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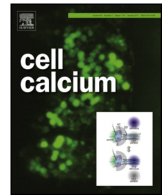

The mitochondrial permeability transition pore is a dispensable element for mitochondrial calcium efflux

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ABSTRACT

The mitochondrial permeability transition pore (mPTP) has long been known to have a role in mitochondrial calcium (Ca^{2+}) homeostasis under pathological conditions as a mediator of the mitochondrial permeability transition and the activation of the consequent cell death mechanism. However, its role in the context of mitochondrial Ca^{2+} homeostasis is not yet clear. Several studies that were based on PPIF inhibition or knock out suggested that mPTP is involved in the Ca^{2+} efflux mechanism, while other observations have revealed the opposite result.

The c subunit of the mitochondrial F_1/F_O ATP synthase has been recently found to be a fundamental component of the mPTP. In this work, we focused on the contribution of the mPTP in the Ca^{2+} efflux mechanism by modulating the expression of the c subunit. We observed that forcing mPTP opening or closing did not impair mitochondrial Ca^{2+} efflux. Therefore, our results strongly suggest that the mPTP does not participate in mitochondrial Ca^{2+} homeostasis in a physiological context in HeLa cells.

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1. Introduction

Mitochondria are intracellular organelles involved in several cellular functions such as ATP production, fatty acid oxidation, Ca^{2+} signaling and cell death. [1–4]. Under pathological conditions, mitochondria are able to adapt by fusion or fission processes and modulate respiratory substrate utilization to regulate ATP production [5]. Furthermore, in cases of severe damage, mitochondria are eliminated by autophagy, and cell death can occur [6]. Thus, these organelles are master regulators of danger signaling [7,8].

A key signaling messenger that is able to transduce life or death signals to mitochondria is intracellular Ca^{2+} [9,10]. The mitochondrial Ca^{2+} uptake and release mechanisms are based on the utilization of gated channels for Ca^{2+} uptake and exchangers for release that are dependent upon the negative mitochondrial membrane potential, which represents the driving force for Ca^{2+} accumulation in the mitochondrial matrix [11]. The mitochondrial Ca^{2+} uniporter (MCU), which is encoded by the recently discovered gene ccdc109a [12,13], is responsible for Ca^{2+} influx, while the mitochondrial Na^+/Ca^{2+} antiporter (NCLX) [14] is responsible for Ca^{2+} efflux. In spite of this information, the mitochondrial Ca^{2+} efflux mechanism has not been completely elucidated. It is mostly accepted that Na^+/Ca^{2+} exchange activity is relevant for excitable cells [15] and that NCLX inhibition or silencing does not completely arrest Ca^{2+} efflux [14], which indicates that other mechanisms are involved in this process. Two mechanisms have been proposed to supplement Ca^{2+} efflux, one based on the H^+/Ca^{2+} antiporter [16] and another based on the mitochondrial permeability transition pore (mPTP) [17], but evidence supporting the latter remains elusive.

The mPTP is a high-conductance channel that is located at the contact sites between the inner and outer mitochondrial membranes. This channel is responsible for the non-selective permeability state of the mitochondrial inner membrane. The transition to this state for small molecules is referred to as the mitochondrial permeability transition (MPT). The molecular composition of the mPTP is not yet clear, but several proteins have been shown to be components that participate in mPTP activity, including voltage-dependent anion channels (VDAC) [18], adenine

Abbreviations: ANT, adenine nucleotide translocase; $[Ca^{2+}]c$, cytosolic calcium concentration; $[Ca^{2+}]_m$, mitochondrial calcium concentration; CsA, cyclosporine A; HK, hexokinase II; H_2O_2, hydrogen peroxide; IB, intracellular milieu; MCU, mitochondrial Ca^{2+} uniporter; NCLX, mitochondrial Na^+/Ca^{2+} antiporter; MPT, mitochondrial permeability transition; mPTP, mitochondrial permeability transition pore; PiC, inorganic phosphate carrier; PPIF, peptidyl prolyl isomerase; siRNAs, small-interfering RNAs; TSPO, peripheral benzodiazepine receptor; RuR, ruthenium red; VDAC, voltage-dependent anion channel.

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nucleotide translocase (ANT) [19], the inorganic phosphate carrier (PiC), [20], peptidyl prolyl isomerase F (PPIF) [21,22], the peripheral benzodiazepine receptor (TSPO) [23], hexokinase II (HK) [24] and several members of the Bcl-2 family [25–27].

Ca^{2+} ions, prooxidant and proapoptotic proteins, a decrease in the mitochondrial membrane potential, pH variations and adenine nucleotides all sensitize the opening of the pore [28,29].

MPT resulting from mPTP opening is usually considered a transducer event in between Ca^{2+} or oxidative signal and different type of cell death [3,30]. Nonetheless several observations have suggested that mPTP is a component of the Ca^{2+} efflux mechanism [31–34] proposing a physiological role for this ambiguous complex. Unfortunately a different amount of studies have proposed the exact opposite [35,36] leaving this supposition still unresolved. Therefore, we focused on the role of the mPTP in mitochondrial Ca^{2+} homeostasis under non-pathological conditions. Recently, we suggested that, similar to PPIF, the c subunit of the F_O ATP synthase constitutes a critical component of the mPTP and that it is required for the MPT, mitochondrial fragmentation and cell death induced by oxidative stress or mitochondrial Ca^{2+} overload [37]. This concept was further confirmed by two different groups after our publication [38,39] and recently reviewed in [40].

By modulating c subunit expression and using pharmacological approaches, we revealed the important finding that the mPTP is not necessary for mitochondrial Ca^{2+} release; therefore, it does not participate in mitochondrial Ca^{2+} homeostasis in a non-pathological context, at least in HeLa cells.

2. Results

2.1. Regulation of mPTP activity by modulating the expression of the F_O ATP synthase c subunit

To study the contribution of mPTP in mitochondrial Ca^{2+} homeostasis, we have investigated the kinetics of the mitochondrial and cytosolic Ca^{2+} influx and efflux. We have recently suggested that the c subunit of the F_1/F_O ATP synthase is a component of the mPTP that is required for the MPT-driven mitochondrial fragmentation that is induced by cytosolic Ca^{2+} overload and oxidative stress [37]. Therefore, we decided to modulate mPTP activity by genetically manipulating the expression of the c subunit in the HeLa human cervical carcinoma cell line. The mammalian ATP synthase c subunit is encoded by three different nuclear genes (ATP5G1, ATP5G2 and ATP5G3) that produce three different protein products. These products differ in their mitochondrial localization sequence but result in the same mature protein after cleavage of the localization peptide [41]. As previously shown, we used a mix of three commercially validated small-interfering RNAs (siRNAs) to silence the three different genes that code for the c subunit (ATP5G1, ATP5G2 and ATP5G3). Analysis of the mRNA levels of the three genes using real-time PCR indicated that the silencing strategy resulted in an approximate reduction of 75% for ATP5G1, 53% for ATP5G2, and 20% for ATP5G3 (Fig. 1Ai), and western blot analysis showed that the protein levels were reduced to nearly 70% of the total protein level (Fig. 1Aii). To monitor the effective state of the mPTP, we used the calcein-Co^{2+} assay during c subunit silencing (Fig. 1B). As previously reported, ATP5G silencing ablated mPTP activity and resulted in increased fluorescence of mitochondrial calcein (Fig. 1B).

By taking advantage of aequorin technology [42], we first measured the mitochondrial and cytosolic Ca^{2+} concentrations after stimulation with 100 M histamine in HeLa cells that were transfected with a control siRNA (siSCR) or a mix of siRNAs that targeted ATP5G1, ATP5G2 and ATP5G3 (siATP5G) for 48 h (Fig. 1C). Silencing of the c subunit caused a non-significant reduction in Ca^{2+} uptake by the mitochondria (peak amplitude: $67.35 \pm 4.41\,\mu M$ vs. $71.08 \pm 3.33\,\mu M$ [siSCR]; $p > 0.05$) and in the Ca^{2+} uptake rate, but no significant variations in the rate of Ca^{2+} release from the mitochondria were observed (Fig. 1D). Furthermore, no significant differences in the cytosolic Ca^{2+} levels (peak amplitude: $2.13 \pm 0.09\,\mu M$ vs. $2.32 \pm 0.08\,\mu M$ [siSCR]; $p > 0.05$) (Fig. 1E) or in the kinetics of Ca^{2+} influx and efflux (Fig. 1F) were detected.

To confirm the experimental method, mitochondrial Ca^{2+} dynamics were monitored in the presence of a well-established blocker of the mitochondrial Ca^{2+} efflux system, CGP37157, which is an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger [43].

HeLa cells were exposed to CGP37157 (10 μM) for 2 min, and Ca^{2+} uptake was induced with histamine (100 μM) in the continuous presence of the inhibitor.

CGP37157 caused an expected increase in Ca^{2+} uptake by the mitochondria (peak amplitude: $109.2 \pm 15.52\,\mu M$ vs. $68.6 \pm 6.81\,\mu M$ [Mock]; $p < 0.05$) (Fig. S1A) and a significant decrease of 31% in the rate of Ca^{2+} release from the mitochondria due to inhibition of the mitochondrial Na^+/Ca^{2+} exchanger (Fig. S1B). The absence of significant differences between the cytosolic Ca^{2+} levels (peak amplitude: $2.84 \pm 0.06\,\mu M$ vs. $2.77 \pm 0.33\,\mu M$ [siSCR]; $p > 0.05$) (Fig. S1C) and Ca^{2+} influx and efflux kinetics (Fig. S1D) confirmed the specificity of CGP37157.

In our experimental settings, HeLa cells appeared to display higher RNA levels of ATP5G1 compared to its homologs. We thus decided to overexpress a Myc-tagged ATP5G1, which was under the control of the cytomegalovirus immediate early promoter, to verify its effect on mitochondrial Ca^{2+} homeostasis.

Overexepression levels were assessed by Western blot and results in dramatic increase in levels of c subunit (Fig. 2A). As expected, ATP5G1 overexpression was sufficient to induce the opening of the mPTP, as confirmed by the calcein-Co^{2+} assay (Fig. 2B).

Overexpression of the c subunit induces a tendency in lower the mitochondrial Ca^{2+} uptake (peak amplitude: $82.34 \pm 5.4\,\mu M$ vs. $93.89 \pm 10.6\,\mu M$ [Mock]; $p > 0.05$), without significantly affect the kinetics of Ca^{2+} influx and efflux (Fig. 2C and D) or cytosolic Ca^{2+} levels (peak amplitude: $3.03 \pm 0.08\,\mu M$ vs. $2.94 \pm 0.07\,\mu M$ [siSCR]; $p > 0.05$) (Fig. 2E and F).

2.2. Modulation by pharmacological treatments of mPTP activity in the regulation of mitochondrial Ca^{2+} influx/efflux

To confirm the absence of an effect of mPTP modulation on Ca^{2+} efflux, we used a pharmacological approach; specifically, Cyclosporine A (CsA) and hydrogen peroxide (H_2O_2) were used as an inhibitor of the mPTP [44] and an inducer of mPTP opening, respectively [45].

CsA (1 μM) was added to HeLa cells 30 min before stimulation with histamine (100 μM) and measurements of $[Ca^{2+}]_m$. As previously reported [37], CsA induced a significant increase in Ca^{2+} uptake (peak amplitude: $82.63 \pm 2.82\,\mu M$ vs. $70.2 \pm 2.65\,\mu M$ [vehicle]; $p < 0.05$) (Fig. 3A) and in the rate of Ca^{2+} accumulation in the mitochondria due to an increase in the mitochondrial membrane potential, which is the driving force for Ca^{2+} accumulation. In contrast, no differences in Ca^{2+} efflux kinetics (Fig. 3B) were observed. The closed state of the mPTP was confirmed using the calcein-Co^{2+} assay (Fig. 3C).

In contrast, to pharmacologically induce mPTP opening, HeLa cells were exposed to H_2O_2 (500 μM) 30 min before stimulation with histamine (100 μM). As expected, in the presence of H_2O_2, a significant decrease in Ca^{2+} uptake by the mitochondria was induced (peak amplitude: $36.52 \pm 3.34\,\mu M$ vs. $70.2 \pm 2.65\,\mu M$ [vehicle]; $p < 0.001$) (Fig. 3D).

Regarding the Ca^{2+} influx/efflux kinetics, a significant reduction of 33% in the rate of Ca^{2+} accumulation into the mitochondria and

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