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The still uncertain identity of the channel-forming unit(s) of the mitochondrial permeability transition pore

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ABSTRACT

Mitochondria from different organisms can undergo a sudden process of inner membrane unselective leakiness to molecules known as the mitochondrial permeability transition (MPT). This process has been studied for nearly four decades and several proteins have been claimed to constitute, or at least regulate the usually inactive pore responsible for this transition. However, no protein candidate proposed as the actual pore-forming unit has passed rigorous gain- or loss-of-function genetic tests. Here we review evidence for -and against- putative channel-forming components of the MPT pore. We conclude that the structure of the MPT pore still remains largely undefined and suggest that future studies should follow established technical considerations to unambiguously consolidate the channel forming constituent(s) of the MPT pore.

1. The mitochondrial permeability transition: from basic science to clinical trials

Early protocols for isolating mitochondria considered calcium a deleterious agent that promoted mitochondrial uncoupling [1]. Hence, most studies typically included calcium-chelating agents in isolation buffers to minimize the occurrence of such uncoupling [2]. Current views acknowledge the presence of a Mitochondrial Permeability Transition (MPT) pore that shifts the mitochondrial inner membrane permeability [3]. When not reversed, channel opening results in the massive release of calcium alongside other metabolites (for a review see [4]). Further outer mitochondrial membrane rupture may result in the release of medium-sized proteins like cytochrome *c*, although it is noteworthy to mention that the MPT pore itself is known to present a molecular exclusion threshold of 1.5 kDa, thereby excluding protein movement through this channel [5,6]. The MPT pore was initially described as a calcium-induced, calcium-release channel [3,7,8] and was later shown to be desensitized to calcium with nanomolar amounts of the cyclic undecapeptide cyclosporin A (CsA) [9]. These phenotypes strongly argued for the participation of the mitochondrial calcium uniporter (MCU) and the mitochondrial isoform of a cyclophilin (CypD) in the regulation of the MPT pore. Indeed, treatment of isolated mitochondria with MCU inhibitors prevented against MPT but only before calcium addition, suggesting a requirement for matrix calcium to activate the MPT pore [10]. CsA has been traditionally used to block the immune response following organ transplantation. Its potent effects on graft rejection require inhibition of cytokine production by blocking specific genes in active T cells [11]. To do this, CsA binds to cytoplasmic cyclophilin(s) and blocks calcineurin phosphatase activity. At high doses (~20 mg/kg), CsA also suppresses JNK- and p38-dependent signaling pathways following immunogen recognition. During pathological conditions, calcineurin/NFAT signaling also mediates cardiac hypertrophy [12,13]. Conversely, the use of CsA as MPT desensitizer to calcium requires concentrations in the nanomolar range [14]. This latter feature has allowed a growing cluster of studies to address the participation of the MPT pore in cell death induced by hypoglycemia, ischemia/reperfusion damage as well as on neurodegenerative and neuromuscular disorders (for a review see [15]). These studies also

Abbreviations: α-MHC, α-myosin heavy chain; ΔΨ, mitochondrial transmembrane potential; ANT, adenine nucleotide translocase; ATR, atractyloside; BKA, bongkrekic acid; Bz-423, benzodiazepine 423; CsA, cyclosporin A; CsABD, cyclosporin A binding domain; CypD, cyclophilin D; CRC, calcium retention capacity; DTG, double transgenic; GST, glutathione *S*-transferase; HS, heat shock; HSP, heat shock proteins; IMM, inner mitochondrial membrane; MCC, multiple conductance channel; MCU, mitochondrial calcium uniporter; MEF, mouse embryo fibroblast; MPT, mitochondrial permeability transition; NEM, *N*-ethylmaleimide; OSCP, oligomycin sensitivity conferral protein; PAO, phenyl arsine oxide; PiC, mitochondrial phosphate carrier; PI, propidium iodide; PIM, protein import machinery; STEMI, segment elevation myocardial infarct; TG, transgenic; VDAC, voltage dependent anion channel

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anticipated the MPT pore as a feasible target to ameliorate such diseases despite divergent outcomes in clinical trials and disease models. For example, following a successful phase 2 trial where patients were administered CsA right before primary percutaneous coronary intervention, the trial subjects presented reduced segment elevation myocardial infarct (STEMI) size [16]. Unexpectedly, the subsequent phase 3 trial (also known as CIRCUS) -where patients were injected with a single dose of CsA- resulted in no improvement in clinical outcomes measured at 1 year after STEMI [17]. Some scientists argued that the lack of a positive outcome may have been due to the fact that CsA is not an irreversible MPT pore inhibitor but rather a 'desensitizer' [18]. Consequently, whether patients with STEMI can present improved primary outcomes with CypD-independent MPT pore inhibitors remain to be tested [19–21].

Studies assessing mitochondrial dysfunction in a miniature swine model of heart failure with preserved ejection fraction showed exacerbated MPT pore activity, which was reversed by low-intensity aerobic interval training [22]. With this in mind, further studies by the same group aimed to assess the potential cardioprotective role of chronical administration of a non-immunosuppressive dose of CsA in the same miniature swine model [23]. These new experiments showed that CsA alleviated the collapse in mitochondrial function detected in animals experiencing heart failure, as measured by Complex I-dependent mitochondrial respiration and susceptibility to MPT. But quite unexpectedly, the authors detected a markedly decreased cardiomyocyte function consistent with impaired *in vivo* systolic and diastolic cardiac function. Such impairment was linked to a systemic hypertensive response of chronic CsA administration during heart failure. The authors concluded that chronic CsA treatment is not a viable therapeutic option during heart failure with preserved ejection fraction in miniature swine [23].

Overall, both studies contributed to the current growing notion that MPT pore desensitization with CsA may not be effective enough to suppress potential pore-dependent pathologies without side effects. Examples of such effects include potential risks of immunosuppression and renal damage [24]. These studies further reinforce the necessity to unveil the pore´s core molecular componentry, which would aim in the design of targeted therapies against the aforementioned pathologies. Consequently, it is the purpose of this review to discuss previous and recent efforts to map the channel-forming component(s) of the MPT pore. Specifically, we will focus on the adenine nucleotide translocase (ANT), the mitochondrial phosphate carrier (PiC), ATP synthase and alternative models still awaiting validation.

2. The adenine nucleotide translocase

Genetic studies designed to determine the participation of several proteins as MPT pore constituents have challenged established paradigms on its structure and has involved several groups around the world [25–32]. It was Hunter and Haworth who first proposed a role of the adenine nucleotide translocator (ANT) as a central component of the MPT pore due to the sensitivity of such process to the selective ligands atractyloside (ATR) and bongkrekic acid (BKA), which sensitize and inhibit calcium-induced pore opening respectively [3,7,8]. Further reports by the Halestrap laboratory found an *in vitro* interaction between the fusion glutathione *S*-transferase-CypD (GST-CypD) and ANT [33]. These experiments highlighted the importance of the redox status of thiol residues for GST-CypD interaction with inner mitochondrial membranes (IMMs). In this work, treatment of isolated mitochondria with diamide (a bifunctional thiol reagent) resulted in an increase in GST-CypD binding to IMMs isolated thereafter, which was absent when diamide was added directly after isolating IMMs [33]. This led the authors to conclude that diamide induced crosslinking of two ANT cysteines required matrix glutathione, which was likely lost during IMM isolation. [14,34]. Noteworthy is the fact that CsA could also abrogate such binding, which suggests that CypD-MPT pore complex(es) could occur through the CsA-binding domain in a process involving the isomerization of proline residues [33]. Indeed, isomerase-inactive mutant CypD is unable to induce MPT opening as assessed by oxidative stress [35]. Modulation of CypD levels is also pivotal for normal mitochondrial function. For example, heart-specific overexpression of wild type CypD results in a severe hypertrophic phenotype resulting in heart dysfunction. For more insight into the molecular aspects of CypD-dependent MPT, we refer the reader to a previous discussion by our group [36].

Studies by different laboratories have also highlighted the importance of discrete cysteine residues controlling MPT [37–39]. While Costantini and colleagues argued that the MPT pore could be induced at two separate sites involving SH groups, one located either at the outer side of the inner mitochondrial membrane and the second one at the level of regulatory proteins in the inter membrane space or outer membrane, the authors showed that ANT dimerization was not required for MPT onset [37]. Indeed, Kowaltowski and collaborators have proposed that phenylarsine oxide (PAO) can induce MPT through its interaction with an extramitochondrial calcium-binding site [40]. These studies argued against previous working models involving dimerization of ANT [41] or even newer ones involving PAO-induced pore opening [19]. In a similar token, studies by Chavez´s group have shown that MPT induced by copper or calcium can be significantly amplified in the presence of 1,10-phenanthroline or ATR [42]. Thiol group-titration revealed that blockage of 5.9 nmol of SH/mg protein was enough to induce pore opening. Further MPT modulation with ATR, ADP and *N*-ethylmaleimide was interpreted as ANT being the pore´s core component. In a subsequent study, the same group found that the dithiol reagent mersalyl induced some sort of CsA-insensitive MPT when incubated alongside PAO [39]. Finally, the same group showed that locking the ANT in the "c" conformation facilitated binding of fluorescent eosin maleimide to a ~30 kDa protein [39]. Although these studies further reinforced the notion that ANT plays a major role in MPT progression, the authors acknowledged that the role of ANT could be accessory.

However, the role of ANT in cell death appears to be more complex than what can be explained by MPT pore opening alone. For example, ANT overexpression in cardiomyocytes results in cell death [43]. Under these conditions, ANT upregulation can induce apoptosis through the activation of pro-apoptotic Bax. The role of Bax as an MPT pore component has been considered in some studies proposing that Bax and Bak may behave as the mitochondrial outer membrane component of the MPT pore [44]. But this hypothesis -at least for Bax- has also been previously ruled out by others showing the dispensability of this BH-3 protein for mitochondria to undergo MPT [45]. Whether Bax forms part of the MPT pore still merits further investigation, especially if we consider the presence of MPT-like structures in model organisms naturally lacking Bax like *Saccharomyces cerevisiae* [46]. Nevertheless, results from Madeo's group suggest hydrogen peroxide may induce cell death through the upregulation of a BH3-only protein in yeast, which can induce CsA-independent swelling in isolated mitochondria (see *Section 3* and [47]).

Although informative, the studies addressed above are not conclusive on the possibility that ANT *per se* can form a calcium-induced calcium-release channel. To address this possibility, Brustovetsky and colleagues showed that purified bovine ANT and recombinant *Neurospora crassa* ANT could form a 600pS pore reminiscent of half of the MPT pore full conductance [48]. These authors also found that locking ANT in the 'm' conformation (with the substrate binding site facing the mitochondrial matrix) with BKA inhibited the channel formed by ANT but not ATR, which blocks the carrier in the 'c' conformation (with the substrate binding site facing the cytoplasm). Noteworthy is the fact that the mitochondrial megachannel (MMC) is thought to be the electrophysiological manifestation of the MPT pore and it usually presents conductances near 1.3 nS [49]. On a related note, Rück and colleagues found that liposome-embedded ANT could