Am J Transl Res 2017;9(7):3345-3359
www.ajtr.org /ISSN:1943-8141/AJTR0030709

Original Article

In vivo opening of the mitochondrial permeability transition pore in a rat model of ventricular fibrillation and closed-chest resuscitation

Iyad M Ayoub[1], Jeejabai Radhakrishnan[1], Raúl J Gazmuri[1,2]

[1]*Resuscitation Institute at Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, USA;*
[2]*Critical Care Medicine, Captain James A Lovell Federal Health Care Center, North Chicago, Illinois, USA*

Received April 17, 2016; Accepted August 11, 2016; Epub July 15, 2017; Published July 30, 2017

Abstract: Opening of the mitochondrial permeability transition pore (mPTP) is considered central to reperfusion injury. Yet, most of our knowledge comes from observations in isolated mitochondria, cells, and organs. We used a rat model of ventricular fibrillation (VF) and closed-chest resuscitation to examine whether the mPTP opens *in vivo* and whether cyclosporine A (CsA) attenuates the associated myocardial injury. Two series of 26 and 18 rats each underwent 10 minutes of untreated VF before attempting resuscitation. In *series-1*, rats received 50 μCi of tritium-labeled 2-deoxyglucose ([^{3}H]DOG) harvesting their hearts at baseline (n=5), during VF (n=5), during resuscitation (n=6), and at post-resuscitation 60 minutes (n=5) and 240 minutes (n=5). mPTP opening was estimated measuring the ratio of mitochondria to left ventricular intracellular [^{3}H]. In *series-2*, rats received 10 mg/kg of CsA or vehicle before resuscitation, measuring mitochondrial NAD^+ content to indirectly assess mPTP opening. In *Series-1*, the mPTP opening ratio *vs* baseline (10.4 ± 1.9) increased during VF (16.8 ± 2.4, *NS*), closed-chest resuscitation (20.8 ± 6.3, $P<0.05$), and at post-resuscitation 60 minutes (20.9 ± 4.7, $P<0.05$) and 240 minutes (25.7 ± 11.0, $P<0.01$). In *series 2*, CsA failed to attenuate reductions in mitochondrial NAD^+ and did not affect plasma cytochrome *c*, plasma cardiac troponin I, myocardial function, and survival. We report for the first time in an intact rat model of VF that mPTP opens during closed-chest resuscitation consistent with previous observations in mitochondria, cells, and organs of mPTP opening upon reperfusion. CsA, at the dose of 10 mg/kg neither prevented mPTP opening nor attenuated post-resuscitation myocardial injury.

Keywords: Cardiopulmonary resuscitation, cyclosporine, deoxyglucose, mitochondria, myocardial ischemia, ventricular fibrillation

Introduction

The term mitochondrial permeability transition pore (mPTP) denotes the formation of a mega pore across the inner and outer mitochondrial membranes through the apposition of mitochondrial proteins enabling the passage of molecules up to 1.5 kDa [1]. The specific proteins involved in pore formation are an issue of continuing scientific debate. The most recent studies proposed that the pore is a heterooligomeric complex composed of three proteins; cyclophilin-D (Cyp-D; a mitochondrial matrix protein), the voltage dependent anion channel (VDAC; an outer mitochondrial membrane protein), and spastic paraplegia 7 (SPG7; an inner mitochondrial membrane protein) [2].

Under physiological conditions, the mPTP may open and close transiently [3, 4] representing a phenomenon known as "flickering" and involved in the regulation of mitochondrial calcium homeostasis [5-7]. mPTP flickering stimulates generation of mitochondrial superoxide, the so-called "superoxide flashes", which is a physiological event that results from functional coupling between transient mPTP opening and electron transport chain dependent reactive oxygen species production [8].

Under pathophysiological conditions, however, the mPTP may open in a sustained manner causing collapse of the proton motive force across the inner mitochondrial membrane required for ATP synthesis by F_o-F_1 ATP synthase [9-11], uncoupling oxidative phosphorylation and leading to energy depletion, cell injury, and eventual cell death [11-15]. Support for this mPTP effect stems from studies in cardiomyocytes subjected to oxidative stress [16-18],

isolated perfused rat hearts subjected to elevated Ca^{2+} levels [14, 19], and studies in an intact rat model of heart failure in which pore opening was measured *ex vivo* after heart removal [20].

However, measuring mPTP opening in isolated mitochondria, cells, and organs simulating ischemia and reperfusion injury may be subject to experimental artifacts limiting extrapolation to *in vivo* settings. Moreover, removal of organs from their natural environment eliminates the influence of adaptive responses affecting ischemia and reperfusion injury such as neuroendocrine response to stress with release of catecholamines and other stress hormones [21].

In vivo measurement of transient mPTP opening accompanied by "superoxide flashes" has been reported recently in mice expressing a superoxide biosensor [22]. However, to the best of our knowledge, measurement of mPTP opening *in vivo* in the setting of ischemia and reperfusion has not been reported. We therefore adapted a technique previously used to measure mPTP opening in isolated rat hearts based on tritium-labeled 2-deoxyglucose ([^{3}H] DOG) [14] and measured mPTP opening in a rat model of ventricular fibrillation (VF) and closed chest resuscitation. We conducted additional experiments to examine whether cyclosporine A (CsA) a putative inhibitor of mPTP opening could elicit favorable myocardial effects in the same rat model, indirectly assessing mPTP opening by measuring mitochondrial NAD^+ levels [23].

Materials and methods

The studies were approved by our Institutional Animal Care and Use Committee and conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Animal preparation

Male retired breeder Sprague-Dawley rats (456 to 543 g) were anesthetized with 45 mg/kg of sodium pentobarbital given intraperitoneally followed by 10 mg/kg given intravenously every 30 minutes to maintain a surgical plane of anesthesia. A 5-F catheter was orally advanced into the trachea and used for positive pressure ventilation during chest compression and the post-resuscitation interval. Proper tracheal placement was verified using an infrared CO_2 analyzer (CO_2 SMO model 7100, Novametrix Medical Systems Inc., Wallingford, Connecticut). A conventional lead II ECG was recorded through subcutaneous needles. For pressure measurement and blood sampling, fluid-filled PE50 catheters were advanced from the right femoral artery into the abdominal aorta and from the left femoral vein into the right atrium. To assess left ventricular (LV) function, another fluid-filled PE50 catheter was advanced from the right carotid artery into the left ventricle. A 3-Fr catheter (model C-PUM-301J, Cook Inc., Bloomington, IN) was advanced through the right external jugular vein into the right atrium, and through its lumen a precurved guidewire was fed into the right ventricle for electrical induction of VF. The guidewire was removed before starting chest compression. For measurement of core temperature and thermodilution cardiac output, a thermocouple probe (0.64-mm diameter, IT-18, Physitemp Instruments, Clifton, NJ) was advanced from the left femoral artery into the thoracic aorta. A 3-F catheter (C-PUM-301J, Cook Inc., Bloomington, IN) was advanced from the left external jugular vein into the right atrium and used for injection of the thermal tracer (200 µl of normal saline at ~24°C) to measure cardiac output. Core temperature was monitored through the thermocouple and maintained between 36.5 and 37.5°C using a lamp.

VF and resuscitation protocols

VF was induced by delivering a 60-Hz alternating current to the right ventricular endocardium (0.1 to 0.6 mA) for 3 minutes after which VF was allowed to continue spontaneously for 7 additional minutes completing a 10 minute interval of untreated VF. Chest compression was then initiated using a pneumatically driven piston device (CJ-80623, CJ Enterprises, Tarzana, CA) centered on the mid-chest and programmed to deliver 200 compressions per minute with a 50% duty cycle. The depth of compression was adjusted to maintain a coronary perfusion pressure between 22 and 24 mmHg by the second minute of chest compression, exceeding the 20 mmHg threshold required for successful resuscitation in rats [24, 25]. The piston travel was measured with a displacement transducer (DSPL, World Precision Instruments Inc., Sarasota, FL). Positive

pressure ventilations were provided with a volume-controlled ventilator (Model 683, Harvard Apparatus, Holliston, MA) set to deliver 25 unsynchronized breaths per minute using a tidal volume of 6 ml/kg and 100% oxygen. Transthoracic defibrillation was attempted after 8 minutes of chest compression by delivering up to two 5-Joules (J) biphasic waveform electrical shocks across the chest wall (Heartstream XL, Philips Medical Systems, Andover, MA) 5 seconds apart. If VF persisted or an organized rhythm with a mean aortic pressure ≤25 mmHg ensued, chest compression was resumed for 30 seconds. The defibrillation-compression cycle was repeated up to three additional times, increasing the energy of individual shocks (if VF persisted) to 7-J for the subsequent two cycles. Successful defibrillation was defined as the return of an organized rhythm with a mean aortic pressure >60 mmHg for >5 minutes. After return of spontaneous circulation, rats were monitored for a maximum of 240 minutes in *series 1* and 360 minutes in *series 2* (see Experimental Series).

In vivo measurement of mPTP opening

We adapted a technique previously developed in isolated perfused rat hearts [14, 26, 27] to measure *in vivo* mPTP opening in our rat model. The technique in the isolated heart involves: *i*) retrograde perfusion of the heart with a [^{3}H]DOG solution mounted in a Langendorff apparatus to enable [^{3}H]DOG uptake by cardiomyocytes through glucose transporters (i.e., facilitated diffusion), *ii)* phosphorylation of uptaken [^{3}H]DOG by hexokinase to [^{3}H]DOG-6-phosphate a form not able to enter the glycolytic pathway trapping [^{3}H]DOG-6-phosphate in the cytosol, iii) entrance of [^{3}H]DOG-6-phosphate to the mitochondrial matrix upon mPTP opening, *iv*) flushing the coronary circuit to remove [^{3}H]DOG from the extracellular space, *v*) trapping of [^{3}H]DOG-6-phosphate in the mitochondria by calcium chelation during tissue processing, and *vi*) measuring mitochondrial and LV intracellular (LV_{IC}) [^{3}H] radioactivity (activity) expressing mPTP opening as the ratio of mitochondria to LV_{IC} [^{3}H] activity [14]. For the present experiments, we loaded the hearts *in vivo* during spontaneous circulation 30 minutes before inducing VF by intravenous injection of [^{3}H]DOG and avoided flushing the coronary circuit to minimize additional *ex vivo* manipulation of myocardial tissue before processing.

Plasma and LV tissue processing: At the end of each experiment and immediately before removing the heart, whole blood was collected, centrifuged for 15 minutes at 1,000 *g*, and 200 µl aliquots of plasma were collected and stored at -80°C. Hearts were excised via a sternotomy and the LV separated, weighed, and homogenized in ice-cold MSH buffer (2.5 ml for 0.1 g tissue) containing (in mM) mannitol 210, sucrose 300, HEPES 5, EGTA 2 at pH 7.4, and BSA 5 mg/ml. EGTA was used to chelate Ca^{2+} sealing the mPTP and thus trapping [^{3}H] DOG-6-phosphate inside the mitochondrial matrix. A 50 µl aliquot of the crude homogenate was used to measure LV [^{3}H] activity and to estimate sample protein content by BCA protein assay (Thermo Scientific Inc., Bannockburn, IL). Mitochondria were isolated by differential centrifugation [28], suspended in 500 µl of MSH buffer and stored at -80°C in separate 100 µl aliquots. From each aliquot, 50 µl was used to measure [^{3}H] mitochondrial activity and the other 50 µl to estimate protein content and quantify citrate synthase (CS) activity [29].

Measurement of [^{3}H] activity in LV homogenate, mitochondrial fraction, and plasma: For [^{3}H] activity measurement in LV homogenate and mitochondrial fraction, suspensions were thawed on ice, mixed with equal volume of 5% perchloric acid ($HClO_4$), and centrifuged at 10,000 *g* for 2 minutes to precipitate proteins. After centrifugation, 50 µl of the supernatant was mixed with 5 ml of scintillation fluid. For [^{3}H] measurement in plasma, samples were thawed on ice and 10 µl was mixed with 5 ml of scintillation fluid. LV tissue homogenate, mitochondrial fraction, and plasma activity were measured as disintegrations per minute (dpm) in a scintillation counter (Beckman Instruments Inc., Irvine, CA) programmed to read each sample for 10 minutes. [^{3}H] activity in LV homogenate, mitochondrial fraction, and plasma was expressed as dpm per ml of the original respective homogenate, mitochondrial fraction, and plasma sample volume (dpm/ml).

Determination of mPTP opening: mPTP opening was determined as the ratio between mitochondrial [^{3}H] activity (normalized to mitochondrial CS; [^{3}H] dpm/IU of CS) divided by LV_{IC} [^{3}H]

activity (normalized to wet weight; [^{3}H] dpm/g wet-weight) ×10^5.

LV_{IC} [^{3}H] activity (dpm/g wet-weight): Because we did not flush the coronary circuit, the LV extracellular activity was presumed equivalent to plasma [^{3}H] (Pl [^{3}H]) activity and subtracted it from the total LV [^{3}H] (LV_T [^{3}H]) activity. We assumed the extracellular fraction of LV tissue to be 0.21 based on previous measurements in the same rat model [30] and determined the LV_{IC} [^{3}H] activity according to equation (*i*):

$$LV_{IC}[^3H]\,(dpm/ml) = [LV_T[^3H]\,(dpm/ml) - (0.21 \cdot Pl[^3H]\,(dpm/ml)]/0.79 \quad (i)$$

Next, the LV_{IC} [^{3}H] (dpm/ml) was divided by LV density estimated to be 1.05 g/ml [31] to obtain LV_{IC} [^{3}H] (dpm/g wet-weight).

Mitochondrial [^{3}H] activity (dpm/IU CS): Mitochondrial CS activity was measured according to Shepherd and Garland [29]. The assay is based on a colorimetric measurement of the molar extinction coefficient of 5-thio-2-nitrobenzoic acid (TNB) by coupling the enzymatic reaction (*ii*) catalyzed by CS (1) to the irreversible chemical reaction (*iii*). The thiol group reacts with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) forming a yellow product whose absorbance correlates linearly with TNB molar concentration.

$$Acetyl\text{-}CoA + Oxaloacnte \xrightarrow{CS} Citrate + CoA\text{-}SH + H^+ + H_20 \quad (ii)$$

$$CoA\text{-}SH + DTNB \rightarrow TNB + CoA\text{-}S\text{-}S\text{-}TNB \quad (iii)$$

For the assay, mitochondrial suspensions thawed on ice were diluted 1:10 with MSH buffer and a 10 µl aliquot transferred to a 1000 µl cuvette with 920 µl of assay buffer composed of 50 mM Tris-HCl, pH 8, 30 mM acetyl-CoA, and 10 mM DTNB. Kinetic activity was measured at wavelength 412 nm (BioMate 3 UV-Vis Spectrophotometer, Thermo Fisher Scientific Inc., Bannockburn, IL) before and after adding 70 µl of 10 mM oxaloacetate (OAA) to the assay reaction. The activity measured before adding the OAA (background) was subtracted from the activity measured after adding the OAA.

Specific enzyme activity was calculated using equation *iv*:

$$CS\ (IU/mg\ protein) = \frac{\Delta A_{412}/min}{\varepsilon^{mM} x l x v} x \frac{V_{cuvette}}{V_{sample} x P_{sample}} \quad (iv)$$

Whereas IU denotes specific activity of CS expressed in international units, ΔA_{412}/min denotes rate of absorbance change measured at 412 nm, ε^{mM} denotes extinction coefficient of TNB at 412 nm and pH 8.1 (13.6 $mM^{-1}cm^{-1}$), *l* denotes optical length using a 1000 µl cuvette (1 cm), *v* denotes stoichiometric number of TNB in the reaction *iii* (1), $V_{cuvette}$ denotes volume of solution in the cuvette (1000 µl), V_{sample} denotes volume of sample in the cuvette (10 µl), and P_{sample} denotes protein concentration in the sample (mg protein/µl).

Mitochondrial [^{3}H] activity (dpm/ml) was first normalized to mitochondrial protein concentration (dpm/mg protein) and then to CS activity by dividing the mitochondrial [^{3}H] dpm/mg protein by CS activity (IU/mg protein) and expressed as dpm/IU of CS. Pore opening was calculated using equation (*v*) and expressed as the ratio between mitochondrial and the left ventricular [^{3}H] activity [14].

$$mPTP\ opening\ ratio = 10^5 x \frac{mitochondrial\ [^3H]\ (dpm/IU\ CS)}{LV_{IC}\ [^3H]\ (dpm/g\ wet\text{-}weight)} \quad (v)$$

Cardiac troponin I measurement

Cardiac troponin I (cTnI) was measured using a commercially available one step "sandwich" enzyme immunoassay method developed for human cTnI (Dimension® clinical chemistry system using Cardiac Troponin-I Flex® reagent cartridge, Dade Behring Inc., UK). The method has excellent reactivity and specificity for rat cTnI, which has 92.8% homology with human cTnI [32-35]. Measurements were made in plasma samples collected at predetermined intervals that had been stored at -80°C.

Plasma cytochrome c measurement

Cytochrome *c* was measured by reverse-phase high-performance liquid chromatography (HPLC) as previously reported [28]. Briefly, arterial blood samples (200 µl) were collected

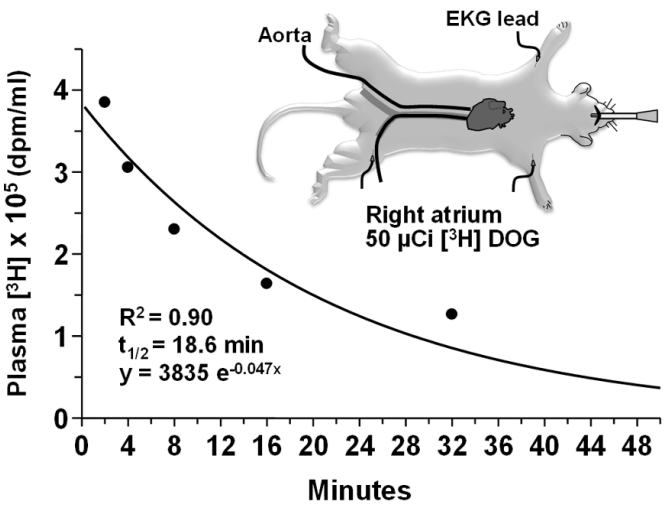

Figure 1. Plasma levels after administration of 50 µCi [^{3}H]DOG into the right atrium. dpm = disintegrations per minute.

into heparinized syringes and centrifuged at 5,000 rpm (2,320 *g*) for 10 minutes at 4°C (Sorvall Biofuge Stratos, Heraeus, Thermo Fisher Scientific Inc., Bannockburn, IL). Plasma was collected and frozen at -80°C until analysis by HPLC. Absorbance was measured at 393 nm. Standard curves (0.2-20 µg/ml) were prepared using rat heart cytochrome *c* dissolved in plasma from sham rats and used for quantification.

Mitochondrial NAD$^+$ measurement

Mitochondrial NAD$^+$ was measured according to Correa *et al* [23]. Briefly, hearts were harvested at the end of the post-resuscitation monitoring period and mitochondria were isolated as described above for measuring [^{3}H] activity. Samples were treated with perchloric acid to release NAD$^+$ and then neutralized with potassium hydroxide (KOH). The amount of NAD$^+$ was determined fluorimetrically by measuring the amount of NADH generated in the NAD$^+$ dependent lactate dehydrogenase (LDH) reaction shown below (*vi*) at excitation 340 nm and emission 460 nm using a microplate reader (Gemini XPS, Molecular Devices, Sunnyvale, CA). The reaction mixture contained (in mM) lactate 2, hydrazine sulfate 300, glycine 870, EDTA 0.19, and LDH 1.2 U, at pH 9.5. The hydrazine was used to drive lactate oxidation forward to completion by removing pyruvate. NAD$^+$ was expressed as pmol/mg mitochondrial protein.

$$Lactate + NAD^+ \xrightarrow{LDH} Pyruvate + NADH \quad (vi)$$

Chemicals

[^{3}H]DOG was purchased from MP Biomedicals. Mannitol, sucrose, HEPES, EGTA, EDTA, BSA, oxaloacetate (OAA), hydrazine sulfate, glycine, EDTA, pyruvate, lactate dehydrogenase, NADH, and lactate were purchased from Sigma-Aldrich, St. Louis, MO. 2-Amino-2-(hydroxymethyl)-1, 3-propanediol, hydrochloride (Tris-HCl), acetyl coenzyme A (acetyl-CoA), 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), Acetonitrile (ACN), trifluoroacetic (TFA) acid, potassium hydroxide (KOH), lactic acid, and perchloric acid were purchased from Thermo Fisher Scientific Inc., Bannockburn, IL. Bradford dye reagent was purchased from Bio-Rad Laboratories, Hercules, CA.

Preparatory experiments

A 2.5 nmol/kg [^{3}H]DOG dose was chosen to achieve a plasma concentration similar to that used by others measuring glucose metabolism in rats without causing adverse metabolic effects [36]. The achieved plasma [^{3}H]DOG concentration has been shown adequate to load the heart [37] given that glucose transporters favor deoxyglucose over glucose. A formulation of [^{3}H]DOG with a specific activity of 40 Ci/mmol (27088S, MP Biomedicals, Santa Ana, CA) was used, delivering 1.25 nmoles (50 µCi) dissolved in 200 µl of 0.9% saline as bolus dose into the right atrium (~100 µCi/kg or 2.5 nmol/kg). In one preparatory experiment, we serially measured arterial plasma [^{3}H] activity during spontaneous circulation at 2, 4, 8, 16, and 32 minutes after injection. As shown in **Figure 1**, plasma [^{3}H] levels decreased over time following a mono-exponential decay function with a half-life of 18.6 minutes attaining a [^{3}H] plasma activity of ~1.3×10^5 dpm/ml at 30 minutes post injection. No adverse hemodynamic or functional effects were observed during this interval. The [^{3}H]DOG dose used was significantly below the dose reported to affect cell glucose transport (~10 mM) [14, 36] but sufficient to load the heart given the exposure for 30 minutes to a concentration enabling facilitated diffusion to occur. Moreover, the subsequent LV tissue analysis in the formal experiments demonstrated quantifiable intracellular [^{3}H] activity.

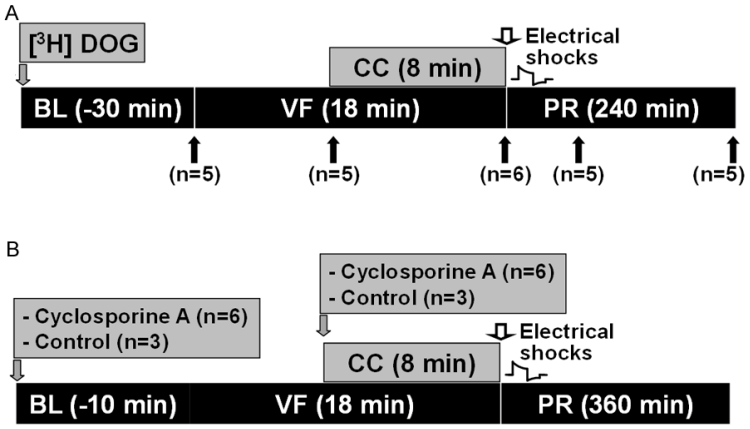

Figure 2. Diagram depicting the experimental protocol. A: Series 1. In vivo measurement of mPTP opening using [^{3}H]DOG. Black arrows represent the times at which the hearts were harvested. B: Series 2. In vivo effect of cyclosporine A during cardiac resuscitation.

Table 1. Hemodynamic and left ventricular effects of [^{3}H]DOG injection

	MAP, mmHg	HR, beats/min	CI, ml/min·kg^{-1}	SVRI, mmHg/ml·min^{-1}·kg^{-1}	LVSWI, gm-m/kg·beat^{-1}
Before	132 ± 11	324 ± 25	143 ± 15	0.92 ± 0.12	0.91 ± 0.15
After	136 ± 11	337 ± 25	144 ± 17	0.95 ± 0.13	0.92 ± 0.15

Measurements were obtained at baseline before and 30 minutes after bolus injection of [^{3}H]DOG (1.25 nmoles) into the right atrium in the 26 rats used for measuring mPTP opening. No differences were observed by one-way repeated-measures ANOVA. [^{3}H]DOG, Tritium-labeled 2-deoxyglucose; MAP, Mean aortic pressure; HR; Heart rate; CI, Cardiac index; SVRI, Systemic vascular resistance index; LVSWI, Left ventricular stroke work index. Mean ± SD.

Experimental series

Series 1: A total of 26 rats (456 to 543 g) received a bolus of 50 μCi [^{3}H]DOG at baseline 30 minutes before the planned induction of VF and were randomized to have their hearts removed immediately before inducing VF (control group; n=5), at the end of untreated VF (n=5), at the end of chest compression (n=6), at 60 minutes post-resuscitation (n=5), or at 240 minutes post-resuscitation (n=5) (**Figure 2**).

Series 2: A total of 18 rats (470 to 516 g) were randomized to receive a bolus of cyclosporine A (10 mg/kg) [39-42] five minutes before inducing VF (n=6), immediately before starting chest compression (n=6), or to receive equal volume vehicle (cremophor EL) control before inducing VF (n=3) or before starting chest compression (n=3) with the investigators blind to the treatment assignment. Resuscitated rats were monitored for up to 360 minutes. Four rats undergoing the same surgical preparation but without inducing VF were monitored for 360 minutes and served as a sham group controlling for the effects of instrumentation and prolonged anesthesia.

Statistical methods

SigmaPlot 11.0 (Systat Software, Point Richmond, CA) was used for statistical analysis. Differences in continuous variables among groups were analyzed by one-way ANOVA using the Holm-Sidak's method for multiple comparisons if overall differences were detected. Differences in continuous variables within groups were analyzed by repeated-measures one-way ANOVA applying Holm-Sidak's correction for multiple comparisons relative to baseline. Alternative nonparametric tests were used if the data failed tests for normality or equal variance. Non-linear regression analysis was performed between variables of interest based. The data was presented as mean ± SD in the text and tables and mean ± SEM in figures. A two-tail p value<0.05 was considered significant.

Results

Series 1

Hemodynamics and LV function: The administration of [^{3}H]DOG into the right atrium had no effects on hemodynamic or LV function (**Table 1**). In addition, the baseline measurements were comparable among the various experimental groups (**Table 2**). During chest compression, adjustments of compression depth successfully maintained an average coronary perfusion pressure above 22 mmHg between minute 2 and minute 8. After return of spontaneous circulation, rats developed characteristic post-resuscitation myocardial dysfunction evidenced by persistently low mean aortic pressure, left ventricular stroke work index, and cardiac index (**Table 2**). Myocardial dysfunction accounted for three

Table 2. Hemodynamic and left ventricular function

	Baseline	VF	Chest Compression		Post-Resuscitation		
	0 min	10 min	4 min	8 min	15 min	60 min	240 min
Mean Aortic Pressure, mmHg							
Baseline	132 ± 11						
VF	143 ± 3	7 ± 1					
CC	143 ± 11	7 ± 2	37 ± 3	38 ± 3			
PR-60	132 ± 12	8 ± 1	38 ± 2	37 ± 4	83 ± 10†	95 ± 13†	
PR-240	129 ± 12	7 ± 1	37 ± 2	39 ± 3	66 ± 20†	94 ± 15†	119 ± 4 [2]
Cardiac Index, ml/min·kg^{-1}							
Baseline	149 ± 18						
VF	136 ± 20						
CC	143 ± 11						
PR-60	146 ± 20				105 ± 46	67 ± 20*	
PR-240	147 ± 18				118 ± 25*	80 ± 13‡	62 ± 6 [2] ‡
Systemic Vascular Resistance Index, mmHg/ml·min^{-1}·kg^{-1}							
Baseline	0.89 ± 0.08						
VF	1.05 ± 0.15						
CC	1.00 ± 0.12						
PR-60	0.91 ± 0.12				0.87 ± 0.35	1.47± 0.47	
PR-240	0.88 ± 0.11				0.54 ± 0.25*	1.17 ± 0.24*	1.90 ± 0.13 [2]†
Left Ventricular Stroke Work Index, gm-m/kg·beat^{-1}							
Baseline	0.91 ± 0.21						
VF	0.93 ± 0.16						
CC	0.93 ± 0.13						
PR-60	0.93 ± 0.15				0.56 ± 0.20‡	0.30 ± 0.12†	
PR-240	0.92 ± 0.14				0.62 ± 0.29*	0.40 ± 0.11*	0.31 ± 0.00 [2]*
Coronary Perfusion Pressure, mmHg							
Baseline	112 ± 11						
VF	124 ± 4	2 ± 1					
CC	125 ± 13	1 ± 0	23 ± 1	24 ± 1			
PR-60	115 ± 8	2 ± 1	24 ± 1	23 ± 2	69 ± 14†	84 ± 12*	
PR-240	112 ± 10	2 ± 1	24 ± 1	24 ± 1	50 ± 21‡	83 ± 16*	106 ± 2 [2]
Coronary Perfusion Pressure/Depth Ratio (mmHg/mm)							
Baseline							
VF							
CC			1.67 ± 0.21	1.57 ± 0.29			
PR-60			1.68 ± 0.28	1.51± 0.32			
PR-240			1.68 ± 0.06	1.53 ± 0.15			

Comparison within each of the 5 groups (Baseline, n=5; VF, n=5; CC, n=6; PR-60, n=5; PR-240, n=5) over the various time events. Numbers in brackets indicate when the sample size decreased from the preceding sample size. VF, Ventricular fibrillation; CC, Chest compression; PR, Post-resuscitation. Mean ± SD. *P<0.05, ‡P<0.005, †P<0.001; PR-15, PR-60 and PR-240 *vs* baseline by one-way repeated-measures ANOVA using Holm-Sidak method for multiple comparisons.

rats dying before post-resuscitation minute 240 yielding an average survival time in the 240 minute group of 202 ± 57 minutes.

mPTP opening: Intact mitochondrial yield measured by CS activity in each experimental group was comparable to that of baseline hearts except for mitochondria isolated from hearts during VF which demonstrated less CS activity (170 ± 18 *vs* 131 ± 7 IU/mg protein, P<0.05). Mitochondrial [^{3}H] activity increased during VF but decreased during chest compression, demonstrating further reduction during the post-resuscitation interval (**Figure 3A**). The LV_{IC} [^{3}H]

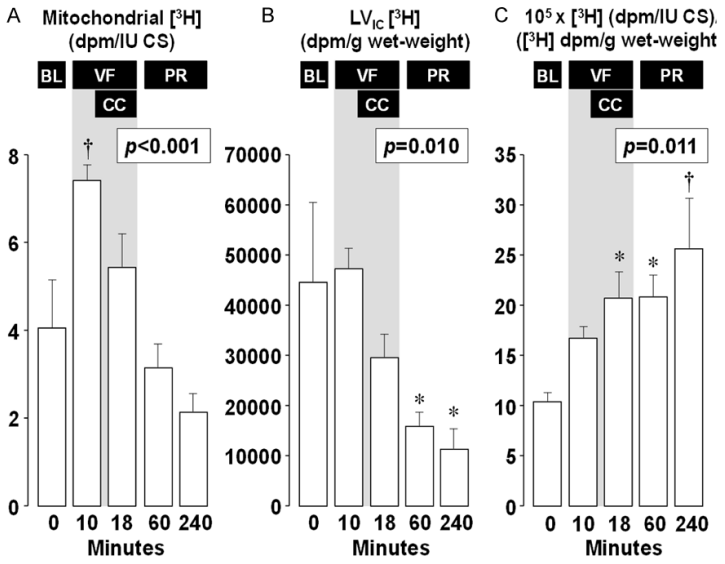

Figure 3. (A) Mitochondrial [^{3}H], (B) left ventricular intracellular [^{3}H] (LV_{IC} [^{3}H]), and (C) their ratio as a measurement of mPTP opening. Mean ± SEM. *P<0.05, †P<0.01 *vs* baseline using one-way ANOVA. CS, Citrate synthase; VF, Ventricular fibrillation; CC, Chest compression; PR, Post-resuscitation.

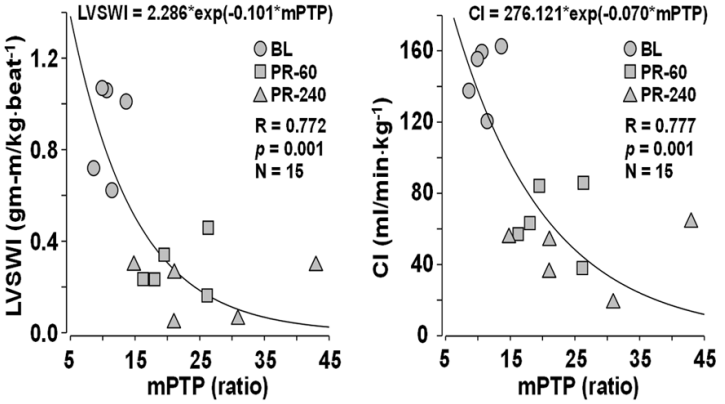

Figure 4. Non-linear regression analysis (exponential decay, 2 parameter) showing the relationship between mitochondrial permeability transition pore opening (mPTP) and left ventricular stroke work index (LVSWI, left) and cardiac index (CI, right) for measurements obtained at baseline (BL), and post-resuscitation at 60 minutes (PR-60) and at 240 minutes (PR-240).

activity remained unchanged during VF but decreased during chest compression (NS) with further reduction post-resuscitation that was statistically significant (**Figure 3B**). The mPTP opening ratio (**Figure 3C**) measured after 10 minutes of untreated VF increased relative to baseline but without attaining statistical significance. During chest compression, the mPTP opening ratio significantly increased and remained elevated during the post-resuscitation interval at 60 minutes with further increase at 240 minutes (**Figure 3C**). Regression analysis assessing the relationship between mPTP opening and both, left ventricular stroke work index and cardiac index, showed a highly significant negative correlation conforming to a mono-exponential decay curve (**Figure 4**).

Series 2

Hemodynamic and LV function: All rats that were treated with cyclosporine A (6 at baseline and 6 during VF) were pooled for the analyses after noticing no differences between subgroups. Similarly, the vehicle treated rats (3 at baseline and 3 during VF) were also pooled. Baseline hemodynamic measurements were comparable between groups (**Table 3**). Treatment with cyclosporine A had no beneficial effects on myocardial distensibility during chest compression as demonstrated by a comparable (cyclosporine A *vs* vehicle) depth of compression (13.9 ± 1.0 *vs* 13.7 ± 0.9 mm) and coronary perfusion pressure to depth of compression ratio (1.7 ± 0.1 *vs* 1.7 ± 0.2 mmHg/mm) averaged over the 2nd and 8th minute of chest compression. All rats were successfully resuscitated with no differences in the energy level of the shock required to terminate VF (cyclosporine A *vs* vehicle; 1.3 ± 0.6 *vs* 1.0 ± 0.6 Joules). However, after return of spontaneous circulation, episodes of recurrent VF requiring post-resuscitation electrical shocks were observed only in vehicle-treated rats (0.0 ± 0.0 *vs* 1.2 ± 1.6 shocks, P<0.05). During the subsequent post-resuscitation interval both groups displayed comparable hemodynamic and LV function (**Table 3**) with comparable survival times (cyclosporine A *vs* vehicle; 321 ± 67 minutes *vs* 331 ± 67 minutes).

Table 3. Baseline and post-resuscitation hemodynamic and LV function

	Baseline	Post-Resuscitation			
	-2 min	15 min	120 min	240 min	360 min
Mean Aortic Pressure, mmHg					
Cyclosporine A	141 ± 14	74 ± 21	102 ± 14	110 ± 31 [10]	75 ± 30 [9]
Vehicle	136 ± 11	84 ± 11	105 ± 17	112 ± 14 [5]	97 ± 35
Cardiac Index, ml/min·kg^{-1}					
Cyclosporine A	155 ± 19	100 ± 37	63 ± 15	56 ± 9 [10]	46 ± 15 [9]
Vehicle	154 ± 16	96 ± 33	62 ± 20	58 ± 13 [5]	52 ± 10
Left Ventricular Stroke Work Index, mmHg/ml·kg^{-1}					
Cyclosporine A	1.08 ± 0.22	0.58 ± 0.24	0.31 ± 0.10	0.33 ± 0.12 [10]	0.21 ± 0.12 [9]
Vehicle	0.97 ± 0.17	0.54 ± 0.19	0.31 ± 0.13	0.29 ± 0.08 [5]	0.23 ± 0.02
+dP/dt$_{max}$, mmHg·sec^{-1}					
Cyclosporine A	3964 ± 826	2695 ± 389	2959 ± 554	3138 ± 1042 [10]	2057 ± 971 [9]
Vehicle	3416 ± 681	2504 ± 472	2986 ± 373	3107 ± 232 [5]	2536 ± 803

N=12 in cyclosporine A group and 6 in vehicle treated rats. Numbers in brackets indicate when sample size decreased from the initial or from the preceding sample size. There were no statistically significant differences between groups. Mean ± SD.

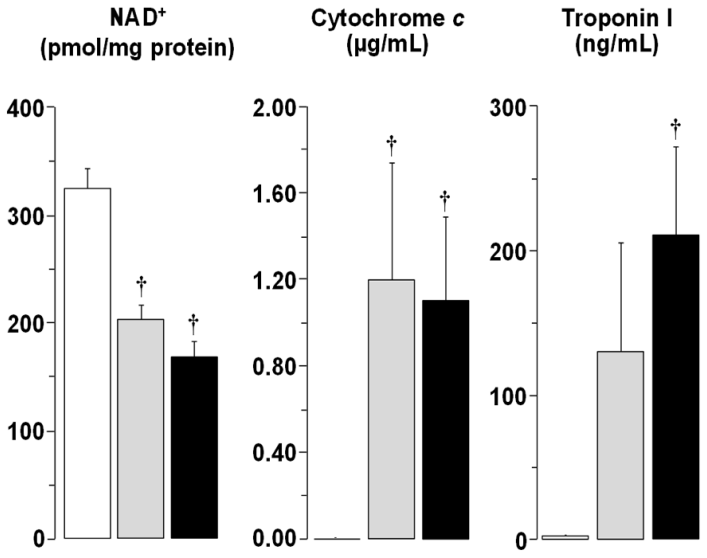

Figure 5. Levels of mitochondrial NAD$^+$, plasma cytochrome c, and plasma cardiac troponin I in sham rats (open bars), and after treatment with vehicle (gray bar) or cyclosporine A (black bars) at 360 minutes post-resuscitation or before death in rats subjected to VF and resuscitation. Mean ± SEM. †P<0.001 *vs* baseline.

Myocardial injury: Treatment with cyclosporine A did not attenuate the release of cytochrome c into the bloodstream (**Figure 5**). Plasma cTnI levels were negligible in sham rats but increased after VF and resuscitation with no differences in levels between cyclosporine A and vehicle treated rats. No difference in NAD$^+$ levels was observed between cyclosporine A and vehicle treated rats. The pooled NAD$^+$ levels of both treatment groups that underwent VF and resuscitation were ~34% lower than sham rats (P<0.001).

Discussion

We have documented *in vivo* opening of the mPTP in rat hearts while undergoing VF-induced cardiac arrest and closed-chest resuscitation, confirming previous *in vitro* and *ex vivo* observations showing mPTP opening upon reoxygenation [43, 18] or reperfusion [14, 9]. We also documented failure of cyclosporine A to prevent mPTP opening based on mitochondrial NAD$^+$ levels and failure to ameliorate mitochondrial and myocardial injury and thereby improve short-term survival.

Our method for *in vivo* measurement of mPTP opening circumvented technical and physiological shortcomings of *in vitro* or *ex vivo* mPTP measurements and enabled for the first time to document mPTP opening during resuscitation from cardiac arrest. With our approach, the key initial step in which [^{3}H]DOG-6-phosphate enters the mitochondria upon mPTP opening occurred in the intact *in situ* heart. In addition, in isolated rat heart experiments, the coronary circuit is

flushed to remove extracellular [^{3}H] before tissue processing. We eliminated this step to minimize processing time and technical confounders and instead calculated and excluded the extracellular [^{3}H] contribution to the total tissue [^{3}H] by concomitant measurement of plasma [^{3}H] and calculating the intracellular [^{3}H] as shown in equation (*i*). The subsequent key step, in which the uptaken [^{3}H]DOG-6-phosphate is trapped in mitochondria by calcium chelation during tissue processing and the ensuing additional steps required for mitochondrial isolation and measurement of the ratio between mitochondrial and intracellular [^{3}H] were the same as previously reported for the isolated heart [27].

Our *in vivo* approach included the influence of the neuroendocrine stress response to cardiac arrest and resuscitation. As part of this response, there is an intense adrenergic surge with increased circulating catecholamines that also contributes to myocardial injury during cardiac resuscitation [44-46]. Increased catecholamine levels can augment myocardial injury by promoting intracellular Ca^{2+} entry and reduce the threshold for mPTP opening [47].

The chronology of mPTP opening in the present study suggests that mPTP opening could have started during the interval of untreated VF when we observed an increase in the mPTP opening ratio (~60% higher than baseline) but without attaining statistical significance. Consistent with the concept that reperfusion after ischemia triggers mPTP opening, gasping that typically occurs after onset of cardiac arrest may generate blood flow in the absence of resuscitation efforts [48, 49]. With the start of chest compression, a statistically significant increase in the mPTP opening ratio was observed that essentially doubled baseline values. The myocardial blood flow during chest compression in our rat model corresponds to ~50% of baseline [50]. The mPTP opening ratio remained high post-resuscitation attaining a level 2.5 fold baseline at 240 minutes, suggesting the possibility of additional opening upon further reperfusion during the post-resuscitation phase.

In previous studies using the same rat model of VF and closed-chest resuscitation, we reported release of cytochrome *c* to the bloodstream detected during chest compression with further increase post-resuscitation, attaining levels that were inversely proportional to myocardial function and survival [28, 51, 52]. These previous observation are consistent with the present ones as mPTP opening would be expected to injure mitochondria resulting in cytochrome *c* release [53-55]. Moreover, in the present study we documented an inverse relationship between the mPTP opening ratio and left ventricular function, again pointing to mitochondrial injury being at the center of post-resuscitation myocardial dysfunction, providing a solid rationale for examining the effect of preventing mPTP opening during cardiac resuscitation [56].

Current efforts to pharmacologically prevent (or increase the threshold for) mPTP opening are focused on cyclophilin-D, which is the only recognized integral protein of the mPTP [2, 57]. Cyclosporine A inhibits cyclophilin-D activity increasing the threshold for mPTP opening [58-61]. Through this mechanism, cyclosporine A has been shown to be protective in isolated cardiomyocytes [16], *in situ* perfused hearts [14], and in intact animal models of myocardial infarction [62, 63, 42] and cardiac arrest [64, 65]. Yet, in the present study cyclosporine A failed to attenuate post-resuscitation myocardial injury with indirect evidence suggesting that it failed to prevent mPTP opening based on mitochondria NAD^+ levels [66]. mPTP opening allows efflux of NAD^+ from the mitochondrial matrix such that mitochondrial NAD^+ levels may be used as indirect marker of mPTP opening. The reduction in mitochondrial NAD^+ after cardiac arrest in our study was ~34% and similar to the 30% reduction reported in isolated rat hearts subjected to global ischemia by Di Lisa *et al* [66]. However, in contrast to our study, the administration of cyclosporine A in the study by Di Lisa *et al* attenuated reperfusion injury and preserved mitochondrial NAD^+.

The failure of cyclosporine A to prevent mPTP opening and ameliorate reperfusion injury in our study was intriguing at the time but consistent with a recent large multicenter, double-blind, randomized clinical trial by Cung et al in which 2.5 mg/kg of cyclosporine A delivered to patients with acute anterior ST-segment elevation myocardial infarction undergoing percutaneous coronary intervention failed to elicit protection from reperfusion injury [67].

There are several possible explanations. Cyclosporine A has a narrow therapeutic index [68] and dosing to achieve the intended effect if challenging. It was also shown by Griffith and Halestrap in isolated cardiac mitochondria that pore opening could become CsA-insensitive under conditions of adenine nucleotide depletion and high matrix [Ca^{2+}] [14], conditions which are present during ischemia [30, 69]. Cyclosporine A has also been reported to induce production of reactive oxygen species and increase cytosolic Ca^{2+} levels potentially counteracting its beneficial effects during ischemia and reperfusion [17, 70]. Moreover, the lipid emulsifying vehicle, cremophor, can accumulate in the inner mitochondrial membrane decreasing the oxidative capacity of respiratory chain complexes I and IV [71-73].

Despite the lack of myocardial protection, cyclosporine A in our study prevented episodes of recurrent VF during the early post-resuscitation period. A similar effect was reported in rats following reperfusion after coronary occlusion [74].

Extrapolation of the present findings to clinical settings is limited by several factors. VF was induced by electrical stimulation, whereas clinically VF typically occurs in patients with underlying coronary artery disease and is often precipitated by coronary occlusion. We cannot exclude that alterations in normal mitochondrial function occurred consequent to electrical stimulation. Inherent to animal models is the use of anesthesia, which exerts independent myocardial protective effects [75]. Despite these limitations, the rat model has been a highly effective translation model for understanding underlying cellular mechanisms during whole body ischemia.

In summary, this is the first study to report *in vivo* mPTP opening during VF and closed-chest resuscitation. These results are consistent with previous observations in isolated mitochondria, cells, and organs in which the mPTP opens during reperfusion. Cyclosporine A did not appear to limit mPTP opening or attenuate post-resuscitation myocardial dysfunction. Further studies are required to determine the therapeutic range and optimal formulation of cyclosporine A during whole body ischemia and reperfusion, or design alternative strategies to increase the threshold for mPTP opening during cardiac resuscitation.

Acknowledgements

This work was supported in part by a National Heart, Lung, and Blood Institute Grant (R01-HL71728-01).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Raúl J Gazmuri, Resuscitation Institute at Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, Illinois 60064, USA. Tel: 224-610-3681; Fax: 847-578-3863; E-mail: raul.gazmuri@rosalindfranklin.edu

References

[1] Halestrap AP. The mitochondrial permeability transition: its molecular mechanism and role in reperfusion injury. Biochem Soc Symp 1999; 66: 181-203.

[2] Shanmughapriya S, Rajan S, Hoffman NE, Higgins AM, Tomar D, Nemani N, Hines KJ, Smith DJ, Eguchi A, Vallem S, Shaikh F, Cheung M, Leonard NJ, Stolakis RS, Wolfers MP, Ibetti J, Chuprun JK, Jog NR, Houser SR, Koch WJ, Elrod JW and Madesh M. SPG7 Is an Essential and Conserved Component of the Mitochondrial Permeability Transition Pore. Mol Cell 2015; 60: 47-62.

[3] Huser J, Rechenmacher CE and Blatter LA. Imaging the permeability pore transition in single mitochondria. Biophys J 1998; 74: 2129-2137.

[4] Huser J and Blatter LA. Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. Biochem J 1999; 343: 311-7.

[5] Bernardi P and Petronilli V. The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. J Bioenerg Biomembr 1996; 28: 131-138.

[6] Vergun O, Votyakova TV and Reynolds IJ. Spontaneous changes in mitochondrial membrane potential in single isolated brain mitochondria. Biophys J 2003; 85: 3358-3366.

[7] Elrod JW, Wong R, Mishra S, Vagnozzi RJ, Sakthievel B, Goonasekera SA, Karch J, Gabel S, Farber J, Force T, Brown JH, Murphy E and Molkentin JD. Cyclophilin D controls mitochondrial pore-dependent Ca(2+) exchange, metabolic flexibility, and propensity for heart failure in mice. J Clin Invest 2010; 120: 3680-3687.

[8] Wang W, Fang H, Groom L, Cheng A, Zhang W, Liu J, Wang X, Li K, Han P, Zheng M, Yin J, Wang W, Mattson MP, Kao JP, Lakatta EG, Sheu SS, Ouyang K, Chen J, Dirksen RT and Cheng H. Superoxide flashes in single mitochondria. Cell 2008; 134: 279-290.

[9] Halestrap AP, Clarke SJ and Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res 2004; 61: 372-385.
[10] Halestrap AP and Pasdois P. The role of the mitochondrial permeability transition pore in heart disease. Biochim Biophys Acta 2009; 1787: 1402-1415.
[11] Bernardi P and Di LF. The mitochondrial permeability transition pore: molecular nature and role as a target in cardioprotection. J Mol Cell Cardiol 2015; 78: 100-6.
[12] Halestrap AP. Calcium-dependent opening of a non-specific pore in the mitochondrial inner membrane is inhibited at pH values below 7. Implications for the protective effect of low pH against chemical and hypoxic cell damage. Biochem J 1991; 278: 715-719.
[13] Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. J Biol Chem 1992; 267: 8834-8839.
[14] Griffiths EJ and Halestrap AP. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. Biochem J 1995; 307: 93-98.
[15] Kristian T, Bernardi P and Siesjo BK. Acidosis promotes the permeability transition in energized mitochondria: implications for reperfusion injury. J Neurotrauma 2001; 18: 1059-1074.
[16] Nazareth W, Yafei N and Crompton M. Inhibition of anoxia-induced injury in heart myocytes by cyclosporin A. J Mol Cell Cardiol 1991; 23: 1351-1354.
[17] Griffiths EJ and Halestrap AP. Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. J Mol Cell Cardiol 1993; 25: 1461-1469.
[18] Assaly R, de TA, Paradis S, Jacquin S, Berdeaux A and Morin D. Oxidative stress, mitochondrial permeability transition pore opening and cell death during hypoxia-reoxygenation in adult cardiomyocytes. Eur J Pharmacol 2012; 675: 6-14.
[19] Prendes MG, Hermann R, Torresin ME, Velez D, Savino EA and Varela A. Role of mitochondrial permeability transition pore and mitochondrial ATP-sensitive potassium channels in the protective effects of ischemic preconditioning in isolated hearts from fed and fasted rats. J Physiol Biochem 2014; 70: 791-800.
[20] Javadov S, Huang C, Kirshenbaum L and Karmazyn M. NHE-1 inhibition improves impaired mitochondrial permeability transition and respiratory function during postinfarction remodelling in the rat. J Mol Cell Cardiol 2005; 38: 135-143.
[21] Hearse DJ and Sutherland FJ. Experimental models for the study of cardiovascular function and disease. Pharmacol Res 2000; 41: 597-603.
[22] Fang H, Chen M, Ding Y, Shang W, Xu J, Zhang X, Zhang W, Li K, Xiao Y, Gao F, Shang S, Li JC, Tian XL, Wang SQ, Zhou J, Weisleder N, Ma J, Ouyang K, Chen J, Wang X, Zheng M, Wang W, Zhang X and Cheng H. Imaging superoxide flash and metabolism-coupled mitochondrial permeability transition in living animals. Cell Res 2011; 21: 1295-1304.
[23] Correa F, Soto V and Zazueta C. Mitochondrial permeability transition relevance for apoptotic triggering in the post-ischemic heart. Int J Biochem Cell Biol 2007; 39: 787-798.
[24] Von Planta I, Weil MH, von Planta M, Bisera J, Bruno S, Gazmuri RJ and Rackow EC. Cardiopulmonary resuscitation in the rat. J Appl Physiol 1988; 65: 2641-2647.
[25] Lamoureux L, Radhakrishnan J and Gazmuri RJ. A Rat Model of Ventricular Fibrillation and Resuscitation by Conventional Closed-chest Technique. J Vis Exp 2015; e52413
[26] Halestrap AP, Connern CP, Griffiths EJ and Kerr PM. Cyclosporin A binding to mitochondrial cyclophilin inhibits the permeability transition pore and protects hearts from ischaemia/reperfusion injury. Mol Cell Biochem 1997; 174: 167-172.
[27] Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH and Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. J Physiol 2003; 549: 513-524.
[28] Radhakrishnan J, Wang S, Ayoub IM, Kolarova JD, Levine RF and Gazmuri RJ. Circulating levels of cytochrome *c* after resuscitation from cardiac arrest: a marker of mitochondrial injury and predictor of survival. Am J Physiol Heart Circ Physiol 2007; 292: H767-H775.
[29] Shepherd D and Garland PB. The kinetic properties of citrate synthase from rat liver mitochondria. Biochem J 1969; 114: 597-610.
[30] Wang S, Radhakrishnan J, Ayoub IM, Kolarova JD, Taglieri DM and Gazmuri RJ. Limiting sarcolemmal Na+ entry during resuscitation from VF prevents excess mitochondrial Ca2+ accumulation and attenuates myocardial injury. J Appl Physiol 2007; 103: 55-65.
[31] Vinnakota KC and Bassingthwaighte JB. Myocardial density and composition: a basis for calculating intracellular metabolite concentrations. Am J Physiol Heart Circ Physiol 2004; 286: H1742-H1749.
[32] Bairoch A and Apweiler R. The SWISS-PROT protein sequence data bank and its new sup-

plement TREMBL. Nucleic Acids Res 1996; 24: 21-25.
[33] O'Brien PJ, Landt Y and Ladenson JH. Differential reactivity of cardiac and skeletal muscle from various species in a cardiac troponin I immunoassay. Clin Chem 1997; 43: 2333-2338.
[34] Fiorillo C, Pace S, Ponziani V, Nediani C, Perna AM, Liguori P, Cecchi C, Nassi N, Donzelli GP, Formigli L and Nassi P. Poly(ADP-ribose) polymerase activation and cell injury in the course of rat heart heterotopic transplantation. Free Radic Res 2002; 36: 79-87.
[35] O'Brien PJ, Smith DE, Knechtel TJ, Marchak MA, Pruimboom-Brees I, Brees DJ, Spratt DP, Archer FJ, Butler P, Potter AN, Provost JP, Richard J, Snyder PA and Reagan WJ. Cardiac troponin I is a sensitive, specific biomarker of cardiac injury in laboratory animals. Lab Anim 2006; 40: 153-171.
[36] McDougal DB Jr, Ferrendelli JA, Yip V, Pusateri ME, Carter JG, Chi MM, Norris B, Manchester J and Lowry OH. Use of nonradioactive 2-deoxyglucose to study compartmentation of brain glucose metabolism and rapid regional changes in rate. Proc Natl Acad Sci U S A 1990; 87: 1357-1361.
[37] Hom FG, Goodner CJ and Berrie MA. A [^{3}H]2-deoxyglucose method for comparing rates of glucose metabolism and insulin responses among rat tissues in vivo. Validation of the model and the absence of an insulin effect on brain. Diabetes 1984; 33: 141-152.
[38] Jenkins AB, Furler SM and Kraegen EW. 2-deoxy-D-glucose metabolism in individual tissues of the rat in vivo. Int J Biochem 1986; 18: 311-318.
[39] Akdemir G, Ergungor MF, Sezer M, Albayrak L, Daglioglu E and Kilinc K. Therapeutic efficacy of intraventricular cyclosporine A and methylprednisolone on a global cerebral ischemia model in rats. Neurol Res 2005; 27: 827-834.
[40] Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D and Ovize M. Postconditioning inhibits mitochondrial permeability transition. Circulation 2005; 111: 194-197.
[41] Huhn R, Heinen A, Weber NC, Hollmann MW, Schlack W and Preckel B. Hyperglycaemia blocks sevoflurane-induced postconditioning in the rat heart in vivo: cardioprotection can be restored by blocking the mitochondrial permeability transition pore. Br J Anaesth 2008; 100: 465-471.
[42] Xie JR and Yu LN. Cardioprotective effects of cyclosporine A in an in vivo model of myocardial ischemia and reperfusion. Acta Anaesthesiol Scand 2007; 51: 909-913.
[43] Shanmuganathan S, Hausenloy DJ, Duchen MR and Yellon DM. Mitochondrial permeability transition pore as a target for cardioprotection in the human heart. Am J Physiol Heart Circ Physiol 2005; 289: H237-H242.
[44] Kern KB, Elchisak MA, Sanders AB, Badylak SF, Tacker WA and Ewy GA. Plasma catecholamines and resuscitation from prolonged cardiac arrest. Crit Care Med 1989; 17: 786-791.
[45] Prengel AW, Lindner KH, Ensinger H and Grünert A. Plasma catecholamine concentrations after successful resuscitation in patients. Crit Care Med 1992; 20: 609-614.
[46] Niemann JT and Garner D. Post-resuscitation plasma catecholamines after prolonged arrest in a swine model. Resuscitation 2005; 65: 97-101.
[47] Izem-Meziane M, Djerdjouri B, Rimbaud S, Caffin F, Fortin D, Garnier A, Veksler V, Joubert F and Ventura-Clapier R. Catecholamine-induced cardiac mitochondrial dysfunction and mPTP opening: protective effect of curcumin. Am J Physiol Heart Circ Physiol 2012; 302: H665-H674.
[48] Yang L, Weil MH, Noc M, Tang W, Turner T and Gazmuri RJ. Spontaneous gasping increases the ability to resuscitate during experimental cardiopulmonary resuscitation. Crit Care Med 1994; 22: 879-883.
[49] Ristagno G, Tang W, Sun S and Weil MH. Spontaneous gasping produces carotid blood flow during untreated cardiac arrest. Resuscitation 2007; 75: 366-371.
[50] Kolarova JD, Ayoub IM and Gazmuri RJ. Cariporide enables hemodynamically more effective chest compression by leftward shift of its flow-depth relationship. Am J Physiol Heart Circ Physiol 2005; 288: H2904-H2911.
[51] Radhakrishnan J, Kolarova JD, Ayoub IM and Gazmuri RJ. AVE4454B--a novel sodium-hydrogen exchanger isoform-1 inhibitor--compared less effective than cariporide for resuscitation from cardiac arrest. Transl Res 2011; 157: 71-80.
[52] Radhakrishnan J, Upadhyaya MP, Ng M, Edelheit A, Moy HM, Ayoub IM and Gazmuri RJ. Erythropoietin facilitates resuscitation from ventricular fibrillation by signaling protection of mitochondrial bioenergetic function in rats. Am J Transl Res 2013; 5: 316-326.
[53] Huang X, Zhai D and Huang Y. Dependence of permeability transition pore opening and cytochrome C release from mitochondria on mitochondria energetic status. Mol Cell Biochem 2001; 224: 1-7.
[54] Hirakawa A, Takeyama N, Nakatani T and Tanaka T. Mitochondrial permeability transition and cytochrome c release in ischemia-reperfusion injury of the rat liver. J Surg Res 2003; 111: 240-247.
[55] Petrosillo G, Di VN, Moro N, Colantuono G, Paradies V, Tiravanti E, Federici A, Ruggiero FM and Paradies G. In vivo hyperoxic precondition-

ing protects against rat-heart ischemia/reperfusion injury by inhibiting mitochondrial permeability transition pore opening and cytochrome c release. Free Radic Biol Med 2011; 50: 477-483.
[56] Jentzer JC, Chonde MD and Dezfulian C. Myocardial Dysfunction and Shock after Cardiac Arrest. Biomed Res Int 2015; 2015: 314796.
[57] Di LF, Carpi A, Giorgio V and Bernardi P. The mitochondrial permeability transition pore and cyclophilin D in cardioprotection. Biochim Biophys Acta 2011; 1813: 1316-1322.
[58] McGuinness O, Yafei N, Costi A and Crompton M. The presence of two classes of high-affinity cyclosporin A binding sites in mitochondria. Evidence that the minor component is involved in the opening of an inner-membrane Ca(2+)-dependent pore. Eur J Biochem 1990; 194: 671-679.
[59] Halestrap AP and Davidson AM. Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 1990; 268: 153-160.
[60] Griffiths EJ and Halestrap AP. Further evidence that cyclosporin A protects mitochondria from calcium overload by inhibiting a matrix peptidyl-prolyl cis-trans isomerase. Implications for the immunosuppressive and toxic effects of cyclosporin. Biochem J 1991; 274: 611-614.
[61] Nicolli A, Basso E, Petronilli V, Wenger RM and Bernardi P. Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. J Biol Chem 1996; 271: 2185-2192.
[62] Argaud L, Gateau-Roesch O, Muntean D, Chalabreysse L, Loufouat J, Robert D and Ovize M. Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury. J Mol Cell Cardiol 2005; 38: 367-374.
[63] Gomez L, Thibault H, Gharib A, Dumont JM, Vuagniaux G, Scalfaro P, Derumeaux G and Ovize M. Inhibition of mitochondrial permeability transition improves functional recovery and reduces mortality following acute myocardial infarction in mice. Am J Physiol Heart Circ Physiol 2007; 293: H1654-H1661.
[64] Huang CH, Tsai MS, Hsu CY, Su YJ, Wang TD, Chang WT and Chen WJ. Post-cardiac arrest myocardial dysfunction is improved with cyclosporine treatment at onset of resuscitation but not in the reperfusion phase. Resuscitation 2011; 82: S41-S47.
[65] Cour M, Abrial M, Jahandiez V, Loufouat J, Belaidi E, Gharib A, Varennes A, Monneret G, Thibault H, Ovize M and Argaud L. Ubiquitous protective effects of cyclosporine A in preventing cardiac arrest-induced multiple organ failure. J Appl Physiol (1985) 2014; 117: 930-936.
[66] Di Lisa F, Menabo R, Canton M, Barile M and Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD^+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. J Biol Chem 2001; 276: 2571-2575.
[67] Cung TT, Morel O, Cayla G, Rioufol G, Garcia-Dorado D, Angoulvant D, Bonnefoy-Cudraz E, Guerin P, Elbaz M, Delarche N, Coste P, Vanzetto G, Metge M, Aupetit JF, Jouve B, Motreff P, Tron C, Labeque JN, Steg PG, Cottin Y, Range G, Clerc J, Claeys MJ, Coussement P, Prunier F, Moulin F, Roth O, Belle L, Dubois P, Barragan P, Gilard M, Piot C, Colin P, De PF, Morice MC, Ider O, Dubois-Rande JL, Unterseeh T, Le BH, Beard T, Blanchard D, Grollier G, Malquarti V, Staat P, Sudre A, Elmer E, Hansson MJ, Bergerot C, Boussaha I, Jossan C, Derumeaux G, Mewton N and Ovize M. Cyclosporine before PCI in Patients with Acute Myocardial Infarction. N Engl J Med 2015; 373: 1021-1031.
[68] Jorga A, Holt DW and Johnston A. Therapeutic drug monitoring of cyclosporine. Transplant Proc 2004; 36: 396S-403S.
[69] Ayoub IM, Kolarova J, Kantola R, Radhakrishnan J and Gazmuri RJ. Zoniporide preserves left ventricular compliance during ventricular fibrillation and minimizes post-resuscitation myocardial dysfunction through benefits on energy metabolism. Crit Care Med 2007; 35: 2329-2336.
[70] Van der TM, Kauffman HF, van der DM, Slebos DJ, Koeter GH, Gans RO and Bakker SJ. Cyclosporin A-induced oxidative stress is not the consequence of an increase in mitochondrial membrane potential. FEBS J 2007; 274: 3003-3012.
[71] Sanchez H, Bigard X, Veksler V, Mettauer B, Lampert E, Lonsdorfer J and Ventura-Clapier R. Immunosuppressive treatment affects cardiac and skeletal muscle mitochondria by the toxic effect of vehicle. J Mol Cell Cardiol 2000; 32: 323-331.
[72] Sanchez H, Zoll J, Bigard X, Veksler V, Mettauer B, Lampert E, Lonsdorfer J and Ventura-Clapier R. Effect of cyclosporin A and its vehicle on cardiac and skeletal muscle mitochondria: relationship to efficacy of the respiratory chain. Br J Pharmacol 2001; 133: 781-788.
[73] N' Guessan BB, Sanchez H, Zoll J, Ribera F, Dufour S, Lampert E, Kindo M, Geny B, Ventura-Clapier R and Mettauer B. Oxidative capacities of cardiac and skeletal muscles of heart transplant recipients: mitochondrial effects of cyclo-

sporin-A and its vehicle Cremophor-EL. Fundam Clin Pharmacol 2014; 28: 151-160.

[74] Arteaga D, Odor A, Lopez RM, Contreras G, Pichardo J, Garcia E, Aranda A and Chavez E. Impairment by cyclosporin A of reperfusion-induced arrhythmias. Life Sci 1992; 51: 1127-1134.

[75] Kato R and Foex P. Myocardial protection by anesthetic agents against ischemia-reperfusion injury: an update for anesthesiologists. Can J Anaesth 2002; 49: 777-791.