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Effects of cyclosporine A and its immunosuppressive or non-immunosuppressive derivatives [D-Ser]^8-CsA and Cs9 on mitochondria from different brain regions

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ABSTRACT

We studied the functional properties of isolated brain mitochondria (BM) prepared from total rat brain (BM_{total}) or from cerebral subregions under basal and Ca^{2+} overload conditions in order to evaluate the effects of cyclosporine A (CsA) in a regiospecific manner. CsA-induced effects were compared with those of two derivatives—the none-immunosuppressive [O-($NH_2(CH2)_5NHC(O)CH_2$)-D-Ser]^8-CsA (Cs9) and its congener, the immunosuppressive [D-Ser]^8-CsA. The glutamate/malate-dependent state 3 respiration of mitochondria (state $3_{glu/mal}$) differed in region-specific manner (cortex > striatum = cerebellum > substantia nigra > hippocampus), but was significantly increased by 1 μM CsA (+21 ± 5%) in all regions. Ca^{2+} overload induced by addition of 20 μM Ca^{2+} caused a significant decrease of state $3_{glu/mal}$ (−45 to −55%) which was almost completely prevented in the presence of 1 μM CsA, 1 μM Cs9 or 1 μM [D-Ser]^8-CsA. Mitochondrial Ca^{2+} accumulation thresholds linked to permeability transition (PT) as well as the rate and completeness of mitochondrial Ca^{2+} accumulation differed between different brain regions. For the first time, we provide a detailed, regiospecific analysis of Ca^{2+}-dependent properties of brain mitochondria. Regardless of their immunosuppressive impact, CsA and its analogues improved mitochondrial functional properties under control conditions. They also preserved brain mitochondria against Ca^{2+} overload-mediated PT and functional impairments. Since Cs9 does not mediate immunosuppression, it might be used as a more specific PT inhibitor than CsA.

1. Introduction

The immunosuppressive peptide generic inhibitor of the prolyl *cis*/*trans* isomerase activity of most human cyclophilins, the cyclic undecapeptide cyclosporine A (CsA), was shown to powerfully decrease the permeability transition pore (PTP) open probability, as shown in liver (Broekemeier et al., 1989; Waldmeier et al., 2002; Petronilli et al., 1993), heart (Halestrap et al., 1997; Novgorodov et al., 1992) and skeletal muscle (Fontanine et al., 1997) mitochondria. Its cytoprotective properties were demonstrated in isolated mitochondria (Broekemeier et al., 1989; Friberg et al., 19991; Petronilli et al., 1993; Novgorodov et al., 1992; Halestrap et al., 1997; Fontanine et al., 1997; Waldmeier et al., 2002; Brustovetsky et al., 2003; Chalmers and Nicholls, 2003; Hansson et al., 2004; Naga et al., 2007), in cellular (Khaspekov et al., 1999; Waldmeier et al., 2002; Bambrick et al., 2006) and animal models (Sullivan et al., 2000; Domañska-Janik et al., 2004). Other authors reported CsA to inhibit Ca^{2+}-induced PTP opening in brain mitochondria (Hansson et al., 2004). However, CsA effects on brain mitochondria are still controversially discussed (Andreyev and Fiskum, 1999; Berman et al., 2000; Brustovetsky and Dubinsky, 2000; Kristian et al, 2000; Chinopoulos et al., 2003; Hansson et al., 2004; Domañska-Janik et al., 2004; Bambrick et al., 2006; Naga et al., 2007;

Abbreviations: BM, brain mitochondria; BM_{total}, total brain mitochondria; BM_{cortex}, cortex mitochondria; $BM_{cerebellum}$, mitochondria prepared from cerebellum; $BM_{hippocampus}$, hippocampual mitochondria; $BM_{striatum}$, striatal mitochondria; $BM_{s.nigra}$, mitochondria prepared from substantia nigra; CAT, carboxyatractyloside; CsA, cyclosporine A; Cs9, [O-($NH_2(CH2)_5NHC(O)CH_2$)-D-Ser]^8-CsA; Cyp-D, cyclophilin D; PT, permeability transition; PTP, permeability transition pore; RCI, respiratory control index; SMM, skeletal muscle mitochondria; state $3_{glu/mal}$, glutamate/malate-dependent state 3 respiration; state 3_{suc}, succinate-dependent state 3 respiration.

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Eliseev et al., 2007). At comparable degrees of Ca^{2+} accumulation, brain mitochondria are more resistant to Ca^{2+}-induced PTP opening than their counterparts in heart or liver (Kristian et al., 2000; Chalmers and Nicholls, 2003; Panov et al., 2004; Eliseev et al., 2007). In addition, several reports have revealed negligible protective effects of CsA on mitochondrial functions and Ca^{2+} overload (Andreyev and Fiskum, 1999; Berman et al., 2000; Kristian et al., 2000; Chinopoulos et al., 2003; Panov et al., 2004). It appears that CsA-induced protection of brain mitochondria against PT might depend on distinct experimental conditions, e.g. media composition (Hansson et al., 2004) and the level of mitochondrial depolarization (Brustovetsky and Dubinsky, 2000). Due to a higher content of cyclophilin D (Cyp-D), synaptic mitochondria, might be more accessible to PT than their non-synaptic counterparts (Naga et al., 2007). It was further reported that CsA protects mitochondria from cortical astrocytes, but not from granule neurons in the cerebellum (Bambrick et al., 2006). There are also hints for distinct mitochondrial vulnerabilities to Ca^{2+} stress in different brain regions (Brustovetsky et al., 2003). CsA has been shown to decrease neuronal cell death in hippocampal cell cultures (Khaspekov et al., 1999). CsA also attenuates acute mitochondrial dysfunction due to traumatic brain injury (Sullivan et al., 2000) or transient cerebral ischemia (Domañska-Janik et al., 2004).

PT plays an important role in mitochondrial cell death (Friberg et al., 1999; Brustovetsky and Dubinsky, 2000; Hansson et al., 2004; Eliseev et al., 2007; Seppet et al., 2009) and in most, if not all neurodegenerative diseases (Kristal et al., 2004; Celsi et al., 2009; Seppet et al., 2009). Thus, PTP inhibitors may promote mitochondrial and cellular protection under pathophysiological conditions and are potentially suitable for the treatment of neurodegenerative diseases (Kristal et al., 2004; Gellerich et al., 2008). Furthermore, neurodegenerative diseases affect often specific brain regions, underlining the need for studies exploring regioselective effects of mitochondrial function, mitochondrial Ca^{2+} overload and the influence of CsA on this parameters.

Via binding cyclophilins, CsA acts in a dual mode. (i) Blockage of cyclophilin-mediated catalysis of peptide bonds preceding specific proline residues of protein ligands, thereby affecting their conformation and function (Fischer et al., 1989) and (ii) inhibition protein phosphatase 2B (calcineurin) by the cyclophilin/CsA complex due to a gain-of-function rendering many cyclosporine derivatives immunosuppressive (Liu et al., 1991). Using a cyclosporine derivative substituted in 6-position or the macrocyclic sanglifehrin, both of which cannot get activated by gain-of-function, the prolyl cis/trans isomerase site of cyclophilins was found to be critical for a selective inhibition of the mitochondrial PTP (Clarke et al., 2002; Griffiths and Halestrap, 1995).

Since the 8-position of CsA is in particular chemically tractable, we have investigated whether the non-immunosuppressive cyclophilin inhibitor Cs9, containing a chemically reactive amino group on a tether (Zhang et al., 2005), affects the functional properties of brain mitochondria in a regiospecific manner. Its parent compound D-Ser^8-CsA (Hu et al., 1995) with CsA-like properties was used as calcineurin inhibiting reference. We also studied total brain mitochondria (BM_{total}) with respect to their stability against Ca^{2+} stress and the effects of cyclosporins. Moreover, we compared the resistance of brain mitochondria prepared from cortex (BM_{cortex}), cerebellum ($BM_{cerebellum}$), hippocampus ($BM_{hippocampus}$), striatum ($BM_{striatum}$), and substratia nigra ($BM_{s.nigra}$) against Ca^{2+} stress. In contrast to previous studies, mitochondrial Ca^{2+} accumulation and oxidative phosphorylation were studied in parallel under Ca^{2+} stress conditions.

2. Materials and methods

2.1. Materials

CsA, ADP (disodium salt), MOPS and rotenone were obtained from Sigma (Taufkirchen, Germany). Cs9 was synthesized according to published procedures (Zhang et al., 2005). Ca-Green-5N was from Molecular Probes (Eugene, OR, USA) and carboxyatractyloside (CAT) was achieved from Calbiochem Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.2. Isolation of mitochondria from different brain regions

Mixtures of non-synaptic and synaptic brain mitochondria from young adult male rats (12 weeks) were isolated as described by Kudin et al., 2004. Immediately after decapitation, brains were rapidly removed, transferred into ice cold MMSE-A solution (225 mM mannitol, 20 mM MOPS, 75 mM sucrose, 1 mM EGTA, pH 7.4). Cortex, cerebellum, striatum, hippocampus and substantia nigra were dissected on ice. All further steps were performed at 4 °C. Tissues were minced and homogenized manually in ice cold MMSE-B solution (MMSE-A, additionally containing 0.05% nagarse, 1 g tissue / 10 ml MMSE-B) in a Dounce homogenizer using four strokes with the A pestle (total clearance 0.12 mm) and eight strokes with the B pestle (total clearance 0.05 mm). Subsequently, 30 ml MMSE-A were added and the homogenate was centrifuged at 2000 *g* for 4 min. The supernatant was passed through a cheese cloth and then centrifuged at 12,000 *g* for 9 min. To permeabilize synaptosomes, the resulting pellet was resuspended in 10 ml MMSE-C (MMSE-A, additionally containing 0.02% digitonin) in a small glass homogenizer, and homogenized manually with 8–10 strokes to obtain a homogenous suspension. This suspension was centrifuged at 12,000 g for 11 min and the pellet was finally resuspended in MMSE-D (225 mM mannitol, 20 mM MOPS, 75 mM sucrose, 0.1 mM EGTA, pH 7.4).

2.3. Oxygen consumption

Mitochondrial respiration was measured using a Clark-type oxygen electrode and a high resolution OROBOROS oxygraph (Gnaiger, 2001) at 30 °C. The oxygen concentration in air-saturated medium was considered to be 200 nmol O_2/ml at 95 kPa. Weight-specific oxygen consumption was calculated from the time derivative of oxygen concentration (DATGRAPH Analysis software, OROBOROS INSTRUMENTS, Innsbruck, Austria). Respiration of mitochondria (0.1 mg protein per ml) was measured in MMMPK buffer containing 5 mM $MgCl_2$, 120 mM mannitol, 40 mM MOPS, 5 mM KH_2PO_4, 60 mM KCl, pH 7.4 using a multiple substrate inhibitor protocol as described previously (Kuznetsov et al., 2008) with sequential additions of 10 mM glutamate, 2 mM malate, 1.5 μM rotenon, 10 mM succinate and 5 μM CAT.

2.4. Ca^{2+} accumulation measurements

Extramitochondrial free Ca^{2+} was monitored with 0.5 μM Calcium Green-5N using a Cary-Eclipse fluorescence spectrophotometer (Varian, Darmstadt, Germany) and excitation and emission wavelengths of 506 and 532 nm, respectively (Gizatullina et al., 2005). Cuvettes were stirred and thermostated at 30 °C. Mitochondrial Ca^{2+} accumulation was measured in MMPK buffer (pH 7.4) containing 120 mM mannitol, 40 mM MOPS, 5 mM KH_2PO_4, 60 mM KCl, 10 mM pyruvate and 2 mM malate. Measurements shown in Fig. 4 were performed in the additional presence of 2 mM Mg^{2+} and 10 μM ADP.

2.5. Protein determination

Mitochondrial protein concentration was determined by the bicinchoninic acid assay (Wiechelmann et al., 1988). Bovine serum albumin was used as standard.