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Influence of anoxia and K_{ATP} channel opener diazoxide on the functions of heart mitochondria

D. M. Kopustinskienė[1*], Ž. Polianskytė[2], A. Toleikis[1]

[1] *Institute for Biomedical Research, Kaunas University of Medicine, Eiveniu 4, LT-3007, Kaunas, Lithuania*
[2] *Department of Biology, Faculty of Nature Sciences, Vytautas Magnus University, Vileikos 8, LT-3035 Kaunas, Lithuania*

We evaluated the ability of diazoxide, the selective opener of mitochondrial K_{ATP} channel, to preserve oxidative phosphorylation in isolated rat heart mitochondria subjected to 20 min of anoxia followed by reoxygenation. Anoxia/reoxygenation significantly decreased the rate of adenosine diphosphate-stimulated oxygen consumption (from 417 ± 16 to 220 ± ± 16 nmol O/min/mg protein), increased the atractyloside-insensitive respiration rate (from 34 ± 2 to 51 ± 5 nmol O/min/mg protein) and had only a negligible effect on the uncoupled respiration rate. Diazoxide (100 μM), present throughout anoxia, had only a tendency to increase the State 3 and uncoupled respiration rates, but did not affect the atractyloside-insensitive respiration of mitochondria. Neither anoxia/reoxygenation nor diazoxide treatment of mitochondria throughout anoxia affected the release of cytochrome c into the incubation medium. Thus, under our experimental conditions, diazoxide has not protected mitochondria from anoxia/reoxygention-mediated damage.

Key words: rat heart mitochondria, diazoxide, anoxia/reoxygenation

INTRODUCTION

Metabolic stress – hypoxia, anoxia, ischemia – causes impairment of energy supply for cardiomyocites in the heart. Mitochondria generate virtually all the ATP in the heart through oxidative phoshporylation, therefore the modulation of their functional activity may be very important for preserving cell viability under stress conditions. Investigations of K_{ATP} channel openers have revealed their cardioprotective action in various models of ischemia/reperfusion (for review, see [1–3]). Although the mechanisms, by which K_{ATP} openers exert their cardioprotective effects have not been clarified yet, it is supposed that the opening of K_{ATP} channels preserves mitochondrial ATP production.

We investigated the influence of anoxia/reoxygenation in the presence and absence of K_{ATP} channel opener diazoxide on the oxygen consumption rate of isolated rat heart mitochondria, respiring on the pyruvate and malate, and the release of cytochrome c from mitochondria into the incubation medium during anoxic incubations.

MATERIALS AND METHODS

The experiments were carried out on mitochondria isolated from male Wistar rat hearts by differential centrifugation procedure. After decapitation, hearts were excised and rinsed in an ice-cold isolation medium containing 220 mM manitol, 70 mM sucrose, 5 mM N-tris[Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) and 0.5 mM EGTA (pH 7.4, adjusted with Trizma base; 2 °C). Mitochondria were isolated in the same medium supplemented with 2 mg/ml bovine serum albumin (BSA; fraction V, A4503, Sigma). The homogenate was centrifuged for 5 min at 750 × g, then the supernatant was centrifuged for 10 min at 6740 × g and the pellet was washed once in the isolation medium without BSA, suspended in it and kept on ice. The mitochondrial protein concentration was determined by the biuret method [4]. The rates of oxygen uptake were recorded at 30 °C with the aid of the Clark-type electrode system in KCl medium (120 mM KCl, 5 mM KH_2PO_4, 5 mM TES and 1 mM $MgCl_2$; pH 7.4, adjusted with Trizma base, 30 °C); 5 mM of both pyruvate and malate were used as substrates. The solubility of oxygen was taken to be 440 ngatoms/ml [5]. The respiration rates were expressed as nmol O/min/mg mitochondrial protein. For the anoxic incubations, mitochondria (2 mg/ml) were added into the closed oxygraph chamber, filled with 1 ml of KCl medium and continuously stirred at 30 °C. Anoxia was reached within 5 min as mitochondria consumed all oxygen in the chamber. The mitochondrial suspension was reoxygenated just after the anoxia was reached or after 20 min of ano-

* Corresponding author. Fax: (+370 7) 796 498. E-mail: daliammk@mail.lt

xic incubation by injecting gas O_2 into the chamber. After the mitochondrial respiration rates were recorded, the suspension was taken out from the chamber and centrifuged for 3 min at 13000 rpm in an Eppendorf centrifuge. The supernatants were used for spectrophotometric measurements of cytochrome c released from mitochondria using a Hitachi 557 spectrophotometer. The results are presented as means ± S. E. of 5 independent experiments.

RESULTS AND DISCUSSION

Our results (Table) show that anoxia/reoxygenation injured the functions of isolated rat heart mitochondria. When anoxia was immediately followed by reoxygenation, the ADP-stimulated respiration rate (V_3) significantly decreased by 26% compared with control (417 ± 16 nmol O/min/mg protein). After 20 min of anoxic incubation followed by reoxygenation, the decrease in this parameter was more pronounced and reached 47% compared with control. The mitochondrial respiration rate in State 3 depends on the activity of the mitochondrial respiratory chain and the components of the phosphorylation system [6, 7]. Thus, the anoxia/reoxygenation-caused decrease in V_3 indicates an impairment of the mitochondrial capacity to phosphorylate ADP.

Anoxia immediately followed by reoxygenation (Table) had no significant effect on the atractyloside-insensitive respiration rate (V_{ATR}) of isolated mitochondria, but significantly increased V_{ATR} by 49% compared with control (34 ± 2 nmol O/min/mg protein) after 20 min of anoxic incubation/reoxygenation. The atractyloside-insensitive mitochondrial respiration rate mainly depends on the passive ion flux through the mitochondrial inner membrane [8]. Thus, we may conclude that anoxia/reoxygenation injures the mitochondrial inner membrane. Both anoxia immediately followed by reoxygenation and the 20 min anoxia/reoxygenation had only a negligible effect on the uncoupled mitochondrial respiration rate (V_{CCCP}), indicating that anoxia/reoxygenation does not impair the activity of mitochondrial respiratory chain.

An intact mitochondrial outer membrane is impermeable for cytochrome c [9]. To evaluate the injury of the mitochondrial outer membrane, we monitored the release of cytochrome c from mitochondria to the incubation medium. Our results (Table) show that neither anoxia/reoxygenation nor diazoxide treatment of mitochondria throughout anoxia affected the release of cytochrome c into the incubation medium. Thus, our results demonstrate that anoxia/reoxygenation-induced impairment of mitochondrial functions differs from ischemia-induced impairment. In contrast to ischemia [9–12], anoxia/reoxygenation treatment of mitochondria does not increase the permeability of the mitochondrial outer membrane and does not decrease the activity of the mitochondrial respiratory chain.

In two papers published by the same group of investigators [13] it has been shown that K_{ATP} channel openers protect isolated mitochondria from anoxia-induced damage, preserving the integrity of the outer mitochondrial membrane, ADP-stimulated mitochondrial respiration, ATP production [13] and reducing mitochondrial reactive oxygen species production at reoxygenation [14]. In contrast to these findings obtained on mitochondria isolated from Sprague–Dawley rat heart [13, 14], under similar experimental conditions we have not observed any cardioprotective effect of diazoxide on mitochondria isolated from Wistar rat heart (Table). Diazoxide (100 μM), present throughout anoxia, had only a tendency to increase the State 3 respiration rate (from 220 ± 16 to 240 ± 17 nmol O/min/mg protein) and the uncoupled respiration rate but did not affect the atractyloside-insensitive respiration rate. The selectivity of K_{ATP} channel openers

Table. **The effect of anoxia/reoxygenation and anoxia/reoxygenation in the presence of diazoxide (100 μM) on the functions of isolated rat heart mitochondria.**
V_2 – the State 2 respiration rate; V_3 – ADP (1.2 mM)-stimulated respiration rate; V_{ATR} – atractyloside (150 μM)-insensitive respiration rate; V_{CCCP} – carbonylcyanide *m*-chlorophenylhydrazone (1 μM)-stimulated respiration rate. Experiments were performed in KCl medium at 30 °C, substrate – pyruvate and malate (5 + 5 mM), n = 5. * p < 0.05 – a statistically significant effect of anoxia/reoxygenation compared to control. The results were analysed with one-way repeated measure analysis of variance (ANOVA) followed by Dunnet post hoc test

	Respiration rate, nmol O/min/mg protein				Release of cytochrome c, nmol/mg protein
	V_2	V_3	V_{ATR}	V_{CCCP}	
Control	31 ± 1	417 ± 16	34 ± 2	442 ± 23	–
Anoxia/ reoxygenation	–	309 ± 14* ↓26%	37 ± 1 ↑9%	406 ± 33 ↓8%	0.175 ± 0.018
Anoxia 20 min/ reoxygenation	–	220 ± 16* ↓47%	51 ± 5* ↑49%	400 ± 27 ↓10%	0.152 ± 0.020
Anoxia 20 min + diazoxide/ reoxygenation	–	240 ± 17* ↓42.5%	49± 5* ↑42%	432 ± 31	0.187 ± 0.016

and blockers highly depends on the experimental conditions [15]. Moreover, response to stress differs not only in the different animal species [16] but even in different strains of rats [17]. These differences should be taken into account while developing new pharmacological agents designed for the protection of the ischemic heart against injuries.

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ANOKSIJOS IR K_{ATP} KANALŲ AKTYVATORIAUS DIAZOKSIDO POVEIKIS MITOCHONDRIJŲ FUNKCIJOMS

S a n t r a u k a

Tyrėme atrankaus mitochondrijų K_{ATP} kanalų aktyvatoriaus diazoksido poveikį oksidaciniam fosforilinimui išskirtose žiurkės širdies mitochondrijose 20 min. anoksijos (reoksigenacijos) metu. Anoksija (reoksigenacija) patikimai sumažino ADP-stimuliuotą mitochondrijų kvėpavimo greitį ir padidino atraktilozidui nejautrų kvėpavimo greitį. Anoksinės inkubacijos metu terpėje buvęs diazoksidas (100 μM) tik truputį padidino trečios metabolinės būsenos ir atskirtą mitochondrijų kvėpavimo greitį, tačiau nepaveikė nejautraus atraktilozidui kvėpavimo greičio. Nei anoksija (reoksigenacija), nei jos metu terpėje buvęs diazoksidas nepakeitė citochromo c išėjimo į inkubavimo terpę. Mūsų eksperimentinėmis sąlygomis diazoksidas neapsaugojo mitochondrijų nuo anoksijos (reoksigenacijos) sukeltų pažeidimų.