Inhibition of mitochondrial permeability transition to prevent the post-cardiac arrest syndrome: a pre-clinical study

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Aims
Resuscitated cardiac arrest (CA), leading to harmful cardiovascular dysfunction and multiple organ failure, includes a whole-body hypoxia–reoxygenation phenomenon. Opening of the mitochondrial permeability transition pore (mPTP) appears to be a pivotal event in ischaemia–reperfusion injury. We hypothesized that pharmacological inhibition of mPTP opening may prevent the post-CA syndrome.

Methods and results
Anaesthetized New Zealand White rabbits underwent a 15 min primary asphyxial CA and 120 min of reperfusion following resuscitation. At reflow, animals received an intravenous bolus of either cyclosporine A (CsA, 5 mg/kg) or NIM 811 (2.5 mg/kg), two potent inhibitors of mPTP opening, or the CsA vehicle (control). Short-term survival, haemodynamics, regional (sonomicrometry), and global cardiac function (dP/dt and aortic flow) were assessed. We measured markers of cellular injuries and/or organ failure, including troponin Ic release, lacticodehydrogenase, lactate, creatinine, and alanine aminotransferase. Cyclosporine A and NIM 811 significantly improved short-term survival, post-resuscitation cardiac function, as well as liver and kidney failure (P < 0.05). CsA and NIM 811 both attenuated in vitro mPTP opening (calcium retention capacity by spectrofluorimetry) and restored oxidative phosphorylation when compared with controls (P < 0.05).

Conclusion
These data suggest that pharmacological inhibition of mPTP opening, added to basic life support, attenuates the post-CA syndrome and improves short-term outcomes in the rabbit model.

Keywords
Cyclosporine A ● Cardiac arrest ● Cardiopulmonary resuscitation ● Ischaemia ● Reperfusion ● Pharmacological post-conditioning

Introduction
Cardiac arrest (CA) is a major public health problem in industrialized countries, with more than 350,000 sudden deaths annually in the USA and in Europe. Asystole and pulseless electrical activity (i.e. non-shockable CA rhythms), associated to the worse outcomes, are the most frequent first documented cardiac rhythms feature after CA. Despite international guidelines for the management of CA, prognosis is poor, not only because the rate of restoration of spontaneous circulation (ROSC) is low, but also because a wide majority of immediate survivors die of post-CA syndrome, i.e. multiple organ failure including cardiovascular dysfunction and irreversible neurological injuries. Even though therapeutic hypothermia may improve neurological outcome following resuscitation from ventricular fibrillation, none of the effective therapy (in addition to basic life support) is actually available to prevent this post-CA syndrome in patients with non-shockable rhythm.

Whatever the aetiology, the consequence of a resuscitated CA is the interruption followed by the restoration of the blood flow to all organs, which represents a global ischaemia–reperfusion insult. Although the whole-body complete ischaemia initially

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causes tissue and organ injuries, a growing body of evidence suggests that reperfusion accompanying ROSC is also responsible for additional mitochondrial dysfunction, including opening of the mitochondrial permeability transition pore (mPTP), reactive oxygen species (ROS) overproduction, or cytochrome c release. Mitochondrial permeability transition is a key event in cell death following ischaemia–reperfusion. In the early minutes of reflow, Ca$^{2+}$ overload, excessive production of ROS and abrupt restoration of pH trigger the opening of the non-specific mPTP leading to cell death. These features of ischaemia–reperfusion are indeed observed in the resuscitated CA. The mPTP, whose structure remains incompletely known, is powerfully inhibited by cyclosporine A (CsA), independently of its immunosuppressive effects, through modulation of cyclophilin D binding to core components of the pore. Accumulating data from our group and others, support pharmacological mPTP inhibition as an ubiquitous effective strategy to prevent irreversible cellular injury induced by ischaemia–reperfusion in different pathophysiological settings such as myocardial infarction, stroke, brain trauma, or organ preservation (heart, liver, kidney, etc.).

Therefore, the objective of the present study was to investigate whether pharmacological inhibition of the mPTP at the onset of the cardiopulmonary resuscitation (CPR) might preserve cardiovascular function and attenuate the post-CA syndrome in the rabbit model.

**Methods**

The investigation conformed to French laws and the revised Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council ‘Guide for the Care and Use of Laboratory Animals’ National Academy Press, Washington, DC, 1996. All experiments were approved by the Lyon I Claude Bernard University Committee for Animal Research.

**Surgical preparation**

Male New Zealand White rabbits (2.5–3 kg) were anaesthetized by intramuscular injection of xylazine (5 mg/kg) and ketamine (50 mg/kg), as previously described. An intravenous (iv) infusion of a mixture of xylazine (25 µg/kg/min) and ketamine (50 µg/kg/min) was then maintained throughout the experiment. After a midline cervical incision, a tracheotomy was performed, and animals underwent mechanical ventilation (Servo Ventilator 900 B, Siemens-Elema, Solna, Sweden) with 30% oxygen. The tidal volume and the respiratory rate were 15 mL/kg and 35 strokes/min, respectively. End-tidal carbon dioxide concentration (EtCO$_2$) and pulse oxymetry (SpO$_2$) were measured continuously and ventilation was adjusted to keep them within the physiological ranges. Body temperature was also monitored continuously using a thermistor inserted into the oesophagus and kept constant by means of a heating pad. An electrocardiogram was recorded continuously through subcutaneous needles.

A 16 G catheter was inserted into the right atrium for administration of drugs and fluids and to measure right atrial pressure (RAP). A 4 F desilet was inserted in the left internal carotid to allow the introduction of a micromanometer-tipped catheter (SPR 330, Millar Instruments, Houston, TX, USA) into the left ventricle (LV) for pressure measurements. A 16 G catheter was also introduced in the right internal carotid to measure aortic blood pressure. A left thoracotomy was performed in the fourth left intercostal space. The pericardium was opened and the heart exposed. A pair of piezoelectric crystals (0.8 mm diameter, Triton Technology, Inc., connected to a sonomicrometer (Model 200, Triton), were implanted longitudinally in the inner third myocardium of the LV anterior wall for measurement of regional myocardial function. The descending thoracic aorta was surgically exposed and a perivascular flowprobe (Transit Time Flowmeter, Triton) placed around the vessel for flow measurements.

A 15 min stabilization period was then observed before experiments.

**Cardiac arrest and resuscitation**

Rabbits were paralysed with an iv injection of pancuronium bromide (50 µg/kg). Primary asphyxial CA was induced by the withdrawal of mechanical ventilation 10 min after curarization (Figure 1). Apnea was confirmed by both absence of respiratory movements and rapid collapse of SpO$_2$. Cardiac arrest was defined as the onset of the decline of mean arterial pressure (MAP) and heart rate (HR) less than 10 mmHg and 30 b.p.m., respectively. Cardiac arrest was maintained untreated for 15 min before resuscitation.

Resuscitation, adapted from international guidelines included: (i) resumption of mechanical ventilation with 100% oxygen, (ii) open chest cardiac massage with dissecting forceps (150–200 min$^{-1}$), and (iii) epinephrine iv boluses (20 µg/kg) every 3 min in the absence of ROSC, following a 1 min period of cardiac massage. Restoration of spontaneous circulation was defined as the return of an organized cardiac rhythm for at least 3 min, with both MAP and HR above 20 mmHg and 100 b.p.m., respectively. In the absence of ROSC after 15 min, resuscitative efforts were stopped: the animal was then declared dead.

**Experimental design**

After the stabilization period, animals were randomly assigned to one of the four following groups (Figure 1): (i) control group (Ctrl) underwent CA with 120 min of reperfusion following resuscitation plus iv bolus injection of the vehicle for CsA; (ii) CsA-treated group: iv bolus of CsA (5 mg/kg) at the onset of the resuscitation (i.e. immediately after restarting ventilation and in the meantime of cardiac massage); (iii) NIM 811-treated group: iv bolus of 2.5 mg/kg NIM 811

**Figure 1 Experimental design.** Controls (Ctrl), cyclosporine A, and NIM 811-treated rabbits underwent 15 min of primary asphyxial cardiac arrest induced by ventilatory arrest (VA) under curarization (C) before cardiopulmonary resuscitation and 120 min reperfusion. ROSC, restoration of spontaneous circulation.
(N-methyl-4-isoleucine-cyclosporine), a non-immunosuppressive derivative of CsA that specifically inhibits mPTP opening.\textsuperscript{17,18} (iv) sham-operated group. Thus, drugs or vehicle was injected similarly in all treated or control animals, respectively, whether or not they resumed some haemodynamics.

Two sets of rabbits were needed to perform all the measurements. A first set (\(n = 7–14/\text{group}\)) was used for complete haemodynamic measurements. The second set (\(n = 8–14/\text{group}\)) was used for blood sampling, pupillary analysis, and mitochondrial assays.

**Haemodynamics and regional contractile function**

Electrocardiogram, aortic blood pressure, sonomicrometry, LV pressure, and its peak positive and negative first derivatives (\(\frac{dP}{dt_{\text{max}}}\) and \(\frac{dP}{dt_{\text{min}}}, \text{respectively}\)) were continuously monitored on a multi-channel recorder (Model 4000, Gould Inc.) and saved on a PC-based data acquisition system supported by IOX software (IOX 1.567, Emka Technologies, France). The coronary perfusion pressure (CPP) was calculated as the difference between diastolic aortic pressure and time-coincident RAP. Aortic blood flow was also recorded using both Triton recorder and IOX software.

For sonomicrometry, LV \(\frac{dP}{dt}\) was used to define the timing of the cardiac cycle for segment length measurements with ultrasonic crystals: end-diastolic length (EDL) was measured at the onset of the rapid increase in LV \(\frac{dP}{dt}\) and end-systolic length (ESL) was measured at peak negative LV \(\frac{dP}{dt}\) (Figure 2A). End-diastolic length and ESL values were used to compute regional shortening fraction (SF) defined as follows: \(SF = (\text{EDL} – \text{ESL})/\text{EDL}\) and data expressed as percentage of baseline values.\textsuperscript{19}

**Markers of myocardial injury, metabolic disorders, and/or organ dysfunctions**

Arterial blood samples (5 mL) were obtained at baseline and at the end of the experimental protocol and assayed at an off-site reference laboratory blinded to clinical data. Troponin Ic, a marker of irreversible cardiac damage, was measured using a chemiluminescence method on an Access II Immunoassay system (Beckman-Coulter, Fullerton, CA, USA). Arterial blood pH was measured with an ABL 5 analyser (Radiometer SA, France). Lactate was determined by enzymatic reaction (Roche Diagnostics, Meylan, France) on a Hitachi 912 Analyser (Hitachi, Mannheim, Germany). Creatinine, lacticodehydrogenase (LDH), and alanine aminotransferase (ALT) were determined with a biochemical multianalyser (Roche Diagnostics). Pupillary diameter was measured using a pupil gauge and reactivity to light defined as present when pupils constricted more than 1 mm: this was considered here as a rough estimate of cerebral damage after CA.

**Mitochondrial isolation**

At the end of the protocol, hearts were harvested while still beating and mitochondria isolated from the LV anterior wall. Mitochondrial fractions were isolated by differential centrifugation as previously described.\textsuperscript{20,21} All operations were carried out in the cold.

![Figure 2](https://academic.oup.com/eurheartj/article-abstract/32/2/226/514298/228)
Heart pieces (0.8–1 g) were placed in isolation buffer containing 70 mM sucrose, 210 mM mannitol, and 1 mM EGTA in Tris/HCl, pH 7.4. The tissue was finely minced with scissors and then homogenized in the same buffer (10 mL/g), using successively a Kontes tissue grinder and a Potter Elvejem. The homogenate was centrifuged at 1300 g for 3 min. The supernatant was poured through cheesecloth and centrifuged at 10 000 g for 10 min. The mitochondrial pellet was suspended and homogenized in the same buffer without calcium chelating agent. Protein content was then routinely assayed according to Gornall’s procedure using bovine serum albumin (BSA) as a standard.22 Purity of isolated mitochondria was assessed by measuring a specific activity of citrate synthase as an inner membrane marker enzyme, quantified in cholate-solubilized mitochondria by measuring the rate of 5,5′-dithiobis(nitrobenzic acid)-reactive reduced coenzyme A (412 nm) at 37°C.23

Brain mitochondria from the cortex were also isolated in a subset of rabbits from the sham, the control, and the CsA-treated group (n = 6–7/group), using the same procedures and buffers. Both heart and brain mitochondria were kept over ice prior to experiments.

**Calcium retention capacity**

Adapted by Ichas et al., calcium retention capacity (CRC) represents a functional test for a quantitative assessment of the in vitro sensitivity of the mPTP to calcium loading.12,20,24 As previously describe, extramitochondrial Ca2+ concentration was recorded using the calcium sensitive probe calcium green-5N (Molecular Probes TM, Eugene, OR, USA), with excitation and emission wavelengths set at 500 and 530 nm, respectively.23 For each of the four groups, mitochondria were isolated as described earlier and separated into two similar aliquots. In one aliquot, CRC was measured with no further intervention, whereas in the other aliquot, CRC was assessed in the presence of 1 μM of CsA in the cuvette, used here to normalize among groups to the maximum sensitivity of cyclophilin D under these experimental conditions. Briefly, isolated mitochondria (250 μg proteins) were suspended in 2 mL buffer (150 mM sucrose, 50 mM KCl, 2 mM KH2PO4 in 20 mM Tris/HCl, pH 7.4). Substrates (5 mM glutamate and 5 mM malate or 10 mM succinate with 1 μM rotenone) were used as electron donors to complex I or complex II, respectively. Mitochondria were gently stirred for 2 min at 25°C. At the end of this pre-incubation period, 10 nmol CaCl2 pulses (i.e. 40 nmol/mg mitochondrial proteins) were performed every minute. As depicted in Figure 3, each calcium pulse is recorded as a peak of fluorescence (i.e. a peak of extramitochondrial calcium concentration). Ca2+ was then rapidly taken up by the mitochondria, resulting in a return of extramitochondrial calcium concentration to near baseline level. Following sufficient calcium loading, extramitochondrial Ca2+ concentration abruptly increased, indicating a massive release of the accumulated Ca2+ by mitochondria due to mPTP opening (Figure 3).12,20 Exposure to CsA or NIM 811 for 1 min before the first Ca2+ pulse, using concentrations ranging from 0.25 to 1 μM, significantly and dose dependently delayed Ca2+ release and Ca2+ induced mPTP opening when compared with untreated mitochondria (data not shown). This demonstrated that abrupt Ca2+ release was induced by mPTP opening.20 The amount of CaCl2 necessary to trigger this massive Ca2+ release (CRC) is used here as an indicator of the susceptibility of mPTP to Ca2+ overload.

**Mitochondrial oxygen consumption**

Mitochondrial respiratory function was measured by the polarographic method of Chance and Williams25 using a Clark oxygen electrode. Freshly isolated heart mitochondria (250 μg proteins) were incubated at 25°C in 2 mL buffer containing 100 mM KCl, 1 mM EGTA, 5 mM KH2PO4 in 50 mM MOPS, pH 7.4 with 1 mg/mL defatted BSA. State 3 (200 μM ADP-stimulated), State 4 (ADP-limited), and respiratory control index (RCI = State 3/State 4) were determined by oxygraphy (Oroboros Oxigraph, Paar, Graz, Austria) with the same energetic substrates as those used for CRC assays. Mitochondria were considered viable for CRC assays when RCI were ≥4. Outer membrane intactness (OMI) was also calculated as the ratio of 8 μM cytochrome c-stimulated respiration on maximally stimulated respiration by 2 mM ADP.

**Chemicals**

NIM 811 was a generous gift of Novartis (Basel, Switzerland). CsA and NIM 811 were dissolved for in vivo use in a mixture of Cremophor EL (polyethoxylated castor oil) with 94% ethanol. All other chemicals were purchased from Sigma Chemical (Sigma-Aldrich, St Louis, MO, USA).

**Statistical analysis**

Data are expressed as mean value ± standard error of the mean (SEM) or number and percentage, as appropriate. Continuous variables were...
analysed by one-way ANOVA. Comparisons between time-based measurements within each group were performed with two-way ANOVA with repeated measures on one factor. Comparisons of categorical variables were performed using a two-sided χ² test or Fisher’s exact test, as appropriate. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined at a value of *P < 0.05.

Results

Eighty-two rabbits were used in the present study. Three animals were excluded because of haemorrhagic shock or ventricular fibrillation during surgery. Results are then presented for the remaining 79 rabbits (sham, *n* = 15; Ctrl, *n* = 28; CsA, *n* = 18; NIM 811, *n* = 18).

Resuscitation and outcomes

All animals underwent a similar duration of asphyxia before CA (i.e. after withdrawal of the mechanical ventilation in paralysed anaesthetized animals), ranging from 379 ± 8 to 397 ± 8 s (Table 1). NIM 811 but not CsA-treated rabbits needed a significantly shorter cardiac massage to recover (Table 1). CsA and NIM 811-treated rabbits required a significantly lesser amount of epinephrine to recover the ROSC (Table 1). Five rabbits in the control group vs. two in the CsA group and one in the NIM 811 group failed to resume a significant haemodynamic status and died of cardiovascular failure (*P = 0.68*). Importantly, the survival rate was improved from 57% in the control group to 88% in the CsA group (*P = 0.02*) and 94% in the NIM 811 group (*P = 0.006*). Moreover, CsA and NIM 811 abolished mortality by sustained shock after ROSC, which occurred in 7/23 rabbits (30%) in the control group (*P = 0.03* and *P = 0.01*, respectively).

Haemodynamics and regional contractile function

Data are presented in each group for all rabbits surviving at 120 min of reperfusion (Figures 2 and 4). All experimental groups displayed 5 min after ROSC a comparable dramatic decrease in MAP, aortic flow, and CPP when compared with baseline or sham (Figure 4). In contrast, HR was barely changed in either group. Whereas no significant change in MAP, aortic flow, and CPP was observed in the control group following resuscitation, all these parameters were significantly improved in both CsA and NIM 811 groups during the reflow period (Figure 4).

A very similar pattern of evolution was observed for segment shortening and LV dP/dt<sub>max</sub> and LV dP/dt<sub>min</sub>, so that at the end of the reflow period, CsA and NIM 811-treated hearts exhibited a significant better function recovery than controls (Figure 2).

Cellular injuries and/or organ dysfunctions

Whereas troponin Ic release was similar at baseline among groups (<0.05 ng/mL), control rabbits exhibited an increase in troponin Ic blood level at 2 h of reperfusion averaging 19.1 ± 4.1 ng/mL when compared with sham animals (1.0 ± 0.6 ng/mL, *P < 0.001* vs. controls) (Figure 5). CsA and NIM 811 significantly reduced this troponin Ic release (Figure 5). As shown in Table 2, at the end of the experiment, the control group displayed a significant decrease in blood pH and increase in blood levels of lactate, LDH, ALT, and creatinine when compared with baseline and sham. In contrast, CsA and NIM 811-treated groups had a significantly limited drop in pH and increase in lactate, LDH, ALT, and creatinine. In the same way, CsA and NIM 811 prevented also mydriasis and pupillary areflexia following CA (Table 3).

Mitochondrial respiration and calcium retention capacity

The mitochondrial protein yield was comparable among the four groups, with mean values ranging from 18.5 ± 1.0 to 19.6 ± 1.6 mg proteins/g of wet weight myocardium. The specific activity of citrate synthase was similar among groups, ranging from 177 ± 126 to 1886 ± 98 mL/mg proteins, thereby confirming the equivalent purity of the mitochondrial isolation in the four groups.

In the control group, State 3 respiration was significantly decreased vs. sham mitochondria when using substrates of complex I, but not when using substrates of complex II (Table 4). CsA and NIM 811 restored this ADP-stimulated oxidative phosphorylation when compared with untreated animals (Table 4).

In the sham group, CRC from heart mitochondria averaged 390 ± 26 and 428 ± 27 nmol CaCl<sub>2</sub>/mg proteins with electron donors to complexes I and II, respectively (*P = 0.34*). As expected, the CRC was significantly reduced in the control group, averaging 212 ± 17 and 218 ± 11 nmol CaCl<sub>2</sub>/mg proteins for complexes I and II substrates, respectively (*P < 0.001* vs. sham) (Figures 3 and 6). In the CsA- and NIM 811-treated groups, the CRC was significantly improved (*P < 0.001*) when compared with the control group, whatever the substrate used (Figure 6). As expected also, CRC was significantly enhanced by the presence of CsA in vitro.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>ROSC, <em>n</em> (%)</th>
<th>Survival, <em>n</em> (%)</th>
<th>Asphyxia before cardiac arrest (s)</th>
<th>Heart massage (survivors) (s)</th>
<th>Epinephrine (survivors) (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>23/28 (78)</td>
<td>16/28 (57)</td>
<td>379 ± 8</td>
<td>171 ± 27</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>CsA</td>
<td>16/18 (88)</td>
<td>16/18 (88)*</td>
<td>386 ± 12</td>
<td>137 ± 21</td>
<td>27 ± 4*</td>
</tr>
<tr>
<td>NIM 811</td>
<td>17/18 (94)</td>
<td>17/18 (94)*</td>
<td>397 ± 8</td>
<td>92 ± 4*</td>
<td>20 ± 1*</td>
</tr>
</tbody>
</table>

ROSC, restoration of spontaneous circulation.

*P < 0.05 vs. Ctrl.
in all of the four groups \((P < 0.001)\). However, no significant difference in CRC was observed among groups when mitochondria were exposed to \textit{ex vivo} addition of CsA (Figure 6).

In mitochondria isolated from the brain, we obtained very similar patterns among the different groups, with CRC averaging 115 ± 13 nmol CaCl2/mg proteins in sham rabbits in comparison with 63 ± 8 nmol CaCl2/mg proteins in controls \((P = 0.003)\). In CsA-treated animals, the CRC, averaging 106 ± 11 nmol CaCl2/mg proteins, was also significantly enhanced when compared with controls \((P = 0.01, P = 0.59\) vs. sham).

**Discussion**

In the present study, we demonstrated for the first time that pharmacological inhibition of mPTP opening at the onset of the CA resuscitation (i) prevents myocardial reperfusion injury and cardiovascular dysfunction, (ii) limits multiple organ failure, and (iii) improves outcomes.

**Post-cardiac arrest syndrome and multiple organ dysfunctions**

As asystole and pulseless electrical activity are the main documented CA rhythm disturbances in humans\(^\text{1,3}\) we developed a prolonged non-shockable CA model.

Multiple organ failure, taking part in the post-CA syndrome, has been recently recognized as a consequence of the systemic ischaemia–reperfusion response after resuscitated CA\(^\text{3}\). Whatever the cause of CA, the duration of untreated CA, and therefore the duration of no flow, is the best predictor of the severity of
this post-resuscitation cardiac failure. Even if post-CA myocardial dysfunction and brain injury are the leading clinical manifestations related to the outcome, all other organ failures (e.g., liver and kidney) should influence the prognosis. In the present study, we therefore aimed at evaluating parameters that may reflect several organ damage following CA. Apart from assessing cardiovascular dysfunction, we measured biomarkers of global homeostasis or specific organ injury at the myocardial, liver, and kidney level, including pH, lactate, troponin Ic, LDH, ALT, and creatinine. However, because both liver and kidney functions were investigated by only one parameter, further investigations will be needed to confirm our results. Even though early neurological prognostication following CA remains challenging, the absence of pupillary light response provides the most reliable predictor of poor outcome (absence of recovery of consciousness or death) and was therefore assessed in our protocol.

In the present study, the untreated primary asphyxial CA resulted in a profound and global cardiovascular dysfunction after ROSC in the control group. In agreement with previous reports, we found using reference methods that standard hemodynamics and both systolic and diastolic regional and global cardiac functions were severely impaired after CPR. It is worth noting that we observed some amount of myocardial necrosis as indicated by troponin Ic release. However, this relatively low blood level of troponin Ic is unlikely to explain the profound cardiovascular dysfunction in control animals. The post-CA syndrome was also characterized by the evidence of myocardial, liver, kidney, and cerebral injury, which, despite early resuscitation treatment, was responsible for a dramatic decrease in short-term survival.

To our knowledge, no intervention has proved to be effective in preventing the whole post-CA syndrome. Therapeutic hypothermia, which has been proposed as a neuroprotective strategy following ventricular fibrillation, did not prevent cardiovascular dysfunction and multiple organ failure. Only few publications have reported interventions able to prevent cardiovascular failure. Ischaemic pre-conditioning, consisting of brief periods of ischaemia–reperfusion preceding resuscitated CA, could reduce the severity of post-resuscitation myocardial dysfunction. Ayoub et al. reported that sodium-hydrogen exchange inhibition has the potential to ameliorate myocardial impairment and survival, whereas Huang et al. described similar effects using erythropoietin.

Based on a concept that has already been investigated by our group and others, especially in the setting of myocardial infarction, we made the hypothesis that mitochondrial permeability transition was involved in each peripheral organ failure.

### Table 2 Biomarkers of metabolic disorders, cellular injuries, and/or organ dysfunction

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>120 min reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>7.44 ± 0.02</td>
<td>7.40 ± 0.03</td>
</tr>
<tr>
<td>Ctrl</td>
<td>7.41 ± 0.02</td>
<td>7.07 ± 0.05†</td>
</tr>
<tr>
<td>CsA</td>
<td>7.42 ± 0.02</td>
<td>7.21 ± 0.03†</td>
</tr>
<tr>
<td>NIM 811</td>
<td>7.41 ± 0.01</td>
<td>7.21 ± 0.02†</td>
</tr>
<tr>
<td><strong>Lactate (mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>2.3 ± 0.3</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>Ctrl</td>
<td>2.1 ± 0.2</td>
<td>15.4 ± 2.2†</td>
</tr>
<tr>
<td>CsA</td>
<td>2.1 ± 0.2</td>
<td>9.2 ± 0.6†</td>
</tr>
<tr>
<td>NIM 811</td>
<td>2.7 ± 0.2</td>
<td>7.5 ± 0.6†</td>
</tr>
<tr>
<td><strong>Creatinine (μmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>84 ± 3</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>Ctrl</td>
<td>79 ± 2</td>
<td>162 ± 8†</td>
</tr>
<tr>
<td>CsA</td>
<td>79 ± 2</td>
<td>140 ± 9†</td>
</tr>
<tr>
<td>NIM 811</td>
<td>80 ± 6</td>
<td>121 ± 8†</td>
</tr>
<tr>
<td><strong>LDH (IU/mL)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>252 ± 24</td>
<td>566 ± 59</td>
</tr>
<tr>
<td>Ctrl</td>
<td>212 ± 27</td>
<td>1327 ± 174†</td>
</tr>
<tr>
<td>CsA</td>
<td>231 ± 17</td>
<td>794 ± 37†</td>
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<tr>
<td>NIM 811</td>
<td>274 ± 22</td>
<td>848 ± 65†</td>
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<td><strong>ALT (IU/mL)</strong></td>
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<tr>
<td>Sham</td>
<td>41 ± 4</td>
<td>42 ± 4</td>
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<tr>
<td>Ctrl</td>
<td>38 ± 9</td>
<td>92 ± 20†</td>
</tr>
<tr>
<td>CsA</td>
<td>40 ± 4</td>
<td>54 ± 7†</td>
</tr>
<tr>
<td>NIM 811</td>
<td>46 ± 6</td>
<td>49 ± 6†</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. baseline.
†P < 0.05 vs. sham.
‡P < 0.05 vs. Ctrl.

### Table 3 Pupillary outcomes

<table>
<thead>
<tr>
<th></th>
<th>Pupillary diameter (mm)</th>
<th>Pupillary reactivity, n (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>120 min reperfusion</td>
</tr>
<tr>
<td>Sham</td>
<td>4.7 ± 0.4</td>
<td>4.7 ± 0.3†</td>
</tr>
<tr>
<td>Ctrl</td>
<td>4.8 ± 0.2</td>
<td>7.0 ± 0.4*</td>
</tr>
<tr>
<td>CsA</td>
<td>5.1 ± 0.3</td>
<td>4.2 ± 0.4†</td>
</tr>
<tr>
<td>NIM 811</td>
<td>4.4 ± 0.4</td>
<td>5.6 ± 0.3*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. baseline.
†P < 0.05 vs. Ctrl.
and sought to determine whether inhibitor of mPTP opening might improve the post-CA syndrome.

**Inhibition of mitochondrial permeability transition pore opening to alleviate the post-cardiac arrest syndrome**

Mitochondrial permeability transition is a crucial event in reperfusion injury. Opening of the mPTP in the inner mitochondrial membrane is favoured by conditions set up by ischaemia–reperfusion, including accumulation of Ca$^{2+}$ in the mitochondrial matrix, overproduction of ROS, adenine nucleotide depletion, and rapid intracellular pH normalization at the time of reoxygenation. This phenomenon results in the collapse of the membrane potential ($\Delta \Psi_m$), uncoupling of the respiratory chain, matrix swelling, and efflux of pro-apoptotic factors (including cytochrome c), which may lead to either apoptosis or necrosis. Although ischaemia per se does not appear to cause mPTP opening, probably because

### Table 4 Oxidative phosphorylation

<table>
<thead>
<tr>
<th></th>
<th>Complex I substrate</th>
<th></th>
<th>Complex II substrate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td>RCI</td>
<td>OMI</td>
</tr>
<tr>
<td>Sham</td>
<td>76 ± 5</td>
<td>11 ± 1</td>
<td>7.0 ± 0.6</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Ctrl</td>
<td>51 ± 6*</td>
<td>9 ± 1</td>
<td>6.2 ± 1.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>CsA</td>
<td>73 ± 3†</td>
<td>10 ± 1</td>
<td>7.5 ± 0.3</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>NIM 811</td>
<td>72 ± 6†</td>
<td>10 ± 1</td>
<td>7.4 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

States 3 and 4 are expressed as nanogram atoms of oxygen/min/mg proteins. RCI, respiratory control index (State 3/State 4); OMI, outer membrane intactness (cytochrome c-stimulated respiration on maximally stimulated respiration by ADP).

*P < 0.05 vs. sham.
†P < 0.05 vs. Ctrl.

#### Figure 6 Calcium retention capacity

Calcium retention capacity was significantly reduced in control animals (Ctrl) vs. shams whatever the substrate used (electron donors to complex I or complex II). As an indicator of mitochondrial permeability transition pore inhibition, calcium retention capacity was restored by cyclosporine A and NIM 811 following cardiac arrest. These differences among groups were prevented by ex vivo addition of cyclosporine A.

*P < 0.05 vs. sham; †P < 0.001 vs. Ctrl.
of the protective effects of intracellular acidosis, it creates the conditions for mPTP opening at reperfusion.\textsuperscript{10,11} Few experiments support the role, in the mediation of the ischaemic preconditioning-induced protection, of a possible transient mPTP opening, associated with a ROS release/signalling.\textsuperscript{11,12} However, accumulating evidence indicates a sustained mPTP opening at reflow (following a prolonged ischaemia) as an effector mechanism in myocardial reperfusion injury.\textsuperscript{9–12,16,17} But also in many pathological conditions such as stroke\textsuperscript{14} or shock in experimental models.\textsuperscript{33}

Our group recently demonstrated for the first time that administration of CsA at the time of reperfusion reduces infarct size in acute myocardial infarction patients.\textsuperscript{13} Since CA can be viewed as a form of multiple organ global ischaemia–reperfusion injury, we hypothesized that at least part of damages observed during the post-CA syndrome may be related to mPTP opening.

In the present study, we demonstrated that CsA and NIM 811 were able to induce a rapid and near-full recovery of haemodynamics after CA and to allow a better resuscitability and less extra-cardiac cellular damage associated to organ failure. Furthermore, our results showing less pupillary areflexia in treated rabbits suggest that CsA derivatives could also prevent post-CA brain injury. As expected, this prevention of the post-CA syndrome, by limiting each organ failure, was associated in our study with an improved short-term survival.

One might question whether inhibition of mPTP opening was specifically responsible for the observed beneficial effects. To address this issue, we first used a specific inhibitor of mPTP opening, i.e. NIM 811,\textsuperscript{16} and second assessed \textit{in vitro} calcium-induced mPTP opening in mitochondria isolated from the post-CA myocardium. Besides its inhibitory effect on the mPTP via binding to cyclophilin D, CsA interacts also with several molecular targets in the cytosol (including cyclophilin A involved in calcineurin-mediated immunosuppression), which may impact on the cellular prosurvival mechanisms.\textsuperscript{9–15} For this reason, we also used the non-immunosuppressive CsA-derivative NIM811 that does not bind to cytosolic cyclophilin A but specifically to the mitochondrial cyclophilin D and thereby inhibits mPTP opening without significant effect on the immune response and the inflammatory reaction.\textsuperscript{17,18,34} The fact that NIM 811 was able to offer similar protection than CsA strongly suggests that mPTP opening is involved in the post-CA syndrome. Using reference methods,\textsuperscript{12,23,35} we further demonstrated that mitochondria isolated from \textit{in vivo} resuscitated control rabbit hearts display an increased susceptibility to mPTP opening when exposed to a Ca\textsuperscript{2+} overload, whereas mPTP inhibitors significantly prevent this phenomenon. Moreover, the shift in CRC we observed in mitochondria following a CA is sensitive to the \textit{ex vivo} addition of CsA, emphasizing a cyclophilin D–dependent mechanism linked to the mitochondrial permeability transition phenomenon. Our results are also in agreement with data from Gazmuri's\textsuperscript{7,36} group, who by measuring cytochrome c release after ventricular fibrillation in rats indirectly suggested a link between mPTP opening and myocardial injury after CA.

In our study, the post-CA syndrome was also associated with a clear alteration of the mitochondrial oxidative metabolism. We noticed an impaired ADP-stimulated mitochondrial respiration in controls when compared with sham. Importantly, this abnormality was abolished by CsA and NIM811. As recently reported by Han et al.,\textsuperscript{37} these results underline the reduction of complex I activity as an important alteration of the mitochondrial oxidative metabolism after CA and resuscitation. These data are also in keeping with reports from other whole-body ischaemia–reperfusion models, such as cardioplegia or septic shock, in which CsA-induced mitochondrial bioenergetic preservation (i.e. ATP synthesis) was related to better cardiovascular function.\textsuperscript{31,38} Altogether, these findings reinforce the proposal to target mitochondrial permeability transition in order to prevent both calcium homeostasis disorders and energy production to improve outcomes after CA.

One may also question whether the overall beneficial effect of CsA and NIM 811 on the post-CA syndrome was related to the direct inhibition of mPTP opening at the level of each organ or a consequence of a better preservation of the cardiovascular function with secondary positive impact on multiple organ perfusion. Importantly, CsA derivatives have been reported to significantly inhibit mPTP opening in the liver and kidney at doses similar to that used in the present study.\textsuperscript{11} We propose that the benefit seen in various organs is likely not a secondary consequence of primary cardiac preservation by mPTP inhibitors. The first point in favour of this is that the absolute amount of myocardial necrosis as indicated by troponin Ic levels is so small (and consequently absolute benefit of treatment) that it is unlikely to explain both intrinsic cardiac depression in controls and improvement in treated animals. Second, in the rabbit model of acute myocardial infarction, we (as well as others) have observed along several previous studies that, even very large, infarcts are quite often associated with a good preservation of haemodynamics.\textsuperscript{12,17,20} Third, we found that CRC was significantly reduced in mitochondria isolated from the brain cortex after CA and that CsA was able to prevent this. In contrast, cortex mitochondria isolated from rabbits that underwent acute myocardial infarction (30 min of coronary artery occlusion and 2 h of reperfusion) without CA displayed no change in CRC (data not shown).

Altogether, these data strongly suggest that mitochondrial permeability transition is triggered by CA in multiple non-cardiac organs and participates to the post-CA syndrome. In the context of the post-CA syndrome, whether hypoxia–reoxygenation is the sole trigger of opening of the mPTP in all organs, and whether global metabolic disorders also play a local role for modulation of mPTP opening in a given cell type, would require additional investigations. Further investigations will be also needed in the future to investigate more extensively the pathways leading to mPTP opening in this model.

**Conclusion**

Our study provides new evidence that at least a significant part of cellular damages, occurring following resuscitation from CA, involves mitochondrial permeability transition, and can be attenuated by specific pharmacological inhibition. Clinical trials are now required to determine whether some prevention in post-CA syndrome and improvement in outcome may be obtained in the
clinical settings through the pharmacological inhibition of the mPTP opening, added to basic life support.

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**References**


