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Inhibition of complex I regulates the mitochondrial permeability transition through a phosphate-sensitive inhibitory site masked by cyclophilin D

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ABSTRACT

Inhibition of the mitochondrial permeability transition pore (PTP) has proved to be an effective strategy for preventing oxidative stress-induced cell death, and the pore represents a viable cellular target for drugs. Here, we report that inhibition of complex I by rotenone is more effective at PTP inhibition than cyclosporin A in tissues that express low levels of the cyclosporin A mitochondrial target, cyclophilin D; and, conversely, that tissues in which rotenone does not affect the PTP are characterized by high levels of expression of cyclophilin D and sensitivity to cyclosporin A. Consistent with a regulatory role of complex I in the PTP-inhibiting effects of rotenone, the concentrations of the latter required for PTP inhibition precisely match those required to inhibit respiration; and a similar effect is seen with the antidiabetic drug metformin, which partially inhibits complex I. Remarkably (i) genetic ablation of cyclophilin D or its displacement with cyclosporin A restored PTP inhibition by rotenone in tissues that are otherwise resistant to its effects; and (ii) rotenone did not inhibit the PTP unless phosphate was present, in striking analogy with the phosphate requirement for the inhibitory effects of cyclosporin A [Basso et al. (2008) *J. Biol. Chem.* 283, 26307–26311]. These results indicate that inhibition of complex I by rotenone or metformin and displacement of cyclophilin D by cyclosporin A affect the PTP through a common mechanism; and that cells can modulate their PTP response to complex I inhibition by modifying the expression of cyclophilin D, a finding that has major implications for pore modulation in vivo.

1. Introduction

The mitochondrial permeability transition (PT) is a sudden increase in the permeability of the inner mitochondrial membrane, which becomes non-selectively permeable to molecules smaller than 1500 Da [1]. It is believed that PT occurs due to the opening of an inner membrane channel regulated by other mitochondrial proteins, which is referred to as the permeability transition pore (PTP). Despite numerous efforts, the exact molecular nature of the PTP remains elusive. The matrix protein cyclophilin D (CypD) is the best defined regulatory component of PT [2–5]. Although the molecular target of CypD on the PTP has not yet been found, occurrence of the PT is easier when CypD binds to the PTP [3]. The classic PT-inhibitor cyclosporin A (CsA) appears to indirectly inhibit PTP opening by detaching CypD from the pore [6,7]. It has recently been shown that CypD ablation, or detachment of CypD from the PTP by CsA, does not prevent PT unless phosphate is present, revealing that CypD masks an inhibitory site for phosphate [8].

Prolonged PT leads to cell death via the release of mitochondrial pro-apoptotic mediators into the cytosol [9], while inhibition of PT prevents oxidative stress-induced cell death [2,4,5], including ischemia-reperfusion injury in humans [10]. Such a therapeutic potential explains why PTP regulation has extensively been studied over the past decades. However, these studies have mainly been performed in rat liver mitochondria [1], with the implicit assumption that PTP regulation is identical in every tissue.

We recently showed that the inhibition of respiratory chain complex I by rotenone, piericidine or metformin [11–13] inhibited PT in U937, KB, HMEC and INS-1 cells [11,13–15], whereas this type of regulation was not observed in rat liver mitochondria [1]. We therefore started an

Abbreviations: ANT, adenine nucleotide translocator; COX, cytochrome oxidase; CRC, Ca^{2+} retention capacity; CsA, cyclosporin A; CypD, cyclophilin D; PT, permeability transition; PTP, permeability transition pore

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extensive study to establish the mechanistic basis for the inhibition of the PT by rotenone. Here we report that PT inhibition by rotenone and metformin is mediated by the same Pi-dependent site unmasked by CsA.

2. Material and methods

2.1. Animals and cell lines

Ppif$^{-/-}$ (CypD null) mice were a generous gift from the laboratory of the late Stanley Korsmeyer [5]. Liver and heart mitochondria were isolated from rat and mouse tissue as described previously [16,17]. Human fibroblasts were prepared from normal skin by trypsin treatment. Human lymphocytes were extracted from whole blood collected in the Grenoble medical centre. OV1 and CLTT cells were generous gifts from Dr J. Benard [18] and Dr Galiana [19], respectively. These cells were grown in a RPMI medium (Gibco) supplemented with 10% fetal calf serum (FCS) and antibiotics. Isolated insulinoma cell lines INS-1, a generous gift of Dr. F. De Fraipont (CHU-Grenoble), were maintained in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Mouse fibroblasts NIH/3T3 (CRL-1658™), human promyeloblasts HL-60 (CCL-240™), human epithelial KB (CCL-17™), human cervix carcinoma Hela (CCL-2™), human erythroleukemia K-562 (CCL-243™), human fibrosarcoma HT-1080 (CCL-121™), human hepatocellular carcinoma Hep G2 (HB-8065™), rat hepatoma MH1C1 (CCL-144™), and rat glioma C6 (CCL-107™) were obtained from ATCC and grown in the medium suggested by the supplier.

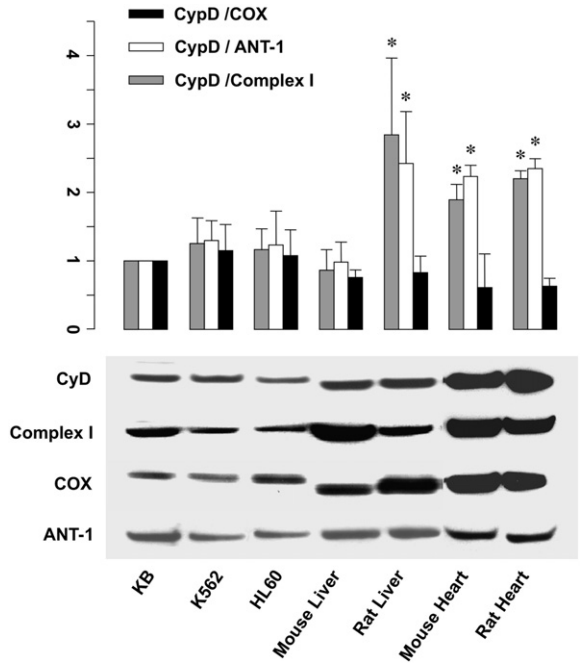

Fig. 2. CypD is more expressed in tissues in which rotenone does not inhibit PTP opening. Representative immunoblot for the quantification of CypD, complex I, cytochrome oxidase, and ANT-1 in the indicated cells. For an easier comparison between the different cell lines, the ratios were normalized (divided by the corresponding ratio observed in KB cells). Results are expressed as mean ± S.E. of five independent experiments. *, $p<0.05$ vs. CypD/COX. Student's *t* test.

2.2. siRNA

Freshly isolated rat hepatocytes were grown in a DMEM medium (Gibco) containing 4.5 g/l glucose supplemented with 25% M199 (Sigma), 10% FCS, 0.2 mg/ml BSA, 10 μg/ml insulin, 2 mM glutamine, and antibiotics (100 U/ml penicillin and streptomycin). After 24 h, the medium was supplemented with 1 μM hydrocortisone, 10 ng/ml EGF, and 1 mg/ml BSA. Rat hepatocytes (1 million cells) were

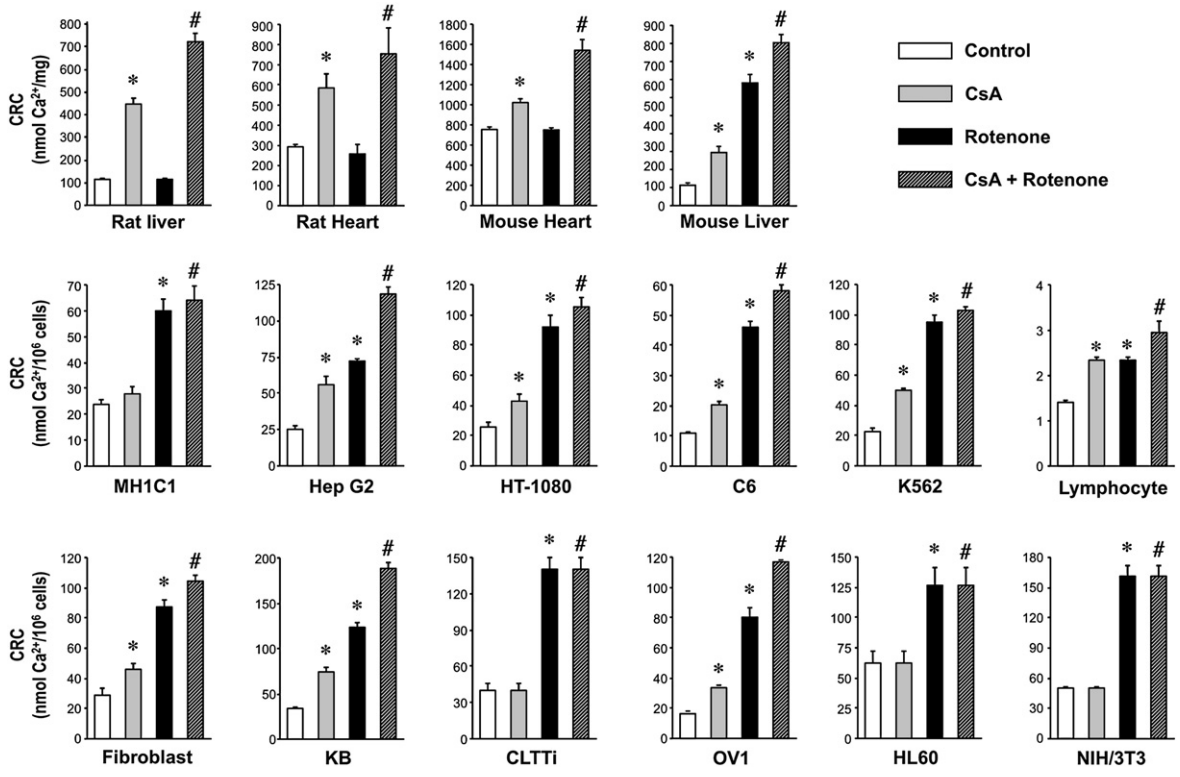

Fig. 1. Rotenone inhibits PTP opening in most of the tested tissues. CRC was measured in the presence of vehicle (control), 1 μM CsA, 1 μM rotenone or 1 μM CsA plus 1 μM rotenone in a medium supplemented with 5 mM succinate-Tris. Results are expressed as mean ± S.E. of at least three independent experiments. *, $p<0.05$ vs. control, #, $p<0.05$ vs. CsA. Student's *t* test.

transfected in a serum and antibiotics-free DMEM transfection medium by cationic liposomes, using Lipofectamine 2000 (Invitrogen). CypD-targeting siRNA oligonucleotides CAAGAGCCCUGAGUUAUGU (for oligo #1) GAGGACUGUUCAGAAAUCU (for oligo #2) and scrambled siRNAs were obtained from Eurogentec. Oligonucleotides (300 pmol) were added to the transfection medium and combined to lipofectamine, according to the manufacturer's protocol. After 20 min of incubation, complexes were added to a 100 mm culture dish containing 5 ml transfection medium. Hepatocytes were then added at a density of 1 million per dish and incubated at 37 °C for 4 h. After this period, the culture medium was supplemented with FCS and antibiotics for 20 h.

2.3. Calcium retention capacity measurement

Unless otherwise specified, liver mitochondria and permeabilized cells were incubated at a pH of 7.4, in a medium containing 250 mM sucrose, 1 mM Pi-Tris, and 10 mM Tris-MOPS, while heart mitochondria were incubated in a medium containing 150 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, and 20 mM Tris/HCl. When the concentration of phosphate was varied, vanadate was added to maintain the total phosphate plus vanadate concentration constant [8]. The incubation medium was supplemented with 5 mM succinate-Tris and 0.25 μM Calcium Green-5N. The final volume was 1 ml at 25 °C. Experiments began by the addition of either 2×10^6 cells followed by 40 μM digitonin, or of 0.5 mg of isolated mitochondria, followed by the addition of vehicle (control), 1 μM CsA, 1 μM rotenone or 1 μM CsA plus 1 μM rotenone. After 2 min of incubation, the Ca^{2+} retention capacity (CRC) was measured by adding sequential Ca^{2+} pulses until PTP opening [20]. Measurements of extramitochondrial Ca^{2+} were performed fluorimetrically (excitation: 506 nm; emission: 530 nm), using a spectrofluorometer equipped with magnetic stirring and thermostatic control [21].

2.4. Respiratory chain activity

Oxygen consumption was measured polarographically at 25 °C using a Clark-type electrode in the medium used for CRC measurement supplemented with 5 mM glutamate-Tris and 2.5 mM malate-Tris.

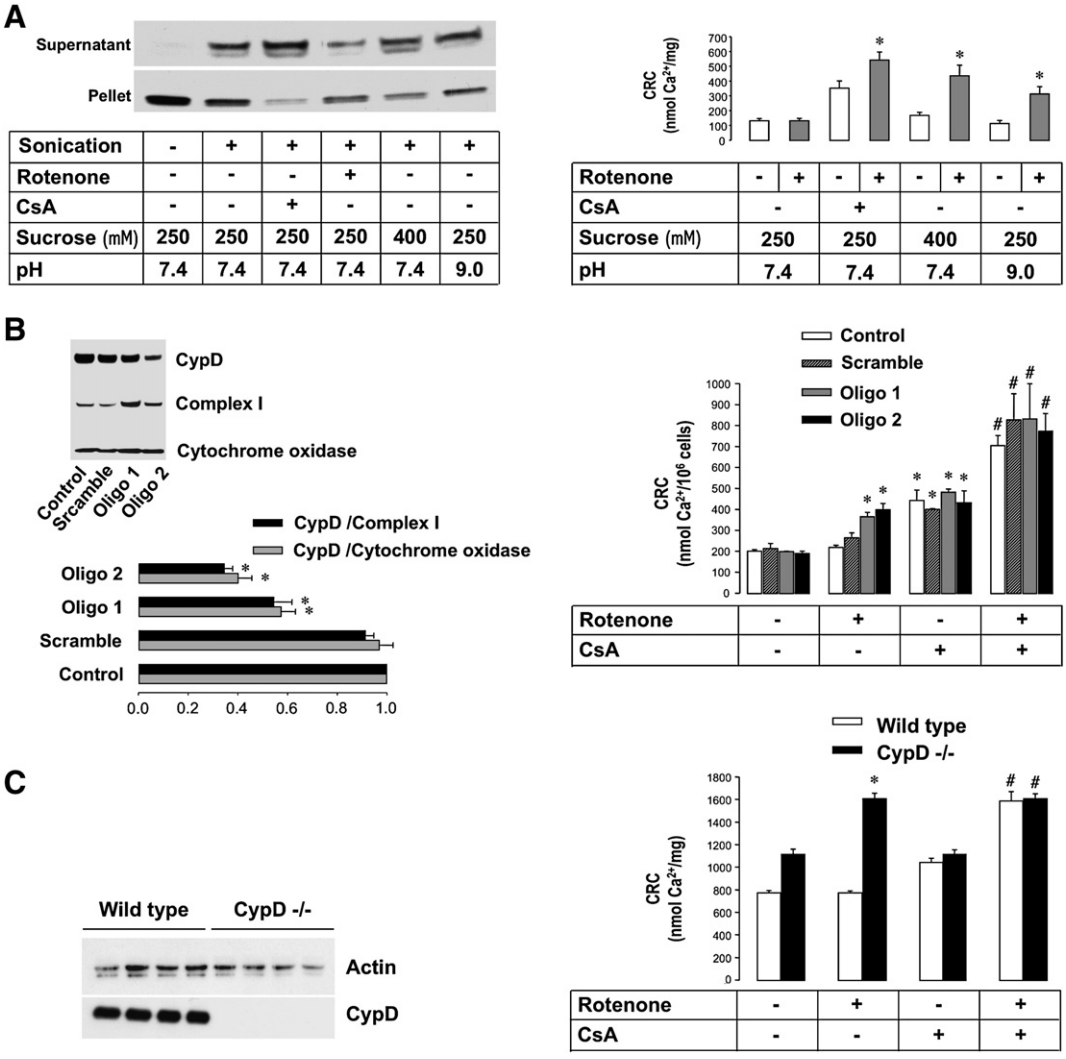

Fig. 3. Detachment of CypD or decrease in CypD content allows rotenone to inhibit PTP opening. A: The measurement of bound (pellet) and unbound (supernatant) CypD and the CRC was performed on rat liver mitochondria incubated in a medium containing the indicated concentration of sucrose in the presence or absence of 1 μM CsA. The pH was as indicated. *, $p<0.05$ vs. the proper control (*i.e.*, without rotenone). Student's *t* test. B: The quantification of CypD, complex I, and cytochrome oxidase was performed on rat hepatocytes transfected with CypD siRNA oligonucleotides. For an easier comparison, the ratios were divided by the proper ratio observed in control conditions (*i.e.*, lipofectamine without oligonucleotides). The CRC measurements were performed on digitonin-permeabilized rat hepatocytes. *, $p<0.05$ vs. control, left panel. Mann–Whitney. *, $p<0.05$ vs. control, right panel. Student's *t* test. C: The quantification of CypD and actin, and the CRC measurements were performed on heart mouse mitochondria isolated from wild type or *Ppif*$^{-/-}$ animals. In all the panels, results are expressed as mean ± S.E. of at least five independent experiments. *, $p<0.05$ vs. control, #, $p<0.05$ vs. CsA. Student's *t* test.

2.5. Protein quantification

Total proteins from cells or mitochondria lysates were separated using 10% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies against human CypD [rabbit polyclonal antibody (Affinity Bioreagent PA1028)], human complex I 39 kDa subunit (mouse monoclonal Molecular Probes A21344), human cytochrome oxidase subunit 4 (cox4) (mouse monoclonal Molecular Probes A21348), ANT-1 (mouse monoclonal Mitosciences MSA02), and actin (mouse monoclonal Sigma A3853). Detection was performed by enhanced chemiluminescence (GE-Healthcare), and densitometry was performed using NIH image software. For the quantification of bound and unbound CypD, mitochondria were broken (sonicated for 30 s twice) and centrifuged for 30 min at 8000 *g*. Supernatants and pellets (resuspended in the same volume) were run on 10% SDS-PAGE gels, blotted and probed with a polyclonal antibody against CypD.

2.6. Quantification of cell death by flow cytometry

Apoptosis analyses were performed with a double-stain system using Annexin V (Interchim) combined with FluoProbes 488 and Propidium Iodide (PI) (Sigma Aldrich). INS-1 cells were detached by trypsinization, washed by centrifugation, and incubated with 100 µl of Annexin V buffer 1× (10 mM HEPES NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$). Cells were then incubated for 15 min at room temperature in the dark in the presence of 5 µl of AnnexinV-FP488. Labeled cells were transferred in a 5 ml propylene tube containing 900 µl PBS. 10 µl from a 1 mg/ml stock solution of PI was added to the suspension and immediately analyzed. Data acquisition (~5000 cells) was carried out using a FACSCAN flow cytometer (Becton Dickinson Biosciences) equipped with a 15-mW argon ion laser tuned at 488 nm, using the Cell Quest Pro software (Becton Dickinson Biosciences). Data were plotted as a function of fluorescence intensity on FL-1 (530 nm/30 nm band-pass filter) (Annexin V) and FL-3 channels (PI) (585–42 nm band-pass filter). The Annexin V^-/PI^- population was regarded as normal healthy cells.

3. Results

Matrix Ca^{2+} is the single most important factor for inducing PT. "PT-inhibitors" and "PT-inducers" are compounds that increase and decrease the amount of Ca^{2+} required to induce PT, respectively [22]. CRC (*i.e.*, the amount of Ca^{2+} required to induce PT) was measured by loading mitochondria or digitonin-permeabilized cells with a train of Ca^{2+} pulses until a fast Ca^{2+} release occurred, which marks the onset of the PT and is accompanied by swelling [21]. As shown in Fig. 1, the complex I inhibitor rotenone did not inhibit PT (*i.e.*, it did not increase the CRC) in rat liver and heart mitochondria, and in mouse heart mitochondria. This is in line with the general literature on the PTP, which has mainly studied rat liver and rat heart mitochondria [1]. In all the other tissues and cell lines tested, however, rotenone was more potent than, or at least as potent as, CsA at PTP inhibition (Fig. 1). This effect was particularly prominent in mouse liver mitochondria, rat hepatoma MH1C1 cells and rat glioma C6 cells, suggesting that the lack of effect of rotenone is not a feature of a particular animal species (rat) or a particular tissue (liver). It is also important to note that CsA is not a universal PT-inhibitor, as it did not prevent PT in CLTTi, NIH/3T3, and HL60 cells (Fig. 1). Overall, this set of data reveals (i) that PTP regulation by rotenone and CsA is different in different tissues and organs, indicating that at least some regulatory features of the PTP defined in rat liver and heart mitochondria may not necessarily apply to other tissues; and (ii) that in tissues or cells where both rotenone and CsA are effective inhibition is additive. Indeed, in preparations where an effect of CsA was detected, the CRC was always higher in the presence of both CsA and rotenone than with CsA alone (Fig. 1). This was particularly evident in mitochondria from rat liver and heart, and from mouse heart, where rotenone alone did not affect the CRC.

CypD is the molecular target of CsA [2–5], while the adenine nucleotide translocator (ANT) and complex I have been suggested to be part of the PTP [14,23]. We next quantified CypD, ANT, complex I, and cytochrome oxidase in several cell lines in which rotenone does or does not regulate PTP opening. The CypD/cytochrome oxidase ratio was the same in all the cells tested, while the CypD/ANT and CypD/complex I ratios were dramatically higher in preparations where rotenone did not inhibit PT in the absence of CsA, as compared to cells in which rotenone alone was effective (Fig. 2). This set of data suggests that the effect of rotenone at PTP regulation might depend on specific interactions between CypD and the pore.

It has been reported that CypD can be detached from the inner membrane of inside-out submitochondrial particles by incubating mitochondria in the presence of CsA [7], in hyperosmotic media [6], or at alkaline pH [7]. We have observed that sonication of mitochondria releases a fraction of CypD, and that this fraction is dramatically increased by treatment with CsA, largely increased by exposure to hypertonic sucrose, and somewhat increased by alkaline pH but not by rotenone (Fig. 3A). Strikingly, rotenone acquired the ability to inhibit the PT in rat liver mitochondria once CypD had been detached by any of the above treatments, the effect being larger after treatment with CsA, intermediate with hypertonic sucrose, and modest but significant with pH 9.0 (Fig. 3A). PT inhibition by rotenone was also observed in permeabilized rat hepatocytes after treatment with specific siRNAs decreasing the expression of CypD (Fig. 3B). In mouse heart mitochondria

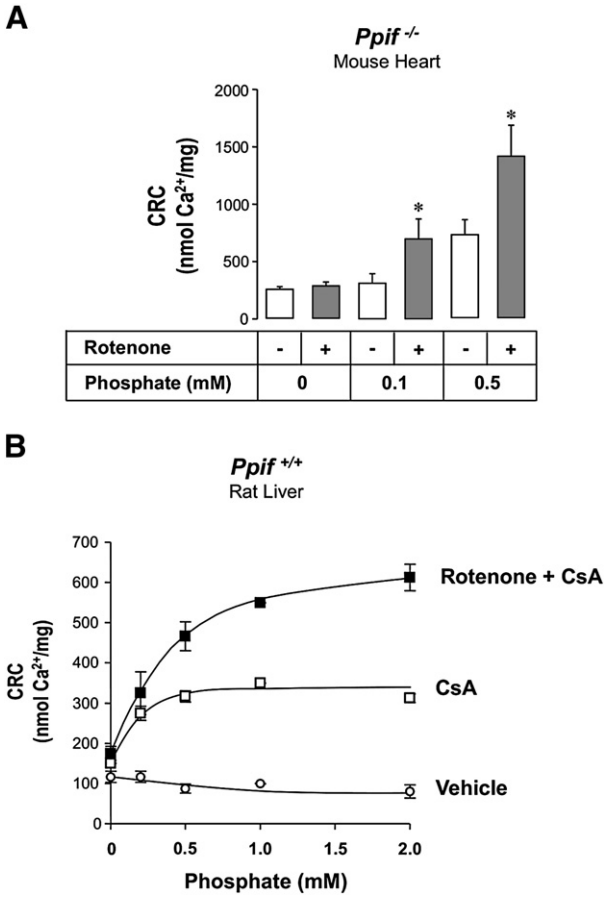

Fig. 4. Rotenone and CsA require phosphate to inhibit PTP opening. The CRC was measured in the presence of 5 mM succinate-Tris at the indicated concentrations of phosphate on heart mouse mitochondria isolated from *Ppif*$^{-/-}$ animals in the presence or not of 1 µM rotenone (panel A) or on rat liver mitochondria in the presence of vehicle (control), 1 µM CsA, or 1 µM CsA plus 1 µM rotenone (panel B). Results are mean ± S.E. of three independent experiments. *, $p<0.05$ vs. without rotenone. Student's *t* test.

rotenone inhibited the PT only in the *Ppif*$^{-/-}$ (CypD null) genotype or after treatment of the wild type littermates with CsA, but not in naïve wild type mitochondria (Fig. 3C).

CypD ablation in mouse heart mitochondria (Fig. 4A) or detachment of CypD by CsA in rat liver mitochondria (Fig. 4B) did not inhibit PT in the absence of phosphate, confirming results obtained with mouse liver mitochondria [8]. Interestingly, rotenone did not inhibit PT unless phosphate was present (Fig.4A,B), suggesting that rotenone also unmasks an inhibitory site for phosphate.

Rotenone inhibited complex I and PT with a similar concentration dependence (Fig. 5A), suggesting that rotenone inhibits PTP opening due to complex I inhibition. Of note, a significant inhibition of PTP opening could be obtained when complex I was inhibited only partially. Indeed, metformin (which partly inhibits complex I activity [11–13]) also increased the CRC in digitonin-permeabilized cells (Fig. 5B). Moreover, it should be mentioned that metformin did not inhibit PTP opening in tissues with high expression of CypD such as rat liver (data not shown), whereas metformin potentiated the action of CsA (Fig. 5B) but not that of rotenone (Fig. 5C), further suggesting that metformin and rotenone have a common mechanism of action.

In order to check whether the synergic action of CsA and complex I inhibition on PTP opening could also be seen in cytoprotection, we exposed INS-1 β-cell to 30 mM glucose, a condition that induces PTP opening and cell death [15]. Hyperglycemia-induced cell death was partially prevented by treatment with CsA or metformin alone, and totally prevented by the combined treatment with both drugs (Fig. 5D).

4. Discussion

In this manuscript we have shown that inhibition of the PT by rotenone is maximal in tissues that express low levels of CypD. Like inhibition by CsA [8], inhibition by rotenone requires Pi, suggesting that rotenone and CsA eventually act on the same inhibitory site, and that the cell can modulate its response to rotenone by shielding this inhibitory site with increased expression of CypD.

The molecular target of CypD for PTP regulation (*i.e.*, the binding site for phosphate) has not been identified yet; and neither transcriptional regulation of the *Ppif* gene (encoding for CypD) nor turnover of CypD have been studied, so the reason why the CypD content varies in different tissues is not known. CypD has a variety of well-characterized mitochondrial targets [24] including: (i) the F_1F_O ATP synthase; binding of CypD is favored by Pi and leads to inhibition of the enzyme, and the effect is counteracted by CsA in striking analogy with the effects on the PTP [25]; (ii) the ANT; this finding was taken to imply that the ANT is part of the PTP [26], but this seems unlikely in view of the fact that the pore is inhibited by CsA in ANT-null mitochondria [27]; (iii) Hsp90 and its related molecule TRAP-1; as CypD bound to the complex is no longer available for PTP opening, CypD displacement from this complex by selective Hsp90 antagonists like Shepherdin reactivates the PTP, which has been successfully used for the selective killing of tumor cells that overexpress TRAP-1 [28]; (iv) the antiapoptotic protein Bcl2; binding could be displaced by CsA resulting in increased tBid-dependent release of cytochrome *c* from mitochondria under conditions that did not cause opening of the PTP [29].

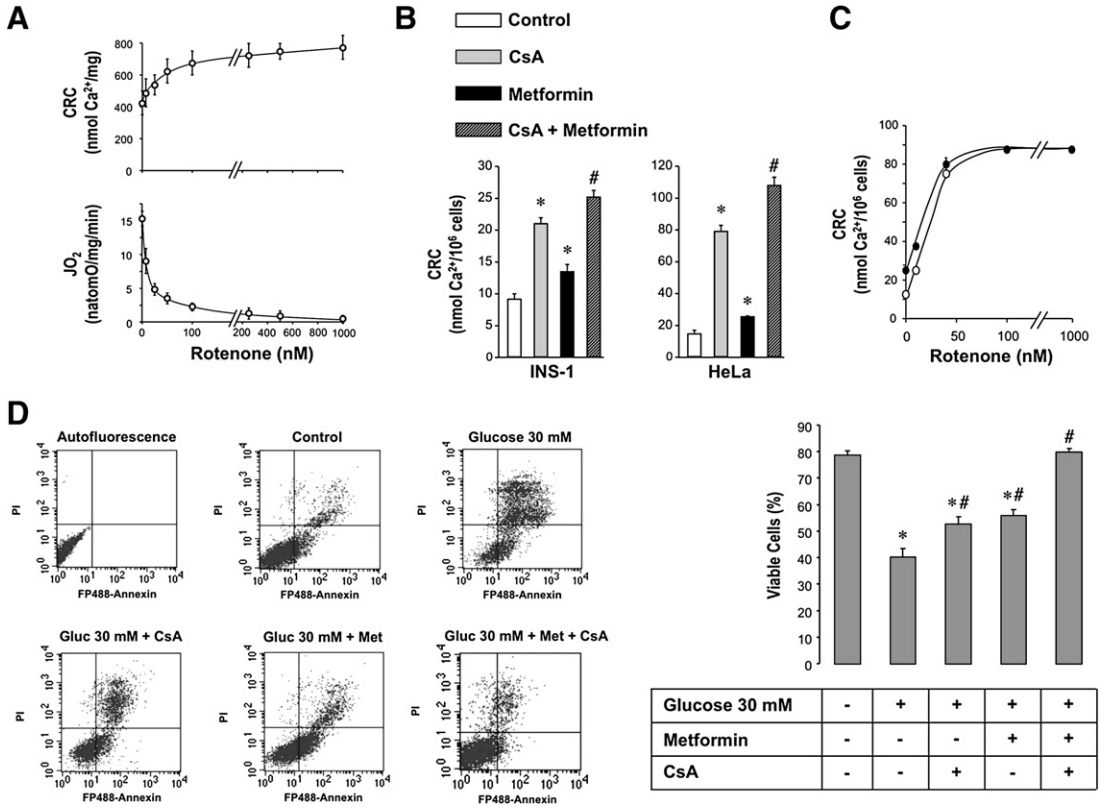

Fig. 5. Mild inhibition of complex I inhibits PTP opening and potentiates the effects of CsA. A: Isolated rat liver mitochondria were incubated in the presence of 1 μM CsA. The oxygen consumption rate in the presence of 5 mM glutamate-Tris plus 2.5 mM malate-Tris (JO_2) and the CRC in the presence of 5 mM succinate-Tris were measured in parallel experiments at the indicated concentration of rotenone. Results are mean ± S.E. of three independent experiments. B: The CRC was measured as in Fig. 1 on digitonin-permeabilized HeLa cells and on digitonin-permeabilized INS-1 cells incubated overnight in the presence or absence of 100 μM metformin. Where indicated, the CRC was measured in the presence of 1 μM CsA. Results are mean ± S.E. of three independent experiments. *, $p<0.05$ vs. control, #, $p<0.05$ vs. CsA. Student's *t* test. C: The CRC was measured as in Fig. 1 at the indicated concentration of rotenone on digitonin-permeabilized HeLa cells incubated overnight in the presence (closed symbols) or absence (open symbols) of 100 μM metformin. Results are mean ± S.E. of three independent experiments. D: INS-1 cells incubated in RPMI 1640 medium supplemented or not with either 1 μM CsA for 1 h, 100 μM metformin for 24 h, or 100 μM metformin for 24 h and 1 μM CsA for 1 h were then incubated in complete RPMI 1640 medium supplemented or not (control) with glucose (30 mM, final concentration) for 72 h. Cell viability was assessed by double labeling as described in Material and methods. Histograms represent the results of three different experiments. Results are mean ± SE; * $p<0.05$ vs. control, # $p<0.05$ vs. Glucose 30 mM. Student's *t* test.

Which, if any, of these interactions is relevant to the site inhibited by rotenone and metformin remains unknown; yet, and irrespective of the site of action, our findings represent a mechanistic advance in understanding of how the PTP is regulated in different tissues. The hypothesis that rotenone regulates PT via a site located outside complex I cannot indisputably be ruled out at present, but this hypothesis seems very unlikely. The effect of rotenone on PTP opening can indeed be traced to complex I inhibition *per se* because a similar outcome was caused by both piericidine [14] and the antidiabetic drug metformin [11,13,15], which partially inhibits complex I [11–13]. Since complex I inhibition affects the redox status of complex I [30–32], we propose a model (see Fig. 6) in which according to its conformation, complex I interacts with the PTP, in turn modulating the number of accessible Pi sites affected by CypD. The early recognition that several ligands of the ANT regulate PT led to proposal that ANT might form the PTP [26]. However, the observation that PT persists in ANT deficient cells questioned this assumption [27]. It was then suggested that ANT is a regulatory component of the PTP, or that PT might involve several different inner membrane proteins. Therefore, an alternative hypothesis should be that complex I might be one of the proteins able to form a nonspecific inner membrane channel depending on its redox status.

CsA is not a natural ligand of eukaryotic cyclophilins, while complex I is subjected to regulation by both covalent and non-covalent modifications [30,33,34]. Our demonstration that complex I impinges on the same site(s) unmasked by CsA (and otherwise shielded by CypD) reveals a novel level of PTP regulation by modulation of the expression levels and/or turnover rate of CypD. As mentioned above, CypD binds to numerous proteins, most probably with different affinities. If we assume that the binding sites on the PTP are of high affinity, once these are saturated a further increase in CypD will not regulate the PTP, unless rotenone unmasks new sites for phosphate. In the scenario proposed in Fig. 6, a decrease in CypD is expected to increase the CRC only once the binding sites of high affinity become vacant, which requires a large decrease in CypD expression in tissues with normally high levels of CypD.

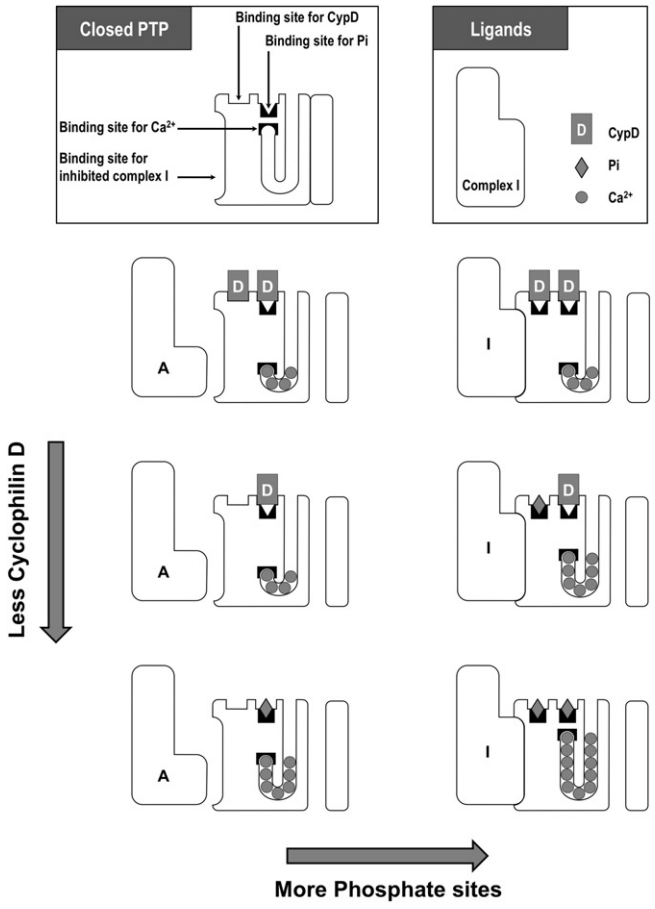

Fig. 6. Model for PTP regulation by Pi, in which the number and the accessibility of Pi sites are modulated by interaction with complex I and CypD, respectively. A: conformation of active Complex I, I: conformation of inactive Complex I. More Pi binds to the PTP, more Ca^{2+} is required to induce PTP opening.

This hypothesis is supported by the findings that a significant decrease in CypD did not inhibit PTP opening in rat liver (*i.e.*, a tissue with high levels of CypD) in the absence of rotenone (Fig. 3B); and that PTP opening was not inhibited in heart mitochondria of *Ppif*$^{+/-}$ mice [2], which is a tissue with high levels of CypD (Fig. 2) where rotenone does not inhibit PTP opening (Fig. 1). On the contrary, PTP opening was inhibited in liver mitochondria of *Ppif*$^{+/-}$ mice [2], *i.e.* a tissue with low levels of CypD (Fig. 2) where rotenone is an effective inhibitor (Fig. 1).

Metformin is the most widely prescribed drug to treat patients affected by type 2 diabetes and is recommended as a first-line oral therapy in both American and European guidelines [35]. This recommendation is based on clinical studies suggesting a reduction of cardiovascular mortality by metformin compared with any other oral antidiabetic treatment [36,37]. This suggests that beside its antihyperglycemic effect, metformin may have other beneficial effects. Both ischemia-reperfusion injury and hyperglycemia-induced cell death have been shown to involve PTP opening [5,10,11,15]. It appears therefore plausible that some of the beneficial effects of metformin are due to the inhibition of PT.

The mechanism of action of metformin in diabetes is widely believed to involve an activation of AMPK [38]. However, this assertion has been questioned by the demonstration that the inhibition of liver neoglucogenesis, which is one of the main effects of metformin in diabetes, persists in liver-specific AMPK-deficient mice [39]. Moreover, the addition of cell-permeant succinate, a substrate bypassing the inhibition of complex I, has been shown to abolish the metformin-induced AMPK activation, suggesting that AMPK activation is due to Complex I inhibition [40].

The finding that the protective effect of rotenone and metformin is additive with that of CsA represents a major advancement that may lead to a better treatment of PTP-dependent diseases with a combination of metformin and cyclophilin inhibitors, a protocol that may prove extremely relevant for diabetes. Mutations of the pancreatic duodenal homeobox gene-1, *Pdx1*, cause heritable diabetes in humans and mice due to increased death of β-cells [41]. The haploinsufficient mouse model displays reduced β-cell mass associated to markers of both β-cell apoptosis and necrosis [42–44].

Genetic ablation of the *Ppif* gene in *Pdx1*$^{+/-}$ mice restored β-cell mass and prevented onset of diabetes, demonstrating that CypD is a viable target for therapy. Our current demonstration that the approved antidiabetic drug metformin hits the same final molecular target on the PTP as does CsA, and that the inhibitory effect is synergistic, opens new perspectives to the study of β-cell death and to effective pharmacological treatments for cytoprotection.

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