Intralipid, a Clinically Safe Compound, Protects the Heart Against Ischemia-Reperfusion Injury More Efficiently Than Cyclosporine-A

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ABSTRACT

Background: We have recently shown that posts ischemic administration of intralipid protects the heart against ischemiareperfusion injury. Here we compared the cardioprotective effects of intralipid with cyclosporine-A, a potent inhibitor of the mitochondrial permeability transition pore opening.

Methods: In vivo rat hearts or isolated Langendorff-perfused mouse hearts were subjected to ischemia followed by reperfusion with intralipid (0.5%, 1% and 2% ex vivo, and 20% in vivo), cyclosporine-A (0.2 μM, 0.8 μM, and 1.5 μM ex vivo and 10 mg/kg in vivo), or vehicle. The hemodynamic function, infarct size, calcium retention capacity, mitochondrial superoxide production, and phosphorylation levels of protein kinase B (Akt)/glycogen synthase kinase-3β (GSK-3β) were measured. The values are mean ± SEM.

Results: Administration of intralipid at reperfusion significantly reduced myocardial infarct size compared with cyclosporine-A (0.5%, 1% and 2% ex vivo, and 20% in vivo), cyclosporine-A (0.8 μM) in protecting the ex vivo heart against ischemia-reperfusion injury, as the rate pressure product at the end of reperfusion was significantly higher (mmHg · beats/min): 12,740 ± 675 [n = 7] vs. 9,203 ± 10,781 [n = 5], P = 0.024), and the infarct size was markedly smaller (17.3 ± 2.9 [n = 7] vs. 29.2 ± 2.7 [n = 5], P = 0.014). Intralipid was as efficient as cyclosporine-A in inhibiting the mitochondrial permeability transition pore opening (calcium retention capacity = 280 ± 8.2 vs. 260.3 ± 2.9 mmol/mg mitochondria protein in cyclosporine-A, P = 0.454, n = 6) and in reducing cardiac mitochondrial superoxide production. Unlike intralipid, which increased phosphorylation of Akt (6-fold) and GSK-3β (5-fold), cyclosporine-A had no effect on the activation of these prosurvival kinases.

Conclusions: Although intralipid inhibits the opening of the mitochondrial permeability transition pore as efficiently as cyclosporine-A, intralipid is more effective in reducing the infarct size and improving the cardiac functional recovery.

What We Already Know about This Topic

• Previous studies have demonstrated that posts ischemic administration of intralipid protects the heart against ischemia-reperfusion injury

• This study compared the cardioprotective effect of intralipid with cyclosporine-A when administered at the onset of myocardial reperfusion

What This Article Tells Us That Is New

• Intralipid is more effective than cyclosporine-A in reducing myocardial infarct size and improving cardiac functional recovery after ischemia-reperfusion

Acute myocardial infarction is responsible for the death of millions of people worldwide each year. Although early reperfusion is the only way to salvage an ischemic organ, during the crucial early moments of reperfusion, significant reversible and irreversible organ damage is initiated, a process referred to as reperfusion injury. The reperfusion injury is sometimes even more damaging than the ischemia itself because of oxidative damage caused by free radicals and calcium overload as a result of reintroduction of blood to the tissue. Pharmacological postconditioning has been used to protect the heart against ischemia-reperfusion (I/R) injury. Cardioplegic arrest and cardiopulmonary bypass are also other key triggers of myocardial injury during cardiac surgery, and multiple techniques have been used to
protect the heart during the surgical requirement for global or regional ischemia. Many pharmacological agents have been shown in experimental studies to have the ability to induce a polarized arrest and to better protect the heart, such as a cardio-selective β1-blocker and the adenosine triphosphate-sensitive potassium channel openers. However, none of pharmacological candidates have been widely accepted. Recently we have shown that postischemic treatment with intralipid, the first safe fat emulsion for human use, improves the cardiac functional recovery of isolated Langendorff-perfused mouse hearts by approximately 4-fold and results in 70% reduction in the myocardial infarct size.

The mitochondrial permeability transition pore (mPTP) is a large nonselective conductance pore located in the inner membrane of mitochondria. Although the exact molecular identity of mPTP has been questioned recently, cyclophilin-D (CypD) is still an established component of mPTP. The mPTP remains closed during ischemia, but opens during the reperfusion period. Opening of the mPTP is favored by events occurring during ischemia and reperfusion, including overproduction of reactive oxygen species and accumulation of Ca2+ in the mitochondrial matrix. Delaying the opening of the mPTP upon reperfusion has been a potential target to reduce myocardial injury. To date, the most specific inhibitor of the mPTP is cyclosporine-A, which acts by directly inhibiting the peptidyl-prolyl cis-trans isomerase activity of CypD, which is a key component of the mPTP. Recently we demonstrated that intralipid inhibits the opening of the mPTP and protects the heart by recruiting the reperfusion injury salvage kinase pathway, phosphatidylinositol 3-kinase/Akt/ERK1, and leading to phosphorylation of GSK-3β.

Here we compared the cardioprotective effect of intralipid with cyclosporine-A when administrated at the onset of reperfusion. Our data revealed that although intralipid inhibits the mPTP opening as efficiently as cyclosporine-A, intralipid is more effective in protecting the heart against I/R injury than cyclosporine-A both in vivo and ex vivo.

Materials and Methods

Animals

Male Sprague-Dawley rats 250–300 g and 3-month-old wild type male mice (C57BL/6) were used. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication No. 85–23, revised 1996; Bethesda, Maryland). The animal protocol received institutional approval by the Animal Research Committee of University of California Los Angeles.

Left Anterior Descending Coronary Artery Occlusion and Measurement of Infarct Size

Male Sprague-Dawley rats were anesthetized with ketamine (80 mg/kg, intraperitoneally) and xylazine (8 mg/kg, intraperitoneally). The rats were intubated and ventilated with a ventilator (CWE SAR-830/P, Ardmore, PA). A catheter filled with heparinized saline was placed into the right carotid artery for the measurement of blood pressure and heart rate. Pressure and heart rate were monitored using a pressure transducer (Power Lab, ADInstruments, Colorado Springs, CO) throughout the experiment. The hearts were exposed through a left thoracotomy in the fourth intercostal space. The pericardium was opened, and a 5.0 Prolene suture was tightened around the proximal left anterior descending coronary artery. Ischemia was confirmed by ST elevation in electrocardiogram.

At the end of the experiment, 2.5 ml of 1% Evans blue dye was injected into the femoral vein, and the myocardial ischemic area at risk was identified as the region lacking blue staining. The ventricles of the hearts were sliced transversely into 2-mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride at 37°C for 15 min to identify the noninfarcted and infarcted areas. The infarcted area was displayed as the area unstained by triphenyltetrazolium chloride. Infarct size was expressed as a percentage of the area at risk.

Langendorff Preparation

Mice were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and heparin (200 U/kg) was injected to prevent blood coagulation. The heart was quickly removed and placed in ice-cold Krebs-Henseleit buffer solution (KH, in mM): glucose 11.1, NaCl 118, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, and CaCl2 2 at pH 7.4 bubbled with 95% O2/5% CO2 at 37°C.

Experimental Protocol

In vivo, we used the well-established method to induce I/R. The heart was subjected to 30 min of ischemia by ligating the proximal left anterior descending coronary artery, followed by 180 min of reperfusion, which was achieved by releasing the tension on the ligature. A bolus of intralipid (20%, 5 ml/kg body weight) or cyclosporine-A (10 mg/kg body weight) were applied via the femoral vein 5 min before reperfusion (fig. 1A). These doses of intralipid and cyclosporine-A have been already used in vivo by our group or others. The rats that died during 180 min reperfusion were excluded from the study (two only in the control group because of a technical problem with the ventilator).

Ex vivo, we used the well-established protocol to induce I/R injury in isolated mouse hearts as shown by our group and others. The heart was connected to the perfusion cannula via the aorta and perfused with KH solution. Once the equilibration was achieved, the aorta was clamped for 20 min to induce global normothermic (37°C) ischemia (the heart was immersed in the 37°C Krebs solution during ischemia), followed by reperfusion for 40 min with KH alone (CTRL group), or with additional of intralipid or cyclosporine-A. The ex vivo hearts that during stabilization had 1) poor contractile function (less than 60 mmHg developed pressure after stabilization), 2) bradycardic (heart rate less

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intralipid = approximately 1%. Our \textit{ex vivo} doses of cyclosporine-A between 0.2 and 1.5 \( \mu \text{M} \) were based on previous \textit{in vitro} studies showing that cyclosporine-A inhibits mitochondrial swelling and loss of membrane potential in a dose-dependent manner with partial inhibition at approximately 0.2 \( \mu \text{M} \) and full inhibition at 1 \( \mu \text{M} \) or more.\cite{21} Cyclosporine-A also protects against apoptosis in a dose-dependent fashion between 0.5 and 2 \( \mu \text{M} \), and starts to present cell toxicity at concentrations greater than 2 \( \mu \text{M} \).\cite{22}

\textbf{Cardiac Functional Measurements}

A catheter (1.4F SPR-671; Millar Instruments, Colorado Springs, CO) connected to a pressure transducer was directly inserted into the left ventricle (LV) to measure left ventricular systolic pressure, left ventricular end-diastolic pressure, and heart rate. The LV developed pressure (LVEDP) was calculated as LVEDP = left ventricular systolic pressure – left ventricular end-diastolic pressure, and rate pressure product (RPP) = heart rate × LVEDP. The maximum rate of LV pressure rise (\( \text{d}P/\text{d}t_{\text{max}} \)) and decline (\( \text{d}P/\text{d}t_{\text{min}} \)) were directly calculated from the selected stable recordings.

\textbf{Myocardial Necrosis}

At the end of the reperfusion, the hearts were cut into four transverse slices and myocardial necrosis was assessed by measurement of the infarct size using triphenyltetrazolium chloride staining. The slices were fixed in 4\% paraformaldehyde, and the area of necrosis was quantified by Photoshop (Adobe Systems Incorporated, San Jose, CA) and expressed as the percentage of total ventricular area.

\textbf{Ca\textsuperscript{2+}-induced Mitochondrial Permeability Transition Preparation of Isolated Mitochondria.} Mitochondria was prepared from the hearts reperfused with KH, 1\% intralipid, or 1.5 \( \mu \text{M} \) cyclosporine-A for 10 min. Briefly, myocardial sections were placed in isolation buffer A containing mM: 70 sucrose, 210 mannitol, 1 EDTA, and 50 Tris-HCl, pH 7.4 at 4\(^\circ\)C. The tissue was finely minced with scissors and homogenized in the same buffer using Kontes and Potter-Elvehjem tissue grinders (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 1,300 \( \times \) g for 3 min; the supernatant was filtered through a cheesecloth and centrifuged at 10,000 \( \times \) g for 10 min. The mitochondrial pellet was resuspended in isolation buffer B containing mM: 70 sucrose, 210 mannitol, 0.1 EDTA, and 50 Tris-HCl, pH 7.4. Mitochondrial protein concentration was measured using the Bradford assay method.

\textbf{Calcium Retention Capacity (CRC)}

\textit{Ca}\textsuperscript{2+} accumulation during reperfusion is one of the major triggers of the opening of the mPTP. Therefore we measured calcium retention capacity (CRC) in the mitochondria isolated from the hearts subjected to I/R. The onset of the mPTP opening was assessed following \textit{in vitro} \textit{Ca}\textsuperscript{2+} overload as previously described.\cite{6} Free \textit{Ca}\textsuperscript{2+} concentration outside

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig1}
\caption{Intralipid reduces the infarct size more efficiently than cyclosporine-A in the \textit{in vivo} ischemia-reperfusion rat model. (A) The left coronary artery was occluded for 30 min followed by 3 h of reperfusion. One single IV bolus of 20\% intralipid (5 ml/kg body weight, intralipid group), cyclosporine-A (10 mg/kg body weight, cyclosporine-A group), and phosphate-buffered saline (control group) was administered 5 min before reperfusion. (B) Representative triphenyltetrazolium chloride-stained heart slices from control, intralipid, and cyclosporine-A group. The white area shows infarcted area, blue area shows noninfarcted area, red and white areas show risk area. (C) Percentage of area at risk divided by left ventricle (AAR/LV), infarct size divided by area at risk (IS/AAR), and infarct size divided by left ventricle in control (IS/LV) (n = 7), intralipid group (n = 7), and cyclosporine-A group (n = 7). \( **P < 0.001 \) versus CTRL, \( \#P < 0.05 \) versus ILP. AAR = area at risk, CsA = cyclosporine-A; CTRL = control; ILP = intralipid; IS = infarct size; LV = left ventricle.}
\end{figure}
the mitochondria was recorded with 0.5 μM calcium green-5N (Invitrogen, Carlsbad, CA) using excitation and emission wavelengths set at 500 and 530 nm, respectively. Isolated mitochondria (500 μg of protein) were suspended in 2 mL buffer C (mm: 150 sucrose, 50 KCl, 2 KH₂PO₄, 5 succinic acid, and 20 Tris-HCl, pH 7.4). Samples were preincubated for 90 s in the spectrofluorometer cuvette, and 10 nmol CaCl₂ pulses were applied every 60 s in the spectrofluorometer. The Ca²⁺ pulses induced a peak of extra-mitochondrial Ca²⁺ concentration that returned to near-baseline level as Ca²⁺ entered the mitochondrial matrix via the Ca²⁺ uniporter. With increasing Ca²⁺ loading, the extra-mitochondrial Ca²⁺ concentration started accumulating, reflecting a lower capacity for mitochondria Ca²⁺ uptake, which was followed by a sustained Ca²⁺ increase, indicating a massive release of the mitochondria Ca²⁺ by the mPTP opening.

The CRC was defined as the amount of Ca²⁺ required to trigger this massive Ca²⁺ release, which was used here as an indicator of the mPTP sensitivity to Ca²⁺. CRC was expressed as nmol of CaCl₂ per mg of mitochondrial protein.

Production of Reactive Oxygen Species in the Heart Tissue and in Isolated Cardiac Mitochondria

Reactive oxygen species (ROS) production during reperfusion triggers the opening of the mPTP. We therefore measured ROS production in the hearts subjected to 20 min ischemia followed by reperfusion for 5 min with KH, 1% intralipid, or 1.5 μM cyclosporine-A.

Dihydroethidium Staining to Measure ROS Production in the Heart Tissue Sections.

Compound-embedded 6 μm heart tissue sections were incubated with 10 μM dihydroethidium in Krebs-HEPES buffer (mm: 99 NaCl, 4.69 KCl, 25 NaHCO₃, 1.03 KH₂PO₄, 5.6 D-Glucose, 20 Na-HEPES, 2.5 CaCl₂, and 1.2 MgSO₄) for 2 h in the dark at room temperature. The sections were then washed three times for 1.5 h in the dark with Krebs-HEPES buffer and mounted with prolong antifade reagent (Invitrogen Carlsbad, CA). Images were acquired with a confocal microscope (Olympus Fluoview, Hicksville, NY).

Electron Spin Resonance to Quantify O₂⁻⁻ Production in Isolated Myocardial Mitochondria.

Superoxide was detected by electron spin resonance as previously reported. The superoxide (O₂⁻⁻) spin probe methoxy carbonyl-2,2,5,5-tetramethyl-pyrrolidine (0.5 mM, Alexis, San Diego, CA) solution was prepared freshly in nitrogen gas-bubbled Krebs-HEPES buffer containing diethyldithiocarbamic acid (5 μM, Sigma-Aldrich, St. Louis, MO) and deferoxamine (25 μM, Sigma-Aldrich). Freshly isolated mitochondria were mixed with the O₂⁻⁻-specific spin probe methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine in the presence or absence of 100 U/ml of superoxide dismutases and loaded in glass capillaries for analysis of O₂⁻⁻ production kinetically for 10 min. The electron spin resonance settings used were as follows: center field, 3475; sweep width, 9G; static field, 3484.981; microwave frequency, 9.75 GHz; microwave power 21.02 mW; modulation frequency 86 KHz; modulation amplitude, 2.47 G; resolution in X, 512; and number of x-scans, 10. The superoxide dismutase inhibitable O₂⁻⁻ signals at 10 min time-point, normalized by protein concentrations, were compared among different experimental groups.

Western Blot Analysis

Intralipid-induced cardioprotection is associated with increased phosphorylation levels of Akt and GSK-3β. Therefore we performed Western blot analysis to examine the possible involvements of Akt and GSK-3β in cardioprotection offered by cyclosporine-A. The entire ex vivo hearts that were reperfused with KH, 1% intralipid, or 1.5 μM cyclosporine-A for 10 min were used for making whole heart lysates, because in this model the whole heart is considered to be the area at risk. Hearts were homogenized at 4°C (mm: 150 NaCl, 50 Tris-HCl, 1 EGTA, 1 EDTA, 1 NaF, 1 PMSF, 1 Na₃VO₄, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate, pH 7.4, supplemented with protease and phosphatase inhibitor cocktails [Roche, San Francisco, CA]). The samples were centrifuged at 12,000 g for 10 min and the supernatants were collected. Protein concentration was measured and 100 μg of total protein was loaded on a 4–20% gradient Tris-HCl SDS polyacrylamide gel, electrotransferred to nitrocellulose paper, blocked with 5% nonfat dry milk in 20 mM Tris-buffered saline with 0.1% Tween and 0.5% Triton X-100, and incubated with primary antibodies. Blots were then indirectly labeled using infrared fluorophore-conjugated secondary antibodies for 1 h at room temperature, and visualized with the Odyssey Imaging System (Li-Cor, Lincoln, NE). Equal loading of protein onto each lane in the gel was confirmed with Vinculin. The proteins were first normalized to their corresponding Vinculin and then the phosphorylated proteins were normalized to their corresponding total protein levels.

Statistics

For cardiac infarct size and CRC, ROS production means were compared between groups using one-way ANOVA. For ex vivo cardiac function, means were compared among doses and over time using two-way repeated measure ANOVA, where both dose and time are repeated factors. Pairwise mean comparisons were judged significant using the Tukey post hoc test. For dose comparisons over time, contrasts were computed under a given ANOVA model to test for trends. A priori sample size/power analysis was not carried out. However, our sample size was intuitively based on our previous experience and our published results. All statistical analyses were performed using SPSS 13.0 (SPSS Inc, Chicago, IL) or SAS 9.3 (SAS Institute, Cary NC). As all outcomes were continuous, results were summarized with means ± standard errors of the mean (SEM). All P values are two-sided, and P < 0.05 was considered statistically significant.
Results

Intralipid Protects the Heart against I/R Injury More Efficiently Than Cyclosporine-A in the In Vivo Rat Model

The cardioprotective effect of intralipid was compared with cyclosporine-A in an in vivo rat model of I/R injury. The area at risk to LV ratio was similar in all groups (62.4 ± 2.0 in CTRL [n = 7], 58.0 ± 3.0 in intralipid group [n = 7], and 59.4 ± 2.8 in cyclosporine-A group [n = 7]), indicating that all three groups were subjected to a comparable degree of ischemic risk. However, the ratio of infarct size to area at risk was significantly smaller in the intralipid group compared with cyclosporine-A (22.9 ± 2.5 in intralipid group vs. 35.2 ± 3.5 in cyclosporine-A group, P = 0.030) (figs. 1B, C). Intralipid and cyclosporine-A both reduced the infarct size significantly compared with the CTRL group (59.8 ± 3.3 in CTRL, P < 0.001). The differences in the infarct size were not because of the hemodynamic changes as the heart rate and mean arterial pressure were not significantly different among the three groups at baseline, during ischemia, and at reperfusion (table 1).

Dose Response Curves to Obtain the Optimal Dose of Postischemic Administration of Intralipid and Cyclosporine-A

Next we compared the cardioprotective effect of intralipid with cyclosporine-A in isolated Langendorff perfused hearts. Figure 2 shows the RPP and infarct size as a function of intralipid (figs. 2A, B) and cyclosporine-A (figs. 2C, D) concentrations. Although 0.5% intralipid induced some degree of protection (RPP = 6,909 ± 2,055 mmHg · beats/min [n = 5]) at 40 min of reperfusion, infarct size = 28.1 ± 4.4 [n = 5]), the cardioprotection achieved by 1% and 2% intralipid were similar and significantly better than 0.5% (1% intralipid: RPP = 12,740 ± 675 mmHg · beats/min [n = 7]) at the end of reperfusion, infarct size = 17.3 ± 2.9 [n = 7]; 2% intralipid: RPP = 12,117 ± 1,527 mmHg · beats/min [n = 5], infarct size = 19.1 ± 2.6 [n = 5]). Cyclosporine-A at 0.2 μM had no apparent cardioprotective effect (RPP = 3,968.6 ± 624 mmHg · beats/min [n = 5] at end of reperfusion, infarct size = 46.7 ± 4.6 [n = 5]). Postischemic administration of 0.8 μM cyclosporine-A significantly improved the cardiac functional recovery and reduced the infarct size compared with 0.2 μM cyclosporine-A (RPP = 9,203 ± 1,078 mmHg · beats/min [n = 5]) at end of reperfusion, infarct size = 27.8 ± 1.6 [n = 5]). Increasing the concentration of cyclosporine-A from 0.8 to 1.5 μM did not result in further cardioprotection as the RPP and the infarct size for 1.5 μM were not significantly different than 0.8 μM (RPP = 8,652 ± 2,182 mmHg · beats/min [n = 5] at end of reperfusion, infarct size = 28.9 ± 2.5 [n = 5]).

Table 1. Systemic Hemodynamics in In Vivo Model of Ischemia-Reperfusion Injury

<table>
<thead>
<tr>
<th></th>
<th>Heart rate (beats/min)</th>
<th>Ischemia</th>
<th>Reperfusion, 1 hr</th>
<th>Reperfusion, 2 hr</th>
<th>Reperfusion, 3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL (n = 5)</td>
<td>296 ± 15</td>
<td>280 ± 10</td>
<td>292 ± 26</td>
<td>286 ± 13</td>
<td>278 ± 19</td>
</tr>
<tr>
<td>ILP (n = 5)</td>
<td>311 ± 16</td>
<td>285 ± 16</td>
<td>281 ± 19</td>
<td>301 ± 10</td>
<td>304 ± 11</td>
</tr>
<tr>
<td>CsA (n = 4)</td>
<td>325 ± 11</td>
<td>296 ± 15</td>
<td>298 ± 17</td>
<td>299 ± 25</td>
<td>302 ± 22</td>
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<tr>
<td>MAP (mmHg)</td>
<td></td>
<td></td>
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<tr>
<td>CTRL (n = 5)</td>
<td>97 ± 4</td>
<td>75 ± 4</td>
<td>79 ± 4</td>
<td>82 ± 13</td>
<td>79 ± 12</td>
</tr>
<tr>
<td>ILP (n = 5)</td>
<td>92 ± 5</td>
<td>79 ± 4</td>
<td>83 ± 6</td>
<td>85 ± 7</td>
<td>83 ± 6</td>
</tr>
<tr>
<td>CsA (n = 4)</td>
<td>93 ± 6</td>
<td>77 ± 6</td>
<td>84 ± 6</td>
<td>88 ± 4</td>
<td>85 ± 3</td>
</tr>
</tbody>
</table>

The heart rate and mean arterial pressure in control, intralipid, and cyclosporine-A groups at baseline, during ischemia, and at reperfusion of 1 hr, 2 hr, and 3 hr. Data are mean ± SEM.

CsA = cyclosporine-A; CTRL = control; ILP = intralipid; MAP = mean arterial pressure.

Fig. 2. Dose response of intralipid and cyclosporine-A in ex vivo ischemia-reperfusion mouse model. (A) Rate pressure product as a function of time in 0.5%, 1%, and 2% intralipid (n = 7). (B) The area of necrosis as the percentage of total ventricular area in 0.5%, 1%, and 2% intralipid group. (C) Rate pressure product as a function of time in 0.2 μM, 0.8 μM, and 1.5 μM cyclosporine-A (n = 5). (D) The area of necrosis as the percentage of total ventricular area in control group, 0.5%, 1%, and 2% intralipid group. **P = 0.022, 2% intralipid versus 0.5% intralipid; ***P = 0.004, 1% intralipid versus 0.5% intralipid; & & P = 0.008, 0.8 μM cyclosporine-A versus 0.2 μM cyclosporine-A; & & & P = 0.009, 1.5 μM cyclosporine-A versus 0.2 μM cyclosporine-A. CsA = cyclosporine-A; ILP = intralipid; RPP = rate pressure product.
Fig. 3. Administration of intralipid at reperfusion improves heart functional recovery against reperfusion injury more efficiently than cyclosporine-A. Representatives of the left ventricular developed pressure and left ventricle pressure rise (dP/dt max) and decline (dP/dt min) as a function of time in control group (A), 1% intralipid (B), and 0.8 μM cyclosporine-A (C). Rate pressure product (D), dP/dt max (E), and left ventricular developed pressure (F) as a function of time in control (closed circles, n = 7), intralipid (open circles, n = 7), and cyclosporine-A group (diamonds, n = 5). *P < 0.05 and **P < 0.001 versus CTRL, #P < 0.05, ##P < 0.01 versus CsA. CsA = cyclosporine-A; ILP = intralipid; KH = Krebs-Henseleit buffer solution; LVDP = left ventricular developed pressure; RPP = rate pressure product.

Intralipid Is More Effective Than Cyclosporine-A in Protecting the Heart against I/R Injury in Ex Vivo Mouse Model

We compared the effect of intralipid and cyclosporine-A on the cardiac functional recovery and infarct size at their optimal dose of 1% and 0.8 μM, respectively. Typical examples of LVDP and dP/dt are shown in figures 3A–C. Although the baseline RPP before ischemia was similar in all groups, the functional recovery was very poor in the CTRL group; RPP was 2,077 ± 100 mmHg · beats/min (n = 7) after 10 min of reperfusion and did not change significantly throughout the 40 min reperfusion (RPP = 2,791 ± 758 mmHg · beats/min [n = 7] at 40 min of reperfusion, fig. 3D). The functional recovery in the intralipid group was much higher, as the RPP was 9,873 ± 995 mmHg · beats/min (n = 7) after 10 min of reperfusion (recovery of 63%), improved further to 11,705 ± 1021 mmHg · beats/min (n = 7) at 20 min of reperfusion (recovery of 75%), and to 12,217 ± 1123 mmHg · beats/min (n = 7) at 30 min and to 12,740 ± 675 mmHg · beats/min (n = 7) after 40 min (recovery of 81%, fig. 3D). The intralipid group also showed a much better LV dP/dt max, LV dP/dt min, and LVDP as the percentage of total ventricular area in infarct zone and the infarct size was also significantly smaller in the intralipid group compared with the cyclosporine-A group. (*P = 0.001, vs. CTRL; #P = 0.014 versus ILP). CsA = cyclosporine-A; CTRL = control; ILP = intralipid.

Intralipid Inhibits the Opening of mPTP as Efficiently as Cyclosporine-A after I/R

Delaying the opening of the mPTP upon reperfusion has been a potential target to reduce myocardial injury. Figures 5A, B represents a typical recording of fluorescence showing the time course of Ca²⁺ concentration in the mitochondrial external medium. In the CTRL group, eight pulses were sufficient to trigger the opening of mPTP. Interestingly, the

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number of calcium pulses required for opening of the mPTP in both intralipid and cyclosporine-A groups were increased to 14 and 13, respectively (fig. 5A). These data suggest that posts ischemic treatment of intralipid increases the resistance of the mPTP to Ca\(^{2+}\) overload to a comparable level with the cyclosporine-A group. In fact, the CRC was about 2-fold higher in intralipid and cyclosporine-A groups compared with CTRL (280 ± 8.2 in intralipid and 260 ± 29 in cyclosporine-A vs. 157 ± 25 nmol/mg protein in CTRL, n = 6, P < 0.001, fig. 5C). As expected, sham hearts had much higher CRC (370 ± 20 nmol/mg protein).

**Inhibition of mPTP Opening by Intralipid is CypD-dependent**

We examined the involvement of CypD in intralipid-induced inhibition of mPTP opening by comparing the CRC after addition of 1.5 μM of cyclosporine-A directly in the cuvette in CTRL, intralipid, and cyclosporine-A groups. Administration of exogenous cyclosporine-A increased the number of Ca\(^{2+}\) pulses required to trigger the opening of mPTP in all groups, but to different extents (figs. 5B, C). In sham and CTRL groups, the addition of cyclosporine-A to the cuvette greatly increased the CRC (from 370 ± 20 to 620 ± 10 nmol/mg protein in sham and from 157 ± 25 to 347 ± 30 nmol/mg protein in CTRL, n = 6, P < 0.001). In intralipid and cyclosporine-A groups, however, the CRC increased mildly to 445 ± 25 and 415 ± 45 nmol/mg protein, respectively, in the presence of cyclosporine-A.

**Posts ischemic Treatment of Intralipid and Cyclosporine-A Decreases ROS Production after I/R**

Next we investigated whether the delay of the mPTP opening in intralipid and cyclosporine-A groups is in part because of decreased ROS/superoxide generation. Dihydroethidium staining of heart sections revealed a significantly lower ROS production in the intralipid and cyclosporine-A groups when compared with CTRL (normalized to CTRL, 0.57 ± 0.04 in intralipid group and 0.49 ± 0.01 in cyclosporine-A, P < 0.001 vs. CTRL, fig. 6A, B). We then examined the superoxide production in isolated cardiac mitochondria from in-
and GSK-3
CTRL;

we examined the possible involvements of Akt and GSK-3
heart homogenates subjected to ischemia-reperfusion injury
from control group, 1% intralipid, or 1.5 μM cyclosporine-A.
(C, D) Western blot analysis of pAkt protein to total Akt (B)
and pGSK-3β to total GSK-3β (D) ratios in control group
(black bars), intralipid group (white bars), and cyclosporine-A
group (gray bars). **P < 0.001 versus control; ##P < 0.001
versus intralipid (n = 4–6/group). Akt = protein kinase B; CsA = cyclosporine-A; CTRL = control; GSK = glycogen
synthase kinase; ILP = intralipid.

Fig. 7. The lack of involvement of protein kinase B (Akt)/
glycogen synthase kinase (GSK) pathways in cyclosporine-
A-induced protection. (A, B) Representative immunoblots of
pAkt and total Akt (A), and pGSK-3β and total GSK-3β (C) in
heart homogenates subjected to ischemia-reperfusion injury
from control group, 1% intralipid, or 1.5 μM cyclosporine-A.
Our previous study has shown that postischemic administra-
tion of intralipid before ischemia in the ex vivo rat model. Postischemic administration of intralipid was also more efficient than cyclosporine-A in improving the heart functional recovery and reducing the infarct size in isolated Langendorff perfused ex vivo hearts. Intralipid is as effective as cyclosporine-A in inhibiting the mPTP opening, most likely by increasing mitochondrial resistance to Ca2+ overload and reducing mitochondrial superoxide production during the first few minutes of reperfusion.

Intralipid Is More Effective Than Cyclosporine-A in Protecting the Heart against I/R Injury

We report that intralipid was more effective than cyclospo-ine-A in protecting the heart against I/R injury because the
infarct size was significantly smaller and the heart functional
recovery indices were all significantly better. Although the
optimal concentration of cyclosporine-A, if applied before
ischemia, has been shown to be 0.2 μM,27,28 here we found
that cyclosporine-A at this concentration does not induce
cardioprotection if applied at the onset of reperfusion.
Interestingly, administration of 1% intralipid at the onset of re-
perfusion rapidly restored heart function, as 40–60% recovery
was observed in the RPP and LVDP parameters within 10
min of reperfusion. On the other hand, the cardiac func-
tional recovery in the cyclosporine-A group during the first
10 min of reperfusion was only 20–30%. Because the first
few minutes of reperfusion are critical in myocardial protec-
tion, intralipid could be an ideal safe pharmacological agent
to rapidly restore the heart function, resulting in smaller
infarct size.

Our data demonstrate that intralipid is a very powerful
postischemic pharmacological agent reducing infarct size
(both in vivo and ex vivo) and improving cardiac contractil-
ity.5 The role of intralipid in preconditioning, however,
seems to be controversial. In the ex vivo model of I/R injury,
intralipid failed to protect the myocardium from contractile
dysfunction when administered 10 min before the onset of
ischemia and throughout the reperfusion phase.29 Adminis-
tration of intralipid before ischemia in an ex vivo model of
myocardial I/R injury was also not able to reduce the infarct
size.30,31 However, in another study from the same group,
intralipid was shown to reduce the infarct size in the ex vivo
model of I/R in rats if administered before ischemia.32 Fur-
ther studies are required to clarify the role of intralipid in
preconditioning.

Intralipid Is as Effective as Cyclosporine-A in Inhibiting mPTP Opening by Increasing Mitochondrial Resistance to Ca2+ Overload and Reducing Mitochondrial Superoxide Production

The opening of the mPTP during reperfusion has been im-
licated in cell death.33,34 Ca2+ accumulation and overproduc-
tion of ROS during reperfusion are the two major trig-
gers of the opening of the mPTP. It has been demonstrated
that ischemic preconditioning and postconditioning induce

Unlike Intralipid, Cyclosporine-A Does Not Induce Akt and GSK-3β Phosphorylation

Intralipid-induced cardioprotection is associated with in-
creased phosphorylation levels of Akt and GSK-3β. Here we
examined the possible involvements of Akt and GSK-3β in
cardioprotection offered by cyclosporine-A. Western blot
analysis revealed that intralipid-induced cardioprotection
was associated with an approximately 6-fold increase in phos-
phorylation of Akt, and an approximately 5-fold increase in
phosphorylation of GSK-3β. Cyclosporine-A, however, did
not affect the phosphorylation levels of Akt and GSK-3β
(fig. 7), because there were no significant differences between
the phosphorylation levels of these prosurvival kinases in
cyclosporine-A and CTRL groups.

Discussion

Our previous study has shown that postischemic administra-
tion of intralipid can protect the heart against I/R injury in
both the in vivo and ex vivo models. Here we compared the
cardioprotective effect of intralipid with cyclosporine-A. Our
data demonstrate that intralipid, a safe fat emulsion for
human use, is more effective than cyclosporine-A in protect-
ing the heart against ischemic I/R injury. A bolus of intralipid
right before the onset of reperfusion resulted in smaller in-
farct size of approximately 40% compared with cyclospo-ine-A in the in vivo rat model. Postischemic administration of intralipid was also more efficient than cyclosporine-A in improving the heart functional recovery and reducing the infarct size in isolated Langendorff perfused ex vivo hearts.
cardioprotection by increasing mitochondrial resistance to Ca\(^{2+}\) overload. Mitochondria are also the major source of ROS generation through their respiratory chain and are also the target organelle of oxidative damage. Decreasing ROS generation during reperfusion has been considered to induce cardioprotection against I/R injury. Recently we showed that intralipid inhibits the opening of the mPTP. Here our data demonstrate that intralipid is as efficient as cyclosporine-A in increasing the mitochondrial calcium uptake for the opening of the mPTP compared with CTRL (fig. 5). We propose that intralipid enhances the homeostasis of cardiomyocytes in the same manner as cyclosporine-A to better regulate calcium overload and therefore increase the threshold for opening of the mPTP. Intralipid exerts its action in a CypD-dependent manner as cyclosporine-A, which results in attenuation of the interaction between CypD with mPTP and therefore increases the mitochondrial CRC.

We also found that ROS generation in the heart tissue, as well as the production of superoxide in cardiac mitochondria during the first 5 min of reperfusion, was significantly reduced by postischemic administration of intralipid. In fact, intralipid was as effective as cyclosporine-A, if not better, in reducing the production of superoxide in mitochondria. Since it is well accepted that overproduction of ROS in the mitochondria is one of the triggers of the opening of the mPTP, reduced superoxide production in the mitochondria by intralipid could delay the opening of mPTP. Cyclosporine-A is currently the most specific inhibitor of the mPTP and has been demonstrated in many species including pigs, as well as in clinical trials, to be cardioprotective. However, cyclosporine-A is known to have undesirable side effects. Intralipid, which has been in clinical use for more than four decades with no known side effects, is as effective as cyclosporine-A in inhibiting the mPTP opening, most likely by increasing mitochondrial resistance to Ca\(^{2+}\) overload and reducing mitochondrial superoxide production during the first few minutes of reperfusion.

**Unlike Intralipid, Cyclosporine-A-induced Cardioprotection Is Not Mediated via Akt/GSK-3β**

Phosphatidylinositol 3-kinase-protein kinase B (PKB)/Akt pathway plays an important role in reperfusion injury. GSK-3β phosphorylation has emerged as an end-effector step where multiple protective signaling pathways converge. We have recently shown that postischemic administration of intralipid increases the phosphorylation levels of Akt and GSK. Here we report that cyclosporine-A-induced cardioprotection is not mediated through Akt/GSK, as cyclosporine-A had no effect on the phosphorylation levels of these kinases. The cyclosporine-A-induced protection of mesenchymal stem cells against apoptosis has also been reported to be independent of Akt/ERK, because cyclosporine-A did not promote the phosphorylation of these prosurvival proteins. The lack of involvement of the Akt/GSK pathway in the cyclosporine- induced cardioprotection could be one of the reasons behind the slow action of cyclosporine-A in improving the posts ischemic heart function.

**Limitations**

To date, cyclosporine-A is known to be the most specific inhibitor of the mPTP. It exerts its action by directly inhibiting the peptidyl-prolyl cis-trans isomerase activity of CypD, which is a key component of the mPTP. Here we report that intralipid inhibits the opening of the mPTP as efficiently as cyclosporine-A. Our previous study demonstrated that intralipid increases the phosphorylation of GSK-3β, leading to inhibition of the mPTP opening. However, it is not clear if intralipid directly inhibits the mPTP in a similar manner as cyclosporine-A. Further experiments using genetically modified mice of mPTP component are required to demonstrate a possible direct action of intralipid on the mPTP.

Our data demonstrate that cyclosporine-A-induced cardioprotection is most likely not mediated via the Akt/GSK-3β pathway. However, we did not examine the involvement of the protein kinase G nor the survivor activating factor enhancement pathway in cyclosporine-A-induced cardioprotection. Because the activation of the reperfusion injury salvage kinases pathway does not seem to be crucial for postconditioning in pigs, it remains to be seen whether reperfusion injury salvage kinases, protein kinase G, or the survivor activating factor enhancement pathways play a role in cyclosporine-A- or intralipid-induced cardioprotection in larger animals.

**Clinical Perspectives**

Acute myocardial infarction is still a major cause of mortality, despite advances in the management of patients. Although prompt reperfusion is critical in salvaging the ischemic heart, it also has the potential to induce reperfusion injury. Currently, there is no effective postischemic pharmacological agent for protecting the heart against the detrimental effects of lethal myocardial reperfusion injury. Delaying the opening of the mPTP upon reperfusion has been a potential target to reduce myocardial injury. Cyclosporine-A, which is one of the most potent inhibitors of mPTP opening, is emerging as a new postconditioning cardioprotective agent and it has been used recently in small clinical trials both in Europe and the United States. Chronic treatment with cyclosporine-A, however, has been associated with a number of potentially serious adverse drug reactions such as high blood pressure, potassium retention, and possibly hyperkalemia, kidney and liver dysfunction, and an increased vulnerability to opportunistic fungal and viral infections. Therefore, because of cyclosporine-A’s undesirable side effects, it is questionable whether cyclosporine-A is an ideal pharmacological postconditioning agent. Intralipid, on the other hand, is a clinically safe lipid emulsion that is generally well tolerated and is widely used in different clinical settings for parenteral nutrition for almost four decades. Our results demonstrate...
that not only is intralipid as potent as cyclosporine-A in inhibiting mPTP opening, but is in fact more effective than cyclosporine-A in reducing the infarct size. Therefore, intralipid could be a better alternative and an ideal safe pharmacological postconditioning agent for targeting the critical first few minutes of reperfusion, to rapidly restore heart function and therefore reduce infarct size. Although our in vivo dose of intralipid at 5 ml/kg is within the range that has been recommended by American Society of Regional Anesthesia and Pain Medicine for rescuing bupivacaine cardiotoxicity in patients, the exact dose of intralipid still needs to be determined in clinical trials for reperfusion injury. Our findings raise the intriguing concept that intralipid could serve as a novel therapeutic agent for acute myocardial infarction patients, which certainly warrants further investigation in the human heart.

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