Assay of mitochondrial functions by resazurin *in vitro*[1]

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KEY WORDS resazurin; drug screening; mitochondria

ABSTRACT

AIM: To study the mechanism of resazurin as indicator of mitochondrial function and to develop a rapid and sensitive assay for measuring metabolic activity of isolated mitochondria from rat liver *in vitro*. **METHODS**: The screening was carried out on 96-well microtitre plates by monitoring fluorescence intensity of resazurin reduced by mitochondria. Experimental conditions were optimized and influences of several inhibitors on mitochondrial function were observed. **RESULTS**: Fluorescence intensity increased in a linear manner when the mitochondrial protein concentration from 5 to 50 μg protein per well was incubated with resazurin (5 μmol/L) during 230 min period at 37 ºC. Edetic acid could promote the reduction of resazurin in mitochondria. The fluorescence intensity decreased greatly after pretreatment with NaN_3, antimycin A, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and oligomycin compared with the control. However, the typical complex I inhibitor, rotenone enhanced the fluorescence intensity without mitochondria. **CONCLUSION**: Using resazurin to determine mitochondrial function is sensitive, inexpensive and could be easily automated for high throughput screening.

INTRODUCTION

It is well known that mitochondria plays a pivotal role in modulating intracellular calcium homeostasis and cell apoptosis. More and more evidences showed that mitochondrial dysfunctions might also exert a profound influence on disorders such as heart failure, diabetes mellitus, and neurodegenerative diseases, *etc*[1-3]. So, it is essential to evaluate the mitochondria functions in physiological or pathological conditions to give more hints developing new drugs. At present, assays of mitochondrial membrane potential[4], membrane integrity[5], respiratory function[6], and activities of mitochondrial respiratory complexes[7] were used for determining mitochondrial function. But these methods could determine the mitochondria function only at static state not dynamic state; and were unable to reflect the overall effect of a compound on mitochondria function. Also complicated operation procedures hindered application of these methods on drug screening.

Resazurin is a redox dye traditionally used for examining the bacterial and yeast contamination of milk and for assessing semen quality. Since resazurin is nontoxic and stable in culture medium, researchers could continuously monitor proliferating cells and investigate cytotoxicity in both conventional and high-throughput applications. This indicator has been used in different types of cells such as hepatic cells[8], lymphocytes[9], cortical, and neonatal rat cerebellum[10]. Resazurin (blue and nonfluorescent) is reduced to resorufin (pink and

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highly fluorescent) by oxidoreductases in mitochondria. So, measurement of resazurin fluorescence is an indicator of mitochondrial function.

In the present study, we developed a novel and sensitive assay for measuring mitochondria function in 96-well microtitre plate and applying in high throughput drug screening with resazurin.

MATERIALS AND METHODS

Materials Resazurin was purchased from Aldrich Chem Ltd. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), oligomycin, antimycin A, and rotenone were obtained from Sigma. Other reagents were of analytic grade.

Preparation of rat liver mitochondria Male Sprague-Dawley rats (250-350 g, Grade II, Certificate No 01-3008) were purchased from the Center of Experimental Animal, Chinese Academy of Medical Sciences.

Rats were decapitated and the livers were removed and homogenized with ice-cold MSETB buffer (in mmol/L: mannitol 210, sucrose 70, edetic acid 0.5, Tris-HCl 10 and 0.2 % bovine serum albumin, pH 7.4)[11]. The homogenate was centrifuged at 1000×*g* for 10 min (SCR20-BA centrifuge, Hitachi, Japan) and the supernatant was centrifuged at 12 000×*g* for 10 min. The pellet was resuspended with MSETB buffer and centrifuged at 12 000×*g* for 10 min twice. The mitochondria suspension obtained by suspending the final pellet with Locke's buffer (in mmol/L: NaCl 154, KCl 5.6, $CaCl_2$ 2.3, $MgCl_2$ 1.0, $NaHCO_3$ 3.6, glucose 5, Hepes 5, pH 7.4). All procedures were performed at 4 ºC. The content of mitochondria was determined as the concentration of proteins measured by Lowry method. Purity of mitochondria were identified with the method of de Duve C[12].

Resazurin reduction assay Mitochondria was added into 96-well plate, incubated with resazurin in a cell culture incubator. Fluorescence intensity was measured (530 nm excitation and 590 nm emission) after incubation by POLARstar microplate reader (BMG galaxy, Germany). Samples containing equal amounts of mitochondrial protein that had been heated to 100 ºC for 10 min prior to the addition of resazurin were used to obtain background signal. The total reaction volume was 100 μL.

Effect of different inhibitors on the mitochondrial functions Mitochondria (5 μg protein/well) were added into 96-well plate, incubated with mitochondrial inhibitors (NaN_3 650 μmol/L, antimycin A 25 mg/L, FCCP 50 μmol/L, oligomycin 250 mg/L, and rotenone 20 μmol/L) at 37 ºC for 30 min in a cell culture incubator. Resazurin 5 μmol/L (final concentration) was pipetted into the wells, and then fluorescence was examined. The plate was incubated for another 60 min, and then fluorescence was measured.

The changing rate=$(F_{60}-F_0)/F_0\times100$ %

F_{60}, F_0 referred to fluorescence of 60 min and 0 min.

Statistical analysis All experiments were replicated three times. For single comparison, difference between groups was assessed by *t*-test. Significance for multiple comparisons was determined by one-way ANOVA. $P<0.05$ was considered statistically significance.

RESULTS

The mitochondria (5 μg protein/well) was incubated with resazurin (5 μmol/L) at different temperature (Fig 1). At 37 ºC, the slope was the highest. The mitochondria could catalyze the reaction at 0 ºC at a low speed.

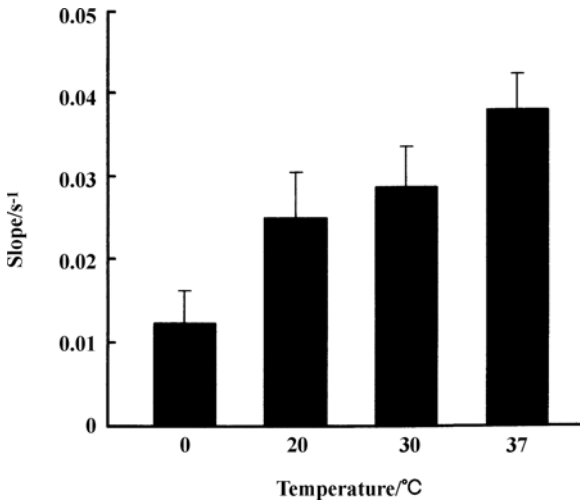

Fig 1. Mitochondria was incubated with resazurin at different temperatures. Fluorescence was measured (530 nm excitation and 590 nm emission). *n*=5. Mean±SD.

Resazurin (5 μmol/L) was reduced by mitochondria (5-50 μg protein/well) during 230 min period in a linear manner (Fig 2) at 37 ºC. Correlation coefficients of time-fluorescence curve of 5 μg and 50 μg mitochondrial protein were 0.998 and 0.996, respectively.

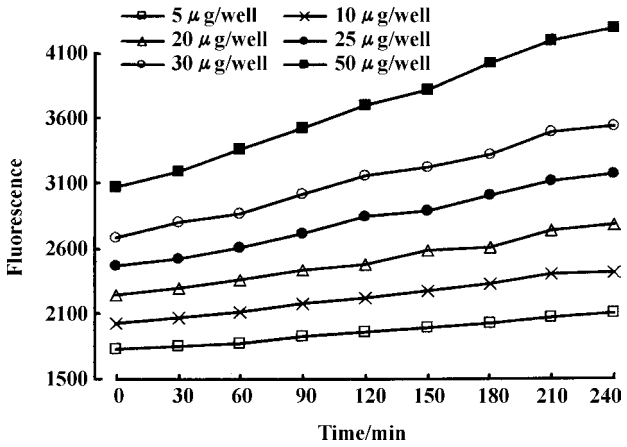

Fig 2. Time-fluorescence curve with different mitochondrial protein. Fluorescence was measured (530 nm excitation and 590 nm emission). *n*=5.

A good linearity in protein-fluorescence curve was acquired during the period of incubation (230 min); its correlation coefficients were 0.993 and 0.990 at 0 and 230 min.

Mitochondria (20 μg protein/well) were incubated with different concentrations of resazurin (5-50 μmol/L) for 30 min (Fig 3). The levels of fluorescence reached the platform in resazurin 40 μmol/L. The tendency was also observed in longer incubation period.

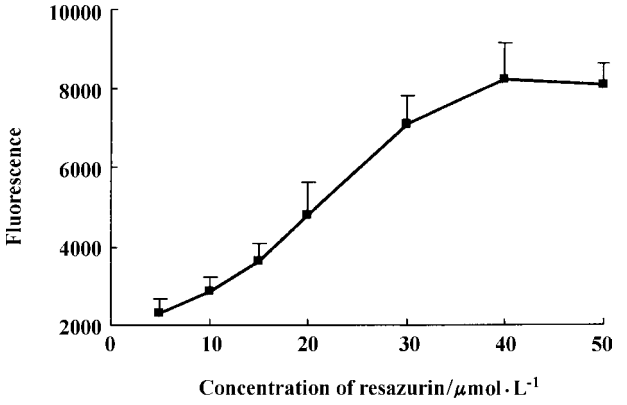

Fig 3. Fluorescence-concentration of resazurin curve. Mitochondria (20 protein μg/well) was incubated with different concentrations of resazurin for 30 min, and then fluorescence was determined (530 nm excitation and 590 nm emission). *n*=5. Mean±SD.

The mitochondria were suspended in Locke's buffer with different concentrations of calcium or edetic acid. Resazurin (5 μmol/L) was reduced by mitochondria (5 μg protein/well) during 4 h. Compared with calcium free Locke's buffer, the reaction rates of resazurin reduction in Locke's buffer with calcium 2.3 mmol/L, 0.23 mmol/L, and 23 μmol/L were low ($P<0.01$, $P<0.05$). Statistical differences were obtained in the reaction rates of resazurin reduction in Locke's buffer with edetic acid 0.5 mmol/L, 50 μmol/L, 5 μmol/L, and 0.5 μmol/L ($P<0.01$ *vs* edetic acid free Locke's buffer (Fig 4, 5).

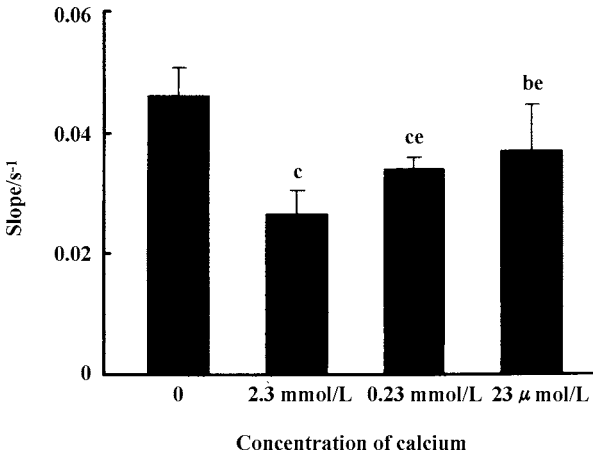

Fig 4. The mitochondria were resuspended in Locke's buffer with different concentrations of calcium. $^bP<0.05$, $^cP<0.01$ *vs* calcium free buffer. $^eP<0.05$ *vs* Locke's buffer with calcium 2.3 mmol/L. *n*=5. Mean±SD.

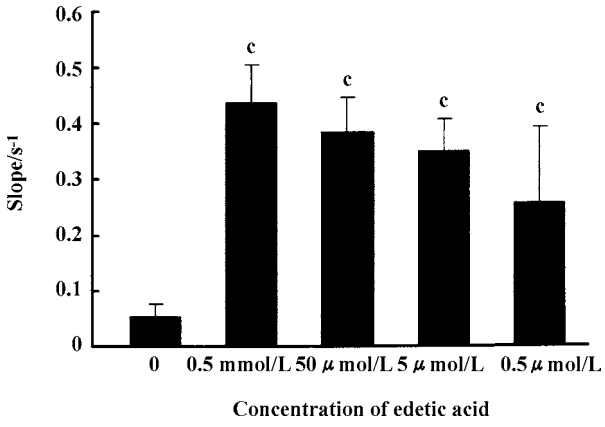

Fig 5. The mitochondria were suspended in Locke's buffer with different concentration of edetic acid. $^cP<0.01$ *vs* edetic acid free buffer. *n*=5. Mean±SD.

After incubation of mitochondria with different kind of inhibitors, the rate of increase was affected significantly (Tab 1). Compared with control group, complexes inhibitor such as NaN_3 (650 μmol/L), antimycin A (25 mg/L), FCCP (50 μmol/L), oligomycin (250 mg/L) restrained the changing rate significantly, while rotenone (20 μmol/L) promoted the reduction of resazurin.

Tab 1. Changing rate of different mitochondrial inhibitor. $^cP<0.01$ *vs* control . *n*=5. Mean±SD.

Group	Concentration	Changing rate/%
Control		17.0±0.9
rotenone	20 μmol/L	31.4±1.3^c
antimycin A	25 mg/L	6.6±1.1^c
NaN_3	650 μmol/L	12.2±0.4^c
oligomycin	250 mg/L	8.1±1.2^c
FCCP	50 μmol/L	8.8±1.2^c

Forty compounds were screened for evaluating their effects on the mitochondrial functions by the above method. One of them, TCJ could promote the reduction of resazurin by mitochondria (slope 0.095 *vs* 0.056, $P<0.01$), and ameliorate the mitochondrial functions inhibited by antimycin (slope 0.078 *vs* 0.046, $P<0.01$) and NaN_3 (slope 0.093 *vs* 0.050, $P<0.01$).

DISCUSSION

Resazurin reduction is a promising new assay *in vitro* which is simple to conduct and amenable to measure continuously in high-throughput manner. However, the evaluation of organelles functions such as mitochondria with resazurin has not been reported. In the present study, we established resazurin reduction assay in mitochondria with a spectrofluorometer plate reader. Resazurin reduction assay could monitor the change of mitochondrial function dynamically. And different from the methods mentioned before, resazurin reduction assay is very simple to operate.

In order to obtain the optimal parameters, temperature, protein concentration, resazurin concentration, and incubation time were observed. It was indicated that at 37 ºC, the maximum reaction rate was obtained. Incubating temperature (30 ºC) could also be applied since minor difference of reaction rate was observed between 37 ºC and 30 ºC. With regard to the resazurin concentration, resazurin 5 μmol/L was chosen for less toxicity and high sensitivity. As for the mitochondrial protein concentration, the slope rate per μg protein was consistent in different concentration.

Because edetic acid is a common reagent used in mitochondria isolation buffer, the effect of edetic acid on resazurin reduction was investigated. The results indicated that edetic acid could promote the reduction of resazurin 5-9 times *vs* edetic acid free system. Since edetic acid could chelate with calcium, the effect of calcium on reduction was further studied. The reaction in Locke's buffer with calcium was inhibited only in a little degree. This kind of decrease could not compensate for increase caused by edetic acid. So it was suggested that edetic acid could promote the reduction of resazurin not by chelating with calcium.

Different kinds of inhibitors were applied to observe effects of them on mitochondrial function including mitochondrial respiratory complexes I (rotenone), III (antimycin A), and IV (NaN_3) inhibitor; uncoupler of oxidative phosphorylation (FCCP); inhibitor of oxidative phosphorylation (oligomycin). It is reasonable to understand the decreased fluorescence caused by inhibitors. During the experiments, we observed that the action times of the inhibitors were different. Antimycin A exerted its effect as early as 30 min after addition of resazurin, while the action time of NaN_3 was retarded. Through the inhibitor, resazurin could provide more information on the metabolic rate and maximal functional capacity of mitochondria exposed to diverse injuries. Furthermore, this method could determine the dynamic changes of mitochondria function. So, evaluating the mitochondrial overall function by resazurin reduction is satisfied.

Moreover, we observed that rotenone, complex I inhibitor, could lead a significant increase in fluorescence. In order to eliminate the possibility of resazurin reacting with rotenone directly, we designed a control group in which resazurin was mixed only with rotenone under the same condition. The significant increasing fluorescence was detected. The result indicated that the marked fluorescence increasing was resulted from the action of rotenone with resazurin. So mitochondria complex I inhibitor rotenone does not suit for this method.

The results of screening for 40 compounds by the method show that the resazurin reduction assay method is a simple and rapid screening method for finding the compounds which could affect mitochondrial functions. Also the mechanism of hit's actions on mitochondria could be investigated by means of different inhibitors and treatments on mitochondria *in vitro*.

In conclusion, resazurin assay was an extremely sensitive, simple and non-toxic procedure to evaluate mitochondrial function. Rat liver mitochondrial was easy to obtain and prepare. Since this assay is a one-step procedure, it can be applied to analyze automati-

cally in a large number of samples.

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