Inhibition of Mitochondrial Remodeling by Cyclosporin A Preserves Myocardial Performance in a Neonatal Rabbit Model of Cardioplegic Arrest

Norihiko Oka, MD; Lixing Wang, MD, Ph D; Wenyu Mi, MD; Christopher A. Caldarone, MD

The Division of Cardiovascular Surgery at the Hospital for Sick Children, University of Toronto

Correspondence:

Christopher A. Caldarone, MD, Division of Cardiovascular Surgery

The Hospital for Sick Children,

555 University Avenue, Toronto, Ontario, Canada, M5G 1X8

Phone: (416) 813-6420

E-mail: christopher.caldarone@sickkids.ca

Abstract

Objective: Mitochondria permeability transition pore (MPTP) opening is associated with apoptotic signaling and alterations in mitochondrial structure and function. We tested whether inhibition of MPTP opening with Cyclosporine A (CsA) preserved mitochondrial structure and function after cardioplegic arrest and whether this preservation is associated with improved myocardial performance.

Methods: Langendorff-perfused neonatal rabbit hearts were perfused for 30 minutes with Krebs-Henseleit (KH) buffer (CCP; n=6) or KH buffer containing 2 μM Cyclosporin A (CCP+CsA; n=6) followed by 60 minutes normothermic crystalloid cardioplegia (CCP) and 60 minutes reperfusion. Control hearts (Non-CCP; n=6) were constantly perfused for 150 minutes without cardioplegic arrest.

Results: In comparison to non-CCP, CCP was associated with Bax translocation to the mitochondria, cytochrome c release, and greater frequency of TUNEL-positive myocytes These changes were also associated with deficits in isolated mitochondrial oxygen consumption at Complex I. CsA pre-treatment minimized or prevented all these findings. Myocardial performance (systolic pressure, maximum positive and negative dP/dt, and elevated LVEDP) at 5, 15, 30 and 60 minutes after reperfusion was diminished in CCP hearts when compared to non-CPB and these deficits could be minimized with CsA pre-treatment. ($P<0.05$ all comparisons)

Conclusions: Cyclosporine A prevents apoptosis-related mitochondrial permeabilization and dysfunction after cardioplegic arrest. This protection is associated with improved myocardial performance. Prevention of mitochondrial MPTP opening is a

valuable target for mitochondrial (and myocardial) preservation after neonatal cardioplegic arrest.

Introduction

As a critical mediator in apoptosis signaling pathways, mitochondria integrate upstream death stimuli and undergo structural and functional remodeling with subsequent transmission of signals to downstream executioner proteins ***(Crow 2004).*** Bax translocation, mitochondrial outer membrane permeabilization, and cytochrome c release directly participate in mitochondrial architectural and functional remodeling after unprotected ischemia/reperfusion ***(Mootha 2001, Appaix 2002, Paradies 2004).*** After protected ischemia/reperfusion (cardioplegic arrest), we have previously reported a similar constellation of apoptosis-related alterations in mitochondrial structure and function in the lamb and piglet myocardium (apoptosis-related mitochondrial dysfunction: ARMD) ***(Hammel 2003, Karimi 2004, Oka 2007)*** and correlated these changes with deficits in mitochondrial electronic transport chain (ETC) activity ***(Caldarone 2004, Wang 2006).*** Because of the close interplay between apoptotic events and mitochondrial dysfunction, and the role of mitochondrial protection in the maintenance of cardiac performance ***(Garlic 1997)***, we hypothesized that 'mitoprotection' conferred by maintenance of outer mitochondrial membrane integrity may be of therapeutic benefit to reduce ARMD and consequently preserve myocardial performance.

The mitochondrial permeability transition pore (MPTP) is a nonspecific pore that may mediate alterations in outer mitochondrial membrane integrity ***(Griffths 1995, Lemasters 1998).*** Pharmacologic inhibition of MPTP opening is associated with myocardial protection against unprotected ischemia/reperfusion injury ***(Baines 2003, Nakagawa 2005).*** Cyclosporin A (CsA), a specific inhibitor of MPTP opening, has been

reported to ameliorate reperfusion injury in a variety of in vitro ***(Akao2003, Halestrap 1997)*** and in vivo ***(Argaud 2005)***animal models of unprotected and protected ischemia ***(Nathan 2005)***.

The current study examines the effects of CsA-mediated MPTP pore inhibition on the relationship between ARMD and global myocardial performance in a neonatal rabbit Langendorf model.

Methods and Materials

Experimental Model

New Zealand White neonatal rabbits (5~6 day; 150-250 g) were anesthetized with sodium pentobarbital (50 mg/kg, IP), heparinized (1000U/kg, IP), and ventilated. The heart was retrogradely perfused *in situ* to avoid ischemia and then excised and mounted on a Langendorff perfusion system, perfused at 75 mmHg with Krebs-Henseleit buffer (KH), equilibrated with 95% O_2/5% CO_2 (pH of 7.35-7.40).

All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC; March 1999).

Experimental Protocol

Two groups of hearts were perfused for a 30 minute stabilization period followed by 60 minutes crystalloid cardioplegic arrest (CCP) and 60 minutes reperfusion. A third group of hearts underwent uninterrupted perfusion (non-CCP, n=6) for 150 minutes.

During the stabilization period, Cyclosporin A (CsA) treated hearts (CCP+CsA, n=6) were perfused with KH in which CsA (0.2 μM) was added to the KHB. Non-treated hearts (CCP, n=6) we stabilized with KH perfusion alone. Cardioplegic arrest for both

groups was induced with crystalloid cardioplegia (65 ml/kg, Plegisol; Hospira, Inc., Lake Forest, IL) at normothermia ***(Feng 2005)***.

CsA was dissolved in DMSO (Fisher Scientific) which was further dissolved into KH at final concentration of 0.05%. The same amount DMSO was added into the KH for the Non-CCP and CCP groups.

At the end of reperfusion, the hearts were rapidly excised. For mitochondrial oxygen consumption and mitochondrial protein extraction, myocardium was fractionated as described ***(Caldarone 2004)***.

A separate set of animals (n=4 for each group) underwent identical procedures after which the left ventricles were sliced and embedded in OCT compound (IMEB, INC., San Marcos), snap frozen in liquid nitrogen, and stored at -80 ˚C for apoptosis and fluorescence immunohistochemistry.

Cardiac Functional Measurements

Isovolumetric measurement of left ventricular (LV) performance was made using a water-filled balloon connected to a pressure transducer (MLT844, ADInstruments, CO, USA) and inserted into the LV across the mitral valve. The volume of the water-filled balloon was adjusted to end diastolic pressure of 0-5 mmHg and kept constant throughout the entire experiment. LV performance was assessed with LV systolic pressure (LVP), LV end-diastolic pressure (LVEDP), LV developed pressure (LVDP), and maximum positive and negative dP/dt. Analog data (HR, LVP, LVDP, LVEDP, ±dP/dtmax) were digitalized and analyzed (Chart IV, ADInstruments, Colorado Springs, CO).

Mitochondrial Isolation

The left ventricle was used for mitochondrial isolation by differential centrifugation ***(Kowaltoski 2001)***. The whole left ventricle (~0.7 g) was placed into 5 ml mitochondrial isolation buffer (MIB) on ice (5 mM MOPS, 2mM Ethylene Glycol Bis-2-Aminoethyl Ether-N,N',N'',n'-Tetraacetic Acid, 70 mM Sucrose and 220 mM Mannitol, 1mM Dithiothreitol , 17 ug/ml phenylmethylsulfonyl fluoride, 8 ug/ml aproptinin and 2 ug/ml leupeptin, pH 7.2) with 0.1% bovine serum albumin (BSA) and finely minced. Tissue was homogenized on ice by using a blade homogenizer and centrifuged (700g x 5 minutes). The supernatant was transferred to a new tube and the same low spin repeated four times (5 minutes each) until no visible pellet was seen. The supernatant from the low spins was centrifuged (8000g x 10min). The pellet was resuspended in 10 ml MIB with 0.1% BSA and centrifuged for another 10 minutes (8000 g). The final pellet was suspended in MIB buffer without BSA for mitochondrial oxygen consumption measurements.

Clark-electrode Oxygen Consumption Measurement

Mitochondrial Complex I, II and IV respiration were measured using a Clark-type oxygen electrode (Instech Laboratories Inc, Plymouth Meeting, PA). Oxygen consumption was measured in the presence of sequential administration of substrates and inhibitors (glutamate/malate for complex I, rotenone/succinate for complex II, antimycin/TMPD/ascorbate for complex IV) added in the following order and final concentration: 2.5mM glutamate, 2.5mM malate, 2mM ADP, 2uM rotenone, 5mM succinate, 1uM antimycin A, 1mM ascorbate with 0.4mM *N,N,N'N'*-tetramethyl-*p*-phenylenediamine (TMPD). Respiration rates are expressed as μmol oxygen $minute^{-1}$ mg^{-1} mitochondrial protein.

Mitochondrial Bax Translocation

Mitochondrial pellets were lysed in a hypotonic buffer (250mM sucrose, 20mM HEPES, 10mM KCl, 1.5 $MgCl_2$ 1 mM EDTA, 1mM EGTA 1 mM PMSF, 8 μg/ml aproptinin, 2 μg/ml Leupeptin [pH 7.4]). After incubation for 30 minutes, the suspension was sonicated and re-centrifuged (13,000g x 30 minutes). The soluble proteins were used for measurement of Bax translocation by western blot. Membranes were probed with anti-rabbit Bax (N-20, 1:1000; Santa Cruz Biotechnology). For protein loading control, the membranes were stripped and re-probed with anti-mouse mitochondrial porin (1:20,000; Molecular Probes, Inc). Secondary antibodies were coupled to horseradish peroxidase (anti-rabbit IgG 1:1000; anti-mouse IgG 1:10,000, Santa Cruz Biotechnology).

Fluorescent Immunohistochemistry and Confocal Imaging

OCT-embedded transverse ventricular slices were cut into 5-μm serial sections fixed in acetone, and blocked. Mouse monoclonal anti-cytochrome c oxidase IV (COX IV) (1:250; BD Biosciences Clonetech, Palo Alto, CA) was used. Sections were incubated with secondary antibody ($Cy^{TM}3$-conjugated donkey anti-mouse IgG, 1:500; Jackson ImmunoResearch Laboratories, INC). Mouse monoclonal anti-cytochrome c (1:200; BD Biosciences Pharmingen, San Diego, CA) was used the primary antibody for cytochrome c staining. Sections were immersed in secondary antibody ($Cy^{TM}2$-conjugated donkey anti-mouse Ig G, 1:200; Jackson ImmunoResearch Laboratories, INC).

Images from at least three different sections were acquired at 64X by a Zeiss LSM510 Multiphoton Laser Scanning Confocal Microscope (Carl Zeiss, Jena, Germany), using the same pinhole setting, pixel format (1024x1024), and scanning data depth (0.8μm). Double fluorescence for green and red channels was imaged using excitation of

Argon-Hene1 at wavelengths of 488 and 530nm. Fifty high power fields from each animal were analyzed by imaging processing software (Volocity 3.0, Improvision, Lexington, MA). High intensity of red and green (above 551 voxels) was regarded as specific immunoreactive signals. The red (COX IV) was set as reference signal and the contribution of the green (cytochrome c) to the colocalization of the both signals was quantified as the overlap coefficient (Kx-green) and used to compare the diffusion of cytochrome c staining between groups ***(Zinchuk 2005)***.

TUNEL Staining

OCT-embedded transverse ventricular slices were cut into 5-μm serial sections. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. After TUNEL, all nuclei were counterstained with DAPI. TUNEL-positive myocytes and the number of total myocytes were counted in 50 random high-power fields (X 600) with a Leica (Deerfield, IL) fluorescent microscope and OpenLab software (Improvision, Lexington, MA). Approximately 10,000 myocytes were examined from at least three different slices from each animal.

Statistics

Data are expressed as mean ± SEM and groups comparisons made with Fisher's LSD ANOVA. A Tukey test was used for multiple post hoc comparisons. A *P* value less than 0.05 was considered statistically significant.

Results

Mitochondrial Bax Translocation

CCP was associated with Bax translocation to the mitochondria (homodimer) in the mitochondrial protein fraction when compared to the Non-CCP myocardium (1.60±0.02 *vs* 1.43 ±0.30 arbitrary units (AU), P<0.01). Bax translocation was reduced after CsA treatment (1.60±0.02 vs 1.47± 0.04 AU, P<0.05) to the levels which were similar to Non-CCP (Figure 1).

Mitochondrial Permeabilization and Cytochrome c Release

Fluorescence immunohistochemistry was used to compare patterns of cytochrome c and COX IV staining (Figure 2). Release of cytochrome c from the mitochondria causes diffusion of the cytochrome c staining in the cytoplasm and a stippled green pattern is produced. This pattern is prominent in the merged images from the CCP myocardium, while in Non-CCP and CCP+CsA myocardium, only faint diffusion of cytochrome c staining in the merged images was found. The contribution of the green color intensity to the colocalization of these color voxels in the merged images were quantified by overlap coefficients (Kx-green). Compared with Non-CCP and CCP+CsA myocardium, lower overlap coefficient (Kx-green) was found in CCP myocardium (P<0.01).

TUNEL Staining

TUNEL-positive nuclei were more frequent in CCP myocardium when compared with Non-CCP and CCP+CsA myocardium (22.5 ± 4.45 *vs* 3 ± 1.29 and 4 ± 2.16 per 10,000 nuclei; P<0.01) (Figure 3). There was no difference between Non-CCP and CCP+CsA (P=0.82).

Clark-Electrode Oxygen Consumption Measurements

Isolated mitochondrial oxygen consumption was assessed during State 2 respiration for mitochondrial complexes I, II and IV and was not different among groups suggesting

comparable levels of mitochondrial integrity after fractionation. Cardioplegic arrest was associated with diminished State 3 respiration and the decrement of State 3 and the ratio of State 3 to State 2 were prevented by pretreatment with CsA ($P<0.01$) (Figure 4 and Table 1). Complex II and Complex IV oxygen consumption rates were not different between groups.

Myocardial performance

There was no significant different change in heart rate among Non-CCP, CCP and CCP+CsA. Left ventricular developed systolic pressure (LVDP), positive and negative maximum dP/dt were significantly depressed at 5, 15, 30, and 60 minutes of reperfusion in the CCP hearts (compared with Non-CCP). LVEDP was significantly increased in CCP when compared with Non-CCP. In contrast, administration of CsA improved all these parameters (Table 2 and Figure 5).

Discussion

A typical pathological change in the post-ischemic myocardium of cardioplegic arrest is manifested by a constellation of apoptotic events ***(Feng 2005)*** and apoptosis-related alterations in mitochondrial structure and function including Bax translocation, mitochondrial permeabilization, cytochrome c release, and deficits in mitochondrial electron transport ***(Caldarone 2004, Wang 2006).*** We have previously defined this constellation of events Apoptosis-Related Mitochondrial Dysfunction (ARMD). The current study demonstrates that MPTP inhibition with CsA prevents ARMD and this prevention is associated with preservation of global myocardial performance.

The etiology of mitochondrial permeabilization during apoptosis is not fully determined. Bax translocation, however, is associated with disruption of outer

mitochondrial membrane integrity and cytochrome c release into the cytoplasm. The mechanism by which Bax induces the permeabilization is not clearly defined and it may act through direct pore-forming capacity ***(Antonnsson 2000)*** or through direct interaction with the mitochondrial permeability transitional pore (MPTP) ***(Shimizu 1999).*** Alternatively, transient MPTP opening may act as an initial event to stimulate Bax translocation to the mitochondria ***(Prechet 2005)*** and, through a positive feedback loop between Bax and the MPTP, stimulate further permeabilization and cytochrome c release ***(Narita 1998).*** In the present study, CsA prevented Bax translocation, suggesting that MPTP opening is an 'upstream' signaling event which is required for Bax translocation and subsequent mitochondrial permeabilization.

Cytochrome c is an important component of mitochondrial oxidative phosphorylation. A relative deficiency of mitochondrial cytochrome c, as may occur after mitochondrial permeabilization, may be responsible for deficits in mitochondrial respiration ***(Mootha 2001).*** Cytochrome c release from mitochondria inhibits mitochondrial respiration due to limitation of electron transport to cytochrome c oxygenase ***(Kuznetsov 2003)*** and cytochrome c release, in turn, stimulates mitochondrial superoxide formation that may amplify the deficits in electron transport chain activity ***(Cai 1998).*** In addition to these upstream apoptotic signals, Ricci *et al* reported that caspase 3, a "downstream" protease activated by cytochrome c release, can directly inhibit electron transport chain activity ***(Ricci 2003).*** In the present study, the prevention of mitochondrial permeabilization with CsA was associated with preservation of mitochondrial function at Complex I, suggesting that loss of reducing equivalents

(cytochrome c) or direct inhibition of electron transport can be prevented with maintenance of mitochondrial outer membrane integrity.

Although the relationship between ARMD and global myocardial performance is not clearly defined, other investigators have demonstrated that inhibition of mitochondrial dysfunction results in the preservation of myocardial function ***(McCully 2002, Javadov 2003).*** Specific inhibition of MPTP by CsA improved the recovery of cardiac function which is associated with reduction of cell death rate in a variety of *in vivo* and in *vitro* ischemia/reperfusion models ***(Argaud 2005, Javadov 2003).*** Therefore, a rationale is provided for a myopreservation strategy focused on maintenance of mitochondrial structural and functional integrity ***(Argaud 2005, Rousou 2004)***. In the current study, cardioplegic arrest induced conspicuous apoptosis-related alterations in mitochondrial structure and function and these changes were correlated with myocardial dysfunction. Pre-treatment with CsA prevents both ARMD and myocardial dysfunction, supporting a causal relationship between ARMD and global myocardial dysfunction..

Conclusion

Inhibition of MPTP opening with Cyclosporin A prevents ARMD and preserves cardiac global performance after cardioplegic arrest. Better understanding of the interaction between apoptotic signals and ARMD, and the correlation of ARMD with myocardial dysfunction will enhance myopreservative strategies for the cardiac surgeon.

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Legends

Figure 1: (A) Representative Western blot of mitochondrial Bax in Non-CCP, CCP and CCP+CsA myocardium; (B) Densitometric measurements of Bax in Non-CCP, CCP, and CCP+CsA hearts. Increased concentrations of Bax (homodimer) in the

mitochondrial fractions normalized by porin content are indicative of translocation of Bax to the mitochondria (*P<0.01 *vs* Non-CCP; †P<0.05 *vs* CCP).

Figure 2: (A) Immunofluorescence of cytochrome c oxidase IV (COX IV) and cytochrome c in the myocardium from Non-CCP, CCP and CCP+CsA myocardium. In each panel, Cox IV (mitochondria) is stained red, cytochrome c is stained green, and the merged mages are shown. Superimposition of red and green staining results in a brownish color that suggests retention of cytochrome c in the Non-CCP and CCP+CsA mitochondria. A fine diffuse green staining can be seen in the merged images of CCP myocardium suggesting mitochondrial release of cytochrome c. Scatterplots display the distribution of red and green voxels in the merge images of Non-CCP, CCP and CCP+CsA, as well as the various degrees of colocalization as shown in orange and yellow. (B) Quantification of the overlap coefficient (Kx-green) in the merge images for Non-CCP, CCP and CCP+CsA groups is shown (*P<0.01 *vs* Non-CCP; $^{\#}$P<0.01 *vs* CCP).

Figure 3: Representative imaging of TUNEL staining. (A) TUNEL-positive nuclei (Green) which are indicated by white arrows; (B) Nuclei DAPI staining (Blue); (C) A merge picture of (A) and (B). (D) Quantitative analysis of TUNEL staining for Non-CCP, CCP and CCP+CsA myocardium (*P<0.01 vs Non-CPB; †P<0.05 vs CCP).

Figure 4: (A) The representative traces of real-time mitochondrial oxygen consumption including complex I, II and IV from Non-CCP, CCP and CCP+CsA; (B) Comparison of the activity of state 3, and the ratio of state 3 to state 2 of complex I, II and IV among the groups (*P<0.01 *vs* Non-CCP; $^{\#}$P<0.01 *vs* CCP).

Figure 5: (A) The representative traces of condensed real-time blood pressure (systolic and diastolic pressure) and first derivatives of LVP (±dP/dt) from Non-CCP,

CCP and CCP+CsA; (B) The changes of systolic pressure, maximum (±dP/dt) and the diastolic pressure at 5, 15, 30 and 60 minutes of reperfusion (*P<0.01 vs Non-CCP; $^{\#}$P<0.01 vs CCP).

Table 1: Isolated mitochondrial oxygen consumption (μmol O_2/min.mg protein) for Non-CCP, CCP and CCP+CsA mitochondria (*P<0.01 vs Non-CCP; †P<0.01 *vs* CCP).

Table 2: Hemodynamics. Values are Mean±SEM; n=6 each group. *P<0.01 vs Non-CCP and †P<0.01 vs CCP.

Table 1: Isolated Mitochondrial Oxygen Consumption

	Complex I			Complex II			Complex IV		
	Non-CCP	CCP	CCP+CsA	Non-CCP	CCP	CCP+CsA	Non-CCP	CCP	CCP+CsA
State 2	0.52±0.05	0.51±0.05	0.49±0.02	0.52±0.05	0.51±0.05	0.49±0.02	0.52±0.05	0.51±0.05	0.49±0.02
State 3	6.21±1.21	2.00±0.28*	5.64±0.82†	3.05±0.38	2.52±0.19	2.77±0.30	9.53±0.47	9.39±0.66	10.42±0.81
Ratio 3/2	11.65±1.15	4.08±0.68*	11.48±1.62†	5.93±0.45	5.29±0.76	5.62±0.54	19.06±1.24	19.07±1.32	21.24±1.62

Table 2: Hemodynamics in Non-CCP, CCP and CCP+CsA Treated Heart

Variables	Baseline			Reperfused								
				15 minute			30 minute			60 minute		
	Non-CCP	CCP	CCP+CsA	Non-CCP	CCP	CCP+CsA	Non-CCP	CCP	CCP+CsA	Non-CCP	CCP	CCP+CsA
HR (bpm)	210±6	230±7	213±6	190±12	230±8	203±8	180±11	236±9	203±8	183±1	230±6	196±8
LVDP (Δ %)	100	100	100	97±1	80±1*	90±3†	95±1	84±0.7*	95±1.2†	95±1	77±1*	94±2†
LVEDP (mmHg)	3.0±0.2	3.1 ±0.0	2.9±0.1	3.1±0.3	17±1.8*	6.7±0.9†	3.0±0.2	11.7±1.6*	3.6±0.2†	3.2±0.3	11.4±1.1*	3.2±0.3†
+dP/dtmax (Δ%)	100	100	100	95±0.9	73±2.6*	86±3.7*†	95±1.1	80±3.3*	94±1.8†	94±1.6	74±3.4*	92±2.3†
-dP/dtmax (Δ%)	100	100	100	95±1.2	63±2.2*	81±2.7*†	94±1.3	73±3.1*	90±1.4†	93±1.0	73±2.5*	93±1.5†