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Cyclosporine A Attenuates Mitochondrial Permeability Transition and Improves Mitochondrial Respiratory Function in Cardiomyocytes Isolated from Dogs With Heart Failure

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Abstract

Objective—We used isolated cardiomyocytes to investigate a possible role of mitochondrial permeability transition pore in mitochondrial abnormalities associated with heart failure.

Methods—Cardiomyocytes were isolated from LV myocardium of normal control dogs and dogs with heart failure produced by intracoronary microembolizations. Mitochondrial permeability transition was measured in isolated cardiomyocytes with intact sarcolemma with and without 0.2 μM Cyclosporin A using calcein AM and the fluorometer. State-3 mitochondrial respiration was also measured with the Clark electrode. Mitochondrial membrane potential was measured with JC-1 probe using the fluorometer. Propidium iodide was used to ensure sarcolemma integrity.

Results—200 minutes after loading with calcein AM, mitochondria of failing cardiomyocytes showed only 50% of maximal level of calcein fluorescence while it remained unchanged in *normal cells. The mitochondrial membrane potential in failing* cardiomyocytes was significantly decreased *by 38% compared to normal cardiomyocytes. Cyclosporine* A significantly slowed the exit of calcein from mitochondria of failing cardiomyocytes *and increased mitochondrial membrane potential by 29%*. State-3 respiration was not affected with *Cyclosporine A* in normal cardiomyocytes while it was significantly increased in failing cardiomyocytes *by 20%*.

Conclusions—Exit of calcein (m.w. 1.0 kDa) from mitochondria of viable failing cardiomyocytes with intact sarcolemma suggests an existence of a reversible transitory permeability transition opening in high conductance mode. Attenuation of calcein exit, $\Delta\Psi_m$ and improvement of state-3 respiration achieved with CsA (0.2 μM) show that permeability transition opening could be a cause of mitochondrial dysfunction described in the failing heart.

Keywords

Permeability transition; Heart failure; Mitochondria

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Introduction

Abnormal mitochondria have long been cited as a key element that may contribute to the progressive worsening of left ventricular (LV) function that characterizes the heart failure state. We previously showed that cardiomyocytes in failing heart exhibit ultrastructural abnormalities of mitochondria including matrix swelling, unfolding of cristae and damage of inner membrane [1]. These morphological abnormatities in mitochondria of the failing heart occur with decreased state-3 respiration in human cardiomyopathy [2] and in experimental canine ischemic cardiomyopathy [3]. The mechanisms of these mitochondrial abnormalities in failing cardiomyocytes remain unknown.

It has been demonstrated that mitochondrial swelling appears in isolated mitochondria with high conductance mitochondrial permeability transition pore (MPTP) openings [4]. MPTP inhibitor cyclosporine A (CsA) completely blocks MPTP and prevents swelling of brain mitochondria induced by calcium insult [5] o**r by** transient ischemia [6]. The role of MPTP in mitochondrial abnormalities described in viable cardiomyocytes of failing heart [1] is not clear. The data about the effect of of CsA in cardiomyocytes with intact sarcolemma are also very limited. The regulation of MPTP in the viable cells with intact plasmalemma is very complex and CsA is unable to control all pro-MPTP factors [7]. MPTPs are Ca^{2+}, redox, voltage, **fatty acids** and pH sensitive. Their opening probability is increased by matrix free [Ca^{2+}], **increased free fatty acids, reactive oxygen species** (ROS), mitochondrial membrane potential ($\Delta\Psi_m$) depolarization, and high pH (>7.0). Binding of CyP-D to adenine nucleotide translocator (ANT), ROS, fatty acids, and atractiliside stabilize ANT in c conformation, which promote MPTP opening. Inorganic phosphate (P_i) and enhanced matrix Ca^{2+} uptake may compete for adenine nucleotide binding sites in ANT and promote MPTP opening [8]. Proapoptotic Bcl family proteins also promote MPTP opening through unknown mechanisms [4]. The most potent inhibitor of MPTP opening, CsA, prevents interaction of CyP-D with ANT but a large elevation of matrix Ca^{2+}. Cardiomyocytes in failing heart exhibit increased intracellular Ca^{2+} resulting in impaired relaxation [9]. Increased ROS may also be present in failing cardiomyocytes [10] due to chronic hypoxia especially in cells located in the areas of intensive interstitial fibrosis [11]. BAX, a promoter of MPTP opening, is also elevated in heart failure against the background of elevated Bcl-2 [12] which appears to attenuate the BAX action to some extent. MPTP opening depolarizes mitochondrial membrane potential ($\Delta\Psi_m$) causing ATP synthase to operate in reverse, which accelerates cellular energy depletion [8].

The direct impact of MPTP on mitochondrial function in failing cardiomyocytes has not yet been determined. If it can be demonstrated that the probability of MPTP opening in failing intact cardiomyocytes is higher than in normal cells, there may be a potential for MPTP inhibitors in the therapy of heart failure. Agents such as CsA may significantly reduce the probability of MPTP opening in intact failing cardiomyocytes by the chelating of CyP-D in spite of existence of other factors promoting MPTP. **The free fatty acids, the major potential side effect of CsA in experiments, could be prevented with trifluoperazine (13).** Whether the reduced probability of MPTP opening can attenuate $\Delta\Psi_m$ and improve mitochondrial function in the population of cardiomyocytes isolated from dogs with heart failure has also not been investigated.

Therefore, in this study, we tested the hypothesis that CsA, a potent blocker of MPTPs [7], can attenuate MPTP opening, protect $\Delta\Psi_m$ and improve mitochondrial respiratory function in failing cardiomyocytes.

Material and Methods

Animal Model

The canine model of chronic heart failure used in this proposal has been fully characterized and previously described in detail [14]. In this model, chronic LV dysfunction and failure are produced by multiple sequential intracoronary embolizations with polystyrene Latex microspheres (70-102 μm in diameter) which lead to loss of viable myocardium and decrease in ejection fraction. Healthy conditioned mongrel dogs, weighing between 20 and 25 kg were used. To produce HF, 7 dogs underwent coronary microembolizations performed one week apart. Microembolizations were discontinued when LV ejection fraction was ≤35% angiographically. *At the time of sacrifice and cardiomyocyte isolation, mean LV ejection fraction was 26 ±2% angiographically*. Microembolizations were performed during cardiac catheterization under general anesthesia and sterile conditions. The anesthetic regimen consisted of a combination of intravenous injections of oxymorphone (0.22 mg/kg), diazepam (0.17 mg/kg), and sodium pentobarbital (150-250 mg to effect). This anesthesia regimen was shown to be effective in preventing the tachycardia, hypertension and myocardial depression seen with pentobarbital alone and does not have a significant effect on global LV function when compared to the conscious state. 5 sham-operated dogs (normal) underwent a similar number of cardiac catheterizations with an equivalent volume of saline administered instead of microspheres.

Isolation of Cardiomyocytes

Cardiomyocytes were isolated from the LV myocardium of 7 dogs with heart failure produced by intracoronary microembolizations. Cardiomyocytes isolated from 7 normal control dogs were used for normal control. Cardiomyocytes were isolated from the LV free wall as previously described [15]. Depending on each protocol, 10 to 20 grams of LV tissue were used to isolate myocytes. In our experience, this method of isolation yields approximately 3×10^6 cardiomyocytes for each gram of tissue. Our yield of rod-shaped, quiescent, calcium tolerant myocytes from cardiomyopathic dog hearts that exclude trypan blue was in the range of 80% to 90%. This range was greater (85-95%) for cardiomyocytes isolated from normal canine hearts. Each gram of tissue is sufficient to plate 30 five-cm^2 petri dishes, each containing 100,000 cardiomyocytes. Thin transmural slices, approximately 0.5mm thick were cut from the tissue block and immediately placed into a saturated 95% O_2 , 5% CO_2 normal Tyrode's solution (4 mM K^+, 2 mM Ca^{2+}) at 37°C. The tissue was then rinsed twice in HEPES solution A (115 mM NaCl, 5 mM KCl, 35 mM sucrose, 10 mM glucose, 10 mM HEPES, and 4 mM taurine, pH 6.95) to remove any residual blood. Each piece of tissue was then placed in a 250 ml polyethylene beaker containing 100 ml of HEPES solution with 15 μm Ca^{2+} (HEPES solution B) and the beakers were placed in a 36°C water bath. A Harvard respirator (maximal displacement 100 ml each) was connected in series to the needle end of five 20-ml plastic syringes (without plunger), with the wide end placed in the polyethylene beaker such that the solution is drawn into the syringe with each pump cycle. The respirator was adjusted to permit the HEPES solution plus tissue to be drawn up to 7/8 of the syringe height, at a rate of 25 cycles/min, a procedure referred to as trituration. A stream of O_2 (100%) was applied continuously to each beaker during the isolation procedure. The tissue was then triturated for 15 min each, once with HEPES solution A for 5 minutes, once with HEPES solution B, and twice with HEPES solution B containing 0.05% collagenase (type 2, Worthington) and 0.13 mg/ml protease (type XIV, Sigma). The dissociated dead cells and debris from the first four triturations were discarded and the cardiomyocytes from the fifth through the ninth trituration were combined. The combined suspension was collected and centrifuged at 500 g for 3 min. The pellet was resuspended in 50 ml HEPES solution B and the resulting suspension placed in a 2 × 50 ml polypropylene tube to stand for 5 min to allow the rod shaped cardiomyocytes to gravity settle. To make cardiomyocytes calcium tolerant, the settled myocytes were

resuspended each time in 50 ml HEPES buffer with increasing concentrations of calcium, from 100, 200, 400, 800, and 1000 μM. The myocytes were allowed 30 min to gravity settle after each calcium buffer change. Finally, the settled cardiomyocytes were resuspended in HEPES buffer containing 1 mM Ca^{++}.

Measurement of Mitochondrial Permeability Transition Pore with Calcein

Calcein AM was used to detect the transient MPTP opening in high conductance mode in intact cells under resting conditions [7]. MPTP opening in high conductance mode allows the unselective diffusion of larger (up to 1.5 kDa) molecules. We proposed that if transient high conductive mode exists in viable failing cardiomyocytes, the use of calcein with molecular weight about 1 kDa for monitoring MPTP opening is ideal choice for detecting it. In such a situation, the higher open probability in transient high conductance mode in single viable failing cardiomyocytes will translate into higher MPTP opening in population of cardiomyocytes when measured with calcein technique. Intact membranes are permeable for esterified calcein AM but not to unesterified calcein. Esterified calcein AM allows lloading of intact cells without permeabilization. Since mitochondrial calcein is always unesterified, the exit of calcein [7] will only reflect the degree of MPTP opening in high conductance mode. When cells are co loaded with calcein AM and $CoCl_2$, calcein fluorescence is quenched in both cytosolic and nuclear compartments. This allows to monitoring of calcein fluorescence in mitochondria of intact cells [7].

Isolated cardiomyocytes were co loaded with 1 μM calcein AM and 1 mM $CoCl_2$ at room temperature in working solution (pH=7.2). To make the relationship between MPTP opening and mitochondrial function more credible, same working solution consisting of 10 mM EGTA-CaEGTA buffer (free Ca^{2+} concentration 100 nM) , 3 mM free Mg^{2+}, 20 mM taurine, 0.5 mM dithiothreitol, 20 mM imidazole, 0.16 M potassium 4-morpholineethanesulfonate and 10 mg/ml fatty acid-free bovine serum albumin was used for both evaluation of state-3 respiratory rate and for MPTP measurement. The rate of calcein loading by mitochondria and the rate of calcein exit through MPTP were measured by recording the fluorescence signal every 5-10 min using Turner Quantech Digital Filter Fluorometer with excitation filter NB490 and Emission filter SC515. The rate of calcein AM loading and exit was calculated as a percent change to maximal fluorescence signal.

Treatment of Isolated Cardiomyocytes

Cardiomyocytes were resuspended in working solution (see above) that mimics some components of normal cytosol. For the current experiment, the cells were stored in working solution at room temperature for up to 200 min. Cardiomyocytes were treated with or without presence of the MPTP inhibitor cyclosporine A at the concentration of 0.2 μM. We used a single dose of CsA (0.2μM) which had been proved to be most effective without producing major side effects. In some treatments, inhibitor of phospholipase A_2 trifluoperazine was used either separately or with CsA to inhibit possible accumulation free fatty acids which can facilitate MPTP opening and limit MPTP inhibition by CsA [13]. To distinguish the specific effect of CsA from its vehicle ethanol, control treatments were performed with ethanol at the concentration that corresponds its presence in 0.2 μM CsA. The final ethanol concentration in all experiments did not exceed 0.1%, which had no measurable effect on the cellular parameters measured.

Mitochondria Respiratory Analysis in Isolated Cardiomyocytes

Mitochondrial respiratory activity was analyzed using a modified version of a previously described procedure [2,3]. HEPES was substituted with working solution described above. The cells were then washed in fresh working solution and placed in the oxygraph chamber for determining mitochondrial respiratory rates. The respiratory rates were determined by a Clark

electrode (Yellow Springs Instruments Co., Yellow Springs, OH, USA) in an oxygraph cell containing isolated cardiomyocytes in 3 ml of the solution B at 34°C with continuous stirring. The solubility of oxygen at 34°C is taken to be 386 ng atoms/ml. Digitonin (0.005%) was added to oxygraph cell to make sarcolemma permeable for ADP. Basal respiratory (V_{SUB}) rate was measured in the presence of 5 mM malate and 5 mM pyruvate. State-3 respiration was measured after addition of 1 mM ADP. The state-3 respiration was blocked with 0.3 mM atractiloside. *CsA in final concentration 0.2 μM was added after addition of ADP*. Respiratory control ratio was calculated as the ratio of state-3 respiratory rate (V_{ADP}) and the respiratory rate after inhibition of state-3 respiration with atractiloside (V_{AT}) [2,3]. *In case CsA was used after ADP, respiratory control ratio was calculated as the ratio of maximal ADP supported respiratory rate achieved after addition CsA and the respiratory rate after inhibition of ADP dependent respiration with atractiloside*. Amount of rod-shaped cardiomyocytes in respiratory chamber was calculated and the rate of respiration was expressed either in ng O_2/min/1×10^6 CM or as a percent change of state-3 respiration after addition of CsA.

Measurement of Mitochondrial Membrane Potential with the Probe JC-1

This assay is based on the ability of the fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide, commonly known as JC-1, to easily penetrate cells and healthy mitochondria. Once inside healthy cells, JC-1 bearing a delocalized positive charge, enters the negatively charged mitochondria where it aggregates and fluorescences red [16]. When the mitochondrial membrane potential ($\Delta\Psi_m$) collapses, JC-1 is distributed throughout the cell in a monomeric form which fluoresces green and the amount of red fluorescence drops [16]. Using the dual fluorescence characteristic of the dye, the changes in the mitochondrial $\Delta\Psi_m$ was assessed by comparing the ratios of 590-600 nm (red)/ 527-534 nm (green) optical densities. MPTP opening causes the loss of $\Delta\Psi_m$, resulting in the red/green optical density ratio drop. This drop corresponds to a reduction in the number of healthy mitochondria able to maintain the negative potential necessary to concentrate JC-1 dye in the red aggregate form.

Commercially available “Mitochondrial Permeability Transition Detection Kits” containing JC-1 reagent was used for this study. Briefly, the cells were incubated at 37°C for 1 h with the corresponding reagent containing JC-1, and then washed twice with PBS and placed in fresh Medium 199 with Earle's salts without serum. After treatment, the working solution was removed by aspiration and the cells were washed again twice with PBS. The cell suspension was transferred to a quartz cuvette, and the fluorescence of cell-associated JC-1 was read in a Turner Quantech Digital Filter Fluorometer at 490 excitation, 515 emission for green, at 540 excitation, and 585 emission for red. The ratios of red/green fluorescent signal intensities were then calculated.

Microscopy

Epifluorescent microscope was used to control calcein AM presence in mitochondria and intactness of sarcolemma during experiment. Bright fluorescence of mitochondria and absence of fluorescence in cytoplasm and nuclei throughout experiment supported the fact that majority of fluorescent signal that registered from the cardiomyocytes was coming from the mitochondria. The 1 μM propidium iodide staining of isolated cardiomyocytes was used to monitor sarcolemmal integrity. Positive staining of cardiomyocyte's nuclei with propidium iodide suggested sarcolemmal damage. Rod-shaped cardiomyocytes showing clear cross striation and intact sarcolemma (no positive nuclei staining with propidium iodide) were suggested to be viable. Percent of viable cardiomyocytes was monitored throughout the experiments.

Data Analysis

All the continuous variable results are presented as mean ± S.E.M. Comparisons of level of calcein fluorescence, $\Delta\Psi_m$ and respiratory rates between normal control cardiomyocytes and cardiomyocytes isolated from heart failure dogs were made using a t-statistic for two means. For this test, a probability of ≤0.05 was considered significant. Comparisons of level of calcein fluorescence, $\Delta\Psi_m$ and respiratory rates between control untreated cardiomyocytes and cardiomyocytes treated with CsA were made using a t-statistic for two means. For this test, a probability of ≤0.05 was considered significant. A one-way analysis of variance was used to determine whether differences exist in calcein fluorescence, $\Delta\Psi_m$ and respiratory rates if effect of treatment with CsA in failing cardiomyocytes compared with control normal cardiomyocytes and at least three parameters namely normal control, heart failure control and heart failure treatment were compared in one group. For this test, significance was set at α=0.05. If significance was achieved, pairwise comparisons were performed among groups using the Student-Newman-Kuels test with a probability value of ≤0.05 considered significant.

Results

Sarcolemma Integrity and Calcein AM Fluorescence in Isolated Cardiomyocytes

Both in normal control cardiomyocytes and in cardiomyocytes isolated from dogs with heart failure the intact rod-shaped cardiomyocytes coloaded with calcein AM and $CoCl_2$ showed bright mitochondrial fluorescence against low background fluorescence of cytoplasm, without fluorescence in nuclei and no propidium iodide staining. Round-shaped cardiomyocytes showed no calcein AM fluorescence and positive nuclear staining with propidium iodide (Fig.1a,1b,1c). Skinning of intact cardiomyocytes incubated in the working solution with 0.005% digitonin did not affect the calcein AM fluorescence in spite of all the cells showing positive nuclei staining with propidium iodide (Fig.1d,1e,1f). Skinning of intact cardiomyocytes incubated in HEPES solution containing 1000 mM Ca^{2+} with 0.005% digitonin lead to complete loss of calcein AM fluorescence and all the cells showed positive nuclei staining with propidium iodide (Fig.1g,1h,1i). Monitoring of calcein AM fluorescence in population of intact normal cardiomyocyres using fluorometer showed that calcein AM fluorescence fell to background levels immediately after cardiomyocytes were skinned with 0.005% digitonin in HEPES solution containing 1000 mM Ca^{2+} while the calcein fluorescence remains unchanged if working solution containing Ca^{2+} chelator EGTA was used (Fig. 2a). These observations suggest that the working solution allows to skin cardiomyocytes making it permeable for external ADP without affecting MPTP. This provided the opportunity to correlate mitochondrial state-3 respiration in skinned cardiomyocytes with MPTP condition in cardiomyocytes with intact sarcolemma.

MPTP in Normal Control Cardiomyocytes versus Failing Control Cardiomyocytes

Incubation of normal control cardiomyocytes with intact sarcolemma in working solution containing 0.5% BSA and Ca^{2+} chelator 10 mM EGTA show slow uptake of calcein AM by mitochondria, calcein fluorescence reached maximal level in 150 min of incubation and kept this maximal level of fluorescence until the end of incubation of 200 min (Fig.2b). Incubation of failing control (untreated) cardiomyocytes with intact sarcolemma in working solution showed faster uptake of calcein AM by mitochondria compared with normal control cardiomyocytes. Mitochondria of failing cardiomyocytes reached maximal level of calcein fluorescence in 40 min of incubation. Calcein AM level in mitochondria of failing cardiomyocytes began to decline in 40-60 min of incubation showing only 50% of maximal level of calcein fluorescence in 160-200 min (Fig.2b). Percent of viable **rod shaped** cardiomyocytes **showing cross-striation and no positive propidium iodide staining** was not significantly changed during experiment **in normal control cardiomyocytes and cardiomyocytes isolated from dogs with heart failure. Incubation of normal control and**

failing cardiomyocytes with intact sarcolemma in working solution did not lead to sarcolemma damage, all the viable rod shaped cardiomyocytes had calcein fluorescence and did not exhibit positive propidium iodide nuclear staining. Furthermore, the decrease in calcein fluorescence in viable cardiomyocytes during experiment was not followed by either significant change in the percentage of viable rod shaped cardiomyocytes or by appearance of positive propidium iodide staining in cardiomyocytes showing positive calcein fluorescence. All of the round shaped cardiomyocytes invariably showed no calcein fluorescence and had positive propidium iodide nuclear stain. This observation shows that round shaped cells did not account for any substantial proportion of the cytosolic calcein pattern observed.

Influence of CsA on MPTP in Normal and Failing Cardiomyocytes

Treatment of normal control intact cardiomyocytes with 0.2 μM CsA did not change the dynamic of both calcein AM efflux or influx (Fig.3a). Combination of CsA with trifluoraperazine and trifuoperazine alone had no effect on calcein AM fluorescence of mitochondria in normal cardiomyocytes (Fig.3a). Treatment of failing intact cardiomyocytes with 0.2 μM CsA did not affect the rate of calcein AM uptake by mitochondria but significantly slowed calcein AM exit compared to untreated failing cardiomyocytes (Fig.3b). Mitochondria of failing cardiomyocytes incubated with 0.2 μM CsA still retained 85% of calcein AM in 160 min of incubation. Combination of CsA with trifluoperazine did not improve the beneficial action of CsA. The trifuoperazine alone had no effect on calcein AM fluorescence of mitochondria in failing cardiomyocytes (Fig.3b). Addition of the CsA vehicle ethanol only in the concentration which corresponds its concentration in 0.2 μM CsA did not affect MPTP either in normal control or failing cardiomyocytes (not shown). Percent of viable cardiomyocytes was not significantly changed during experiment. **Treatment of cardiomyocytes did not lead to any significant change in the percentage of viable rod shaped cardiomyocytes showing mitochondrial calcein fluorescence and negative propidium iodide nuclear staining**.

Mitochondrial Membrane Potential

Failing cardiomyocytes showed a significant decrease of the ratio of fluorescence excitation compared with normal control cardiomyocytes. Incubation of failing cardiomyocytes with 0.2 μM CsA for 200 min. significantly increased the ratio of fluorescence excitation, but did not reach levels seen with normals. (Fig.4a). T**he J-aggregate formation is largely membrane potential dependent and a reduction in the plasma membrane potential leads to a reduction in J-aggregate formation and accordingly to a reduction in the ratio of fluorescence excitation due to shift of fluorescence maxima to a longer wavelength** [17]. I**t was also demonstrated that fluorescence of J-aggregates responds linearly to an increase in membrane potential** [17]. **To establish an empirical calibration curve between the shift in the ratio of fluorescence excitation energy and $\Delta\Psi_m$, we assumed that the $\Delta\Psi_m$ contributes most of the energy stored in the gradient at 150 mV in normal mitochondria** [18]. **Accordingly, the $\Delta\Psi_m$ in the population of failing untreated cardiomyocytes was about 92 and the $\Delta\Psi_m$ in the population of failing cardiomyocytes treated with CsA was about 118** (Fig.4).

Mitochondrial Respiration

Addition of 0.2 μM CsA significantly increased state-3 respiration in failing cardiomyocytes measured after addition of 1 mM ADP (Fig.5a). Respiratory control ratio was also *significantly* increased with CsA (Fig.5b).

Discussion

Novel Aspects

Chronic alterations in mitochondrial permeability due to MPTP have been described in previous studies mostly performed in aging rodent [19-24]. In this study, we have demonstrated for the first time increased MPTP opening in the population of viable cardiomyocytes isolated from dogs with *chronic* heart failure *without acute ischemia or calcium overload. This was demonstrated by using esterified calcein AM which is permeable to intact membranes. Esterified calcein AM allows loading of all the compartments of intact cells without permeabilization. Calcein becomes unesterified in the mitochondrial matrix. It therefore becomes impermeable to intact mitochondrial membrane and it's exit from mitochondrial matrix completely depends on the degree of MPTP opening and the size of unesterified calcein molecule* [7]. Since this increased *MPTP opening* was recorded in viable cardiomyocytes with intact sarcolemma by using calcein with molecular weight about 1 kD, it confirms the existence of reversible transient MPTP opening in high conductance mode in heart failure. This occurs along with decreased ΔΨm in the same population of viable failing cardiomyocytes.

Treatment of MPTP with CsA and ΔΨm

The MPTP can operate under two distinct modes: low conductance mode and high conductance mode [25]. A low conductance state, that allows the diffusion of small ions like Ca^{2+}, is pH operated, promoting spontaneous closure of the channel. In normal cells, Ca^{2+} -induced release of Ca^{2+} from mitochondria following propagation of depolarization and Ca^{2+} waves from one mitochondrion to another depends on the transient opening of MPTP operating in a low conductance mode [26]. In normal cardiomyocytes, such MPTP opening can transiently depolarize mitochondrial membrane causing a mitochondrial Ca^{2+} efflux during contraction [26]. In high conductance mode, that allows the unselective diffusion of big (up to 1.5 kDa) molecules, MPTP opening fall into two classes: transient and long-lasting. The long-lasting MPTP opening is irreversible. This irreversible high conductance mode takes part in the cascade leading to cell death either through necrosis [27] or apoptosis [28]. In heart failure, this process may result in apoptosis of some cardiomyocytes [28,29].

In the present study, we show that cardiomyocytes isolated from dogs with heart failure exhibit faster uptake and exit of calcein by mitochondria of failing cardiomyocytes when compared to normal cardiomyocytes due to increased probability of MPTP opening in failing cardiomyocytes. It is not clear why uptake *of membrane permeable esterified calcein AM* in failing cardiomyocytes is faster when compared to normal control cardiomyocytes. *Since the exit of calcein from mitochondria (7) reflects the degree of MPTP opening in high conductance mode*, this explains why the calcein fluorescence remains for a long time at maximal level without any change in normals while it declines in failing cardiomyocytes.

Like other ion channels, MPTP open and close stochastically, namely randomly involving chance and probability (8). In high conductance mode, the rapidity with which electron transport regenerates $\Delta\Psi_m$ when the MPTP transiently closes will play a critical role in determining whether it remains closed or reopens. *On the other hand, higher the frequency of intermittent opening of MPTP in high conductance mode the less time is left for $\Delta\Psi_m$ to be regenerated. It is likely that increased probability of MPTP opening in transient high conductive mode is the one of the main causes for decreased $\Delta\Psi_m$ in failing cardiomyocytes. This suggestion is supported by the data of this report that attenuation of MPTP opening by CsA leads to attenuation of $\Delta\Psi_m$ as well*. This interplay may thus determine whether MPTP opening are transient and reversible or long-lasting and irreversible. In the current study, long-term incubation of failing cardiomyocytes with CsA slowdown calcein AM exit from

mitochondria and partially restored $\Delta\Psi_m$. This reflects the attenuation of MPTP opening probability in high conductance mode, which positively affect $\Delta\Psi_m$ as well. *This finding shows that progressive loss of* $\Delta\Psi_m$ *observed in failing cardiomyocytes is at least partially caused by reversible intermittent opening of MPTP*. Long-term treatment with CsA did not restore MPTP probability opening and $\Delta\Psi_m$ to the normal level. *One can suggest that this gap between partially restored MPTP and* $\Delta\Psi_m$ *and their level in normal cells represents irreversible component of MPTP opening in high conductance mode which can not be reversed by CsA and finely lead to cardiomyocyte apoprosis described in failing heart* [12,29].

MPTP and Mitochondrial Abnormalities

The failing cardiomyocytes show decreased $\Delta\Psi_m$. Reduced $\Delta\Psi_m$ decreases the extramitochondrial phosphorylation potential and adversely impacts the ability of the cell to function [30]. Under these types of conditions, mitochondrial creatine kinase can no longer operate in the reverse direction, but rather works together with extramitochondrial creatine kinase in the forward direction, resulting in the loss of creatine kinase system ADP-transport function [31]. Addition of 0.2 μM CsA increased state-3 respiration and respiratory control ratio. *It is very likely that improved state-3 respiration could be a direct result of attenuation of MPTP and ΔΨm due to treatment of failing cardiomyocytes with CsA*. **As we showed before, 0.2 μM CsA significantly increased cytochrome c oxidase dependent mitochondria respiration in the presence of uncoupler N,N,N,N′,N′-tetramethyl-p-phenylenediamine and an artificial electron donor ascorbate (32). It suggests that CsA attenuates electron transport chain on the level cytochrome c oxidase rather than phosphorylation apparatus**.

Treatment of MPTP with CsA and Free Fatty Acids Accumulation

Linking MPTP opening to mitochondrial damage, a substantial number of publications have described protective effects of MPTP inhibitor CsA on cells subjected to a variety of injurious conditions. Such studies have been conducted on hepatocytes [13], cardiomyocytes [25] and other types of cells [33]. The cell injury studies have normally been conducted with time frames of a few hours, whereas the inhibitory action of CsA on MPTP opening in isolated hepatocyte mitochondria is gradually lost as the time frame exceeds about 15 min [34]. Free fatty acids accumulate in cyclosporine A-treated isolated mitochondria due to activation of phospholipase A which limits pore inhibition by CsA [33]. The use of CsA in combination with phospholipase A_2 inhibitor trifluoperazine did not affect the inhibitory effect of CsA in failing cardiomyocytes as has been described in mitochondria isolated from hepatocytes [13]. Keeping in mind that all the experiments were performed on cardiomyocytes with intact sarcolemma, it is unlikely that the composition of respiratory solution used for calcein AM loading had significant impact on fatty acids accumulation. It is more likely that 0.2 μM CsA does not activate phospholipase A_2 in intact cardiomyocytes enough to block MPTP inhibitory action of CsA.

Study Limitations

At this time, it is not possible to completely translate the data obtained from isolated cardiomyocytes into action of CsA *in situ* in failing heart where failing cardiomyocytes are subjected to continuous chronic hypoxia, elevated levels of angiotensin-II , cateholamines, TNFα and calcium overload [14,35,36]. Additional investigations are required to understand the feasibility of use of MPTP blockers in the treatment of heart failure. CsA has been shown to protect myocytes from reperfusion injury [37]. However, CsA has a relatively narrow therapeutic window and side effects that make it unsuitable for routine use. These may be predominenetly due to the action of CsA to inhibit calcineurin, a Ca^{2+}-dependent protein phosphatase [38]. In addition, cytosolic CyP, which is also inhibited by CsA, may play a protective role against ischemia/reperfusion injury, since antisense knockout of CyP

desensitizes myocytes to hypoxic damage [39]. *It is also possible that MPTP opening recorded in cardiomyocytes isolated from myocardium of dogs with heart failure at least partially depends on an increased frailty of failing cardiomyocytes to the cell isolation process* [40]. *Unfortunately it is not feasible yet to monitor simultaneously mitochondrial function and PTP opening in the intact organ* [41] *to confirm or to deny such a scenario*. Nevertheless, CsA still can be useful for investigation into the potential importance of MPTP in pathogenesis of heart failure.

Conclusions

Exit of calcein (m.w. 1.0 kDa) from mitochondria of viable failing cardiomyocytes with intact sarcolemma suggests an existence of a reversible transitory permeability transition opening in high conductance mode. Attenuation of calcein exit, $\Delta\Psi_m$ and improvement of state-3 respiration achieved with CsA (0.2 μM) show that permeability transition opening could be a cause of mitochondrial dysfunction described in the failing heart. In conclusion, our observations show that CsA attenuates of MPTP opening in failing cardiomyocytes. Attenuation of MPTP opening improves $\Delta\Psi_m$ and increases state-3 respiration. This suggests that MPTP attenuation could be a potential target in treatment of chronic heart failure.

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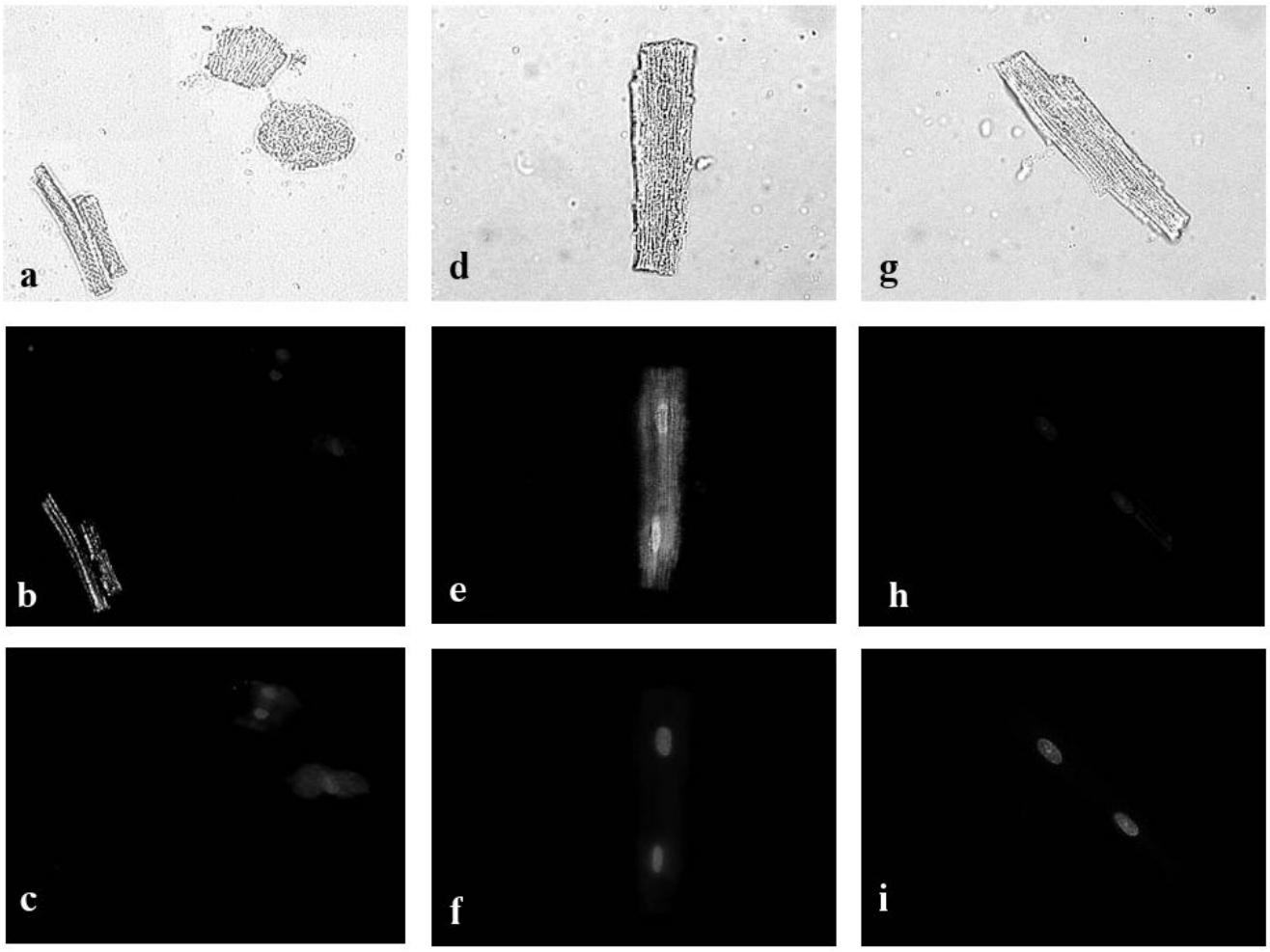

Fig.1.

Isolated cardiomyocytes loaded with calcein AM and double-stained with propidium iodide: a,b,c – intact isolated cardiomyocytes, a – routine microscopy, b – same cardiomyocytes under fluorescein light, c – same cardiomyocytes under rhodamine light; d,e,f – cardiomyocyte skinned with 0.005% digitotin in working solution, d – routine microscopy, e – same cardiomyocyte under fluorescein light, f – same cardiomyocyte under rhodamine light; g,h,i – cardiomyocyte skinned with 0.005% digitotin in HEPES, g – routine microscopy, h – same cardiomyocyte under fluorescein light, i – same cardiomyocyte under rhodamine light.

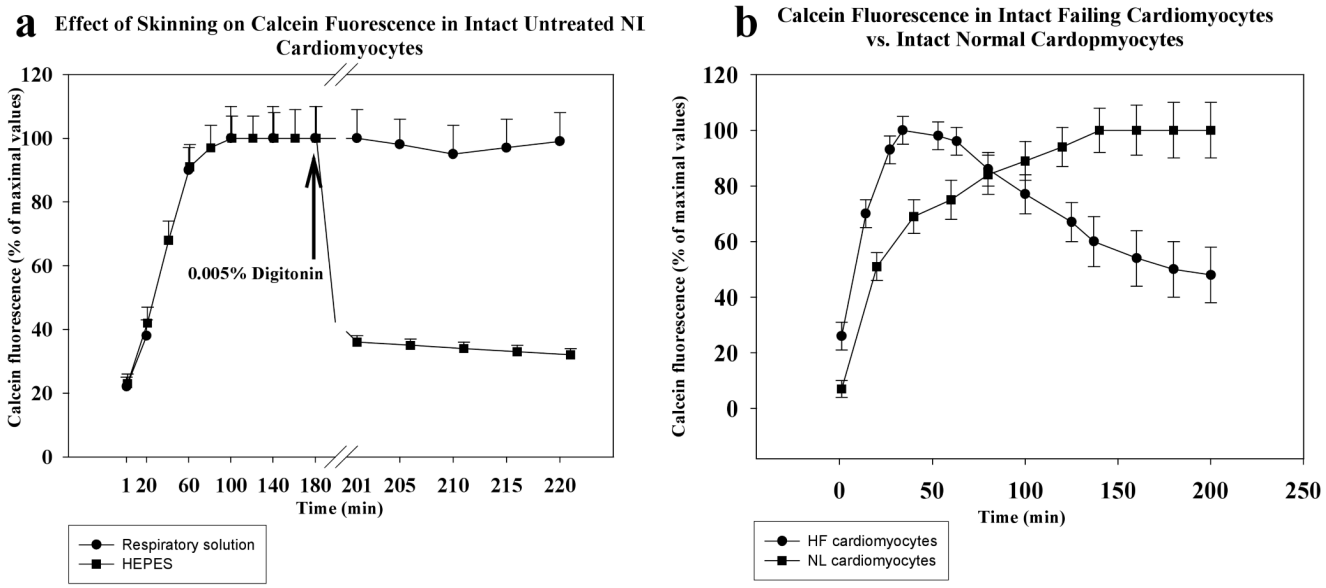

Fig.2.

Dynamic of Calcein AM fluorescence in normal and failing isolated cardiomyocytes: a – effect of skinning on calcein AM fluorescence in normal cardiomyocytes (*n=7*), working solution (●)(*n=7*) versus HEPES (■)(*n=7*) ; b – dynamic of calcein AM fluorescence in failing cardiomyocytes (●)(*n=7*) versus normal cardiomyocytes (■)(*n=7*) with intact sarcolemma.

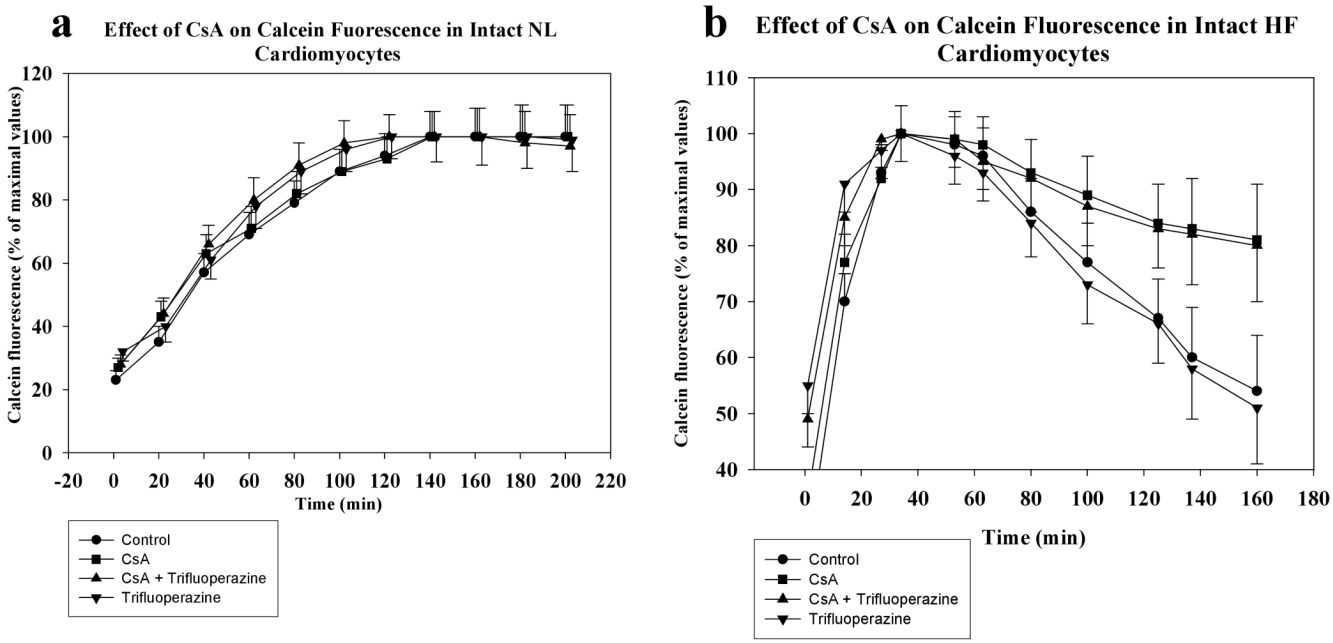

Fig.3.

Effect of CsA on calcein AM fluorescence: a – dynamic of calcein AM fluorescence in normal intact cardiomyocytes (*n=7*); b - dynamic of calcein AM fluorescence in failing intact cardiomyocytes (*n=7*). ●- untreated cardiomyocytes (*n=7*), ■ – cardiomyocytes treated with 0.2 μM CsA(*n=7*), ▲ – cardiomyocytes treated with CsA + trifluoperazine(*n=7*), ▼- cardiomyocytes treated with trifluoperazine(*n=7*).

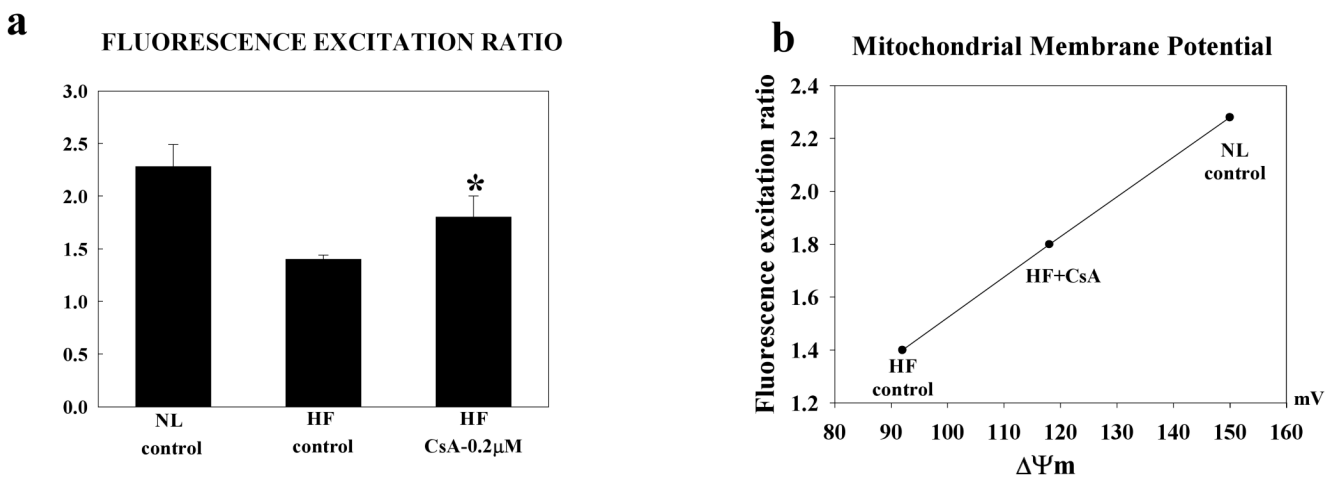

Fig.4.

Effect of CsA on mitochondrial membrane potential ($\Delta\Psi_m$) *in normal control cardiomyocytes* (*n=7*) *and in failing cardiomyocytes* (*n=7*). **a – Fluorescence excitation ratio.** *The fluorescence excitation ratio in each case was calculated as a the ratios of red/green fluorescent signal intensities and was represented as relative numbers of the ratios of intensity units*. *=p<0.5 compared to untreated HF cardiomyocytes; **b – Relationship between fluorescence exitation ratio and mitochondrial membrane potential. The $\Delta\Psi_m$ in failing cardiomyoctes was calculated empirically taking the $\Delta\Psi_m$ in normal controls for 150 mV (see results)**.

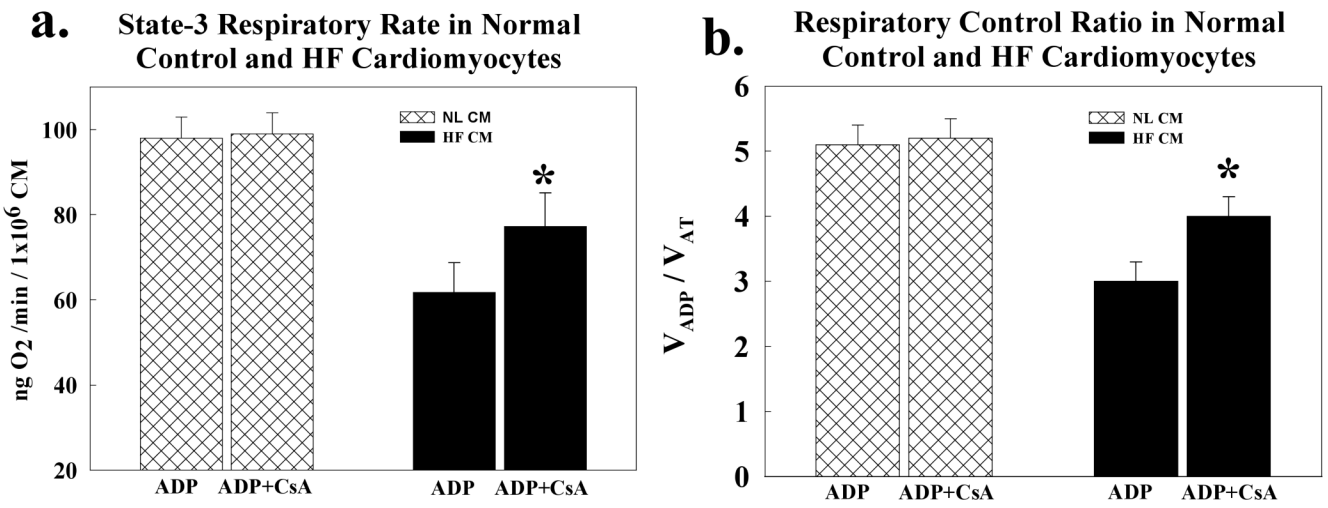

Fig.5.

Effect of CsA on mitochondrial respiratory parameters in normal control (*n=7*) and failing cardiomyocytes (*n=7*): a – CsA *significantly* increases state-3 respiration in failing cardiomyocytes *and does not affect state-3 respiration in normal control cardiomyocytes*. ; b – CsA *significantly* increases mitochondrial respiratory control ratio in failing cardiomyocytes *and does not affect respiratory control ratio in normal control cardiomyocytes. V_{ADP} – state-3 respiratory rate after addition 1 mM ADP; V_{AT} – respiratory rate after addition 0.3 mM atractiloside. *=p<0.05 compared to HF untreated cardiomyocytes*.