Mitochondrion 12 (2012) 120–125

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

The roles of phosphate and the phosphate carrier in the mitochondrial permeability transition pore

Pinadda Varanyuwatana, Andrew P. Halestrap *

School of Biochemistry and The Bristol Heart Institute, Medical Sciences Building, University of Bristol, Bristol BS8 1TD, UK

ARTICLE INFO

Article history:
Received 25 October 2010
Received in revised form 8 February 2011
Accepted 29 April 2011
Available online 8 May 2011

Keywords:
Permeability transition pore
Heart
Reperfusion injury
Phosphate
Calcium

ABSTRACT

Phosphate activation of the mitochondrial permeability transition pore (MPTP) opening is well-documented and could involve the phosphate carrier (PiC) that we have proposed is the pore's cyclophilin-D binding component. However, others have reported that following CyP-D ablation Pi inhibits MPTP opening while cyclosporine-A (CsA) inhibits MPTP opening only when Pi is present. Here we demonstrate that Pi activates MPTP opening under all energised and de-energised conditions tested while CsA inhibits pore opening whether or not Pi is present. Using siRNA in HeLa cells we could reduce PiC expression by 65–80% but this inhibited neither mitochondrial calcium accumulation nor MPTP opening.

1. Introduction

The mitochondrial permeability transition pore (MPTP) is a non-selective pore in the inner mitochondrial membrane (IMM), permeable to all solutes <1.5 kDa. It opens in response to high matrix calcium concentrations but the sensitivity to [Ca^{2+}] is greatly enhanced by oxidative stress, phosphate and adenine nucleotide depletion (Bernardi et al., 2006; Crompton, 1999; Halestrap, 2009). These conditions occur when cells are stressed such as occurs during reperfusion following a period of ischemia, and it is now widely accepted that MPTP opening is a critical mediator of necrotic cell death under these circumstances (Basso et al., 2008; Crompton, 2000; Di Lisa & Bernardi, 2009; Halestrap, 2010; Halestrap & Pasdois, 2009). Indeed, inhibition of MPTP opening, either pharmacological by MPTP inhibitors such as cyclosporine A (CsA) and sanglifehrin A (SfA) or indirectly by decreasing oxidative stress and calcium overload (e.g. by ischemic preconditioning) provide strong protection against reperfusion injury (Bernardi et al., 2006; Halestrap, 2010; Halestrap et al., 2004).

1.1. The molecular composition of the MPTP

Despite many years of research in several laboratories the detailed molecular mechanism of the MPTP remains uncertain although some proteins have been shown to play important roles. Thus, inhibition of MPTP opening by CsA and SfA implicated the matrix peptidyl-prolyl cis-trans isomerase activity of cyclophilin D (CypD), and this was subsequently confirmed through the use of CyP-D knockout mice (see (Azzolin et al., 2010; Halestrap, 2009)). However, genetic or pharmacological ablation of CyP-D does not abolish MPTP opening but rather decreases its sensitivity to [Ca^{2+}]. This implies that CyP-D plays a facilitating rather than an essential role in MPTP opening. A role of the adenine nucleotide translocase (ANT) was implicated by the inhibitory effects of adenine nucleotides and bongkrekic acid, a ligand of the ANT that traps the ANT in the "m" conformation, and the activating effect of carboxyatractyloside (CAT) that traps the ANT in the "c" conformation (see (Halestrap & Brenner, 2003; Klingenberg, 2008)). These data were taken as evidence that the ANT might be the pore forming component of the MPTP, but subsequently the use of mitochondria from ANT knockout mice cast doubt on this conclusion. Thus mitochondria from these mice still displayed Ca-induced MPTP opening that was enhanced by oxidative stress, but pore opening was less sensitive to [Ca^{2+}] and was no longer sensitive to adenine nucleotides and CAT (Kokoszka et al., 2004). These data can be interpreted in two ways. Either the ANT may play only a regulatory role or the ANT might be able to form the pore but in its absence other proteins could take over this function.

Abbreviations: ANT, adenine nucleotide translocase; BKA, bongkrekic acid; CAT, carboxyatractyloside; CRC, calcium retention capacity; CsA, cyclosporine A; CyP-D, cyclophilin-D; PEG, poly(ethylene glycol); IMM, inner mitochondrial membrane; MPT, mitochondrial permeability transition; MPTP, mitochondrial permeability transition pore; NTA, nitrilotriacetic acid; OMM, outer mitochondrial membrane; PiC, mitochondrial phosphate carrier; PPIase, peptidyl-prolyl cis-trans isomerase; ROS, reactive oxygen species; SfA, sanglifehrin A; VDAC, voltage activated anion channel.

* Corresponding author. Tel.: +44 117 3312118; fax: +44 117 3312168.
E-mail address: A.Halestrap@Bristol.ac.uk (A.P. Halestrap).

1567-7249/$ – see front matter © 2011 Elsevier B.V. and Mitochondria Research Society. All rights reserved.
doi:10.1016/j.mito.2011.04.006

1.2. The role of the mitochondrial phosphate carrier in MPTP formation

Recent work performed in this laboratory has provided strong evidence that the mitochondrial phosphate carrier (PiC) may play a key role in MPTP formation (Leung et al., 2008). Thus we were able to demonstrate CsA-sensitive binding of CyP-D to the PiC whilst the modification of thiol groups on the PiC by oxidative stress and phenylarsine oxide (PAO) correlated with MPTP opening. Furthermore, the sensitivity of MPTP opening to inhibition by N-ethylmaleimide and ubiquinone 0 (UQ_0) matched their ability to inhibit phosphate transport into mitochondria. UQ_0 also prevented PAO from activating pore opening and inhibited the binding of the PiC to immobilised PAO. In the light of these data we suggested that the PiC might be a key component of the MPTP that undergoes a calcium-induced conformational change to induce pore formation. This conformational change might be enhanced by an interaction of the PiC with the "c" but not the "m" conformation of the ANT (Halestrap, 2009, 2010; Leung et al., 2008). We further proposed that binding of Pi to the PiC might provide an explanation for the ability of Pi to activate pore opening (Leung et al., 2008). Such activation of the MPTP by Pi in energised mitochondria has been well documented (Al-Nasser & Crompton, 1986; Chalmers & Nicholls, 2003; Crompton et al., 1988; Crompton & Costi, 1988; Roos et al., 1980), and we demonstrated a similar effect in de-energised mitochondria (Halestrap & Davidson, 1990).

However, more recent data from Bernardi's laboratory has suggested that the effects of Pi may be more complex (Basso et al., 2008; Di Lisa & Bernardi, 2009). To measure MPTP opening, these authors assayed the calcium retention capacity (CRC) of energised liver mitochondria from wild-type and CyP-D knockout mice in the presence or absence of CsA and phosphate. This assay determines the amount of calcium that must be taken up by the mitochondria before the MPTP opens and then releases the accumulated calcium. Since an anion is required to support energised calcium uptake into the mitochondria under these conditions, when phosphate was omitted, the phosphate analogues arsenate (Asi) or vanadate were added in its place. It was found that in the absence of Pi, ablation of CyP-D genetically (CyP-D knockdown) or pharmacologically (CsA addition) did not lead to an observable increase in CRC whereas in the presence of Pi it did. The authors interpreted these data as demonstrating that Pi actually inhibits (rather than activates) pore opening but that this effect is overcome by the presence of CyP-D, accounting for the protective effects of CsA in the presence of Pi. Since this effect of Pi was not shared by arsenate or vanadate, which are also substrates for the PiC, their data would argue against a role for the PiC in the MPTP. However, we have presented extensive data to show that under de-energised conditions in KSCN medium, the MPTP is inhibited by CsA in the absence of Pi (Griffiths & Halestrap, 1991; Halestrap et al., 1997; Halestrap & Davidson, 1990), which would argue against a requirement for Pi to demonstrate CsA inhibition of the MPTP. In this paper we confirm that Pi is not required to demonstrate CsA-sensitive pore opening in either energised or de-energised mitochondria incubated in standard KCl media. We also explore the use of siRNA knockdown to provide further evidence for a role for the PiC in the MPTP.

2. Materials and methods

2.1. Preparation of mitochondria

Mitochondria were prepared from livers of 250 g male Wistar rats following homogenisation in sucrose isolation buffer (300 mM sucrose, 10 mM Tris–HCl, 2 mM EGTA, pH 7.4) in a Dounce Potter homoginizer and purified by Percoll® density-gradient centrifugation as in Halestrap and Davidson (1990).

2.2. Measurement of MPTP opening in de-energised mitochondria

2.2.1. De-energised swelling

This was determined by following the decrease in light scattering (monitored as A_{520}) as described previously (Halestrap et al., 1997; Halestrap and Davidson 1990). Mitochondria were incubated at 25 °C and 1 mg/ml in de-energised assay buffers containing 150 mM KCl or KSCN, 20 mM MOPS, 10 mM TRIS, 2 mM nitrilotriacetic acid (NTA), 0.5 μM rotenone, 0.5 μM antimycin A, 2 μM A23187, and when required either 20 mM Pi or 20 mM Asi. Swelling of mitochondria was initiated with 50 μM free [Ca^{2+}] and A_{520} was monitored continuously in a spectrophotometer with computerised data acquisition.

2.2.1. Shrinkage assay

This technique was employed to determine the sensitivity of MPTP to [Ca^{2+}] and [Pi] and was carried out as previously described (Connern and Halestrap 1994; Connern and Halestrap 1996). Mitochondria were incubated for 20 min at 30 °C and 2 mg/ml under standard de-energised KSCN buffer (as above) but without added NTA or A23187 and with addition of 1 mM $CaCl_2$. Any residual swelling was terminated by an addition of 1.2 mM EGTA, which also resealed swollen mitochondria. The resulting swollen mitochondria were collected by centrifugation at 12,000×*g* for 10 min and resuspended at 2 mg/ml in either de-energised KCl or KSCN buffer without added NTA or Ca^{2+} ionophore. In order to ensure equilibrium of matrix with the new buffer, the swollen mitochondria were incubated again at 30 °C supplemented with 1 mM $CaCl_2$. After 3 min, 1.2 mM EGTA was added to reseal the mitochondria before centrifugation at 12,000×*g* for 10 min. The swollen mitochondria were resuspended at 30 mg/ml in either de-energised KSCN or KCl buffer containing 2 mM NTA and 2 μM A23187.

The extent of MPTP opening in these pre-swollen mitochondria was determined by addition of poly(ethylene glycol), PEG 2000, to induce shrinkage. Initially 2 mg of swollen mitochondria was added to 3 ml of assay buffer containing the required free [Ca^{2+}] and [Pi] or [Asi]. Free [Ca^{2+}] was calculated as described in Rutter and Denton (1988) assuming the same binding constant of Ca^{2+} to Asi as to Pi. Our own measurements with Fura-6F (see below) suggested that this was a reasonable assumption. Shrinkage was initiated after exactly 1 min of incubation by a rapid addition of 0.5 ml 50% (w/v) PEG (to give a final PEG concentration of 7% w/v) and continuously monitored (10 data points per second) as an increase in A_{520}.

2.3. Determination of MPTP opening in energised mitochondria

Simultaneous measurement of extramitochondrial [Ca^{2+}] and mitochondrial membrane potential was performed using Fura-6F (Molecular Probe, F-15178) and Rhodamine-123 (Molecular Probe, R22420) in a multiwavelength fluorimeter (Cairn Instruments). Excitation was at 340 and 380 nm for Fura-6F and 490 nm for Rhodamine-123 with 90° fluorescence emission detected by a photomultiplier using a 520 nm bandpass filter. A second photomultiplier detected 90° light scattering at 490 nm. Excitation filters were contained in a spinning wheel rotating continuously at 24 Hz. Liver mitochondria (1 mg/ml) were incubated at 30 °C within a stirred cuvette containing 3 ml assay buffer containing 120 mM KCl, 10 mM MOPS, 5 mM L-glutamate, 2 mM L-malate, 20 μM EGTA, 1 μM Fura-6F, 100 nM Rhodamine-123 and either 1 mM Pi or 1 mM Asi, pH 7.2. Additions of Ca^{2+} were made as required through an injection port.

2.4. siRNA-knockdown of the PiC and assay of MPTP opening in HeLa cells

HeLa cells were cultured as described previously (Ullah et al., 2006). The siRNA used against the human PiC was 5′-CUGGCGCA-CAUCACUAUAUdTdT-3′ and was obtained from Sigma Gynosis who