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Methyl-beta-cyclodextrin induces mitochondrial cholesterol depletion and alters the mitochondrial structure and bioenergetics

Wieslaw Ziolkowski [a,b,*], Michał Szkatula [c], Artur Nurczyk [a], Takashi Wakabayashi [d], Jan Jacek Kaczor [a], Robert Antoni Olek [a,b], Narcyz Knap [c], Jedrzej Antosiewicz [a,b], Mariusz Roman Wieckowski [e], Michal Wozniak [c]

[a] Department of Biochemistry, J. Sniadecki Academy of Physical Education and Sport, K. Gorskiego 1, 80-336 Gdansk, Poland
[b] Department of Bioenergetics and Physiology of Exercise, Medical University of Gdansk, Debinki 1, 80-210 Gdansk, Poland
[c] Department of Medical Chemistry, Medical University of Gdansk, Debinki 1, 80-210 Gdansk, Poland
[d] Department of Cell Biology and Molecular Pathology, Medical University of Gdansk, Debinki 1, 80-210 Gdansk, Poland
[e] Department of Biochemistry, Nencki Institute of Experimental Biology, Pasteur 3, 02-093 Warsaw, Poland

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This paper is dedicated to our mentor and friend Prof. Jerzy Popinigis, who has inspired us to engage in the mitochondriology

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ABSTRACT

There is growing evidence of mitochondrial membrane raft-like microdomains that are involved in the apoptotic pathway. The aim of this study was to investigate the effect of methyl-beta-cyclodextrin (MβCD), being a well-known lipid microdomain disrupting agent and cholesterol chelator, on the structure and bioenergetics of rat liver mitochondria (RLM). We observed that MβCD decreases the function of RLM, induces changes in the mitochondrial configuration state and decreases the calcium chloride-induced swelling. These data suggest that disruption of mitochondrial raft-like microdomains by cholesterol efflux on one hand impairs mitochondrial bioenergetics, but on the other hand it protects the mitochondria from swelling.

1. Introduction

There are at least four main membrane lipid microdomains located within the plasma membrane, mitochondrial membranes, golgi apparatus and endoplasmic reticulum [1–4]. The plasma membrane "lipid rafts" consist of cholesterol and sphingolipids [1].

There is also growing evidence suggesting the existence of mitochondrial raft-like microdomains [4,5], which are involved in the signaling pathways controlling the apoptotic process [6,7].

Even if the cholesterol content in mitochondria is lower than in other organelles, its appropriate level for mitochondrial raft-like microdomains seems to be crucial [4,5]. In rat liver mitochondria (RLM), cholesterol is mainly distributed within the areas of outer membrane (OM), involved in creation of mitochondria contact sites [8], joining mitochondrial membranes, where voltage-dependent anion channel (VDAC) is localized on the inside or outside of cholesterol-containing lipid raft-like areas [9].

Mitochondrial contact sites are also associated with the mitochondrial permeability transition pore (mPTP) being a multiprotein complex, comprising, among others, adenine nucleotide translocator (ANT), VDAC, peripheral benzodiazepine receptor, creatine kinase, hexokinase II, cyclophiline D, and BAX/Bcl-2-like proteins. Activation of mPTP is associated with mitochondrial

Abbreviations: ANT, adenine nucleotide translocator; COX, cytochrome *c* oxidase; CSA, cyclosporin A; DPM, disintegrations per minute; GD3, disialoganglioside; MAM, mitochondria-associated membranes; MβCD, methyl-beta-cyclodextrin; mDRMs, mitochondrial detergent-resistant membranes; mPTP, mitochondrial permeability transition pore; OM, outer membrane; RCI, respiratory control index; RLM, rat liver mitochondria; t-Bid, pro-apoptotic protein; VDAC, voltage-dependent anion channel

* Corresponding author at: Department of Biochemistry, J. Sniadecki Academy of Physical Education and Sport, 1 K. Gorskiego St., 80-336 Gdansk, Poland. Fax: +48 585547387.
E-mail address: wiech@awf.gda.pl (W. Ziolkowski).

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depolarization, uncoupling of oxidative phosphorylation, swelling of mitochondria and release of death-promoting factors like cytochrome *c* [10]. There are many factors implicated in mPTP regulation, i.e., oxidative stress [11], mitochondrial GSH depletion, Ca^{2+} and Pi, lowering of extramitochondrial ATP [12], and ADP level [13], collapse of mitochondrial membrane potential, and decrease of matrix pH [14,15]. In addition, it has been shown that mitochondrial raft-like microdomain specific lipids play a key role in the regulation of mPTP [5]. Furthermore, it has been proposed that lipid microdomains, enriched in cholesterol and ceramide, could coexist as structural elements with some mPTP-forming proteins, and with members of the Bcl-2 family [16]. In the reperfused rat heart model, the release of cytochrome *c* from mitochondria correlates with Bax insertion into the mitochondrial detergent-resistant membranes (mDRMs), which are abundant in VDAC, ANT, cholesterol and ceramide [16].

Methyl-beta-cyclodextrin (MβCD) belongs to the β-cyclodextrin family, being capable of removing cholesterol from the plasma membrane [17]. The methylated form is more efficient cholesterol chelator than non-methylated β-cyclodextrin [18]. MβCD molecule has an internal hydrophobic cavity that can accommodate normally insoluble compounds such as cholesterol. The surface hydrophilicity of cyclodextrin-cholesterol complex accounts for its solubility in aqueous solutions [19]. Garofalo et al. [4] reported that MβCD-induced disruption of raft-like microdomains in isolated mitochondria from human lymphoblastoid CEM cells prevented mitochondria depolarization and cytochrome *c* release after treatment with disialoganglioside (GD3) or t-Bid, pro-apoptotic protein. Also in another study, an inhibitory effect of MβCD on rBAX-induced opening of mPTP was observed [5]. MβCD lowered cholesterol and ganglioside content in the mitochondria of control rat kidney cortex. Similar resistance to mPTP opening induced by rBAX, was observed in kidney mitochondria from hypothyroid rats that were characterized by lower cholesterol and ganglioside content [5]. Overall, these data suggest that lipid composition of mitochondrial membrane raft-like microdomains may play an important role in the regulation of early stages of apoptosis.

Therefore, we hypothesized that the disruption of mitochondrial raft-like microdomains by cholesterol removal could affect the mitochondrial structure and energetic metabolism.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for BSA (Merck, Damstadt, Germany).

2.2. Animals

All experiments were approved by the Local Ethical Committee at the Medical University in Gdansk (Consent No. 13/2007).

Wistar rats weighing 250–300 g were housed in an environmentally controlled room (23 ± 1 °C, 12 h/12 h light–dark cycle), and received standard rat chow and water ad libitum.

2.3. Isolation of RLM

Mitochondria were isolated as described earlier [20]. The quality of mitochondrial preparation was checked by Western-blotting analysis. Further purification of crude mitochondria, and isolation of mitochondria-associated membranes (MAM) fraction and pure mitochondria were performed as previously described [21]. Mitochondrial and MAM lysates (40 μg of protein/lane) were separated by SDS–PAGE on 10% gel and transferred onto PVDF membrane (BioRad). Membranes were blocked using 2% non-fat milk in TBS buffer with 0.01% Tween-20 for 1 h. The purity of mitochondrial and MAM fractions was checked using specific antibodies against ADP/ATP carrier (ANT), plasma membrane Ca^{2+}-ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) followed by incubation with secondary HRP-conjugated antibodies. During the experiment, mitochondria were kept in the form of suspension containing 30–50 mg of protein per 1 ml. The protein content was estimated according to the method of Lowry et al. [22].

2.4. Cholesterol estimation

Isolated RLM (1 mg of protein/ml) were incubated with control respiration buffer or with the buffer containing 2% or 4% MβCD. After 5 min of incubation, samples were centrifuged at 10 000 g for 10 min, and mitochondrial cholesterol was determined as previously described [23].

2.5. Mitochondrial respiratory activity assay

Mitochondrial oxygen uptake was measured at 25 and 37 °C with the Clark oxygen electrode using Gilson Polarograph. The respiration medium contained 170 mM sucrose, 15 mM KCl, 5 mM potassium phosphate, 0.1 mM EDTA, 0.1% BSA, and 5 mM succinate (pH 7.4) was used as an oxidizable substrate. Mitochondria (1 mg of protein/ml) were incubated for 2.5 min in the medium without (control) or in the presence of 2% or 4% of MβCD. Respiratory control index (RCI) and ADP/O were measured and calculated from oxygen electrode traces as previously described [24].

2.6. Mitochondrial enzyme activities measurement

The activity of cytochrome *c* oxidase (COX) was measured according to [25] and the activity of rotenone-insensitive NADH cytochrome *c* reductase was assessed according to [26]. Enzymatic activities measured in mitochondrial pellet (1 mg of protein/ml) obtained after 5 min incubation with respiration buffer were expressed as 100%. The activity of the enzymes assessed in other cases were compared to the control and expressed as percentage of control activity.

2.7. Swelling of the RLM

The measurement of RLM swelling was performed spectrophotometrically as previously described [27]. $CaCl_2$ (100 μM) was used as mPTP opening inducer and cyclosporin A (CSA, 1 μM) as mPTP opening inhibitor. 1 mg of RLM was added to the buffer supplemented with 1 mM succinate and 1 μM rotenone in the presence or absence of 2% or 4% of MβCD.

2.8. EM analyses

Mitochondria (1 mg of protein) were incubated for 5 min with control buffer or buffer supplemented with 2% or 4% of MβCD, or 0.25% digitonin, then fixed and processed for electron microscopy as previously described [28].

2.9. Data analysis

Statistical analysis was performed using a dedicated software package (Statistica v. 7.0, Stat Soft Inc.). Results are expressed as mean ± standard error of the mean (S.E.M.). Differences between means were tested using one-way ANOVA test. The statistical significance was established at $P < 0.05$.