 3.5. In contrast, the relative oxygen consumption rate (*RCR*) in states 3 and 4 was about 10.

Figure 3a shows a linear relationship between the concentration of mitochondria and ATP formation in state 3 respiration. This indicates that 17.4 μmol of ATP/mg of mitochondrial protein was produced in 10 min. Since heat and ATP production are both proportional to mitochondrial concentration, it follows that a linear relationship would exist between the heat and ATP production. In fact the coefficient of correlation for that relationship was 0.995, as shown in Fig. 3b.

Mitochondrial Dysfunction Caused by *in Vitro* Treatment In state 3 respiration, 2,4-dinitrophenol (DNP),

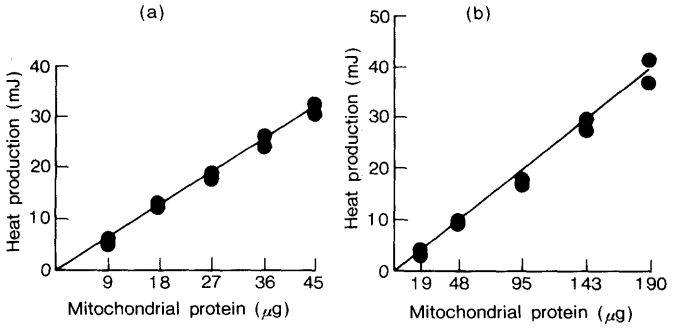

Fig. 2. Heat Production in State 3 (a) and State 4 (b) Respiration at Various Concentrations of Rat Heart Mitochondria

Heat production was measured at 37 °C for 10 min, calculated as shown in Fig. 1.

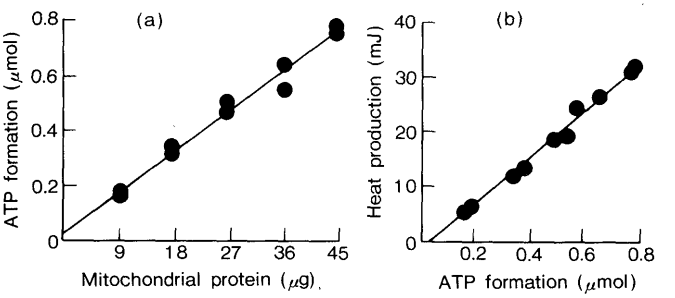

Fig. 3. ATP Formation in State 3 Respiration at Various Concentrations of Mitochondria (a) and the Relationship between Heat Production and ATP Formation in State 3 Respiration (b)

ATP formation was determined for the same preparation used to measure the heat production after heat production was measured at 37 °C for 10 min.

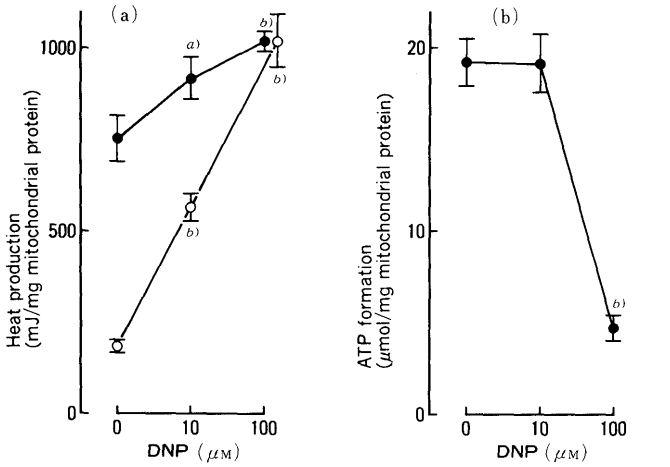

Fig. 4. Effect of DNP on Heat Production (a) and ATP Formation (b) in Normal Rat Ventricle Mitochondria

All values are the mean ± S.D. ($n=5$ each group). *a*) $p<0.01$, *b*) $p<0.001$ *vs.* control value. ▼, state 3; ▽, state 4.

an uncoupler of mitochondrial oxidative phosphorylation, inhibits the ATP synthesis without affecting the oxygen consumption. Furthermore, it increases the oxygen consumption and causes a respiratory burst in state 4 respiration.[6-8] Thus, DNP causes dissipation of energy as heat.

As shown in Fig. 4, DNP did in fact increase the heat production in state 3 respiration. This increase can be explained by the uncoupling effect when ATP synthesis was inhibited at 100 μM DNP. But at 10 μM, DNP has no effect on ATP formation. So it is unclear why heat production was also increased at 10 μM DNP, as Fig. 4 shows.

Table I. Effect of Antimycin A_1 on the Heat Production and ATP Formation of Normal Rat Ventricle Mitochondria

	Heat production (mJ/mg mitochondrial protein)		ATP formation (μmol/mg mitochondrial protein)
	State 3	State 4	
Control	630±40	159±7	13.7±1.0
Antimycin A_1	76± 2[a)]	70±6[a)]	0.9±0.1[a)]

Data represent mean±S.D. (n=4—10, each group). *a)* $p<0.001$ *vs.* control value.

Although this can not be explained in thermodynamic terms, this calorimetric method may still be more sensitive than the polarographic method in detecting the dysfunction of mitochondrial state 3 respiration caused by DNP.

Furthermore, DNP also increased the heat production in state 4 respiration (Fig. 4). This can be easily explained: DNP increases the oxygen consumption in state 4, which entails the loss of energy.

When DNP was applied to rat liver mitochondria, a similar result was obtained (data not shown).

Antimycin A_1, an inhibitor of the electron transport system, inhibits the oxygen consumption in both states 3 and 4 respiration.[9,10] So it follows that heat production in both states will be suppressed. In fact, as shown in Table I, antimycin A_1 at a concentration of 100 nM decreased the heat production in both states 3 and 4 respiration. ATP formation in state 3 respiration was also depressed.

Nakamura and Matsuoka[11] previously reported the simultaneous measurement of temperature change and oxygen consumption in a reaction medium of rat liver mitochondria. Their results also showed the increase of enthalpy change per gram atom of oxygen consumed in state 4 respiration treated with DNP. This coincides with our results. However, the precision of the two methods is considered to be different for the following reason.

Nakamura and Matsuoka reported that the possible proton translocation or production in mitochondria and the resulting protonation reaction of Tris (ΔH(protonation)$=-47.5$ kJ/mol)[12] or Hepes (ΔH(protonation)$=-20.96$ kJ/mol)[13] during the respiratory process did not make a major contribution to the total heat production in the reaction of mitochondrial oxidative phosphorylation. However, we detected a difference in the heat production measured in Tris and Hepes buffers, which had a different enthalpy change in the protonation. As shown in Table II, heat production in state 3 respiration was about 1.17 times higher in Hepes-buffered solution than in Tris-buffered solution, whereas the amounts of ATP formed during measurement were almost the same in both buffers. Hence, the difference of heat production can be ascribed to the difference in enthalpy change in the protonation of these buffers. This suggests that our calorimetric method is more precise than the method of Nakamura and Matsuoka.

According to the calculation method of Kodama *et al.*,[14] the proton consumption in this reaction is about 0.27 mol/mol of ATP formed.

Mitochondrial Dysfunction Caused by *in Vivo* Treatment A large amount of isoproterenol administration (600 mg/kg, s.c.) in the rat causes myocardial infarction[15,16] followed by mitochondrial dysfunction.[17] In our study, the oxygen consumption in state 4 respiration, measured by the polarographic method after a 24-h administration of isoproterenol, was significantly increased, while in state 3 respiration, oxygen consumption was unaffected and the P/O ratio was not changed (data not shown). This indicates that mitochondrial respiration undergoes a partial burst, but the phosphorylation reaction is preserved.

Table II. Heat Production and ATP Formation in State 3 Respiration in Hepes or Tris-Buffered Solution

	Heat production (mJ/mg mitochondrial protein)	ATP formation (μmol/mg mitochondrial protein)
Tris	820±65	21.3±1.1
Hepes	1026±17[a)]	20.1±2.6

Data represent mean±S.D. for four determinations. *a)* $p<0.001$ *vs.* Tris-buffered solution.

Table III. Mitochondrial Function in Isoproterenol (ISO)-Induced Myocardial Ischemia

	Heat production (mJ/mg mitochondrial protein)		ATP formation (μmol/mg mitochondrial protein)
	State 3	State 4	
Control	686±120	176±22	16.8±1.7
ISO-treated	604± 66	213±22[a)]	15.5±2.0

Heat production in states 3 and 4 respiration, and ATP formation in state 3 respiration were measured in normal or isoproterenol-treated rat ventricle mitochondria. Data represent mean±S.D. for ten determinations. *a)* $p<0.01$ *vs.* control value.

Table III shows the change in mitochondrial function in isoproterenol-treated rats measured by the calorimetric method. Heat production in state 4 respiration was significantly increased, but heat production and ATP formation in state 3 respiration were not changed significantly. It is a novel finding that the mitochondrial dysfunction caused by isoproterenol can be detected by the calorimetric method. These observations were in accordance with the result using the polarographic method, and suggest that the calorimetric method described here can also be used to detect the mitochondrial dysfunction induced by *in vivo* treatment.

In summary, measurement of heat production is a uniquely precise method for evaluating the functional state of mitochondria, and has the potential to yield novel information not easily obtainable by other analytical methods.

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