Archives of Biochemistry and Biophysics 558 (2014) 87–94

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Carbenoxolone induces permeability transition pore opening in rat mitochondria via the translocator protein TSPO and connexin43

Tamara Azarashvili [a,b,*], Yulia Baburina [a], Dmitry Grachev [a], Olga Krestinina [a], Vassilios Papadopoulos [c], John J. Lemasters [a,d,e], Irina Odinokova [a], Georg Reiser [b]

[a] Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Institutskaya Str., Pushchino, Moscow Region 142290, Russia
[b] Institut für Neurobiochemie, Otto-von-Guericke-Universität Magdeburg, Medizinische Fakultät, Leipziger Str. 44, 39120 Magdeburg, Germany
[c] The Research Institute of the McGill University Health Center, 2155 Guy Street, Suite 500, Montreal, Quebec H3H 2R9, Canada
[d] Department of Drug Discovery & Biomedical Sciences, Medical University of South Carolina, DD504 Drug Discovery Bldg., 70 President St., MSC 140, Charleston, SC 29425, USA
[e] Department of Biochemistry & Molecular Biology, Medical University of South Carolina, DD504 Drug Discovery Bldg., 70 President St., MSC 140, Charleston, SC 29425, USA

ARTICLE INFO

Article history:
Received 8 April 2014
and in revised form 18 June 2014
Available online 1 July 2014

Keywords:
Carbenoxolone
Protoporphyrin IX
TSPO
Connexins
Mitochondria
Permeability transition pore

ABSTRACT

Ca^{2+}-induced permeability transition pore (mPTP) opening in isolated rat brain mitochondria is promoted through targeting of connexin43. After a threshold Ca^{2+} load, mitochondrial membrane potential drops and efflux of accumulated Ca^{2+} from the mitochondrial matrix occurs, indicating the mPTP opening. Specific antibodies were used to assess the role of the translocator protein (18 kDa; TSPO) and connexin43 in swelling of isolated rat liver and brain mitochondria induced by carbenoxolone and the endogenous TSPO ligand protoporphyrin IX. Mitochondrial membrane potential, Ca^{2+} transport and oxygen consumption were determined using selective electrodes. All the parameters were detected simultaneously in a chamber with the selective electrodes. The phosphorylation state of mitochondrial protein targets was assessed. We report that Ca^{2+}-induced mitochondrial swelling was strengthened in the presence of both carbenoxolone and protoporphyrin IX. The carbenoxolone- and protoporphyrin IX-accelerated mPTP induction in brain mitochondria was completely prevented by antibodies specific for the mitochondrial translocator protein (TSPO). The anti-TSPO antibodies were more effective than anti-connexin43 antibodies. Moreover, carbenoxolone-stimulated phosphorylation of mitochondrial proteins was inhibited by anti-TSPO antibodies. Taken together, the data suggests that, in addition to acting via connexion43, carbenoxolone may exert its effect on mPTP via mitochondrial outer membrane TSPO.

Introduction

Carbenoxolone (Cbx),[1] a substance from medicinal licorice, is the water soluble 3-hemisuccinate of glycyrrhetinic acid (succinyl ester of glycyrrhetinic acid), which is used in the treatment of gastric ulcer and as a therapy for polyarthritis and rheumatoid arthritis [1,2]. It has been demonstrated that Cbx and 18β-glycyrrhetinic acid affect mitochondrial function by modulating the mitochondrial permeability transition pore (mPTP). Energy-dependent accumulation of calcium ions in mitochondria over a certain threshold or by oxidative stress causes increases in permeability of the mitochondrial inner membrane due to the formation of non-selective pores, which permits passage of molecules with a molecular mass of <1.5 kDa and causes dissipation of the membrane potential ($\Delta\Psi_m$), swelling of mitochondria and release of apoptotic factors. The composition of mPTP as well as the regulatory mechanism of pore

* Corresponding author at: Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Institutskaya Str., 3, Pushchino, Moscow Region 142290, Russia. Fax: +7 4967 330553.

E-mail addresses: tamara.azarashvili@gmail.com (T. Azarashvili), byul@rambler.ru (Y. Baburina), grachew@rambler.ru (D. Grachev), krestinina@rambler.ru (O. Krestinina), vassilios.papadopoulos@mcgill.ca (V. Papadopoulos), JJLemasters@musc.edu (J.J. Lemasters), odinokova@rambler.ru (I. Odinokova), georg.reiser@med.ovgu.de (G. Reiser).

[1] *Abbreviations used:* ACBD3, acyl-CoA binding domain containing protein 3; ANT, adenine nucleotide translocase; Cbx, carbenoxolone, (3β)-3-[(3-carboxypropanoyl)oxy]-11-oxoolean-12-en-30-oic acid; CsA, cyclosporin A; Cx, connexin; MBP, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PAP7, PBR (TSPO)-associated protein 7; PK11195, 1-(2-chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide; PKA, protein kinase A; PKC, protein kinase C; PPIX, protoporphyrin IX; mPTP, mitochondrial permeability transition pore; RBM, rat brain mitochondria; RLM, rat liver mitochondria; Ro 5-4864, 7-chloro-1,3dihydro-1-methyl-5-(p-chlorophenyl)-2H-1,4-benzodiazepine; ROS, reactive oxygen species; TPP, tetraphenylphosphonium; TSPO, translocator protein (18 kDa); VDAC, voltage-dependent anion channel; $\Delta\Psi_m$, mitochondrial transmembrane potential.

http://dx.doi.org/10.1016/j.abb.2014.06.027
0003-9861/

function are not fully established. Therefore, the search for the components of mPTP and the identity of factors modulating its operation is an ongoing effort. In particular, Cbx was shown to be able to initiate mPTP opening in rat liver mitochondria (RLM), inducing swelling, collapse of $\Delta\Psi_m$, sulfhydryl and pyridine nucleotide oxidation, reactive oxygen species (ROS) generation, and release of both cytochrome *c* and apoptosis inducing factor [3–5].

Recently, we compared the mechanism of action of Cbx on Ca^{2+}-induced mPTP opening in rat brain mitochondria (RBM, synaptic and non-synaptic) and rat liver mitochondria (RLM), in an attempt to identify the mitochondrial target of Cbx [6]. Our data showed that Cbx altered the parameters of mPTP function by shortening the lag time of MPT onset (lowering the capacity to retain Ca^{2+} in the matrix) and initiating Ca^{2+}-induced Ca^{2+} efflux from the mitochondrial matrix [6]. Cbx increased Ca^{2+}-induced high amplitude swelling of both RBM and RLM. Cbx-stimulated Ca^{2+} efflux and Ca^{2+}-induced high amplitude swelling of mitochondria were CsA sensitive [6]. These effects of Cbx were not linked to ROS production, however, connexin43 (Cx43) was identified to be the target of Cbx [6].

Connexins (Cx) are a family of proteins that form gap junction megachannels that mediate intercellular communication and allow inorganic ions and small organic signaling molecules to diffuse rapidly and directly from the cytoplasm of one cell to another [7]. The presence of connexin43 (Cx43) in mitochondria has been reported [7–11] and it was proposed that Cx43 may function in protective preconditioning mechanism [8,11]. Cbx is a universal effective water-soluble blocker of gap junctions [2]. The presence of Cx43 in mitochondria suggested that connexins might be the target for Cbx in mitochondria. Indeed, we detected Cx43 in rat brain and heart mitochondria, but not in liver mitochondria. However, Cx26 and Cx32 were found in rat liver mitochondria and may also be targets for Cbx [6].

Cbx being a gap junction inhibitor has a structural similarity to the steroids [12]. The initial steps of steroidogenesis take place in the mitochondria of steroid producing tissues, including adrenals, gonads, placenta, brain, and liver [13,14]. In these tissues, steroid formation is initiated with the transfer of the substrate cholesterol from intracellular stores to the inner mitochondrial membrane. Cholesterol transport into mitochondria is mediated by the translocator protein (18 kDa) TSPO, previously known as the peripheral-type benzodiazepine receptor, a high affinity drug and cholesterol-binding protein present in the outer mitochondrial membrane [15,16]. Cholesterol binding to TSPO occurs at the cholesterol binding amino acid consensus sequence -L/V-(X1–5-Y-(X)1–5-R/K- [15,16]. Interestingly, a comparable cholesterol binding amino acid consensus sequence (CRAC motif) was detected in both Cx43 and Cx32 [17,18].

TSPO has been implicated in mPTP functions [14,19–21]. TSPO-associated mitochondrial proteins have been described, including the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT) [22–24], which both are considered to be major modulators of mPTP. Modulation of mPTP by chemicals opening or closing the channel alters the ability of steroidogenic cells to form steroids [14]. Moreover, TSPO ligands have been shown to modulate mPTP function [19,25]. We also reported that TSPO ligands modulate in a Ca^{2+}- and CsA-dependent manner the phosphorylation of 43–46 kDa, 21 kDa and 17 kDa proteins, as well as of a 3.5 kDa peptide. The phosphorylation status of these proteins and peptide was shown to change depending on the opened/closed state of the pore [26]. These phosphoproteins were identified: 46 kDa phosphoprotein is 2′,3′-cyclic nucleotide-3′-phosphodiestearase [27], 21 kDa and 17 kDa phosphoproteins are isoforms of myelin basic protein (MBP) [28], and the 3.5 kDa phosphopeptide is subunit *c* of ATP synthase [29]. Incubation of the rat brain mitochondria (RBM) with anti-TSPO antibodies specifically prevented these phosphorylations, suggesting that TSPO participates in the modulation of mPTP opening. It was previously reported that in the presence of the anti-TSPO antibody there was strong suppression of the Ca^{2+} efflux rate occurring after the threshold Ca^{2+} addition [19]. The rates of both Ca^{2+} uptake and Ca^{2+} efflux were similar in control RBM. However, in anti-TSPO-treated RBM, the rate of Ca^{2+} uptake increased and the maximal Ca^{2+} efflux rate diminished. Additionally, Ca^{2+} efflux occurred after a prolonged lag phase [19].

In the present study we investigated the role of TSPO and connexin43 in mediating Cbx- and PPIX-dependent induction of mPTP opening in mitochondria. The results suggest that TSPO and connexin43 mediate the effects of Cbx on mPTP.

Materials and methods

Rat liver mitochondria isolation

RLM were isolated from livers of adult Wistar rats by differential centrifugation, as previously described [6]. The homogenization buffer used contained 250 mM sucrose, 70 mM mannitol, 10 mM Tris–HCl buffer (pH 7.4), 0.01% BSA (fraction V, fat acid free), and 1 mM EGTA. BSA and EGTA were omitted from the second washing solution. Mitochondria were suspended in solution without BSA and EGTA. Protein concentration in mitochondrial suspensions was 60–65 mg/ml. All animal procedures were approved by the ethics committee of the German federal state of Sachsen-Anhalt and are in accordance with the European Communities Council Directive (86/609/EEC).

Rat brain mitochondria isolation

Adult Wistar rats were fasted overnight before decapitation and isolation of mitochondria. In brief, brains were rapidly removed (within 30 s) and placed in ice cold isolation buffer, containing 0.32 M sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 0.2% BSA, and 10 mM Tris–HCl (pH 7.4). The tissue was homogenized in a glass homogenizer, using a ratio of brain tissue to isolation medium of 1:10 (w/v). The homogenate was centrifuged twice at 2000×*g* for 3 min. The supernatant of mitochondria obtained after centrifugation of the 2000×*g* was sedimented at 12,500×*g* for 10 min. RBM were suspended in the isolation buffer and purified on Percoll gradients [30]. Thereafter, obtained fractions (myelin fraction, synaptic and non-synaptic mitochondria) were washed out and re-suspended with isolation buffer without BSA and EGTA. All solutions used for mitochondrial isolation were ice cold, and manipulations were carried out at 4 °C. All chemicals used for mitochondria isolation and incubation were of analytical grade and purchased from Sigma–Aldrich Chemie, Steinheim, Germany.

Assessment of mitochondrial function

Parameters of mPTP opening were measured simultaneously in a temperature-controlled chamber with installed selective electrodes. $\Delta\Psi$m was determined by measuring mitochondrial uptake of tetraphenylphosphonium ions (TPP^+) using a TPP^+-selective electrode (Nico-Analyt, Moscow, Russia). Ca^{2+} transport was determined with a Ca^{2+}-sensitive electrode (Nico-Analyt, Moscow, Russia), and oxygen consumption rate was detected with a Clark-type O_2 electrode in a 2-ml cell chamber. Mitochondria (2 mg protein/ml) were incubated at 25 °C in the medium containing 120 mM KCl, 10 mM Tris–HCl (pH 7.4), 0.4 mM K_2HPO_4, 5 mM potassium succinate, and 2.5 μM rotenone. TPP^+ and Ca^{2+} concentration changes in the mitochondrial incubation medium were recorded on a linear scale. mPTP opening in RBM was induced by