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Short communication

Microcalorimetric studies of the inhibition of sodium azide on the mitochondrial metabolism of fish liver tissue

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Abstract

The metabolic thermogenic curves of mitochondria isolated from fish liver tissue have been determined by an LKB 2277 Bioactivity Monitor. The metabolism activity of mitochondria inhibited by sodium azide has also been studied. The thermogenic curves can be divided into four parts: the lag phase, active recovery phase, stationary phase, and decline phase. From these thermogenic curves, the recovery rate constant k_1, decline rate constant k_2, the maximum heat production rate P_m, heat output Q are obtained. The metabolic heat released, time of each phase, rate constants, and shape of the thermogenic can be significantly influenced by sodium azide added. These results suggest that sodium azide inhibits the metabolism of mitochondria, and the inhibition is incomplete. The inhibitory effect reaches the maximal value with the exactly sodium azide concentration of 2μg mol^{-1}. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Mitochondria; Microcalorimetry; Metabolism; Sodium azide

1. Introduction

Mitochondria produce most of the cell's energy by oxidative phosphorylation, a process in which electrons are passed along a series of carrier molecules called the electron transport chain. These electrons are generated from reduced nicotinamide adenine dinucleotide (NADH), which is produced by oxidation of nutrients such as glucose, and are ultimately transferred to molecular oxygen. The electron transport requires the consecutively actions of five respiratory enzyme complexes located in the mitochondrial inner membrane. The passage of electrons between these complexes releases energy that is stored in the form of a proton gradient across the membrane and is then used by ATP synthase to make ATP from ADP (adenosine 5′-diphosphate) and phosphate.

Azide is an inhibitor of electron transport chain. It is the inhibitor of cytochrome oxidase (complex IV), which is one of the five respiratory enzyme complexes located in the mitochondrial inner membrane. It blocks the electron transport between cytochromes aa3 and O_2 in the respiratory chain. Recent studies show azide has more effects. Azide do not affect mtDNA copy number or mRNA levels in myoblasts [1], and appropriate concentration of azide can result in a rapid increase in K (ATP) channel activity in the Cambridge rat insulinoma G1 cells [2].

The second law of thermodynamics states that spontaneous reactions occur in directions that increase

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the overall disorder of the universe. A consequence of this is that with each energy transfer some energy is lost to the chaotic motion of molecules that we measure as temperature. Currently used biological reaction microcalorimetry systems have a resolution of 0.2 $\mu W\ ml^{-1}$. They are very suitable for measurements of heat production of slight exothermic or endothermic processes, such as the heat production of anaerobic microbial, cell cultures or organelles. Calorimetry is completely nonspecific which often is a valuable property when the method is used for the monitoring of complex and poorly characterized biological systems. However, for many applications, it would be of great value if the calorimetric signal contains specific information concerning a given property of a biological system. With the inherent high specificity of such systems this can often be achieved by specific activation or inhibition of the metabolic processes on line.

In this paper, the thermogenic curves of the metabolism of mitochondria isolated from *Carassius auratus* liver with the inhibition of sodium azide were studied by using an LKB 2277 Bioactivity Monitor. We found that sodium azide inhibits the metabolism of mitochondria, but the inhibition is incomplete and the effect of inhibitor reaches its maximum value at the exactly concentration of 2 $\mu g\ ml^{-1}$.

2. Experimental

2.1. Materials

C. auratus was supplied by the Institute of Life Science, Wuhan University.

Isolating medium was 0.25 $mol\ l^{-1}$ sucrose (A.R., Chemical reagent factory of Guangzhou), 1 $mmol\ l^{-1}$ EDTA (A.R. Chemical reagent factory of Shantou), 10 $mmol\ l^{-1}$ Tris–HCl (A.R. Xinhua Chemical reagent of Shanghai), pH 7.4. The medium was sterilized at 12°C for 30 min.

Sodium azide, A.R., Shanghai stock, supply and marketing agency of chemical reagent.

Biuret reagent contained per 1000 ml: $CuSO_4 \cdot 5H_2O$ (A.R., No. 3 Chemical reagent factory of Shanghai) 1.5 g, $NaKC_4H_4O_6 \cdot 4H_2O$ (A.R., Medicine corporation of China) 6.0 g, NaOH (A.R., No. 3 chemical reagent factory of Tianjin) 10.0 g.

2.2. Equipment

A microcalorimeter, LKB 2277 Bioactivity Monitor was used to obtain the thermogenic curves of the mitochondria. The microcalorimeter was thermostated at 28°C. The signal was recorded by means of an LKB 2210 recorder (1000 mV range). For more details of the performance and construction of the instrument, see [3,4].

2.3. Isolation of mitochondria

Mitochondria were isolated by first removing the liver from the *C. auratus* and washing with sterile isolating medium. The liver was then weighed, homogenized and centrifuged at 4000 rpm for 15 min. The clear supernatant was centrifuged again for 15 min at 4000 rpm. The sediment was discarded after each step. The clear liquid was the centrifuged twice at high speed (10000 rpm) 15 min each time to deposit the mitochondria as sediment. This was resuspended in the isolating medium for measurements. All the above operations were performed aseptically at 273–277 K.

2.4. Experimental determination

Using UV spectrophotometry and biuret reagent, we maintained the equal concentration of mitochondria protein at 3.9 $mg\ ml^{-1}$ by adding the isolating medium.

The thermogenic curves of mitochondria were recorded using sealed glass ampoules, one containing a reference solution such as double distilled water, the other containing the sample (suspension of mitochondria). Each ampoule contained a 1 ml sample or reference and 2 ml of air. The sodium azide was added into the sample at the beginning of the experiment. The final concentration of sodium azide is 0, 1, 2, 3, 5 $\mu g\ ml^{-1}$, respectively.

3. Results and discussion

The thermogenic curves for mitochondria under different concentration of sodium azide are shown in Fig. 1.

As previously reported [5], analysis of the thermogenic curves reveals four phases: lag phase, activity

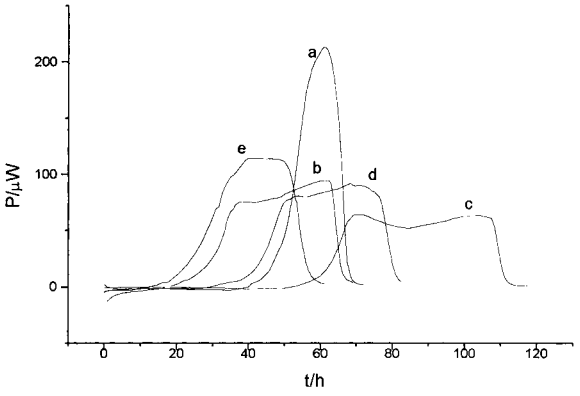

Fig. 1. Thermogenic curves of mitochondria metabolism with different concentration of sodium azide. (a) control; (b) 1 μg ml^{-1}; (c) 2 μg ml^{-1}; (d) 3 μg ml^{-1}; (e) 5 μg ml^{-1}.

recover phase, stationary phase, and decline phase. As seen from the thermogenic curves, the activity recovery phase and decline phase when sodium azide added are S-shape. This indicates that the change of metabolism activity was limited. The 'logistic equation' is the classical equation to describe this type of thermogenic curves. In the activity recovery phase and the decline phase, we calculated the activity recovery rate and decline rate by using the 'logistic equation' [6].

$$\frac{dp}{dt} = kP(1 - SP) \quad (1)$$

where k is recovery rate constant or decline rate constant, P the heat production rate and S is the factor of limitation.

The integral form of Eq. (1) is:

$$\ln\left[\frac{P}{(1 - SP)}\right] = \ln\left[\frac{P_0}{(1 - SP_0)}\right] + kt \quad (t = 0, P = P_0) \quad (2)$$

where P_0 is the heat production rate when t equals 0.

The recovery rate constant and decline rate constant according to Eq. (2) were calculated for all experiments. The values are shown in Table 1.

As we can see from the thermogenic curves and data, the shapes of the thermogenic curves at different concentration of sodium azide are very similar. They all have a distinct stationary phase, and it differs from that of the control. The shape of thermogenic curve of control appeared to have no stationary phase.

The time of the metabolism power can be detected were increased from about 32 to 57, 62, 53 and 50 h corresponding to the concentration of sodium azide at 1, 2, 3 and 5 μg ml^{-1}, respectively.

(Fig. 2 show the recovery rate constant at different concentration of sodium azide). The maximal thermal powers are lower than that of control when the sodium azide were added. The activity recovery constants in Table 1 (and Fig. 2) show that the relation between inhibitory effect and the concentration is not linear. The inhibitory action reached its maximum when the concentration of sodium azide was exactly 2 μg ml^{-1}.

It should be noted that although the time of metabolism and the maximal thermal power of mitochondria varied with different concentration of sodium azide, the total heat output remained nearly the same (shown in Table 1).

Table 1
Values of metabolism parameters of *C. auratus* liver mitochondria at 28°C

C_{NaN_3} (μg ml^{-1})	Phase	k (min^{-1})	S (μW^{-1})	R	Maximum power, P_m (μW)	Total heat output, Q (J)
Control	Recovery	0.0056	0.0042	0.997	213.2	10.41
	Decline	−0.0172	0.0046	−0.993		
1	Recovery	0.0039	0.0671	0.999	93.9	9.78
	Decline	−0.0196	0.0105	−0.993		
2	Recovery	0.0031	0.0000	0.984	63.8	10.81
	Decline	−0.0168	0.0149	−0.987		
3	Recovery	0.0034	0.0032	0.989	91.2	10.57
	Decline	−0.0123	0.0106	−0.996		
5	Recovery	0.0037	0.0076	0.999	113.8	10.80
	Decline	−0.0127	0.0085	0.999		

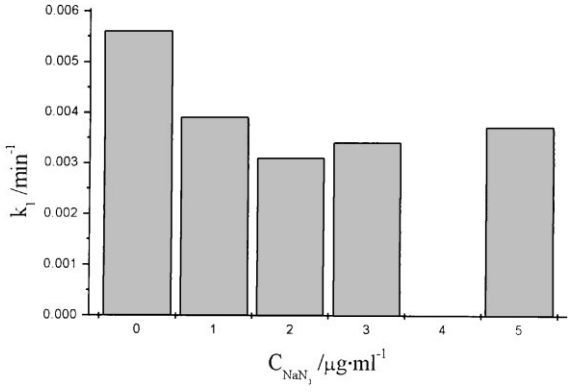

Fig. 2. Comparison of the recovery rate constant (k_1, min^{-1}) of different concentration of sodium azide.

We know that sodium azide is a complex IV (cytochrome oxidase) inhibitor. The substrate of cytochrome oxidase, cytochrome c, is a water-soluble hemoprotein that donates electrons on the cytoplasmic side of the mitochondrial inner membrane [7]. These electrons are transferred to the active site, which contains a heme iron and a copper [8], and they are used to reduce O_2 into two water molecules. The protons needed for this reaction are taken from the mitochondrial matrix side through two channels. The same channels are used to pump one proton per electron across the membrane.

Azide can combine with the ferri form of the enzyme, so it decreases the concentration of the enzyme combined with O_2, and results in the decrease of thermal power.

It must be realized that, in many cells and tissues, only part of the respiration is inhibited by sodium azide, regardless of the concentration [9]. The inhibition by sodium azide is incomplete. We thought that it was because sodium azide is a competitive, reversible inhibitor of cytochrome oxidase. So most of the nutrients still can be utilized through oxidation processes in mitochondria, and the total heat production remained the same (about 10 J). Due to the different recovery activity rates, the metabolism time is longer when the recovery activity rate is slower.

As to the non-linearity relation between inhibitory effect and the azide concentration, it is because sodium azide can act upon mitochondria in many ways. It has been reported [1] that at low concentrations azide inhibited cytochrome oxidase activity without changes in bioenergetics (either lactate production or creatine phosphorylation) or mRNA for mitochondrial enzymes while higher azide concentrations resulted in changes in bioenergetic parameters and increases in steady state COX II (cytochrome oxidase II) mRNA levels. We believe it was the cause of this phenomenon.

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