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

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Abstract

Ca²⁺-induced permeability transition pore (mPTP) opening in isolated rat brain mitochondria is promoted through targeting of connexin43. After a threshold Ca²⁺ load, mitochondrial membrane potential drops and efflux of accumulated Ca²⁺ 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, Ca²⁺ 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 Ca²⁺-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 connexin43, carbenoxolone may exert its effect on mPTP via mitochondrial outer membrane TSPO.

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Keywords

Carbenoxolone; Protoporphyrin IX; TSPO; Connexins; Mitochondria; Permeability transition pore

Introduction

Carbenoxolone (Cbx),¹ a substance from medicinal licorice, is the water soluble 3-hemisuccinate of glycyrrhetic acid (succinyl ester of glycyrrhetic 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 18 β -glycyrrhetic 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 (Ψ_m), swelling of mitochondria and release of apoptotic factors. The composition of mPTP as well as the regulatory mechanism of pore 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 Ψ_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 Ca²⁺-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 Ca²⁺ in the matrix) and initiating Ca²⁺-induced Ca²⁺ efflux from the mitochondrial matrix [6]. Cbx increased Ca²⁺-induced high amplitude swelling of both RBM and RLM. Cbx-stimulated Ca²⁺ efflux and Ca²⁺-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

¹Abbreviations used: ACBD3, acyl-CoA binding domain containing protein 3; ANT, adenine nucleotide translocase; Cbx, carbenoxolone, (3 β)-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-1,3-dihydro-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; Ψ_m , mitochondrial transmembrane potential.

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'-phosphodiesterase [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. Ψ_m was determined by measuring mitochondrial uptake of tetraphenylphosphonium ions (TPP⁺) using a TPP⁺-selective electrode (Nico-Analyt, Moscow, Russia). Ca²⁺ transport was determined with a Ca²⁺-sensitive electrode (Nico-Analyt, Moscow, Russia), and oxygen consumption rate was detected with a Clarktype O₂ 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 K₂HPO₄, 5mM potassium succinate, and 2.5 μM rotenone. TPP⁺ and Ca²⁺ concentration changes in the mitochondrial incubation medium were recorded on a linear scale. mPTP opening in RBM was induced by threshold Ca²⁺ load (100 nmol Ca²⁺ per mg protein), as described elsewhere [6,19,26,31,32]. Anti-TSPO and anti-connexin43 antibodies were applied 10 s following the addition of RBM into the buffer and approximately 4 min before the addition of Ca²⁺ to the incubation medium to investigate effects on the Ca²⁺-induced mPTP opening. The anti-TSPO antibody that we successfully used in similar studies [15,19] was raised against amino acids 9–27 of the mouse TSPO sequence (VGLTLVPSLGGFMGAYFVR) [18] and affinity purified on a column with immobilized peptide antigen. Anti-connexin43 antibody was obtained from BD Biosciences (Becton-Dickinson, Heidelberg, Germany).

Swelling of RLM was measured as a change in light scattering of the mitochondrial suspension at 540 nm (A540) using a Tecan I-Control infinite 200 spectrophotometer at 25 °C. Standard incubation conditions for swelling assay were 125 mM KCl, 10 mM Tris, 0.4

mM KH_2PO_4 , 5 mM succinate, 5 μM rotenone. The protein concentration used was 0.5 mg/ml. RLM swelling was initiated by the addition of 75 μM Ca^{2+} into the standard medium (2 ml).

Phosphorylation, electrophoresis, and immunoblot analysis of mitochondrial proteins

For the protein phosphorylation studies, aliquots of the mitochondrial suspensions were supplemented with a mixture of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham Biosciences, UK) and unlabeled $\text{Mg}^{2+}\text{-ATP}$ to achieve final concentrations of 2 mM Mg^{2+} , 400 μM ATP, and 5–7 μCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Oligomycin (1.5 μM) was also added to prevent ATP hydrolysis by $\text{F}_0\text{F}_1\text{-ATPase}$. The samples of mitochondria were incubated for 3 min at room temperature (21–23 °C), and the protein phosphorylation reaction was stopped with sample solubilization buffer, followed by heating at 100 °C for 3 min [26,31]. To separate the mitochondrial proteins, the solubilized mitochondrial suspensions containing 15 μg of protein per lane were separated by SDS polyacrylamide gel electrophoresis (SDS–PAGE) carried out using 4% concentrating and 15% or 10% separating gels. Radioactive bands were visualized by exposure to Kodak X-Omat AR-5 film. For quantitative evaluation of the protein phosphorylation, the films were scanned with a BioRad densitometer (GS-100 Calibrated, Munchen, Germany), and changes in protein phosphorylation were calculated as percent of specific controls for each phosphoprotein band.

For the Western blot studies, 20 μg mitochondrial proteins was separated by 15% SDS–PAGE and transferred to nitrocellulose membranes. TSPO was detected using the above described antibodies (1:1000 dilution). Immunoreactive protein bands were visualized using the ECL system of Super-Signal West Pico (Pierce, Rockford, IL, USA).

For statistical analysis, relative levels of protein phosphorylation were expressed as means $\pm$ SD from at least 3–4 independent experiments, each performed in duplicate. Significance was determined by using Student's *t* test. A value of $P < 0.05$ was considered to be significant (asterisk indicates $P < 0.05$). Analysis of variance with Bonferroni *post hoc* comparison was used with statistical significance at $P < 0.05$ (indicated by #).

Results

Cbx and PPIX effects on Ca^{2+} -induced swelling of RLM

We previously demonstrated that Cbx causes Ca^{2+} -induced swelling of mitochondria, which correlates with a decrease of Ψ_m [6]. Cbx (1 μM) also accelerates Ca^{2+} -induced swelling of RLM, and this effect is further enhanced in the presence of 10 μM Cbx. Cbx-stimulated acceleration of RLM swelling is CsA sensitive [6]. Because Ca^{2+} -induced swelling is a parameter of mPTP function, we determined if the TSPO ligand, PPIX, altered Cbx-activated RLM swelling by measuring swelling as a decrease of absorbance at 540 nm. RLM swelling under control conditions or in the presence of added PPIX (1 μM) and Cbx (1 μM) without Ca^{2+} was not observed (Fig. 1A, traces 1–3). Ca^{2+} -induced swelling shown in trace 4 (Fig. 1) was accelerated by 1 μM PPIX (trace 6), and the effect was more pronounced in the presence of 1 μM Cbx (trace 5). Addition of both PPIX and Cbx to RLM led to a significant enhancement of RLM swelling (Fig. 1, trace 7). The diagram in Fig. 1B shows the average half time ($T_{1/2}$) to reach 50% of the maximal swelling of RLM. The parameter

$T_{1/2}$ strongly decreased in the presence of Cbx (4- to 4.5-fold), and this effect was doubled in the presence of PPIX and Cbx, resulting in overall 10-fold reduction of $T_{1/2}$ parameter compared to control Ca^{2+} -induced swelling.

Next, we investigated PPIX- and Cbx-stimulated mPTP opening in RBM and examined the effect of anti-TSPO antibodies on the induction of mPTP opening induced by the test compounds. In these experiments, we used non-purified mitochondria, which contain both pools of RBM (synaptic and non-synaptic RBM). The presence of TSPO in both pools of mitochondria was determined by Western blot analysis. For these studies, non-purified mitochondria were separated on Percoll gradients, and the presence of TSPO was analyzed in synaptic and non-synaptic mitochondria and the myelin fraction. Fig. 2 demonstrates the presence of TSPO in both pools of mitochondria. The bar graph in Fig. 2 demonstrates that TSPO levels were 15-fold higher in synaptic mitochondria compared to the myelin fraction and 3-fold higher compared to non-synaptic mitochondria. Since a small pool of intact mitochondria was earlier found in the myelin fraction [33], the mitochondrial extract used to test the effects of PPIX and Cbx contained all fractions of RBM.

Effects of anti-TSPO antibody on PPIX-induced mPTP opening in RBM

The effect of PPIX on RBM functions exerted via TSPO is shown on Fig. 3 using the TSPO ligand PPIX and anti-TSPO antibodies. The functional state of the mitochondria was determined by simultaneous measurements of Ψ_m and Ca^{2+} transport. Effects of PPIX and anti-TSPO antibodies on Ca^{2+} accumulation and Ca^{2+} -induced mPTP opening in RBM were investigated. Mitochondria maintained a high Ψ_m in the presence of succinate and rotenone. As shown in Fig. 3A, an initial pulse of Ca^{2+} (added to RBM) induced a short, reversible increase in oxygen consumption rate and a sharp decrease in Ψ_m , as measured by the membrane potential indicator TPP⁺. This was followed by the restoration of Ψ_m . In addition, Ca^{2+} rapidly accumulated into the mitochondrial matrix. However, the second addition of Ca^{2+} resulted in a decrease in the rate of Ca^{2+} influx ($V_{in} \text{Ca}^{2+}$, see Fig. 3A), the short restoration of Ψ_m , and an enhanced oxygen consumption rate in state 4. Together, these changes indicate uncoupling of the energy transforming systems in the inner mitochondrial membrane and initiation of mPTP opening. Indeed, within approximately 6 min (400–450 s) after the second pulse of Ca^{2+} , complete dissipation of Ψ_m and Ca^{2+} efflux from the mitochondrial matrix occurred. CsA, a specific blocker of mPTP opening, was used to confirm that mPTP was responsible for the observed Ca^{2+} -induced Ca^{2+} efflux. In the presence of CsA, the second addition of Ca^{2+} did not lead to Ca^{2+} efflux and Ψ_m dissipation (not shown).

In the presence of 1 μM PPIX, mPTP opening in RBM was found to be facilitated. Ca^{2+} -induced Ca^{2+} efflux and dissipation of the Ψ_m was observed also after the second addition of Ca^{2+} (Fig. 3B). However, restoration of Ψ_m was not observed, and lag-time period was rather short, within 1 min, demonstrating PPIX-dependent acceleration of mPTP opening. We also examined the effect of the anti-TSPO antibody on PPIX-stimulated opening of mPTP. As shown in Fig. 3C, the addition of anti-TSPO antibody (1:1000) prevented the PPIX-activated mPTP opening. Interestingly, under these conditions the ability to retain Ca^{2+} at the threshold Ca^{2+} concentration was also increased in the presence of PPIX with

anti-TSPO antibody. Pore opening was observed after the second Ca^{2+} addition, and lag phase time was prolonged to the same extent as control under the same threshold Ca^{2+} concentration. Addition of anti-TSPO antibody alone to RBM significantly prevented the mPTP opening (Fig. 3D) and prolonged lag phase time to 10 min. Namely, lag phase time was increased in comparison with control condition (6 min). BSA added to mitochondrial suspension at the same concentrations as antibodies did not have any effect (not shown).

Effect of anti-TSPO antibody on Cbx-induced mPTP opening in RBM

To test the hypothesis that TSPO mediates the effects of Cbx on mPTP, we examined the effects of the anti-TSPO antibody on the Ca^{2+} -induced mPTP opening in RBM by Cbx. The inhibitory effects of the anti-TSPO antibody on the mPTP opening were recently described [19]. Here we demonstrated the combined effect of Cbx and anti-TSPO antibody. Fig. 4A shows that in controls a second Ca^{2+} pulse led to initiation of mPTP opening. In the absence of Cbx, opening of mPTP began within 280 s after the second addition of Ca^{2+} . Addition of 1 μM Cbx to the mitochondrial suspension led to a decrease from 300 s to 50–80 s of the lag phase before the release of Ca^{2+} from mitochondria (Fig. 4B), indicating strong acceleration of mPTP opening. As previously shown, Cbx-stimulated Ca^{2+} efflux from RBM is CsA sensitive [6]. The presence of anti-TSPO antibody (1:1000) also increased acceleration of mPTP opening. Cbx-stimulated Ca^{2+} efflux from RBM was found to be CsA sensitive [6]. The presence of anti-TSPO antibody (1:1000) increased the lag time from 280 s to 500 s, preventing mPTP opening (Fig. 4C). Addition of anti-TSPO antibody to Ca^{2+} -treated (control) RBM prevented stimulation of mPTP opening, prolonging a lag time to 370 s (Fig. 4D). In contrast to anti-TSPO antibody, anti-Cx43 antibody failed to inhibit the mPTP opening in the presence only of threshold Ca^{2+} concentrations. Fig. 5 shows the analysis with the average action of the anti-TSPO and anti-Cx43 antibodies used for the induction of mPTP opening stimulated by Cbx. Both antibodies suppressed Ca^{2+} influx in the presence as well as in the absence of Cbx. Ca^{2+} release was inhibited by both the anti-TSPO and anti-Cx43 antibodies in Cbx treated mitochondria, suggesting TSPO as a potential Cbx target [6].

Effect of Cbx on protein phosphorylation in RBM

Taking into consideration that licorice extract (where Cbx is present) inhibits cAMP phosphodiesterase activity, we examined whether Cbx is able to modulate protein phosphorylation in RBM. Fig. 6 shows a semi-quantitative analysis of the phosphoprotein bands from SDS-PAGE after separation of Ca^{2+} -loaded RBM in the presence or absence of Cbx and anti-TSPO antibody. We investigated the concentration-dependent effect of Cbx on protein phosphorylation and found that Cbx at 1 μM and 10 μM significantly stimulated the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into 43–46 kDa proteins (where Cx43 and CNP were found) [6,27], 17 kDa proteins (myelin basic protein isoforms) [28], as well as of 3.5 kDa (subunit *c* of ATP synthase) (Fig. 6A). When Ca^{2+} -loaded RBM were treated with Cbx (under conditions of mPTP acceleration), the phosphorylation of all proteins mentioned was increased. Moreover, PPIX at 1 μM combined with 1 μM Cbx induced an additive stimulating effect on phosphorylation of the same proteins (not shown). The carbenoxolone-stimulated phosphorylation of mitochondrial proteins was inhibited by anti-TSPO antibodies (Fig. 6B).

Discussion

Cbx, a substance from medicinal licorice, induces mPTP opening in rat liver mitochondria, stimulating mitochondrial swelling and Ψ_m collapse. These effects are accompanied by peroxide generation and sulfhydryl and pyridine nucleotide oxidation, presumptive indicators of oxidative stress [3–5]. It was suggested that Cbx induces ROS production that is critical for the induction of mPTP opening in the presence of Ca^{2+} [5]. This was a logical conclusion, because mPTP is formed in the inner mitochondrial membrane in response to Ca^{2+} overload or oxidative stress. Recently we showed that Cbx is able to accelerate mPTP opening in rat brain mitochondria [6]. The examination of Cbx-induced ROS production in RBM showed that in Ca^{2+} -overloaded RBM the level of H_2O_2 is rather low, and generation of H_2O_2 by RBM is not enhanced in the presence of 1 μM Cbx [6]. Instead, Cx43 was found to be the target of Cbx, which at 1 μM concentration facilitates the Ca^{2+} -induced CsA sensitive mPTP opening [6].

As a potential gap junction inhibitor, Cbx has structural similarities to corticosteroids [12]. The rate-limiting step of steroidogenesis is the transport of cholesterol from intracellular stores into mitochondria, where it is converted to pregnenolone at the mitochondrial inner membrane. Cholesterol import and transfer from the outer to the inner membrane is mediated by TSPO, previously known as the peripheral-type benzodiazepine receptor, which is situated in the outer membrane and can modulate mPTP functions [19–25]. Therefore, we hypothesized that TSPO might also be a target of Cbx. First, we compared the effect of 1 μM PPIX (a TSPO ligand) and 1 μM Cbx (gap junction inhibitor) on Ca^{2+} -induced swelling of RLM, one of the mPTP parameters. We showed that swelling of RLM was stimulated by the TSPO ligand PPIX and that Cbx significantly potentiated this effect (Fig. 1). PPIX and Cbx enhanced the effect of each other. To examine the effect of Cbx on brain mitochondrial function, we used a whole mitochondrial fraction containing synaptic and non-synaptic pools of mitochondria as well as mitochondria associated with the myelin fraction and demonstrated the presence of TSPO in all pools of mitochondria by immunoblot analysis (Fig. 2).

Investigation of mPTP function in RBM detected by simultaneous measurement of the transmembrane potential (Ψ_m), Ca^{2+} transport, and oxygen consumption revealed that PPIX and Cbx induced mPTP opening, which was strongly prevented by the addition of a specific anti-TSPO antibody directed against amino acids 9–27 of the TSPO sequence. Ca^{2+} -induced, CsA-sensitive mPTP is a multiprotein complex formed in the inner mitochondrial membrane after excessive Ca^{2+} accumulation or as a response to oxidative stress. The mPTP opening leads to the release of pro-apoptotic factors and the initiation of programmed cell death. The exact composition of this non-selective mitochondrial pore and the mechanisms of regulation of mPTP are still not fully understood. Cyclophilin D and the Pi carrier are considered to be involved in mPTP function. However, doubt has been raised about the essential role of ANT and VDAC, which are considered to be the major modulators of the permeability of the inner and outer membranes [34–41]. Recently, new potential structural components of the mPTP have been reported such as the ATP synthase dimer [42] and the subunit *c* of Fo-ATP synthase [43,44].

Also, a critical point in mPTP function is the presence of TSPO. We recently showed that anti-TSPO antibodies prevent mPTP opening more effectively than CsA, indicating the possible direct participation of TSPO in mPTP regulation [19]. The efficacy of anti-TSPO antibodies to prevent the induction of mPTP opening is stronger than anti-Cx43 antibodies in Cbx-treated mitochondria. The anti-Cx43 antibody does not influence mPTP opening in the presence of threshold Ca^{2+} in control RBM (without Cbx), suggesting that Cx43 is not a structural element of the pore complex. However, it could be an additional target of Cbx in mitochondria. Although the monoclonal anti-Cx43 antibody is ineffective in preventing Ca^{2+} -induced mPTP opening in rat brain mitochondria, it completely abolishes the acceleration of mPTP opening by Cbx. Taken together, these results suggested that Cx43 may interact with mPTP through TSPO.

TSPO contains a cholesterol binding amino acid consensus sequence -L/V-(X1-5-Y-(X)1-5-R/K- CRAC motif [18]. Interestingly, a comparable cholesterol binding amino acid consensus sequence is detected in Cx43 [45]. We recently reported that TSPO ligands, such as PK11195, Ro 5-4864, and PPIX are able to induce mPTP opening in RBM [26,31]. In addition, here we shown that PPIX- and Cbx-dependent swelling of Ca^{2+} overloaded RLM were further increased in the combined presence of PPIX and Cbx. These data suggest that TSPO ligands and Cbx might have the same target, and this target might be the CRAC domain. Taking into consideration that the CRAC domain is present in TSPO, Bax and Cx43, it is possible that this domain might participate in regulation of the channel/pore activity and permeability of the mitochondrial membranes.

It should be noted that in Ca^{2+} -overloaded RBM, when mPTP was opened, the phosphorylation status of mitochondrial membrane proteins is changed. This phosphorylation is modulated by TSPO ligands (PK 11195, Ro5-4864, and PPIX) [19,26,32]. The molecular mass of the phosphorylated proteins is 43–46 kDa (where Cx43 was found), 17 kDa and 21.5 kDa (identified by 2D electrophoresis and mass spectrometry analysis as phosphorylated isoforms of MBP associated with mitochondria [28]), 3.5 kDa (identified as subunit *c* of FoF_1 -ATP synthase [29]). Modulation of the phosphorylation level of these proteins is Ca^{2+} - and CsA-dependent, supporting their relationship with mPTP function [26,31,32,46]. Interestingly, while Cbx lowers threshold calcium concentration in purified synaptic and non-synaptic mitochondria, in the present study we found acceleration of mPTP opening in non-purified RBM without changing of threshold calcium. This is likely due to the presence of CNP and MBP in the myelin fraction which are able to prevent mPTP opening [33] and reduce the stimulating effect of Cbx.

We subsequently examined the effect of Cbx on protein phosphorylation and observed that Cbx was able to stimulate the 43–46 kDa, 17 kDa, and 3.5 kDa proteins phosphorylation in RBM loaded with threshold Ca^{2+} in a manner similar to TSPO ligands. This demonstrates that the phosphorylation of 43–46 kDa, 17 and 21 kDa proteins is modulated by Cbx probably via TSPO. Moreover, anti-TSPO antibody modulated the Cbx-stimulated phosphorylation of these proteins, indicating the existence of common targets for TSPO ligands and Cbx (Figs. 4 and 5).

The activating effect of Cbx on protein phosphorylation could be partly explained by the fact that licorice extract (where Cbx is present) possesses the ability to inhibit cAMP phosphodiesterase activity [47]. Cbx is known to upregulate Cx43 expression via the protein kinase A (PKA)-dependent pathway [48]. The function of TSPO was also reported to be regulated by PKA-dependent phosphorylation [49]. Although the direct interaction of TSPO with Cx43 has not been established yet, it is likely that TSPO participates in modulating the phosphorylation status of 46 kDa, 17–21, subunit *c*, and Cx 43 proteins. This conclusion is supported by the fact that TSPO interacts with a protein binding to the regulatory PKA subunits, RI α and RII α , thus acting like an A-kinase anchoring protein. This protein, originally named PAP7 for PBR-associated protein 7, was recently found to be a member of the acyl-CoA binding domain (ACBD) containing family of proteins, specifically ACBD3 [50]. Previously, it was considered that PBR is tight protein complex, composed of VDAC, ANT and TSPO [22–24]. One component of this complex – ANT – can be involved in another super-complex, the ATP synthasome, comprised of ANT, the Pi carrier and the ATP synthase [51,52]. Taking into consideration our own data and existing literature we propose a working scheme (Fig. 7) where the PKA-ACBD3-TSPO-VDAC complex transduces signals that modulate mitochondrial function, thus termed transduceosome [53–55]. This hypothesis explains the regulation of the phosphorylation of the above mentioned proteins. Consistent with this possibility, when PAP-7/ACBD3 is associated with the mitochondria, it can serve as a target for PKA in TSPO-containing mitochondria [56]. The signal of phosphorylation could be translated from TSPO to VDAC and then to ANT [19]. ANT, being a part of the ATP synthasome, can contact subunit *c* of the Fo-ATP synthase [51,52]. Thus, the phosphorylation status of subunit *c* coupled with its pore forming properties [32,46] can be modulated. It is also likely that PAP7/ACBD3 might bind phosphodiesterase as an anchoring protein. In that case, the Cbx-induced inhibition of phosphodiesterase activity could increase the local concentration of cAMP and, in turn, activate PKA, thus providing an explanation for the local regulation of mPTP function. The molecular mechanism of such path of regulation is needed to be examined in further investigations. In summary, the results presented herein indicate that the effect of Cbx on mitochondrial function might be exerted through regulation of mPTP opening coupled with phosphorylation/ dephosphorylation of mitochondrial proteins. TSPO is key player in these events.

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Fig. 1.

Effect of Cbx and PPIX on Ca²⁺-induced swelling in rat liver mitochondria (RLM).

Mitochondrial swelling was measured as described in Materials and methods. Cbx (1 μ M) and PPIX (1 μ M) were applied 20 s after the addition of RLM. (A) Trace 1 – control RLM; trace 2 – RLM treated with 1 μ M Cbx; trace 3 – RLM treated with 1 μ M PPIX; traces 4–7 – RLM in the presence of threshold Ca²⁺ treated with 1 μ M Cbx (trace 5), 1 μ M PPIX (trace 6) and 1 μ M PPIX plus 1 μ M Cbx (trace 7). (B) Comparison of the half-time ($T_{1/2}$) for reaching 50% of the maximal Ca²⁺-induced swelling of RLM in the absence and presence of 1 μ M Cbx, 1 μ M PPIX, and 1 μ M Cbx plus 1 μ M PPIX. Values represent means $\pm$ SD from three independent experiments. * $P < 0.05$, vs. Ca²⁺ load induced swelling, determined by Student's *t*-test.

Fig. 2.

Level of TSPO in fractions obtained after separation of rat brain mitochondria on Percoll density gradients. (A) Aliquots of mitochondrial fractions (20 μ g of protein per lane) were separated by 15% SDS-PAGE gels, followed by Western blots. The membrane was probed with anti-TSPO antibody (1:1000). (B) Quantitation of immunostaining using computer assisted densitometry. Bar graphs represent TSPO immunoreactivity in the myelin fraction (M), synaptic mitochondria (S), and non-synaptic mitochondria (N) (three independent isolations).

Fig. 3. Effect of PPIX and anti-TSPO antibody on Ca^{2+} transport, membrane potential and oxygen consumption. Mitochondrial parameters were measured as described in Materials and methods. (A) Control RBM, (B) 1 μM PPIX-treated RBM, (C) 1 μM PPIX and anti-TSPO antibody (1:1000) treated RBM, (D) anti-TSPO antibody treated

Fig. 4. Effect of Cbx and anti-TSPO antibody on Ca^{2+} transport, membrane potential and oxygen consumption. Mitochondrial parameters were measured as described in Materials and methods. (A) Control RBM, (B) 1 μM Cbx-treated RBM, (C) 1 μM Cbx and anti-TSPO antibody (1:1000) treated RBM, (D) anti-TSPO antibody treated RBM. The arrows show where CaCl_2 (100 μM) was added to the mitochondrial suspension.

Fig. 5.

Effect of anti-TSPO antibody and anti-Cx43 antibody on Cbx-induced mPTP opening.

Mitochondrial parameters ($v_{st.2}$, $V_{in}^{Ca^{2+}}$, T_{lag} and $V_{out}^{Ca^{2+}}$) were calculated as described in [19]. Anti-PBR/TSPO antibody (1:1000), anti-Cx43 (1:500), and 1 μ M Cbx were used.

Column 1 – respiratory rate state 2, column 2 – Ca^{2+} influx rate ($V_{in}^{Ca^{2+}}$), column 3 – lag phase – time period between addition of threshold Ca^{2+} load and initiation of Ca^{2+} efflux (T_{lag}), and column 4 – the Ca^{2+} efflux rate ($V_{out}^{Ca^{2+}}$). Parameters for RBM in the mPTP opened state were taken as 100%. Values represent means $\pm$ SD from four independent experiments. * $P < 0.05$ vs. control, determined by Student's t -test.

Fig. 6.

Combined effects of Cbx and anti-TSPO antibody on protein phosphorylation in RBM loaded with threshold Ca²⁺. RBM were pre-incubated with and without additions and loaded with threshold Ca²⁺ as described in Fig. 3. The 20 μl aliquots of control and Cbx-treated RBM were taken after mPTP opening (approximately 5 min after incubation of RBM was started), their phosphorylation with [γ-³²P]ATP was started and then treated for PAGE as described in Materials and methods. (A) Relative levels of ³²P-incorporation into phosphoproteins of Ca²⁺-loaded RBM in the presence of different concentrations of Cbx (100 nM, 500 nM, 1 μM, and 10 μM). (B) Relative levels of ³²P-incorporation into phosphoproteins of Ca²⁺-loaded RBM in the presence of 1 μM Cbx and 1 μM CsA as well as anti-TSPO-antibody (1:1000 dilution). Levels of protein phosphorylation of RBM in the PTP-opened state were taken as 100% control. Values represent means ± SD from 3–4 independent experiments. **P* < 0.05, vs. control, determined by Student's *t*-test; #ANOVA with Bonferroni *post hoc* comparison with statistical significance at *p* < 0.05. RBM. The arrows show where CaCl₂ (100 μM) was added to the mitochondrial suspension.

Fig. 7.

Hypothetical scheme of carboxoxolone interaction with TSPO and mPTP. TSPO is located in the outer mitochondrial membrane where it associates with both VDAC and PAP7/ACBD3 [23,50]. TSPO and VDAC functions are known to be regulated by PKA [50], which binds to PAP7/ACBD3. Thus, the cAMP signal from PAP7/ACBD3 might be translated to TSPO, and as a signaling molecule, TSPO might further translate the signal to VDAC or ANT. In turn, ANT been part of the ATP synthasome [51,52] can translate the phosphorylation signal to subunit *c* of the Fo-ATP synthase modulating mPTP [43]. Based on data presented in this manuscript and the literature, we propose that Cbx binds to TSPO or PAP7/ACBD3 to suppress cAMP hydrolysis, thus increasing the local concentration of

cAMP and inducing/ increasing TSPO phosphorylation. This event, in turn, would modify the activity of VDAC/ANT/mPTP and/or connexin channels.

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