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Original article

Evaluation of Porin Interaction with Adenine Nucleotide Translocase and Cyclophilin-D Proteins after Brain Ischemia and Reperfusion

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Abstract

Objective (s)
Porin is a mitochondrial outer membrane channel, which usually functions as the pathway for the movement of various substances in and out of the mitochondria and is considered to be a component of the permeability transition (PT) pore complex that plays a role in the PT. We addressed the hypothesis that porin interacts with other mitochondrial proteins after ischemic injury.
Materials and Methods
For this purpose, we used *in vivo* 4-vessel occlusion model of rat brain and porin purification method by hydroxyapatite column. After SDS gel electrophoresis and silver nitrate staining, Western blotting was done for porin, adenine nucleotide translocase and cyclophilin-D proteins.
Results
Porin was purified from mitochondrial mixture in ischemic brain and control groups. Investigation of interaction of adenine nucleotide transposes (ANT) and cyclophilin-D with porin by Western blotting showed no proteins co-purified with porin from injured tissues.
Conclusion
The present study implies that there may not be interaction between porin, and ANT or cyclophilin-D, and if there is any, it is not maintained during the purification procedure.

Keywords: Ischemia, Mitochondrial permeability transition pore, Porins, Reperfusion

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Introduction

Mitochondria play a crucial role in cell death. These organelles can play a critical role in the progression of necrotic cell death by activation of phospholipases, nucleases and proteases (1, 2), and apoptotic cell death by releasing proapoptotic proteins, such as cytochorome C, apoptosis-inducing factor (AIF), and Diablo-SMAC from the intermembrane space into the cytosol in response to a number of death-promoting stimuli (3-5). The mitochondrial permeability transition pore (MPTP) is a nonspecific pore, permeable to all molecules of less than 1.5 kDa, and opens under circumstances of excessive load of calcium (6). In ischemia/reperfusion, the MPTP has been identified as a major participant in the mitochondrial pathways of cell death (6-11). During ischemia a decreasing in intracellular pH prevents MPTP opening (12). However, in the reperfusion phase there is a rapid increase of reactive oxygen species (ROS) formation and the pH returns to normal level, exciting MPTP opening and hence cell death (1).

The molecular identity of the mitochondrial permeability transition pore has not been established and its structure is controversial. The MPTP is assumed to be due to the formation of dynamic multiprotein complexes at outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) contact sites (13). Biochemical and pharmacological evidence show that porin or voltage-dependent anion channel (VDAC) in the OMM is a component of the mitochondrial permeability transition pore (14). During ischemia/reperfusion this protein is directly involved in mitochondrial dysfunction, neuronal impairment, apoptosis and necrosis (15, 16) and thereby could be a therapeutic target to prevent cellular degeneration (15).

Adenine nucleotide translocase (ANT) in IMM is other presumptive component of MPTP and is thought to play a dual role in the transport of ADP and ATP across the mitochondrial inner membrane and an important mediator of apoptosis (17, 18). It is likely that the Ca^{2+} requirement for the induction of the mitochondrial permeability transition pore opening might be due to the Ca^{2+} dependent interaction between cyclophilin-D (CyP-D) resident in the mitochondrial matrix space and ANT in inner mitochondrial membrane (19). Different investigations have shown that CyP-D can bind tightly to a VDAC/ANT complex and are constituents of the MPTP (20-24). In spite of these studies, VDAC and ANT- knockout mice studies has shown that the VDAC and ANT are non-essential components of the MPTP that may be dispensable for MPTP-associated cell death (25, 26). However, the molecular identity of the MPTP has remained uncertain.

In this study, we have isolated porin from an outer membrane of mitochondria by hydroxyapatite/celit chromatography. In order to confirm porin binding with ANT and cyclophilin-D after brain ischemia and reperfusion, porin was purified from mitochondrial proteins and then its binding with ANT and cyclophilin-D was confirmed by immunoblotting.

Materials and Methods

Rat brains mitochondria were prepared from male Wistar rats 18-30- days- old. Animal care and handling was performed in accordance with the rules approved by the local research council at University of Toronto, Canada.

Animal model

Surgery was done in two groups: ischemic and sham groups. Transient brain ischemia was produced using the *in vivo* 4-vessel occlusion model (27). Briefly, in this method on the first day the experiment animals were anaesthetized with halothane 2.0% and 70% nitrous oxide (N_2O) in oxygen (O_2), placed on a stereotaxic frame and the vertebral arteries were eletrocoagulated through the alar foraminae of the first cervical vertebrae. On the following day, a temporary occlusion was produced by clamping of both common carotid arteries for 30 min and so completing

the 4-vessel occlusion (carotid+vertebral). Animals that did not lose the righting reflex or convulsed during the ischemic insult were excluded from the experiments. Reperfusion was introduced by re-infusing the shed blood and by releasing the clamps placed around the carotid arteries. Animals were sacrificed after 60 min of reperfusion and the brain was removed. Sham-operated rats were treated the same as controls.

Porin purification

Rat brain mitochondria were prepared from Wistar rats 18-30 olds, as described by De Pinto *et al* (28) and Perez Velazquez *et al* (15). Brain tissue was homogenized in 0.25 M sucrose solution, containing 10 mM Tris-HCL, 0.5 Na EDTA, pH 7.4, and centrifuged at 2000 g for 3 min. Supernatants were separated and centrifuged at 12,500 g for 8 min, and the pellet resuspended in a solution containing 3% Ficoll, 0.12M mannitol, 0.03 M sucrose, 25 µM Na EDTA, pH 7.4. The solution containing the resuspended pellet was layered on to a similar volume of a solution containing 6% Ficoll, 0.24 M mannitol, 0.06 M sucrose and 50 µM Na EDTA and centrifuged at 11,500 g for 30 min. The mitochondrial pellets were then resuspended in the initial solution and centrifuged at 12,500 g for 10 min. All procedures were carried out at 4 °C. The protease inhibitors leupeptin (1 mM), phenylmethyl-sulfonyl fluoride (PMSF, 0.23 mM) and pepestatin were added to all solutions. Mitochondrial proteins were then extracted from isolated mitochondria using a solubilization buffer containing 3% Triton X-100, Tris–HCl, EDTA into hydroxyapatite/celite column. Protein collections were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Developing of gel was done by silver nitrate kit (Amersham Biosciences). Briefly at first the gel was fixed with fixing solution (ethanol and glacial acetic acid) for 30 min. After washing, sensitizing solution (ethanol, glutardialdehyde, sodium thiosulphate, and sodium acetate) was added for 30 min and after washing, silver solution was added for 20 min and was developed with developing solution (sodium carbonate and formaldehyde). The purification process and silver nitrate staining were repeated three times.

Immunoblotting

The proteins in mitochondria and cytosol were separated by 10% SDS-PAGE and were electrophoretically transferred to PVDF membranes (Bio-Rad). Membrane was probed with the indicated antibodies at 4 ºC overnight. The primary antibodies were either a 1:10000 dilution of rabbit VDAC1 [VDAC1 (N-18), USA], ANT (University of Bristol, UK), and cyclophilin-D (abcam, USA). Detections were carried out by alkaline-phosphatase conjugated goat anti-mouse IgG (1:3000) (Promega, Madison, USA) and processed using the protoblot II alkaline- phosphatase system (Promega, Madison, USA). The immunoblotting study was repeated three times.

Results

There is particularly strong evidence that porin is one of component of the PT pore (9, 14). Accordingly, a porin purification was done for the identification of porin binding proteins as candidate constituents of the pore.

We examined mitochondrial porin at protein levels in normal and ischemic brains. In order to identify mitochondrial porin, a Triton X-100 extract of mitochondrial protein was subjected to chromatography on a hydroxyapatite/celite column. As shown in Figures 1 and 2, porin immunoreactivity was evident as a single 30 kDa band detected by Western blot analysis. The mitochondrial porin was successfully overexpressed in sham control group mitochondrial proteins fraction (second fraction) (Figure 1). In the ischemia group, porin specific band was observed with a polyclonal anti-porin antibody in second fraction of porin purification process (Figure 2).

In order to study the interactions of porin with ANT and cyclophilin-D, we used Anti–ANT and Anti cyclophilin-D for total mitochondrial proteins, first and second fractions after the chromatography. ANT was strongly expressed in the mitochondrial proteins mixture and can be seen that a single 30 kDa band was recognized in Western blots of

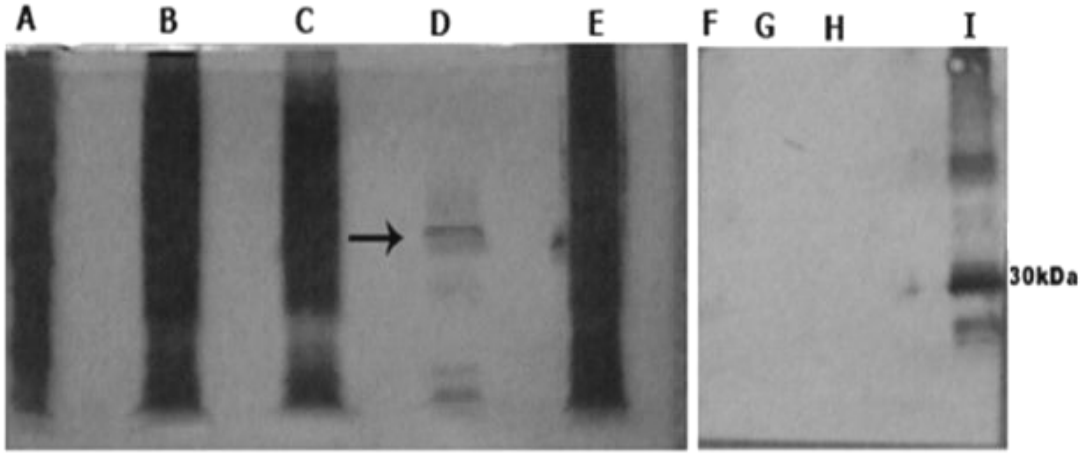

Figure 1. Silver staining (left) and Western blotting (right) of mitochondrial proteins from rat brain of sham group. Lanes A and F are rainbow marker. Lanes B and C are first fraction of mitochondrial proteins after chromatography. Lanes D, G, and H are second fraction of mitochondrial proteins after chromatography. Arrow show porin purification after second fraction. Lanes E and I are mitochondrial proteins mixture. Anti ANT has recognized ANT protein in mitochondrial proteins mixture (lane I) in level of 30 kDa.

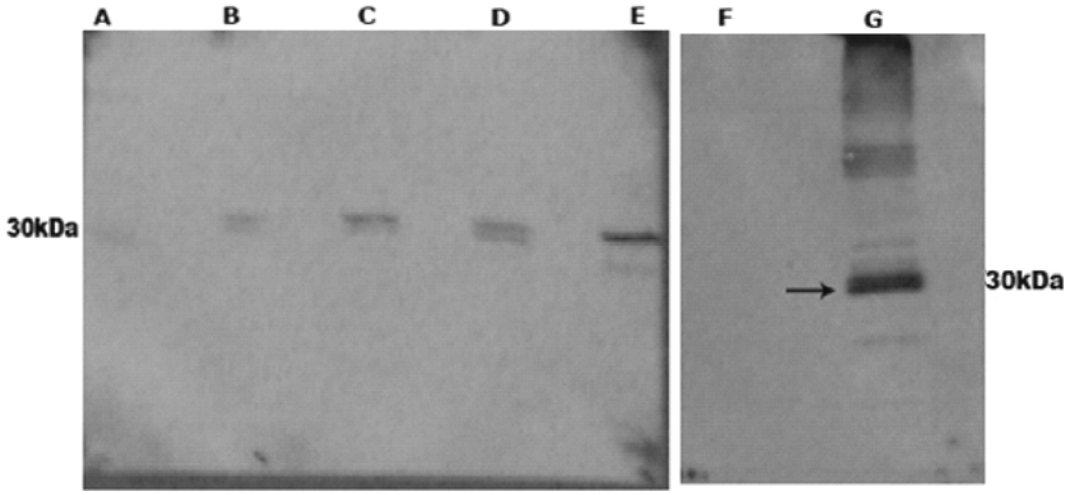

Figure 2. The anti-peptide polyclonal anti-porin antibody has recognized porin (left) and anti ANT has recognized ANT protein in Western blots after brain ischemia. Lane A is rainbow marker, lanes B, D, and F are second fraction of mitochondrial proteins after chromatography. Lane C is first fraction of mitochondrial proteins after chromatography. Lane E and G are mitochondrial protein mixture. Arrow shows ANT in level of 30 kDa in mixture of mitochondrial protein. After porin purification interaction porin and ANT did not occur (lane F).

total mitochondrial proteins in sham control group (Figure 3, lane C). It was not expressed in the mitochondrial protein mixture after ischemia/reperfusion (Figure 2). Western blotting showed that cyclophilin-D was expressed in mixture of mitochondrial proteins in ischemic brains (Figure 4, lane G) and virtually no band was seen in first and second fractions of the mitochondrial proteins in ischemic brain (Figure 4, lane E and F). These data did not reveal ANT and cyclophilin-D proteins contemporary with porin in second fraction of porin purification process and so no interactions have occurred between these proteins in either control and ischemic brain.

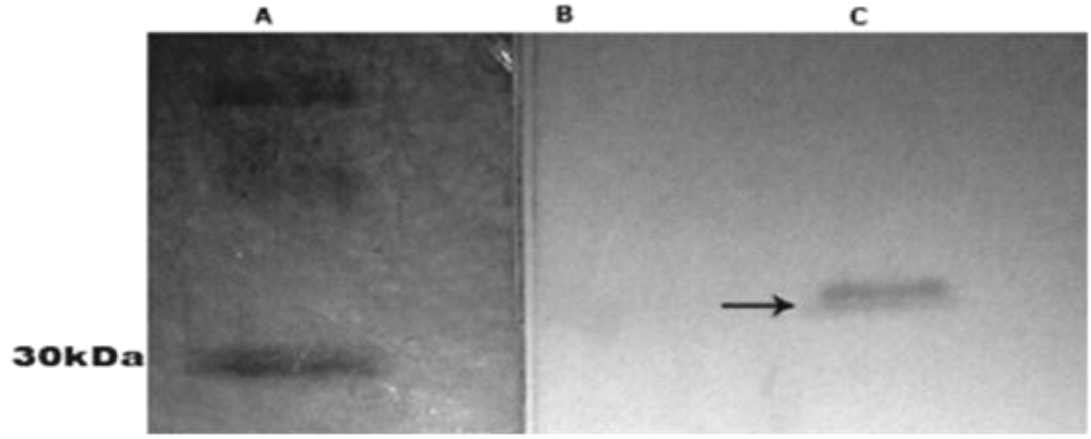

Figure 3. Silver staining (left) and Western blotting (right) of mitochondrial proteins from normal rat brain. Lane B is rainbow marker, lane A is second fraction of mitochondrial protein after chromatography. Porin purification is seen in the level of 30 kDa. Lane C is mixture of mitochondrial protein. Anti-ANT has recognized ANT protein (Arrow), and is not seen in the second fraction.

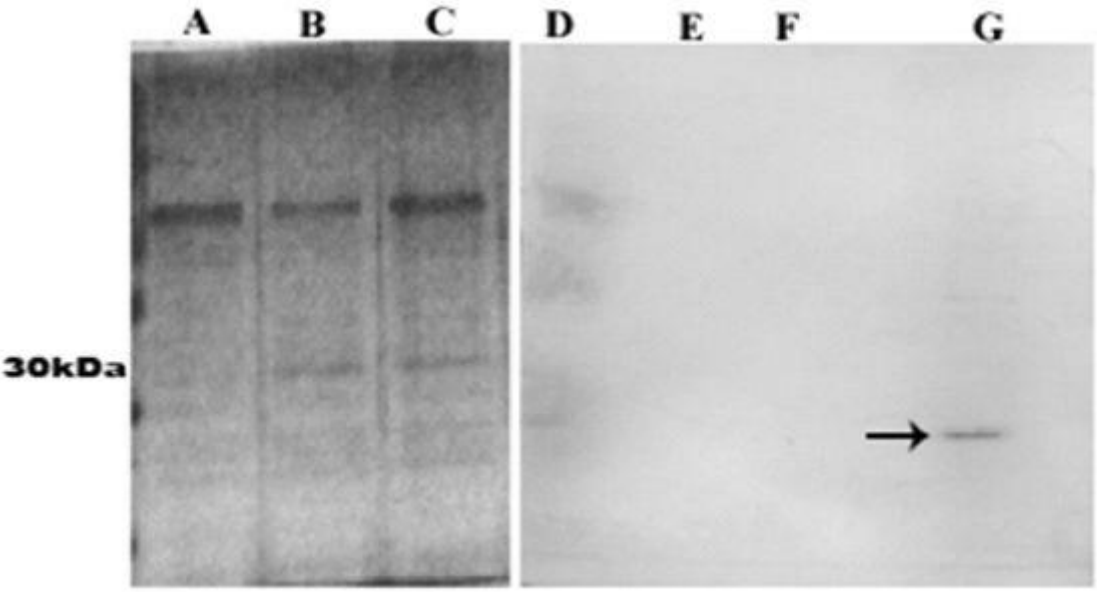

Figure 4. Silver staining (left) and Western blotting (right) of mitochondrial proteins from rat brain. Lanes A and B are second fraction of mitochondrial proteins from ischemic rat brain and lane C is second fraction of sham operated brain. Porin purification is seen in the level of 30 kDa. Lane D is rainbow marker. Anti cyclophilin-D has recognized cyclophilin-D protein in mitochondrial protein mixture of ischemic rat brain (lane G, arrow) and lanes E and F are the first and second fractions of mitochondrial proteins from ischemic rat brain. Anti cyclophilin-D has not recognized cyclophilin-D in either fraction. After porin purification interaction of porin and cyclophilin-D did not occur (lane F).

Discussion

In this study, we have evaluated porin purification from mitochondria of rat brain by hydroxyapatite/celit chromatography after *in vivo* ischemic/reperfusion, and its analysis by Western blotting to define interaction of porin with ANT and cyclophilin-D. Attention has been paid to porin because of some studies suggesting its critical role in MPTP structure (9, 14, 16, 20, 29).

In the present study, porin was purified according to the method of de Pinto by hydroxyapatite/celite chromatography. Porin solubilization with respect to total mitochondrial membrane protein is more efficient with the more hydrophobic detergents

such as Triton (30). Detergent in our study was Triton X-100 and porin purification was done efficiently.

Our study did not show binding of porin with ANT and cyclophilin-D in the first or second soluble fractions; however Western blot technique confirmed existence of these proteins in the mitochondrial mixture. These data do not demonstrate the notion of a functional complex between these components. There are various evidence that VDAC, ANT and CyP-D are constituents of the PT pore. Studies using anti-VDAC antibodies have provided experimental evidence for a direct role of the VDAC in the MPTP (31). Perez Velazquez *et al*. showed that porin is a crucial protein involved in mitochondrial and cell dysfunction, and it is conceivable that antibodies can be used as therapeutic agents (29). Under ischemic/reperfusion of high matrix $Ca^{2+}$and/or oxidative stress (2) porin interacts with ANT from IMM and cyclophilin-D from mitochondrial matrix and form MPTP (9-11, 13, 20). It is thought that these proteins may be cross-linked through protein-protein interaction by formation of disulfide bridges (32).

More direct evidence show that the ANT might bind CyP-D were provided through the use of a CyP-D affinity column (glutathione S-transferase (GST) pull-down assays) (6). The role of Cyp-D in regulating the MPT is based on the observation that cyclosporin A (CsA), a specific inhibitor of the cyclophilin family, blocks the MPT. 'Pulldown' experiments have revealed that cyclophilin-D binds specifically and tightly to a VDAC/ANT complex and it does so even in the presence of cyclosporin A (20). Reconstitutions of Ca^{2+}activated, CSA-inhibited PT pore activity from purified ANT, VDAC and CyP-D in liposomes (20, 33) and black lipid membranes (34) have confirmed that these components form the PT pore and binding of the voltage dependent anion channel (VDAC) to ANT and CyP-D. However, our research similar to some other studies (21, 35, 36) have suspected this hypothesis. Differences between these studies may reflect the different detergent solubilisation routine and kind of mitochondria. Woodfield *et al* (21) and present study used Triton X-100 and liver mitochondria and brain mitochondria respectively rather than the heart mitochondria and chaps detergent for solubilisation used by Crompton *et al* (20). Today there is some evidence that ANT is not normally the major pore forming component of the MPTP, but rather plays only a regulatory role (2). Whether ANT has an obligatory pore-forming role has been questioned (19). Cyclophilin-D facilitates the pore formation by its the peptidyl–prolyl cis–trans isomerase activity (6) and probably is mitochondrial permeablization modulating and defending (37). Recently Baines *et al* has shown that mitochondria lacking all isoforms of porin exhibit normal pore opening and are still sensitive to ischemia/reperfusion (25). Furthermore genetic studies have shown that mitochondria lacking VDAC1 still exhibited a normal cyclosporine-sensitive MPTP response (38).These evidence shows that probably porin is not an essential component of the MPTP.

Our study does not suggest co-purification porin with ANT and cyclophilin-D. Probably after ischemia/reperfusion interaction between these proteins does not occur or porin purification procedure separates contact sites between these proteins. It seems that more documents is needed for determination of MPTP structure. Recently Halestrap proposed that MPTP is enhanced by an interaction of the mitochondrial phosphate carrier (PiC), a ~30 kDa inner mitochondrial membrane protein, with the "c" conformation of the ANT (36). However, more evidence is needed to prove this model.

Conclusion

Considering our findings in the present study, we can conclude that there may not be interaction between porin and ANT or cyclophilin-D following brain ischemia/reperfuion, or if there is any, it is not maintained during the purification procedure.

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