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Classification of rat liver mitochondrial porousness progress pore by utilizing mitochondrial expanding measure

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Abstract

The mitochondrial permeability transition (MPT) is considered to contribute substantially to the regulation of normal mitochondrial metabolism and plays as an important mediator of cell death. MPT is regulated in a tissue specific manner. The importance of this work is highlighted by the fact that knowledge regarding liver MPT may serve as important determinants of the physiological state of the liver. Our results suggest that liver mitochondria exhibit a cyclosporin A sensitive, exogenous calcium mediated full scale MPT opening, indicative of classical MPT. Transmission electron microscopy suggests a homogeneous mitochondrial population. Complex II substrate (succinate) in comparison to complex I substrate (NADH) induces increased MPT opening. Reactive Oxygen Species induces liver mitochondrial MPT opening and is abrogated by ascorbic acid and -tocopherol. Liver MPT is sensitive to redox modulation. Reducing and oxidizing agents decrease and increase MPT opening respectively. MPT of liver mitochondria show typical pH dependence, acidic and alkaline pH induces MPT closure while, maximal MPT opening and calcium sensitivity was achieved at a pH range of ~7.2-7.4. ATP inhibited, while ADP activated MPT opening. Thus, our study demonstrates that all the major MPT functions in normal liver mitochondria could be determined by simple mitochondrial swelling assay under well-defined conditions, thereby suggesting its application in therapeutic diagnostics.

Keywords: Mitochondria, mitochondrial permeability transition, swelling assay, oxidants, reductants, calcium, cyclosporin A, pH.

INTRODUCTION

Mitochondrial permeability transition (MPT) is the opening of a permeation pathway of the inner mitochondrial membrane allowing the diffusion of solutes of molecular mass up to ~1500 Da, caused by an opening of specific nonselective proteinaceous pores in the inner mitochondrial membrane (Paolo et al., 2006; Crompton, 1999). Minimum molecular constituents of the regulated MPTP in mammals are believed to be the voltage dependent anion channel (VDAC),

The adenine nucleotide translocators (ANT) and cyclophilin D (Yoshida and Takshi, 2005) . MPT has recently attracted renewed attraction because of its proposed role in the regulation of normal mitochondrial metabolism and apoptosis (Adam and Lech, 2002; Jae et al., 2003). Specific features of MPT show significant tissue-specific variability (Brustovetsky and Dubinsky, 2000; Andreyev and Fiskum, 1999). Increased permeability of the inner membrane is initiated by an increased level of intramitochondrial Ca^{2+} and is regulated by multiple effectors, including inorganic phosphate, the redox state of pyridine nucleotides and thiols (oxidative stress), membrane potential, cyclosporin A, and other factors (Fontaine et al., 1998; Balaban et al., 2003; Mario et al., 2005; John and Ekhson, 2006). The most significant and well documented deviation from classical MPT was reported in brain tissue (Berman et al., 2000). The MPT has many recognized modulators and inhibitors.

Equal contribution by both authors

Abbreviations: Mitochondrial permeability transition (MPT), arsenic trioxide (ATO), dithiothreitol (DTT), reactive oxygen species (ROS), ascorbic acid (AA), calcium (Ca^{2+}), cyclosporin A (CsA).

MPT is also involved in the regulation of apoptosis. Mitochondrial proapoptotic factors such as cyt c, apoptosis inducing factor, and Smac/Diablo are normally confined to the mitochondrial matrix. Once released, supposedly through the MPT into the cytosol, cyt c binds with Apaf-1 which prompts the activation of caspases in the presence of ATP/dATP (Petronilli et al., 2001). MPT may be an effector mechanism that could explain the involvement of mitochondria in many pathological condi-tions and high prevalence diseases for e.g. myocardial ischemia-reperfusion injury, liver diseases, neuropathy and muscular dystrophies (Weiss et al., 2003). The MPT attracted renewed attention because of its proposed roles in many models of apoptosis (Watts and Jeffrey, 2003). Hence, the regulation of MPT needs to be studied in depth. Until now, to the best of our knowledge a complete functional characterization of liver mitochondrial MPT has not been performed by using a simple assay method. In the present study, we attempted to characterize the major functions of the liver mitochondrial MPT by using spec - trophotometric assay of mitochondrial swelling in details.

Experimental procedures

Materials

Calcium chloride ($CaCl_2$), cyclosporin A (CsA), bovine serum albumin (BSA), arsenic trioxide (ATO), potassium dichromate ($K_2Cr_2O_7$), reduced glutathione (RG), 3' dithiothreitol (DTT), ethylene glycol- bis(2- aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), succinic acid, Bradford reagent and phenylmethylsulfonyl-fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraaceticacid (EDTA), N-2-hydroxy-ethyl-pipe-razine-N-2-ethanesulfonic acid (HEPES) buffer, reduced nicotinamide adenine dinucleotide (NADH) and sucrose were purchased from Hi media Laboratories Pvt. Ltd. (Mumbai, India). Unless otherwise mentioned; all other chemicals were purchased from Sigma (St. Louis, MO, USA).

Animals

Male Sprague Dawley rats (200 – 250 g) were maintained following the CPCSEA guidelines and with prior permission from the Insti-tute's Animal Ethics Committee. Animals were maintained in the Institute's climate controlled (22 ± 1ºC with 12 h dark and light cycle) animal house facility and were allowed free access to food (standard laboratory rat-chow) and water. Rats were sacrificed by decapitation and liver was quickly excised.

Isolation of rat liver mitochondria

Excised liver from Sprague Dawley rat was chopped in ice- cold homogenization buffer pH 7.5 (250 mM sucrose, 0.1 mM EDTA and 20 mM HEPES-HCl pH 7.4, 0.1 mM PMSF, 10 mM KCl, 1.5 mM $MgCl_2$) and centrifuged at 1,000 *g* for 10 min at 4^0C. Mitochondria were isolated by subjecting the supernatant to centrifugation at 17,000 *g* for 10 min at 4^0C. The pellet was resuspended in the isolation medium (1 g tissue/10 ml media) and then centrifuged at 17,000 *g* for 10 min at 4^0C and isolated mitochondria were resuspended in a minimal volume of homogenizing buffer pH 7.5 (Lass et al., 1997, Paul et al., 2007). Fresh mitochondria were used for each experiment. Protein concentration was determined by following the Bradford method, using BSA as standard (Bradford, 1976). The isolated mitochondria were characterized by respiratory ratio, succinate dehydrogenase activity and transmission electron microscopy (David et al., 1973).

Mitochondrial swelling assay

The sensitivity of the mitochondrial pore transition pore was deter-mined by studying the rate of change in absorbance at 540 nm under energized and deenergized condition using Perkin Elmer (Lambda 25, UV- Vis) spectrophotometer (Juanita et al., 2005). Mitochondria (500 g/ ml) were incubated at 30^0C in respiration buffer pH 7.2 (125 mM sucrose, 50 mM KCl, 5 mM HEPES, 2 mM KH_2PO_4 and 1 mM $MgCl_2$) and were energized with 20 mM succinate to support swelling using varying concentrations of Ca^{2+} and CsA as positive and negative control.

Mitochondrial swelling was also confirmed by transmission elec-tron microscopy (TEM). Mitochondrial samples (1 mg/ml) intended for TEM were run using the same protocols as for the spectropho-tometric method. Treated/untreated samples were rapidly chilled and centrifuged in a Sigma 1-14 centrifuge, 7000 *g* for 7 min, fixed with modified karnovsky's fixative, washed in phosphate buffer twice and post fixed in 1% osmium tetraoxide. Samples were progressively dehydrated, embedded in araldite, ultrathin sections (60 - 80 nm) were cut, stained in uranyl acetate and lead citrate and then examined by a Philips CM-10, TEM) operated at 80 kV (Juanita et al., 2005).

Statistical analysis of data

Experiments were repeated for at least three times. Results were represented as data expressed as Mean ± S.E., n = 5; otherwise representative data are presented.

RESULTS

Isolated mitochondria were characterized using TEM, respiratory ratio and activity of different electron transport chain (ETC) complexes (data not shown). Mitochondrial isolation for obtaining intact and functional mitochondrial MPT is very tricky and thus, was standardized using different methods of mitochondrial isolation. The optimum results thus obtained were set for further experiments. Isolated rat liver mitochondria (500 µg/ml) was added to respiration buffer and incubated with varying concen-trations of Ca^{2+}, at 30^0C. Addition of Ca^{2+} to liver mito-chondrial suspension resulted in rapid, almost instanta-neous, decrease in light scattering (Figure 1A). The decrease in light scattering was more pronounced with higher Ca^{2+} doses, a dose-dependent effect. The amount of swelling increased from 5 - 10 % (5 µM Ca^{2+}) to about 75 - 80% (400 µM Ca^{2+}). Chelating agents like EGTA are supposed to chelate the intracellular Ca^{2+} ions and thus prevents the Ca^{2+} induced opening of the MPT. Sequestering of the cytosolic Ca^{2+} by EGTA (0.1 – 100 M) resulted in a recovery of the light scattering value (~6 - 33% respectively) (Figure 1B) which was counte-racted by the large amount of Ca^{2+} (data not shown). Mitochondria need to be incubated for at least 1 min to

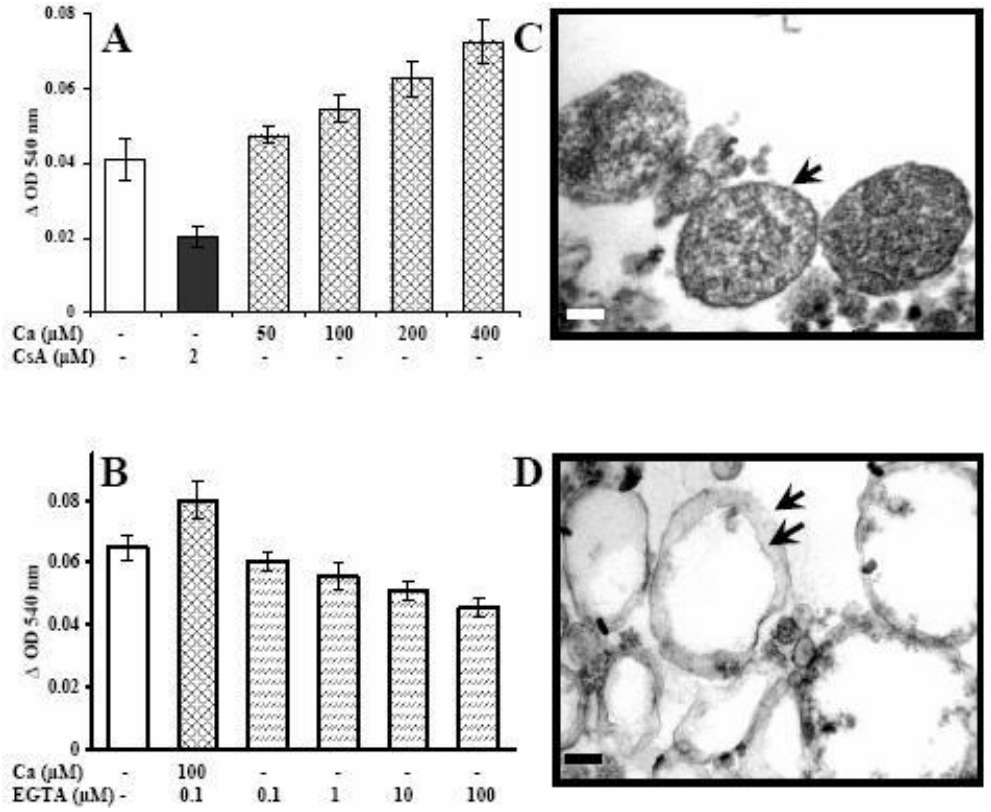

Figure 1. Transient calcium-induced permeability transition of respiring liver mitochondria. (A) Bar graphs representative of changes in absorbance (OD 540 nm) up to 8 min, at 30^{o}C. A decrease in scattering reflects mitochondrial swelling. Mitochondria (1 mg/ml) were suspended in respiratory buffer pH 7.2 containing 20 mM succinate to induce energy dependent swelling. Ca^{2+} (5-400 µM) was added in the presence and absence of CsA (2 µM). (B) Effect of Ca^{2+} sequestering by an excess of EGTA (0.1-100 µM) on mitochondrial swelling. Mitochondria (1mg/ml) were suspended in respiratory buffer pH 7.2 at 30°C and incubated with EGTA for 1 min and then 20 mM succinate was used to energize the mitochondrial suspension and mitochondrial swelling was observed at 540 nm at 30°C. In EGTA treated mitochondria no external Ca^{2+} was added. Data expressed as Mean ± S.E., *n* = 4. (C) Determination of mitochondrial swelling by TEM. Experiments for electron micrographs were run as above, with a protein concentration (1mg/ml). Untreated mitochondrial preparations fixed with karnovsky fixative, post fixed in osmium tetroxide followed by staining with uranyl acetate and lead citrate were examined with a Philips Morgani, (CM-10.TEM) operated at 60-80 hz. Micrograph of isolated rat liver mitochondria illustrates the purity of mitochondrial fraction. Arrows indicate examples of mitochondria with normal dense structure with closely apposed inner and outer mitochondrial membrane. Bar, 0.25 µm. (D) TEM image of isolated rat liver mitochondria after 5 min Ca^{2+} (400 µM) exposure. Arrows indicate swollen mitochondria, with separated inner and outer mitochondrial membrane, ruptured cristae, and non-dense matrix. Whole mitochondrial population undergoes swelling. Data shown is representative of three independent experiments. Bar, 0.25 µm.

allow EGTA to chelate the internal reserve of Ca^{2+}. As external Ca^{2+} was not added in the EGTA treated samples, EGTA could only chelate the intracellular pool of calcium which compromises a non significant amount and thus a small decrease in absorbance was observed (Figure 1B). The Ca^{2+} induced swelling was further confirmed using TEM. TEM experiments suggests that mitochondrial population are homogenous regarding the gating potential at which pore opening occurs in different mitochondrial subpopulations as evident from Figures 1C and 1D. All mitochondria appeared swollen and exhibited a loss of the typical cristae structure and no intermediate state could be detected. CsA displayed a protective effect and reduce mitochondrial swelling. Liver mitochondria show CsA sensitive MPT. Titration with Ca^{2+} showed CsA insensitivity and vice versa (data not shown). To specifi-cally characterize MPT using the spectrophotometric method in liver mitochondria, we characterized the basic features of the MPT.

Substrate dependence of the MPT opening in mammalian liver mitochondria was studied. NADH and succinate were used as complex I and II substrate respectively, to

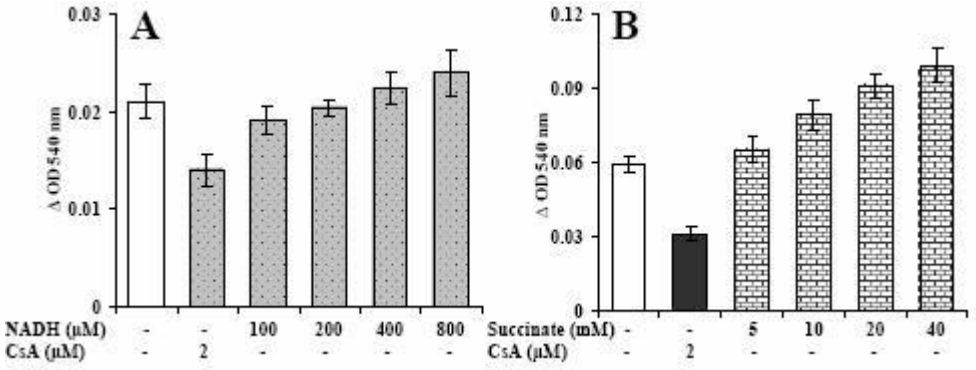

Figure 2. Effect of complex I and II respiratory substrates on Ca^{2+} (200 μM) induced mitochondrial MPT induction. **(A)** Mitochondria (1 mg/ml) were suspended in respiratory buffer pH 7.2 at 30°C. NADH (200-800 μM) (complex I substrate) was used to induce mitochondrial swelling and was observed spectrophotometrically at 540 nm at 30°C. As a control CsA (2 μM) was used. Data shown is representative of five independent experiments and expressed as Mean ± S.E. (**B)** Experiment was conducted under similar conditions only succinate (5-40 mM) (complex II substrate) was added in place of NADH and the mitochondrial swelling was observed. The amplitude of swelling was observed for 8 min.

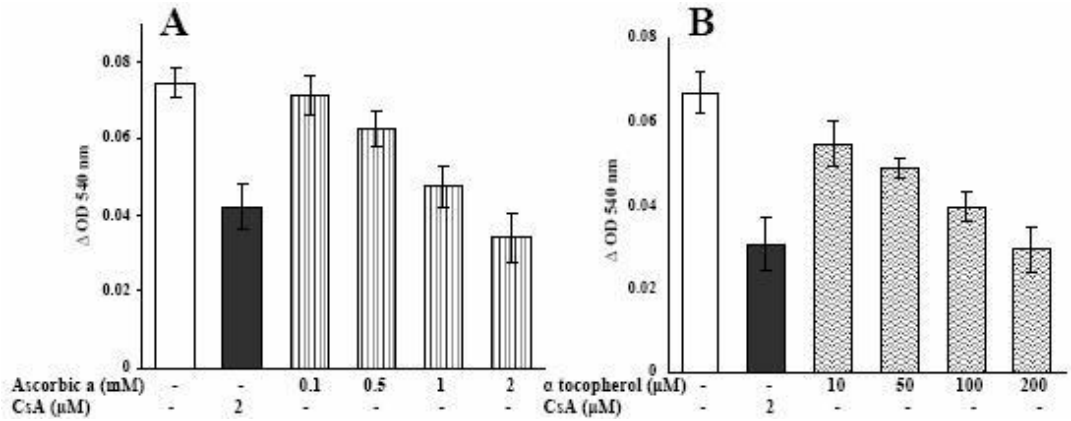

Figure 3. Effect of antioxidants on rotenone (5 μM) mediated mitochondrial MPT induction. (A) Mitochondria (1 mg/ml) were energized with succinate (20 mM) in respiration buffer pH 7.2. at 30°C. MPT status in presence of ascorbic acid (100 μM - 2 mM) was studied on rotenone-induced MPT opening. Control experiment here denotes maximal rotenone-induced mitochondrial swelling. Ascorbic acid scavenges the rotenone-induced ROS and abrogates MPT induction. CsA (2 μM) was used to check the functioning of MPT. Data expressed as Mean ± S.E., *n* = 5. (B) Effect of antioxidant tocopherol on the MPT opening was studied using different concentrations (10-200 μM). Mitochondria (1 mg/ml) were similarly treated as above and succinate was used to energize the mitochondrial suspension.

check the effectivity of different substrates on MPT opening. The probability of MPT in mammalian liver mitochondria was higher when substrates are provided to respiretory complex II rather than complex I. NADH shows poor response (Figure 2A), while succinate induced full MPT opening (Figure 2B). Mitochondrial ETC-mediated ROS have been implicated in membrane potential ($_m$) depolarization and MPT opening and the effect of antioxidants on rotenone- induced opening of liver mitochondrial MPT was studied. Ascorbic acid and tocopherol prevent the swelling of mitochondria in dose dependent manner as shown in Figure 3 (A, B) respectively. The liver MPT was further characterized by studying the effect of oxidizing agents that are known to react with vicinal thiols of ANT of open MPT pore. Arsenic trioxide (ATO) and potassium dichromate were used as oxidizing agents and their effect on mitochondrial swelling was observed Figure 4 (A, B). Similar trend was attained with both the oxidant.

Similarly, reducing agents e.g. dithiothreitol (DTT) and reduced glutathione (RG) were used as reducing agents and the MPT status was studied in their presence. RG and DTT exhibited decrease in MPT opening (~ 42 and 28% for 100 M of RG and DTT respectively) Figure 4 (C, D) respectively. It is interesting to note that, these re-

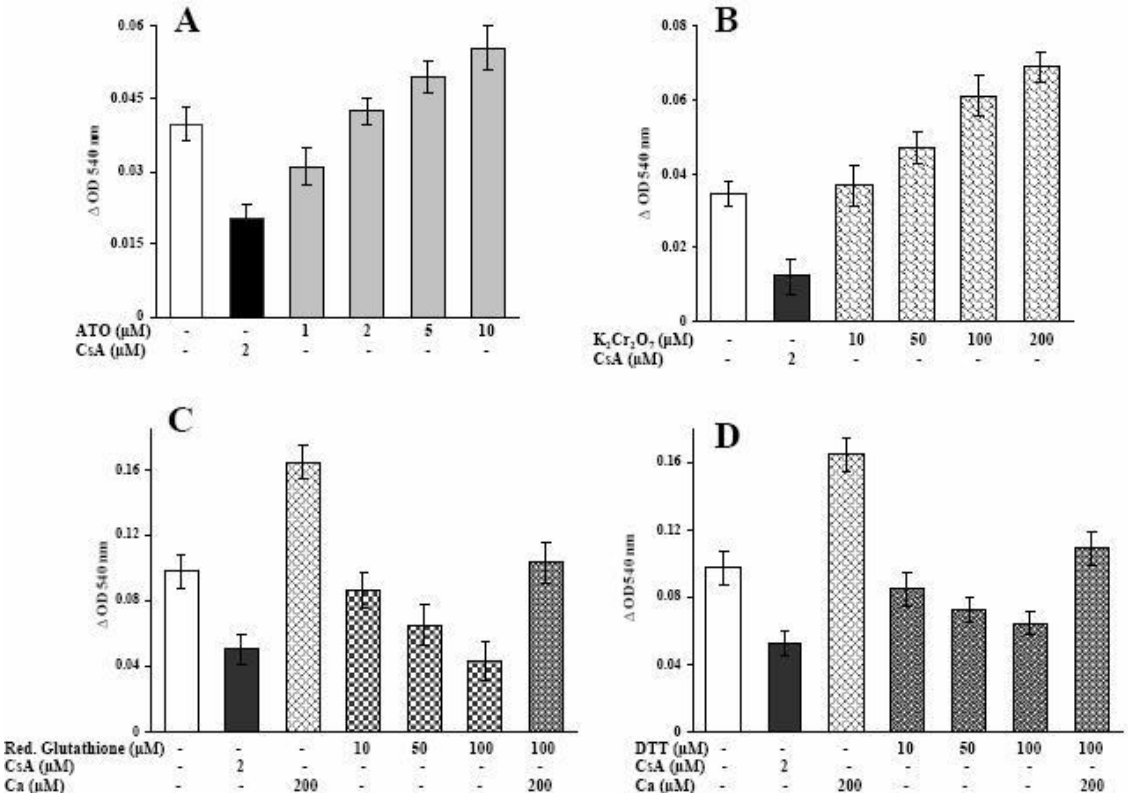

Figure 4. Effect of oxidants and reductants on succinate induced mitochondrial MPT induction. (A) Mitochondria (1 mg/ml) were suspended in respiratory buffer pH 7.2, 20 mM succinate was used to energize the mitochondrial suspension and the effect of ATO (1-10 μM) on mitochondrial swelling was observed spectrophotometrically at 540 nm at 30°C. CsA (2 μM) was used to check the functioning of MPT. (B) Effect of oxidant ($K_2Cr_2O_7$) on the functioning of MPT. Mitochondria (1 mg/ml) were suspended in respiratory buffer pH 7.2, 20 mM succinate was used to energize the mitochondrial suspension and the effect of $K_2Cr_2O_7$ (10-200 μM) on mitochondrial swelling was observed spectrophotometrically at 540 nm at 30°C. Data expressed as Mean ± S.E., *n* = 4. (C) Mitochondria (1 mg/ml) were treated similarly and succinate was used to energize the mitochondrial suspension and the effect of reducing agents RG (10-100 μM) on mitochondrial swelling was observed. Effect of RG (100 μM) on the Ca^{2+} induced MPT was also observed. CsA (2 μM) was used to check the functioning of MPT. (D) Mitochondria were similarly treated and succinate was used to energize the mitochondrial suspension and the effect of reducing agent DTT on mitochondrial swelling was observed. Effect of DTT on Ca^{2+} induced MPT was also checked. Data expressed as Mean ± S.E., *n* = 4.

reductants nullify the effect of Ca^{2+} on MPT opening to greater extent than untreated case. The pH of respiratory medium plays an important role in opening and closing of MPT. Lower pH (6.0 - 7.0) blocks MPT while higher extra cellular pH (7.4 - 8.0) does not increase the MPT opening (Figure 5). Our result suggested that MPT opens maximally at pH 7.2 - 7.4. The calcium sensitivity also lies in the same pH range. Adenine nucleotides significantly modulate the MPT status. Kinetics of MPT opening was changed by the addition of 50 - 250 μM ATP. ATP causes closing of MPT in a dose dependent manner (Figure 6A). On the contrary, ADP causes MPT pore opening (Figure 6B). Thus we can say that liver mitochondrial MPT was fully characterized using the spectrophotometric method.

DISCUSSION

MPT opening has emerged as either an accomplice or a central mechanism in the pathophysiology of a large number of diseases (Weiss et al., 2003), thus demanding a proper assay method. Liver is a very important organ responsible for detoxification of several toxic compounds. Therefore elucidation and characterization of the mechanism of liver mitochondrial MPT modulation is important. To check for functional MPT, different concentrations of Ca^{2+} were used to induce MPT. The mitochondrial swelling was also greatly inhibited in the presence of 2 μM CsA. Thus liver mitochondria possess Ca^{2+} sensitive, CsA sensitive MPT as also evident from other studies (Han et al., 2001). Ca^{2+} mediated MPT opening was sensitive towards complex II substrate (succinate) as compared to complex I substrate (NADH). Though Fontaine et al, 1998 reported that increased electron flux through the complex I favors MPT opening at a given Ca^{2+} load (Fontaine et al., 1998). The accessibility of external NADH to the mitochondrial matrix may be low

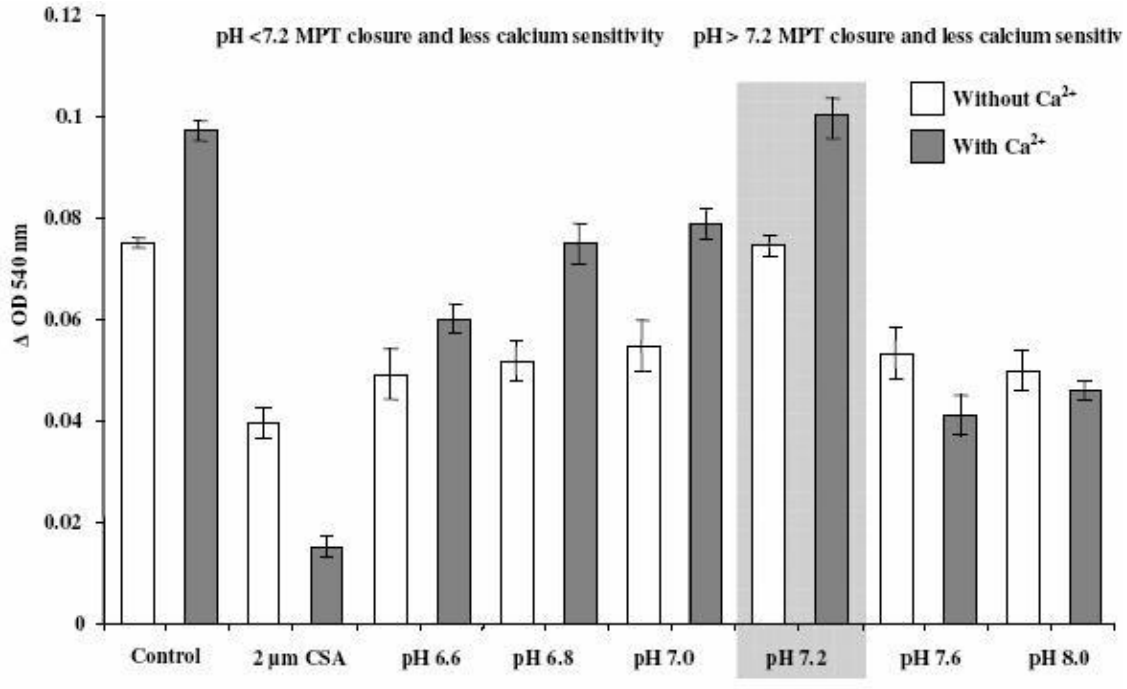

Figure 5. Effect of extramitochondrial pH on succinate induced mitochondrial MPT induction. Mitochondria (1 mg/ml) were suspended in phosphate buffer of different pH range (pH 6.6-8.0), 20 mM succinate was used to energize the mitochondrial suspension and both calcium induced and non-induced mitochondrial swelling was observed spectrophotometrically at 540 nm at 30°C. The calcium sensitivity was studied at different pH. Data expressed as Mean ± S.E., *n* = 5.

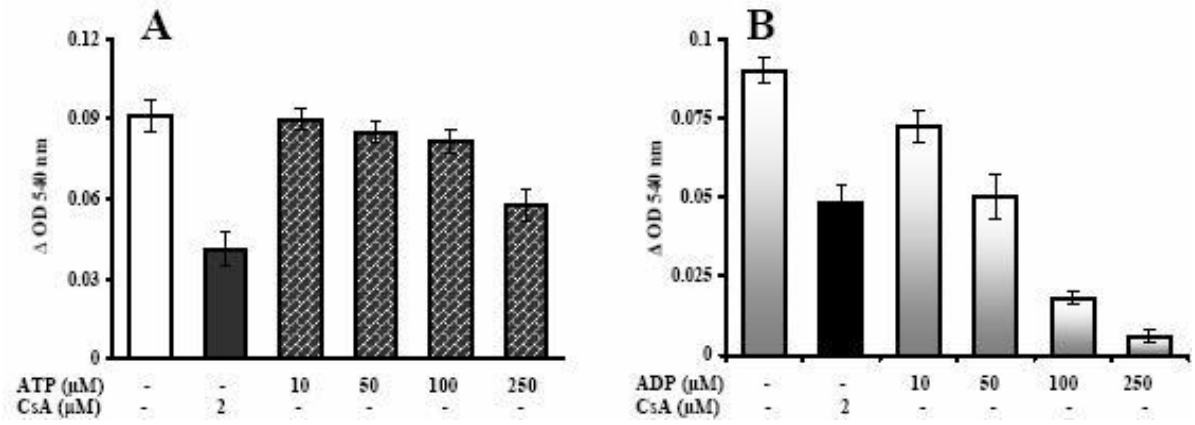

Figure 6. Effect of adenine nucleotides on succinate induced mitochondrial MPT induction. (A) Mitochondria (1 mg/ml) were suspended in respiration buffer pH 7.2 and 20 mM succinate was used to energize the mitochondrial suspension and the effect of Adenine nucleotide triphosphate (ATP) (10-250 µM) on mitochondrial swelling was observed spectrophotometrically at 540 nm. Data expressed as Mean ± S.E., *n* = 3. (B) Mitochondria were similarly treated and the effect of adenine dinucleotide triphosphate (ADP) (10-250 µM) on mitochondrial swelling was observed spectrophotometrically at 540 nm. CsA (2 µM) was used to check the functioning of MPT. Data expressed as Mean ± S.E., *n* = 3.

may lead to low complex I mediated energization. The role of Ca^{2+} on MPT of liver mitochondria induction was further illustrated by using EGTA which chelates/ depletes the physiological pool of matrix Ca^{2+} which is an essential permissive factor for MPT opening. Antioxidants like ascorbic acid and tocoperol are reported to scavenge ROS in the cell. Hence we used rotenone a standard ROS generator and operated by MPT opening

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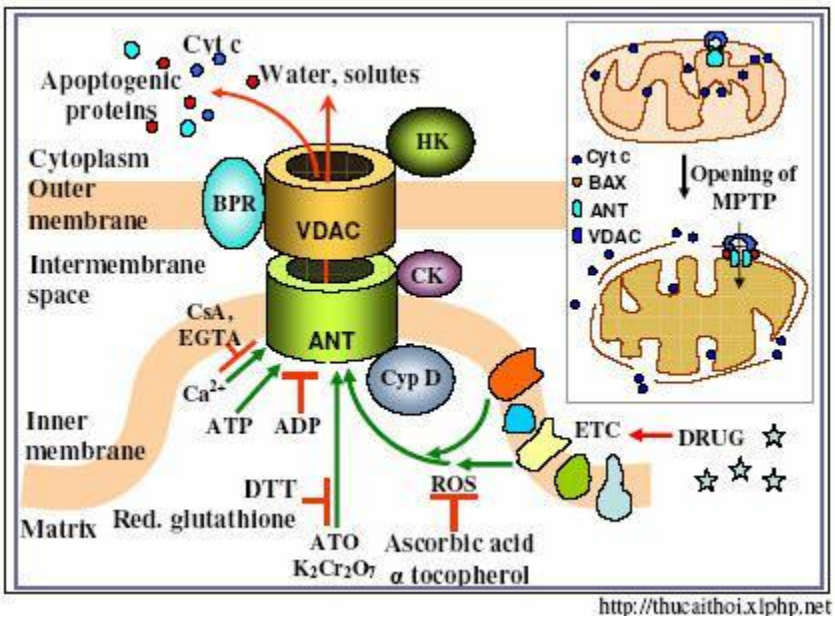

Figure 7. Cartoon model of the effect of several factors on liver mitochondrial MPT induction. The model shows components of MPT and the negative and positive modulation of MPT by different factors. Inset shows the phenomenon of MPT opening that leads to the mitochondrial matrix swelling and the release of apoptogenic proteins that causes subsequent apoptosis.

(Isenberg and Klaunig, 2000). Ascorbic acid and tocoperol prevented rotenone-induced MPT opening by scavenging ETC induced ROS, suggesting a role of mitochondrial ETC-induced ROS in the oxidation of vicinal thiol's of ANT (Han et al., 2001). Thus ROS scavengers have a definite role to play in preventing drug-induced hepatotoxicity. The redox status of mitochondria also plays a critical role in opening and closing of MPT. In the reduced state of mitochondria, MPT closes while in oxidized conditions it opens. Costantini et al. (1996) have provided data to suggest that 2 distinct thiol groups are implicated in modulating MPT activity (Costantini et al., 1996). The oxidizing agents are reported to internally cross-link the ANT between Cys^{160} and Cys^{257} leading to the binding of cyclophilin D (CyP-D) to Pro^{62} and subsequent dimerization of VDAC to ANT and MPT opening (Halestrap et al., 1997; Torok and Joshi, 1985). When the effect of Ca^{2+} on reducing agents was studied it opened the MPT partially. This site is reported to be sensitive to oxidation of glutathione (Chernyak and Bernardi, 1996; Costantini et al., 1996). ATO and $K_2Cr_2O_7$ may be considered to act in a similar manner. DTT and reduced glutathione being reducing agents may reduce the site/help in keeping the site reduced by reducing the glutathione pool (Halestrap et al., 1997). Thus, DTT and reduced glutathione probably prevents the cysteine groups from being oxidized, protects disulphide bond formation between them and prevent MPT opening. Hence reducing agents may serve as important choices for drug/ alcohol induced liver cell death or in neurological diseases. Adenine nucleotides, ATP and ADP were found to effectively decrease and increase MPT opening respectively. The effect of pH on MPT opening is quite interesting. Our result correlated well with that of Qian et al. (1997) who reported, cultured rat hepatocytes during anoxia showed acidosis (pH 6.0) followed by MPT and after reperfusion (pH 7.4) shows MPT opening (Qian et al., 1997). Interestingly the calcium sensitivity of MPT pore also lies in the same pH range. In energized mitochondria, acidic matrix pH promotes MPT opening as more P_i uptake occurs and more ATP formation (Zemfira et al., 2005), Figure 7.

In summary, liver cell mitochondria, in addition to classical Ca^{2+}-induced MPT also exhibit its modulation by different agents like antioxidants, oxidants and reducetants. Our study demonstrates a complete functional characterization of liver MPT by using different modulators can be used for conducting basic research as well as to treat different disease conditions.

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