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Cyclophilin D-Sensitive Mitochondrial Permeability Transition in Adult Human Brain and Liver Mitochondria

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Abstract

The mitochondrial permeability transition (mPT) is considered to be a major cause of cell death under a variety of pathophysiological conditions of the central nervous system (CNS) and other organs. Pharmacological inhibition or genetic knockout of the matrix protein cyclophilin D (CypD) prevents mPT and cell degeneration in several models of brain injury. If these findings in animal models are translatable to human disease, pharmacological inhibition of mPT offers a promising therapeutic target. The objective of this study was to validate the presence of a CypD-sensitive mPT in adult human brain and liver mitochondria. In order to perform functional characterization of human mitochondria, fresh tissue samples were obtained during hemorrhage or tumor surgery and mitochondria were rapidly isolated. Mitochondrial calcium retention capacity, a quantitative assay for mPT, was significantly increased by the CypD inhibitor cyclosporin A in both human brain and liver mitochondria, whereas thiol-reactive compounds and oxidants sensitized mitochondria to calcium-induced mPT. Brain mitochondria underwent swelling upon calcium overload, which was reversible upon calcium removal. To further explore mPT of human mitochondria, liver mitochondria were demonstrated to exhibit several classical features of the mPT phenomenon, such as calcium-induced loss of membrane potential and respiratory coupling, as well as release of the pro-apoptotic protein cytochrome c. We concluded that adult viable human brain and liver mitochondria possess an active CypD-sensitive mPT. Our findings support the rationale of CypD and mPT inhibition as pharmacological targets in acute and chronic neurodegeneration.

Key words: ischemia; oxidative stress; mitochondria; traumatic brain injury; traumatic spinal cord injury

Introduction

Activation of mitochondrial permeability transition (mPT) is considered to be a major cause of cell death under a variety of pathophysiological conditions, including ischemia/reperfusion injury, neurodegenerative disease, traumatic brain injury (TBI), muscular dystrophy, and drug toxicity (Bernardi et al., 2006; Halestrap and Pasdois, 2009; Kroemer et al., 2007; Liu and Murphy, 2009; Mbye et al., 2009; Millay et al., 2008; Nicholls, 2009; Russmann et al., 2009). Provided that findings in animal models can be translated to human disease, pharmacological inhibition of mPT offers a promising therapeutic target for the treatment of these disorders (Baines, 2010; Cook et al., 2009; Morota et al., 2009; Waldmeier et al., 2003).

The mPT is defined as a sudden increase in inner mitochondrial membrane permeability causing loss of ion homeostasis and the proton motive force required for ATP synthesis. The matrix protein cyclophilin D (CypD) is a peptidylprolyl cis-trans isomerase that regulates mPT and facilitates its activation by calcium. Animals lacking CypD display increased resistance to ischemic insults, muscular dystrophies, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (Baines et al., 2005; Du et al., 2008;

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Forte et al., 2007; Martin et al., 2009; Nakagawa et al., 2005; Schinzel et al., 2005), and the CypD inhibitor cyclosporin A (CsA) and its analogs have displayed neuroprotective effects in several animal models of acute neurological damage and chronic neurodegenerative disease. CsA treatment has also demonstrated promising results in initial clinical trials of TBI (Empey et al., 2006; Hatton et al., 2008; Mazzeo et al., 2008), as well as myocardial reperfusion injury (Mewton et al., 2010; Piot et al., 2008). Preserving the integrity of mitochondrial membranes through inhibition of mPT has been put forward as the central mechanism for the neuroprotective and cardioprotective effects of CsA, even though the drug has several pharmacological targets. Further, it has not been established whether cellular calcium overload and oxidative stress can trigger the mPT phenomenon in adult human mitochondria similarly to that described in mitochondria derived from animal tissues. It has also been suggested that CypD is downregulated in neurons during development, which would decrease the sensitivity of the mPT to calcium, and prohibit the use of CypD as a pharmacological target in disorders of the adult central nervous system (CNS) (Eliseev et al., 2007).

The purpose of this study was to determine whether a CypD-sensitive mPT exists in adult human brain and liver mitochondria, and thus constitutes a relevant pharmacological target in diseases for which mPT has been implicated in the pathogenesis. Further, if present, the objective was to evaluate whether human mPT displays analogous functional characteristics and is modulated by endogenous regulators and oxidants similarly to mitochondria from animal tissues.

Methods

Tissue samples

In order to obtain fresh human tissue for functional mitochondrial analyses, brain samples were collected from five patients undergoing neurosurgery, and liver tissue from seven patients undergoing liver resection (see Table 1 for patient demographics). Tissue that would otherwise have been discarded was transferred into ice-cold isolation buffer and rapidly prepared for mitochondrial isolation. Only the morphologically normal parenchyma surrounding the resected tumors was used. The human study was approved by the Ethical Committee of Hachioji Medical Center, Tokyo Medical University, permit number 12-01, and complied with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects, and the EU Convention for the Protection of Human Rights and Dignity of the Human Being with Regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine.

Mitochondrial isolation

Isolation of human brain mitochondria was achieved using a discontinuous Percoll gradient according to the method of Sims and Anderson (Sims and Anderson, 2008), with slight modification as previously described (Hansson et al., 2008), and the three density gradient layers consisted of 12%, 19%, and 40% Percoll, respectively. Liver mitochondria were isolated using differential centrifugation including a 19% Percoll step (Hansson et al., 2008).

Mitochondrial permeability transition assays

A Perkin-Elmer luminescence spectrometer (LS-55B; Emeryville, CA) with a temperature-controlled cuvette holder was used for all fluorescence and light scattering experiments. De-energized experiments were performed at 28°C in a 150 mM KCl-based buffer containing 0.5 μM rotenone, 0.2 mg/mL antimycin A, 2 μM calcium ionophore A23187, 0.5 mM PPi, and 2 mM nitrilotriacetic acid. Swelling and calcium retention capacity (CRC) experiments using respiring mitochondria were performed at 37°C in a 125 mM KCl-based buffer, including 2 mM Pi (K), 200 μM ATP, 50 μM ADP, 1 mM $MgCl_2$, 5 mM malate, and 5 mM glutamate. The extent of swelling was calculated as the calcium-induced decrease in light scattering compared to that by the ionophore alamethicin (10 μg/mL; Hansson et al., 2004a). Due to some variability between mitochondrial preparations, the Ca^{2+} concentrations administered to the mitochondrial suspensions ranged from 80 to 150 μM, to reach a comparable inter-experimental control response. The same concentration was used for all experiments of a particular preparation. Mitochondrial calcium uptake and release were monitored by the excitation ratio (ex. 340/380 nm, em. 509 nm) of the extramitochondrial calcium-sensitive fluorescent probe Fura 6F (250 nM). The brain mitochondrial suspensions were infused with 77–200 nmol $CaCl_2$/(mg×min). In one set of experiments (Fig. 1B), the infusion speed was initially at the lowest range, but then doubled following infusion of 2.5 μmol/mg $CaCl_2$ in order to reduce

Table 1. Patient Demographics, Indications for Surgery, and Origin of Tissue Samples

Brain (B) or liver (L) sample	*Gender*	*Age (y)*	*Indication for surgery*	*Origin of tissue*
B1	Male	45	Arteriovenous malformation	Right temporal cortex
B2	Male	72	Intracerebral hemorrhage	Caudate putamen
B3	Male	42	Subarachnoid hemorrhage	Right frontal cortex
B4	Male	81	Intracerebral hemorrhage	Right frontal cortex
B5	Male	62	Glioma	Right temporal cortex
L1	Male	58	Metastatic colon cancer	Quadrate lobe
L2	Male	60	Metastatic colon cancer	Quadrate lobe
L3	Male	65	Metastatic colon cancer	Left lateral segment
L4	Male	57	Metastatic colon cancer	Left lateral segment
L5	Male	56	Bile duct cancer	Quadrate lobe
L6	Female	80	Metastatic liver carcinoma	Quadrate lobe
L7	Female	74	Metastatic liver carcinoma	Quadrate lobe

experimental time. Liver mitochondria were infused with 50 nmol $CaCl_2$/(mg×min). CRC was calculated as the amount of infused calcium from the start of mitochondrial calcium uptake, until start of maximal calcium release. Rhodamine 123 (100 nM) was used to assess mitochondrial membrane potential, with excitation and emission set to 490 nm and 528 nm, respectively.

Mitochondrial respiration

Respiratory activities of mitochondrial preparations were measured by determining oxygen consumption in airtight chambers at 30°C, using Clark-type oxygen electrodes (Hansatech, Norfolk, U.K.). First, 100 μg brain or 200 μg liver mitochondria were suspended in 400 μL respiration medium consisting of 5 mM malate, 5 mM glutamate, 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/L BSA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM Pi (K), and 20 mM K-HEPES (pH 7.1; Kuznetsov et al., 2004). Respiratory control ratios (RCR) were calculated as the ratio of oxygen consumption during active phosphorylation in the presence of ADP (state 3) to the resting rate after ADP was consumed (state 4), or after addition of 1 μg/mL oligomycin (state 4_{oligo}).

Electron microscopy

Mitochondrial samples were immersed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 2 h, washed in phosphate buffer for 20 min, immersed for 2 h in 1% osmium tetroxide in phosphate buffer, and then dehydrated with graded alcohol and embedded with embedding medium. Sections 50 nm in size were prepared in Reichert-Jung Ultracut-E and stained with 4% uranylacetate, followed by 0.5% lead citrate. Electron micrographs were obtained using a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan).

Cytochrome c release

Liver mitochondrial samples were prepared similarly to the light scattering experiments, and were exposed to 200 μM Ca^{2+} with or without mPT inhibitors. An ELISA kit for detection of human cytochrome c (Cyt c; Quantikine®; R&D Systems, Inc., Minneapolis, MN) was employed to measure Cyt c release as described previously (Hansson et al., 2008).

Immunoblotting

Samples of isolated mitochondria were boiled with 2×SDS sample buffer for 10 min. Total protein (12–24 μg/sample) was separated on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA), transferred to PVDF membranes, and blocked overnight with 5% skim milk in phosphate-buffered saline. A laboratory-generated (F. Shibazaki), and a commercial (PA1-028; Pierce Thermo Fischer Scientific, Rochester, NY), primary rabbit polyclonal antibody against human CypD were used. Anti-tubulin antibody (Sigma-Aldrich, St. Louis, MO), and anti-ANT mouse monoclonal antibody (Calbiochem, San Diego, CA) were used for internal control. The primary antibodies were incubated at room temperature for 1 h. Anti-horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Pierce Thermo Fischer Scientific) were incubated at room temperature for 1 h, and then washed six times with phosphate-buffered saline containing 0.1% Tween 20, and detected on Western blots by the SuperSignal West Dura chemiluminescence detection system (Pierce Thermo Fischer Scientific).

Statistical analysis

All liver mitochondrial experiments were replicated in at least 3–4 separate mitochondrial preparations. Data are presented as means ± standard error of the mean (SEM), and were generally evaluated with analysis of variance (ANOVA), followed by Dunnett's *post-hoc* test. Paired comparisons were performed for CRC experiments, as all evaluations of treatment effects were performed using mitochondria from the same individuals. The effect of CypD inhibition on brain mitochondrial CRC was evaluated in four, and reversible swelling in three, separate preparations. The level of statistical significance was set at 5%. Other swelling experiments, respiration, and CRC experiments for phenylarsine oxide were only replicated in two separate brain mitochondrial preparations due to limited size of the samples, and no statistical analyses were performed.

Results

Mitochondrial permeability transition in human brain and liver mitochondria is modulated by cyclophilin D and oxidative stress

The isolation procedure resulted in a yield of 0.13–0.97 mg brain mitochondria and 2.4–21 mg liver mitochondria from the different tissue samples (0.13–0.95 g brain tissue and 0.4–3.9 g liver tissue). Due to the restricted availability and variation in the amount of utilizable brain tissue obtained, we were unable to do full sets of experiments on the brain mitochondrial preparations. To investigate the presence of mPT in adult human mitochondria, suspensions of brain and liver mitochondria were exposed to continuous infusions of calcium, and CRC was determined. CRC is a quantitative mPT assay that measures the amount of calcium mitochondria can retain before induction of mPT causes release of the sequestered calcium (Chalmers and Nicholls, 2003; Hansson et al., 2010). In a physiological medium containing adenine nucleotides and P_i, the CypD inhibitor CsA significantly increased CRC in both brain (2.34 ± 0.23 and 2.99 ± 0.34 μmol Ca^{2+}/mg mitochondria, for control and 1 μM CsA, respectively), and liver mitochondria (1.02 ± 0.27 and 1.51 ± 0.20 μmol Ca^{2+}/mg mitochondria, for control and 1 μM CsA, respectively; Fig. 1A–D). Immunoblots confirmed the presence of CypD in the isolated mitochondrial preparations (Fig. 1E). Oxidative stress is considered to sensitize mitochondria to mPT activation through oxidation of critical thiol groups on the mPT pore components (Halestrap et al., 1997; Petronilli et al., 1994). The vicinal thiol reagent phenylarsine oxide (PhAs; 1 μM) demonstrated a clear tendency toward reduced CRC in brain mitochondria (Fig. 1C, $n = 2$), and a significant reduction of CRC in liver mitochondria (0.39 ± 0.16 μmol Ca^{2+}/mg; Fig. 1D, $n = 3$). The oxidant *tert*-butyl hydroperoxide (tBOOH; 500 μM) also significantly reduced CRC in liver mitochondria (0.79 ± 0.22 μmol Ca^{2+}/mg; Fig. 1D).

Loss of respiratory coupling following calcium-induced permeability transition

In order to assess the functional integrity of isolated mitochondrial preparations, respiration experiments were performed. Both brain and liver mitochondria demonstrated

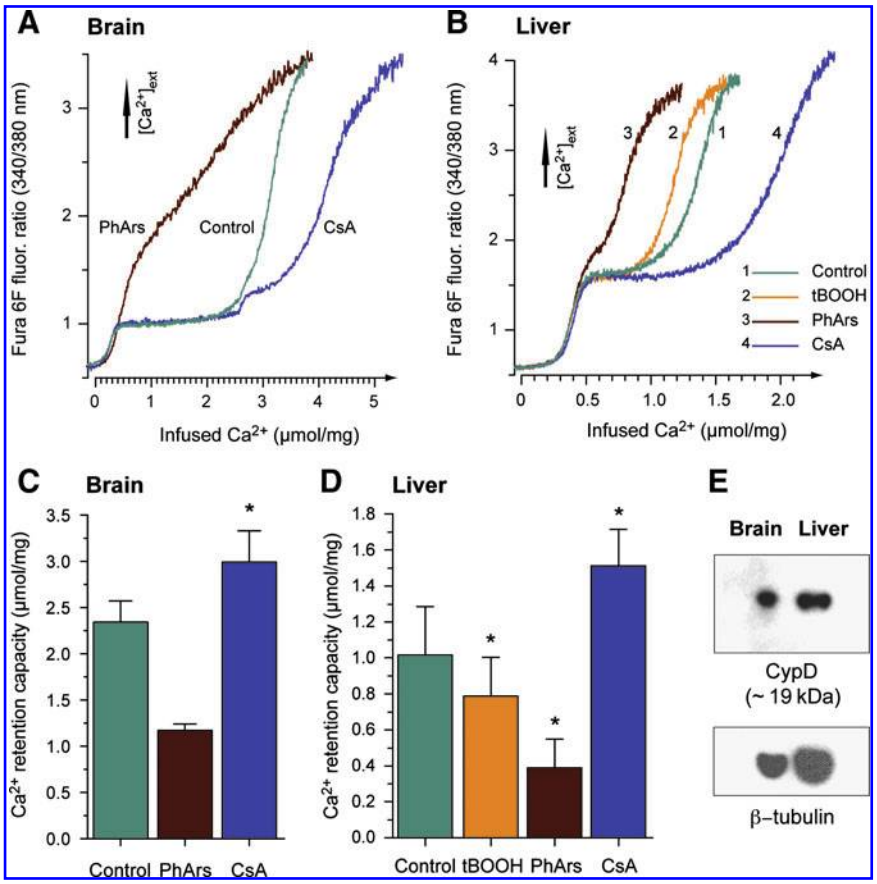


FIG. 1. Modulation of calcium retention capacity in human brain and liver mitochondria by cyclophilin D (CypD) inhibition and oxidants. (**A** and **B**) Representative traces of human brain and liver mitochondrial calcium handling during continuous calcium infusion. Changes in fura 6F fluorescence intensity correspond to the level of extramitochondrial [Ca^{2+}]. Experiments were performed with or without presence of the CypD-inhibitor cyclosporin A (CsA, 1 μM), the vicinal thiol reagent phenylarsine oxide (PhArs, 1 μM), and *tert*-butyl hydroperoxide (tBOOH, 500 μM, tested in liver mitochondria only). (**C** and **D**) Calculated mitochondrial calcium retention capacity. Values are means $\pm$ standard error of the mean (SEM; * in **C** indicates $p < 0.05$ by paired *t*-test, $n = 4$ for control and CsA; PhArs was only tested twice in brain mitochondria and no statistical evaluation was performed; * in **D** indicates $p < 0.05$ by repeated-measures ANOVA with Dunnett's *post-hoc* test, $n = 3$). (**E**) Western blots of CypD and β-tubulin in isolated human brain and liver mitochondria (12 μg/lane and 22 μg/lane were used for brain and liver mitochondria, respectively).

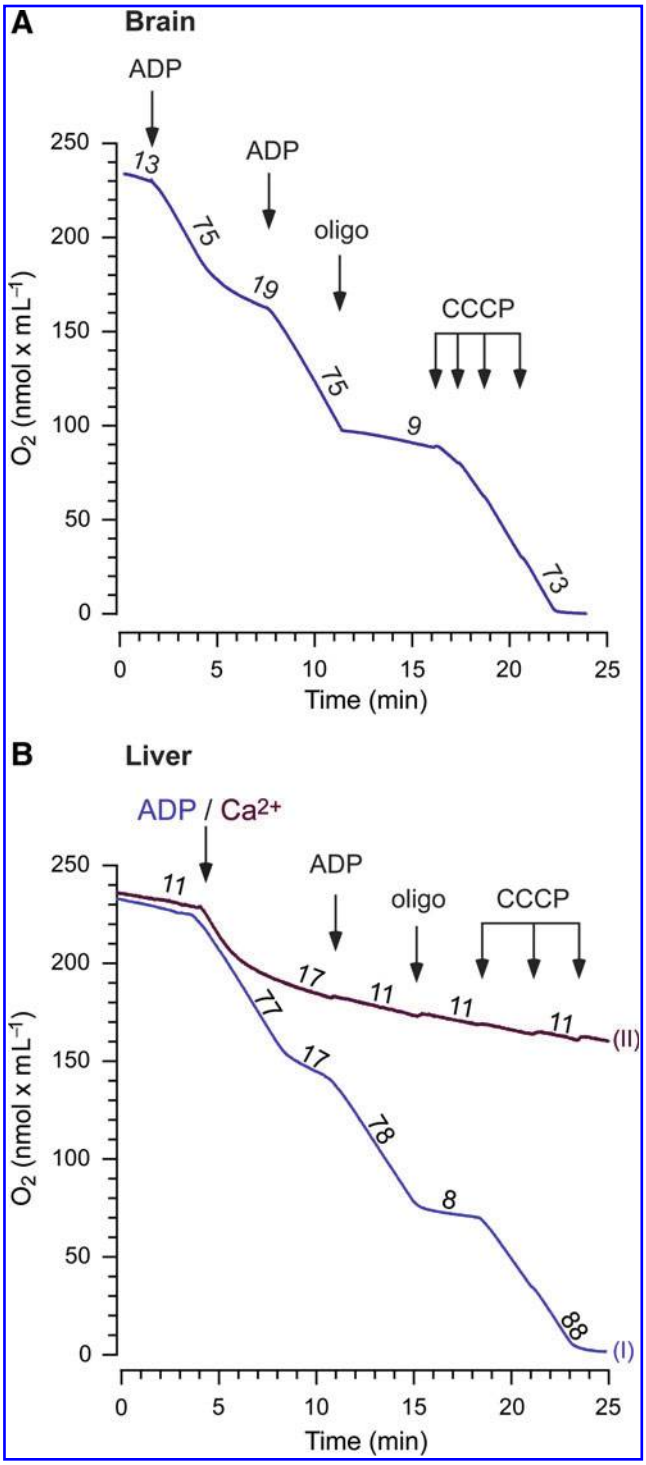
well-coupled respiration (Fig. 2). Mean respiratory control ratios (RCR) of liver mitochondria were 5.28 ± 0.59 and 9.92 ± 1.2 for state 3/state 4 ($n = 5$), and state 3/state 4_{oligo} ($n = 4$), respectively. Brain mitochondria demonstrated an RCR of 3.9 in one complete experiment (Fig. 2A), and 12.6 in a second experiment with a lower concentration of mitochondria (which tended to overestimate the ratio; data not shown). Following exposure to Ca^{2+}, liver mitochondria demonstrated respiratory inhibition and loss of respiratory coupling, as evidenced by a lack of stimulatory responses upon addition of ADP or the protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone) (Fig. 2B).

Inhibition of calcium-induced swelling, membrane potential loss, and Cyt c release by cyclosporin analogs and adenine nucleotides in human liver mitochondria

To further evaluate the presence and characteristics of mPT in human mitochondria, mitochondrial morphology, membrane potential, and Cyt c release were examined following calcium exposure. Without cyclosporin analogs present, calcium induced an extensive degree of mitochondrial swelling, $69.0 \pm 6.3\%$ compared to that of the non-specific ionophore alamethicin, and mitochondrial membrane potential was essentially lost (Fig. 3A–C). Electron micrographs confirmed a dramatic change in mitochondrial morphology following calcium exposure, including matrix swelling and disruption of the outer mitochondrial membrane (Fig. 3D). The CypD inhibitor CsA, and the non-immunosuppressive cyclosporin analog D-MeAla^3 EtVal^4-cyclosporin (NI-Cs), virtually abolished the calcium-induced swelling ($3.67 \pm 7.1\%$ and $5.94 \pm 12.1\%$, respectively; Fig. 3A and C), and largely prevented the loss of membrane potential (Fig. 3B). Electron micrographs of mitochondria exposed to calcium in the presence of CsA did not display any overt morphological alterations (Fig. 3D). Calcium exposure also induced an extensive Cyt c release ($37.1 \pm 3.1\%$ and $8.43 \pm 3.8\%$ of total Cyt c content for mitochondria with and without 200 μM Ca^{2+}, respectively;

$p < 0.05$), which was significantly inhibited by ADP and CsA ($14.2 \pm 1.2\%$ of total Cyt c content; Fig. 3E).

In de-energized mitochondria calcium-induced mPT is independent of respiration-driven electrophoretic Ca^{2+} uptake, as the anion equilibrates over the mitochondrial inner membrane through a calcium ionophore, and more direct pharmacological interactions with the components of the mPT pore complex can be studied. The CypD inhibitors CsA and NI-Cs (both at 1 μM), as well as the adenine nucleotides ADP and ATP (both at 100 μM), almost completely prevented the swelling induced by 200 μM Ca^{2+} in de-energized liver mitochondria (Fig. 4A and B).



Reversible calcium-induced swelling and inhibition of swelling by mPT modulators in human brain mitochondria

To explore the characteristics of mPT-mediated swelling in human brain mitochondria, the samples were exposed to either transient or long-term calcium exposure. In one set of experiments, 200 μM Ca^{2+} was administered to mitochondria, EGTA was added after 1 min to chelate the calcium, and the initial light-scattering decrease by calcium was significantly reversed, from $33.9 \pm 5.2\%$ to $15.9 \pm 4.1\%$ (of alamethicin-induced swelling, $n = 3$; Fig. 5A). The mitochondria also underwent a pronounced swelling response following a second calcium addition. In another set of experiments human brain mitochondria were exposed to calcium with or without presence of 1 μM CsA and 100 μM ADP. The mPT modulators delayed and partially prevented the swelling response following an addition of a total of 200 μM Ca^{2+} ($n = 2$, no statistical analysis was performed; Fig. 5B).

Discussion

Mitochondrial dysfunction and activation of mPT are thoroughly implicated in several disorders of the CNS and other organs. Here we demonstrate that both human brain and liver mitochondria exhibit several classical characteristics of the mPT phenomenon following calcium overload. A number of previous conclusions on mPT drawn from animal studies are thus validated in adult human brain and liver mitochondria.

The molecular basis of mPT has been a matter of debate for a couple of decades and has yet to be fully resolved. Seminal studies by Hunter and Haworth established that mPT was induced by Ca^{2+} and inhibited by ADP, NADH, and Mg^{2+}, and they suggested that mPT may be caused by the regulated opening of a non-specific channel (Haworth and Hunter, 1979; Hunter and Haworth, 1979a). Several lines of evidence pointed toward adenine nucleotide translocator (ANT) as the inner mitochondrial membrane protein mediating mPT

FIG. 2. Coupled respiration in isolated human brain and liver mitochondria with respiratory inhibition following calcium-induced permeability transition. (**A** and **B**) Respiratory control was evaluated by measuring oxygen consumption of mitochondria oxidizing 5 mM malate and glutamate during and after ADP phosphorylation (250 μM ADP). A second addition of ADP (1 mM) was followed by administration of the ATP synthase inhibitor oligomycin (oligo, 1 μg/mL), and titration of the protonophore CCCP, 0.5 μM per addition. In liver mitochondria, the first ADP addition (trace I) was replaced by 1 mM $CaCl_2$ (trace II) to induce mPT. Calcium induced an initial stimulation of respiration, followed by respiratory inhibition and abolished respiratory control. The numbers indicate rates of respiration [nmol O_2/(min×mg mitochondria)] from one experiment in brain mitochondria, and means from 4–5 experiments in liver mitochondria (CCCP, carbonyl cyanide m-chlorophenylhydrazone; ADP, adenosine diphosphate; ATP, adenosine triphosphate; $CaCl_2$, calcium chloride; mPT, mitochondrial permeability transition). Color image is available online at www.liebertpub.com/neu.

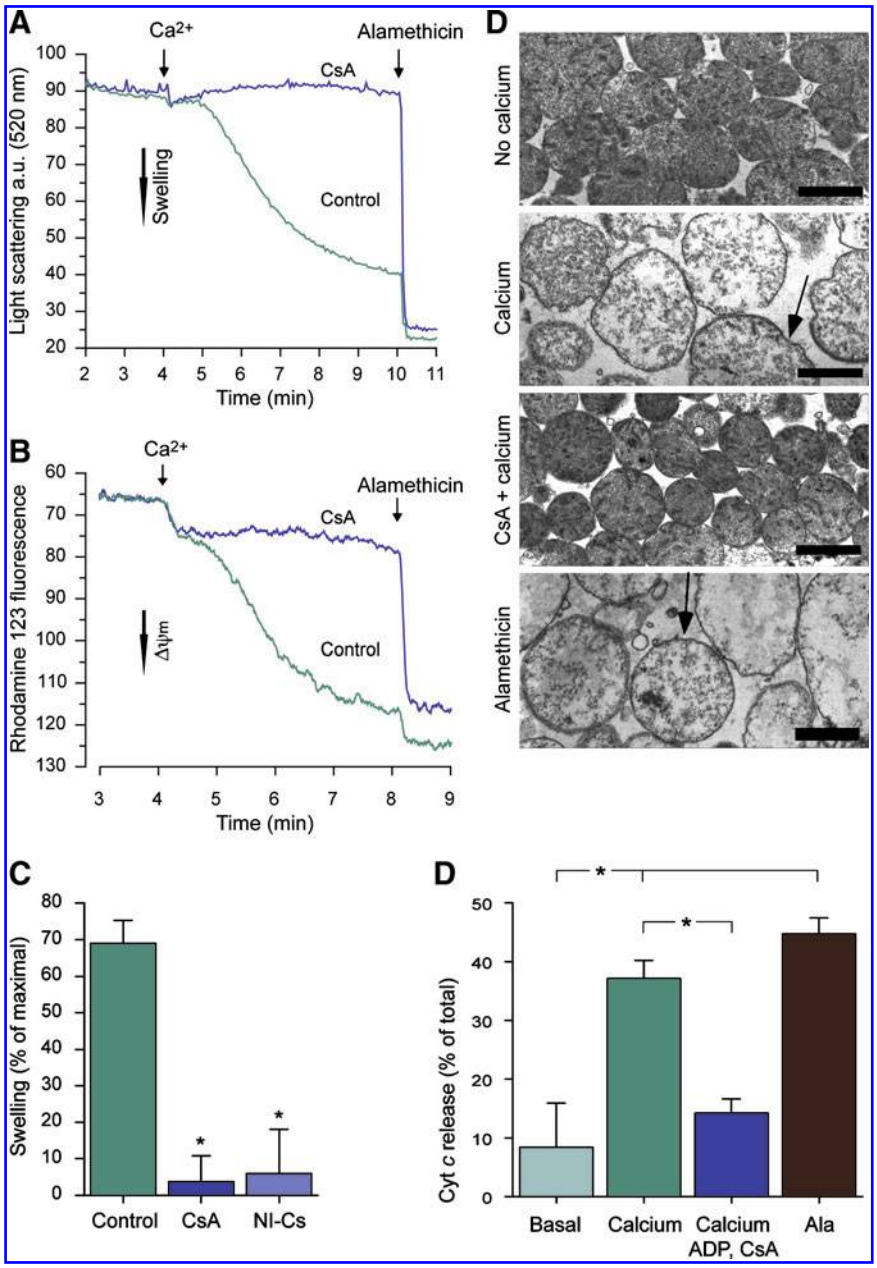


FIG. 3. Cyclophilin D (CypD)-sensitive swelling, membrane potential dissipation, and cytochrome c release in human liver mitochondria. Respiring human liver mitochondria were exposed to 100 μM Ca^{2+} with or without the presence of CypD inhibitors, and were monitored by following changes in (**A**) light scattering, or (**B**) rhodamine 123 fluorescence. Representative traces of control runs and experiments using the CypD inhibitor cyclosporin A (CsA; 1 μM) are shown. (**C**) The degree of calcium-induced mitochondrial swelling compared to that induced by the ionophore alamethicin (expressed as a percentage of maximal) was calculated from light-scattering traces for control, CsA, and for the non-immunosuppressive cyclosporin analog D-MeAla^{3}EtVal^4-cyclosporin (NI-Cs; 1 μM), using 80–150 μM Ca^{2+} to induce permeability transition (*$p < 0.05$ compared to control by analysis of variance [ANOVA] with Dunnett's *post-hoc* test, $n = 3$–4). (**D**) Electron micrographs prepared from light-scattering experiments demonstrating gross morphological swelling and disruption of the outer mitochondrial membrane induced by Ca^{2+} (arrows). A similar appearance is seen following alamethicin exposure. In presence of CsA, no apparent morphological change was induced by Ca^{2+} (scale bars = 1 μm). (**E**) Cytochrome c (Cyt c) release from mitochondria incubated in medium only (control), exposed to 200 μM Ca^{2+} with or without the mPT inhibitors ADP (200 μM) and CsA (1 μM), and following alamethicin permeabilization (*$p < 0.05$ between groups by ANOVA with Bonferroni's *post-hoc* test, $n = 4$; ADP, adenosine diphosphate; mPT, mitochondrial permeability transition; a.u., arbitrary units). Color image is available online at www.liebertpub.com/neu.

(Bauer et al., 1999; Brustovetsky and Klingenberg, 1996; Halestrap and Davidson, 1990; Hunter and Haworth, 1979a). The immunosuppressant CsA was later found to inhibit mPT activation (Crompton et al., 1988), and the effect was attributed to inhibition of the matrix peptidylprolyl cis-trans isomerase CypD, and its interaction with the inner membrane component of mPT (Halestrap and Davidson, 1990). Genetic knockout studies have confirmed an important regulatory

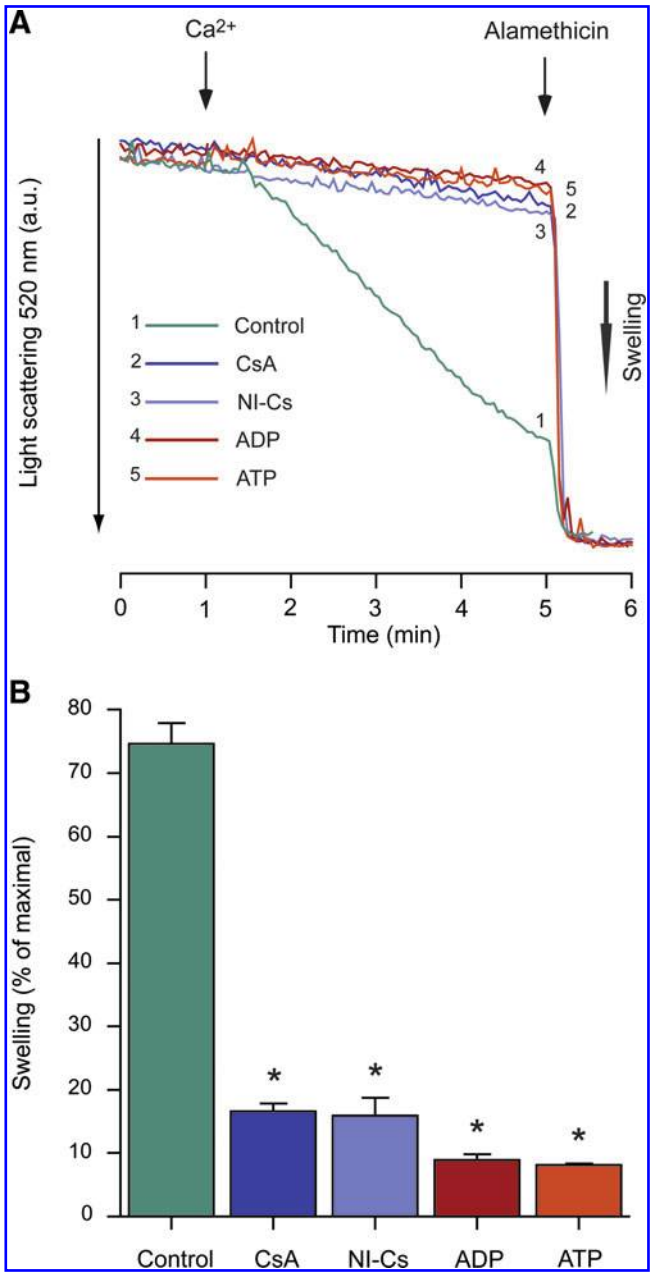


FIG. 4. Inhibition of permeability transition by cyclosporin analogs and adenine nucleotides in human liver mitochondria. (**A**) Representative traces of light-scattering changes of human liver mitochondria exposed to 200 μM Ca^{2+} under de-energized conditions, in presence of the cyclophilin D (CypD) inhibitors cyclosporin A (CsA; 1 μM), or D-MeAla^{3}EtVal^4-cyclosporin (NI-Cs; 1 μM), or the endogenous mPT modulators ADP or ATP (both at 100 μM). (**B**) Calculations of swelling relative to that induced by alamethicin (percentage of maximal; $^*p < 0.05$ compared to control by analysis of variance with Dunnett's *post-hoc* test, $n = 3$; ADP, adenosine diphosphate; ATP, adenosine triphosphate; mPT, mitochondrial permeability transition; a.u., arbitrary units).

role of CypD and ANT, but have questioned that ANT is essential for mPT pore formation (Baines et al., 2005; Basso et al., 2005; Kokoszka et al., 2004; Nakagawa et al., 2005; Schinzel et al., 2005). Recent evidence has also suggested a role of the phosphate carrier in mPT pore formation and regulation (Alcala et al., 2008; Leung et al., 2008), but this awaits the close evaluation that has been performed for ANT and CypD. The present results demonstrate that both human brain and liver mitochondria exhibit several defining characteristics of the mPT phenomenon, such as calcium-induced swelling and calcium-induced calcium release. Human liver mitochondria also demonstrated calcium-induced loss of membrane potential and respiratory coupling, as well as release of Cyt c. These processes were repressed by CypD inhibitors and adenine nucleotides, supporting important roles of CypD and ANT in mPT activation in human mitochondria. Further, oxidative stress is considered to sensitize rodent mitochondria to mPT activation through oxidation of critical thiol groups on the mPT pore components (Halestrap et al., 1997; Petronilli et al., 1994), and both PhArs and tBOOH sensitized human liver mitochondria to mPT.

Corresponding to the intricate regulation of mPT, several classes of drugs have been shown or suggested to inhibit mPT (Kroemer et al., 2007; Zoratti and Szabo, 1995). One difficulty when attributing pharmacological effects to mPT inhibition is that drug effects are often non-specific, both at the mitochondrial level and *in vivo* (Morota et al., 2009). Another obstacle is that a seemingly beneficial effect on certain mPT characteristics may be caused by inhibition of otherwise vital mitochondrial functions, and the net outcome on mitochondrial integrity may be negative (Mansson et al., 2010). The most common evidence for mPT as a mediator of cell death, and thus its potential as a pharmacological target, derives from studies using CypD inhibitors such as CsA. Even though the target of CsA in mitochondria is specific, in contrast to several other proposed mPT inhibitors (Mansson et al., 2010; Morota et al., 2009), the effect *in vivo* is not. In order to determine the possible contribution of calcineurin inhibition to the effect of CsA, non-immunosuppressive cyclosporin analogs have been used, but they also inhibit other cyclophilins throughout the cell (Hansson et al., 2004b; Matsumoto et al., 1999; Mbye et al., 2009). A second obstacle when evaluating this class of drugs for neuroprotection is their limited penetration across the blood–brain barrier (BBB; Tsuji et al., 1993), unless measures are taken to facilitate CNS entry. Nevertheless, CsA and its analogs have been among the most convincing and broadly effective group of drugs displaying neuroprotective properties in several diverse models of acute and chronic neurological disease. Pharmacological studies using CypD inhibitors in animal models have in particular implicated mPT in the pathogenesis of focal and global ischemia (Domanska-Janik et al., 2004; Matsumoto et al., 1999; Uchino et al., 1998; Yoshimoto and Siesjö, 1999), hypoglycemic brain damage (Friberg et al., 1998), TBI (Buki et al., 1999; Mbye et al., 2009; Sullivan et al., 2000), and ALS (Karlsson et al., 2004; Keep et al., 2001; Kirkinezos et al., 2004).

More specific evidence for a pathogenic role of mPT and CypD in neurological disorders has been obtained through genetic knockout studies. Animals lacking CypD have displayed increased resistance to cerebral ischemia, supporting the pharmacological studies using CypD inhibitors in different cerebral ischemia models (Schinzel et al., 2005). CypD deletion has also been found to be beneficial in animal models of multiple sclerosis and Alzheimer's disease (Du et al., 2008; Forte et al., 2007).

In contrast to the conclusions of pharmacological studies using CypD inhibitors in animal models of neurological

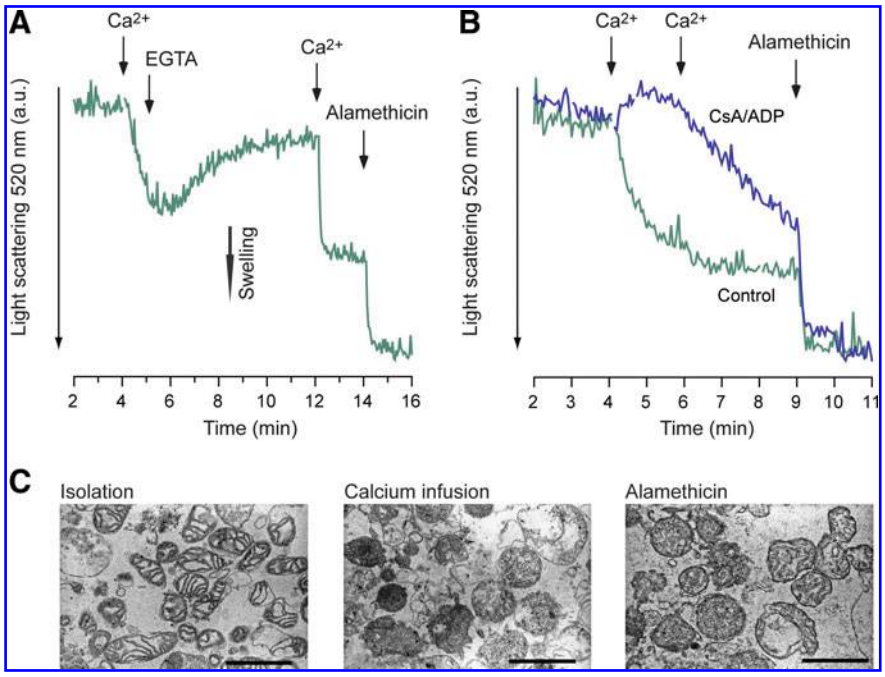


FIG. 5. Reversible calcium-induced mPT and inhibition of swelling by mPT inhibitors in human brain mitochondria. (**A**) Representative traces ($n = 3$) of reversible swelling in human brain mitochondria induced by 200 μM Ca^{2+}, followed by 400 μM of the Ca^{2+} chelator EGTA. Swelling was induced a second time by Ca^{2+} (1 mM), and mitochondria were then permeabilized by the non-specific ionophore alamethicin. (**B**) Representative traces ($n = 2$) of Ca^{2+}-induced swelling with or without the presence of the endogenous mPT inhibitor ADP (100 μM), and the cyclophilin D (CypD) inhibitor cyclosporin A (CsA; 1 μM). Mitochondria were challenged with two additions of 100 μM Ca^{2+}, followed by exposure to alamethicin. (**C**) Electron micrographs of brain mitochondria following isolation, calcium infusion, and alamethicin exposure [scale bars = 1 μm; ADP, adenosine diphosphate; mPT, mitochondrial permeability transition; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; a.u., arbitrary units]. Color image is available online at www.liebertpub.com/neu.

disorders, several studies using isolated mitochondria have questioned a prominent role of mPT in the CNS. Brain mitochondria have been argued to be insensitive to mPT and swelling (Berman et al., 2000), or to be relatively resistant to mPT induction (Andreyev and Fiskum, 1999), and CsA has been suggested to be a less potent inhibitor of mPT in brain mitochondria compared to mitochondria from other organs (Brustovetsky and Dubinsky, 2000; Kristal and Dubinsky, 1997). Other studies have provided evidence and argued for a qualitatively and pharmacologically similar mPT phenomenon in rodent brain mitochondria to that in the more commonly studied heart and liver mitochondria (Chalmers and Nicholls, 2003; Hansson et al., 2004a). Further, it has been suggested that CypD is downregulated in the mature rodent brain (Eliseev et al., 2007). If CypD is downregulated in the adult human brain, there would be no rationale for using CypD inhibitors in patients with neurological disorders. Here we confirm the presence of CypD in human adult brain and liver mitochondria. Further, the CypD inhibitor CsA was found to have a significant effect on mitochondrial CRC, a quantitative assay for mPT, in adult human brain as well as liver mitochondria.

Induction of mPT is transient when inducing factors are removed *in vitro* (Hunter and Haworth, 1979b). Reversible mPT-mediated swelling has previously been demonstrated in rodent brain mitochondria (Hansson et al., 2004a), and reversible mPT-dependent remodeling of mitochondria has also been described in cultured hippocampal neurons in models of excitotoxicity (Shalbuyeva et al., 2006). Using *in vivo* imaging with two-photon microscopy, loss of mitochondrial membrane potential has been demonstrated to occur within 1–3 min of global cerebral ischemia. The mitochondrial dysfunction was recovered rapidly upon reperfusion, and was blocked by CsA, indicating that mPT activation is an early reversible event that could trigger delayed cell death (Liu and Murphy, 2009). Previous studies support this conclusion, as mitochondria have been demonstrated to accumulate calcium shortly after ischemia (Zaidan and Sims, 1994), and to undergo transient swelling following ischemia (Petito and Pulsinelli, 1984). Moreover, early CsA administration following reperfusion prevents early Cyt c release, and dramatically reduces delayed neuronal cell death in animals subjected to global ischemia (Domanska-Janik et al., 2004; Uchino et al., 1998). As demonstrated here, mPT activation and swelling are reversible events in human brain mitochondria, supporting the conclusion drawn from animal studies of reversible mPT in the pathogenesis of delayed neuronal death in cerebral ischemia.

As stated above, there is extensive documentation of a neuroprotective effect of CsA in different animal models of TBI. The shear forces will cause an immediate, but also a delayed, cellular injury and disruption of the BBB (Buki and Povlishock, 2006). In contrast to other neurological indications, mPT inhibition can be achieved in brain parenchyma via systemic CsA administration, due to the disruption of the BBB. Promising animal data have influenced two independent groups to initiate NIH-sponsored human clinical trials of

CsA administration to patients with severe TBI in the United States. The initial studies in patients with TBI show that CsA is well tolerated, enters the CNS, and demonstrates a dose-related improvement in favorable outcome (Hatton et al., 2008; Mazzeo et al., 2008, 2006). Although mPT inhibition may not be the only target for CsA, these clinical trials in TBI, as well as the promising effect of CsA against myocardial reperfusion injury (Mewton et al., 2010; Piot et al., 2008), are the first human studies testing the hypothesis of mPT-mediated injury in human disease.

The major challenge of the present study was to obtain viable brain tissue and viable mitochondria for functional analyses. Therefore, it was not feasible to address all aspects of mPT in brain mitochondria. Further, brain tissue samples were derived from patients with neurological conditions requiring neurosurgery (Table 1), and it is unknown how these acute conditions may affect the evaluation of mPT and expression of CypD. In summary, we have provided evidence that human brain and liver mitochondria possess a CypD-sensitive permeability transition. Taking the limitations mentioned above into account, the findings presented here support the rationale of CypD inhibition as a pharmacological target in patients with acute neurological damage and chronic neurodegenerative disease.

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Author Disclosure Statement

E.E. is co-founder and officer, and M.J.H. is a stockholder of Maas Biolab, LLC, and NeuroVive Pharmaceutical AB (publ), which hold intellectual property rights and develop the use of cyclosporins as cyclophilin D inhibitors for neurological treatment. The other authors have nothing to disclose.

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