Phosphorylation of GSK-3β Mediates Intralipid-induced Cardioprotection against Ischemia/Reperfusion Injury

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ABSTRACT

Background: Intralipid (Sigma, St. Louis, MO), a brand name for the first safe fat emulsion for human use, has been shown to be cardioprotective. However, the mechanism of this protection is not known. The authors investigated the molecular mechanism(s) of Intralipid-induced cardioprotection against ischemia/reperfusion injury, particularly the role of glycogen synthase kinase-3β (GSK-3β) and mitochondrial permeability transition pore in this protective action.

Methods: In vivo rat hearts or isolated Langendorff-perfused mouse hearts were subjected to ischemia followed by reperfusion with Intralipid (1% in ex vivo and one bolus of 20% in in vivo) or vehicle. The hemodynamic function, infarct size, threshold for the opening of mitochondrial permeability transition pore, and phosphorylation levels of protein kinase B (Akt)/extracellular signal-regulating kinase (ERK)/GSK-3β were measured.

Results: Administration of Intralipid at the onset of reperfusion resulted in approximately 70% reduction in infarct size in the in vivo rat model. Intralipid also significantly improved functional recovery of isolated Langendorff-perfused mouse hearts as the rate pressure product was increased from 2,999 ± 863 mmHg*beats/min in the control group to 13,676 ± 611 mmHg*beats/min (mean ± SEM) and the infarct size was markedly smaller (18.3 ± 2.4% vs. 54.8 ± 2.9% in the control group, P < 0.01). The Intralipid-induced cardioprotection was fully abolished by LY294002, a specific inhibitor of PI3K, but only partially by PD98059, a specific ERK inhibitor. Intralipid also increased the phosphorylation levels of Akt/ERK1/glycogen synthase kinase-3β by eightfold, threefold, and ninefold, respectively. The opening of mitochondrial permeability transition pore was inhibited by Intralipid because calcium retention capacity was higher in the Intralipid group (274.3 ± 8.4 nM/mg vs. 168.6 ± 9.6 nM/mg in the control group).

Conclusions: Postischemic treatment with Intralipid inhibits the opening of mitochondrial permeability transition pore and protects the heart through glycogen synthase kinase-3β via PI3K/Akt/ERK pathways.

CORONARY heart disease remains the leading cause of morbidity and mortality in Western countries. The best hope of salvaging viable myocardium after a coronary occlusion is by rapid reperfusion of the ischemic myocardium, either by thrombolysis or primary percutaneous coronary intervention. Although reperfusion restores blood flow, oxygen, and nutrients to the cardiac muscle, it also has the potential to induce reperfusion injury. Postconditioning of the heart with brief episodes of reperfusion/occlusion at the onset of reflow has been shown to limit infarct size.2,3

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However, this approach is not practical for patients treated with thrombolytic agents and therefore a more generic pharmacologic postconditioning is still needed. The ideal pharmacologic candidates need to be safe and effective when administered during the first few minutes of reperfusion by inducing cellular protection or enhancing myocardial tolerance to ischemia/reperfusion injury. Several drugs have yielded encouraging results in animals and a few have been tested in humans; however, none of these modalities has been widely accepted.4–6

Lipids and in particular polyunsaturated fatty acids have received special cardiovascular research attention because polyunsaturated fatty acid-rich diets are associated with a decreased risk of coronary artery disease.7,8 Acute application of polyunsaturated fatty acids to cardiomyocytes has also been shown to shorten action potential duration and this could account for the antiarrhythmic mechanism of the polyunsaturated fatty acids.9 Intralipid (Sigma, St. Louis, MO) is a brand name for the first safe fat emulsion for human use; Intralipid 20% is an emulsion of soybean oil (20%), egg yolk phospholipids (1.2%), and glycerol (2.2%). Intralipid has been widely used in patients who need total parenteral nutrition and as a vehicle for different drugs such as propofol. It has been shown recently that postischemic administration of Intralipid protects the isolated rat heart against ischemia/reperfusion injury.10 However, the molecular mechanism in which Intralipid mediates cardioprotection is completely unknown.

Here we found that postischemic administration of Intralipid protects the heart both in the in vivo rat model as well as in isolated mouse hearts. We then investigated the mechanism of Intralipid-induced cardioprotection. Our data revealed that postischemic treatment of Intralipid inhibits the opening of the mitochondrial permeability transition pore (mPTP) and protects the heart by recruiting the reperfusion injury salvage kinase pathway phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt)/extracellular signal-regulating kinase (ERK)-1 leading to phosphorylation of glycogen synthase kinase-3β (GSK-3β).

Materials and Methods

Animals
Male Sprague-Dawley rats 250–300 g and male mice (C57BL/6) 2–3 months old were used. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animal protocol was approved by the Animal Research Committee, University of California, Los Angeles, California.

Left Anterior Descending Coronary Artery Occlusion and Measurement of Infarct Size
Male Sprague-Dawley rats were anesthetized with ketamine (80 mg/kg intraperitoneal) and xylazine (8 mg/kg intraperitoneal). The rats were intubated and ventilated with a ventilator (CWE SAR-830/P, Ardmore, PA). 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 the electrocardiograph. The heart was subjected to 30 min of ischemia, followed by 180 min of reperfusion, which was achieved by releasing the tension on the ligature. An Intralipid bolus (20%, 5 ml/kg body weight) was applied via the femoral vein 5 min before reperfusion. The same volume of phosphate buffered saline was given in the control group (fig. 1A).

At the end of the experiment, 2.5 ml 1% Evans Blue dye was injected into the femoral vein and the myocardial ischemic area at risk (AAR) 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 (TTC) at 37°C for 15 min to identify the noninfarcted and infarcted areas. The infarcted area was displayed as the area unstained by TTC. Infarct size was expressed as a percentage of the AAR.

Langendorff Preparation
Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), and heparin (200 IU/kg) was injected to prevent blood coagulation. The heart was quickly removed and perfused through the aorta in a Langendorff apparatus with Krebs Henseleit bicarbonate buffer solution (KH) (mM): glucose 11.1, NaCl 118, KCl 4.7, MgSO4 1.2,
LV pressure rise (dP/dtmax) and decline (dP/dtmin) were directly induced necrosis in the mouse model as used by our group during ischemia. 40 min of reperfusion was sufficient to cause the heart was immersed in the 37°C Krebs solution (Invitrogen, Carlsbad, CA) using excitation and emission wavelengths set at 500 and 530 nm, respectively. Isolated mitochondria (500 μg protein) were suspended in 2 ml buffer C (150 mM sucrose, 50 mM KCl, 2 mM KH$_2$PO$_4$, 5 mM succinic acid, and 20 mM Tris-HCl, pH 7.4). Samples were preincubated for 90 s in the spectrofluorometer cuvette, and CaCl$_2$ pulses (2 μl 10 mM stock solution) were applied every 60 s in the spectrofluorometer. The Ca$^{2+}$ pulses induced a peak of extramitochondrial Ca$^{2+}$ concentration that returned to near-baseline level as Ca$^{2+}$ entered the mitochondrial matrix via the Ca$^{2+}$ uniporter. With increasing Ca$^{2+}$ loading, the extramitochondrial Ca$^{2+}$ concentration started accumulating, reflecting a lower capacity for mitochondria Ca$^{2+}$ uptake, which was followed by a sustained Ca$^{2+}$ increase indicating a massive release of the mitochondria Ca$^{2+}$ by the mPTP opening. The Ca$^{2+}$ retention capacity (CRC) was defined as the amount of Ca$^{2+}$ required to trigger this massive Ca$^{2+}$ release, which was used here as an indicator of the mPTP sensitivity to Ca$^{2+}$. CRC was expressed as μM of CaCl$_2$ per mg of mitochondrial protein.

Heart Functional Measurements
A catheter (1.4F Millar SPR-671) connected to a pressure transducer (Power Lab, AD Instruments Inc., Colorado Springs, CO) was directly inserted into the left ventricle (LV) to measure left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and heart rate (HR). The LVDP was calculated as LVDP = LVSP – LVEDP and the rate pressure product (RPP) as RPP = HR × LVDP. The maximum rate of LV pressure rise (dP/dt$_{max}$) and decline (dP/dt$_{min}$) were directly calculated from the selected stable recordings.

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 TTC staining. 11 Because the heart was immersed in the 37°C Krebs solution during ischemia, 40 min of reperfusion was sufficient to induce necrosis in the mouse model as used by our group as well as other groups. 11,12 The amount of necrosis was similar for reperfusion of 40 and 90 min (data not shown). The slices were fixed in 4% paraformaldehyde, and the area of necrosis was quantified by Photoshop and expressed as the percentage of total ventricular area.

Ca$^{2+}$-induced Mitochondrial Permeability Transition Preparation of Isolated Mitochondria. Mitochondria is prepared as previously described. 3 Briefly, myocardial sections of ex vivo hearts reperfused for 10 min (approximately 0.15–0.22 g) were placed in isolation buffer A containing 70 mM sucrose, 210 mM mannitol, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4 at 4°C. The tissue was finely minced with scissors and homogenized in the same buffer A (1 ml buffer/0.1 g of tissue) using Kontes and Potter-Elvehjem tissue grinders (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged at 1,300 g for 3 min; the supernatant was filtered through a cheesecloth and centrifuged at 10,000 g for 10 min. The mitochondrial pellet was resuspended in isolation buffer B containing 70 mM sucrose, 210 mM mannitol, 0.1 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Mitochondrial protein concentration was measured using the Bradford assay.

Calcium Retention Capacity. The onset of the mPTP opening was assessed following in vitro Ca$^{2+}$ overload as previously described. 13 Free Ca$^{2+}$ concentration outside 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.
was purchased from Invitrogen (Carlsbad, CA) and was used at 45 μM according to the data sheet provided by Invitrogen stating that LY294002 at 50 μM completely abolishes the PI3 k activity without apparent cell toxicity. 2′-Amino-3′-methoxyflavone (PD98059) was purchased from Invitrogen and was used at 10 μM, a concentration that has been used previously by many investigators in isolated Langendorff-perfused hearts to explore the role of ERK signal transduction pathway.\(^{16–18}\) The primary antibodies used were anti-ERK1/2 (rabbit polyclonal), antiphospho ERK1/2 (Thr202/Tyr204, mouse monoclonal), anti-AKT (rabbit polyclonal), phospho AKT (Ser 473, rabbit polyclonal), anti-GSK-3β (rabbit monoclonal), antiphospho GSK-3β (Ser 9, rabbit polyclonal), and mouse monoclonal antivinculin. All antibodies were purchased from Cell Signaling (Danvers, MA) with the exception of antivinculin, which was purchased from Sigma Chemical Company. The secondary antibodies were goat antirabbit Alexa 680 from Invitrogen and goat antirabbit IR Dye 800 CW from the Odyssey\textsuperscript{®} Imaging System.

**Statistical Analysis**

For the in vivo study, which has only two groups, means were compared using the Student t test. For the ex vivo studies with four or five groups, mean profiles over time were compared across groups using repeated-measure ANOVA methods. Under the ANOVA model, pairwise mean comparisons were judged significant using the Tukey studentized range criterion. These criteria prevents the type I error from exceeding the nominal α = 0.05 level for the outcome. SPSS, version 13.0 (SPSS Inc, Chicago, IL) was used to carry out the computations. Because all outcomes were continuous, results were summarized with means ± SEMs.

**Results**

**Intralipid Protects the Heart against Ischemia/Reperfusion Injury in the In Vivo Rat Model**

It has been previously shown that postischemic administration of Intralipid can protect the heart against ischemia/reperfusion injury in isolated rat hearts.\(^{10}\) Here we first examined whether Intralipid can also protect the heart against ischemia/reperfusion injury in the in vivo rat model. The left coronary artery was occluded for 30 min followed by 3 h of reperfusion. One single bolus of PBS or Intralipid was applied through the femoral vein 5 min before the reperfusion (fig. 1A). The AAR to LV ratio was similar in both groups (62.1 ± 2.4 in the control group [n = 6] vs. 60.1 ± 2.6 in the Intralipid group [n = 6]), indicating that the two groups were subjected to a comparable degree of ischemic risk (fig. 1B). However, the infarct size was significantly smaller in the Intralipid group in comparison with the control group; the ratio of infarct size to AAR was 20.7 ± 1.3 in the Intralipid group versus 61.8 ± 3.1 in the control group (P < 0.01), and the ratio of infarct size to LV was 12.4 ± 0.6 in the Intralipid group versus 38.1 ± 1.5 in the control group (P < 0.01) (fig. 1C and D). These data demonstrate that only one bolus of Intralipid right before reperfusion is sufficient to protect the heart against ischemia/reperfusion injury in vivo.

**Postischemic Administration of Intralipid Protects Isolated Mice Hearts against Ischemia/Reperfusion Injury**

To explore the molecular mechanism in which Intralipid mediates protection against ischemia/reperfusion injury, we used the Langendorff-perfused isolated mouse heart rather than the in vivo model of ischemia/reperfusion injury because the former can be more easily manipulated to unravel the key mechanisms mediating Intralipid-induced cardioprotection. We first examined if Intralipid could also protect mouse hearts against ischemia/reperfusion injury as in rats. We used the well-established protocol to induce ischemia/reperfusion injury in isolated mouse hearts\(^{11,12}\) (fig. 2A). Similar to rats, postischemic administration of Intralipid in mice significantly improved the functional recovery (RPP = 13,676 ± 611 mmHg·beats/min [n = 6] vs. 2,999 ± 863 mmHg·beats/min in the control group [n = 6]) [P < 0.01] at 40 min reperfusion, fig. 2B). The Intralipid group also showed a much better LV dP/dt\(_{\text{max}}\) and LV dP/dt\(_{\text{min}}\) and LVDP compared with control hearts (fig. 2, C and D and Table 1). The infarct size was also significantly smaller in the Intralipid group (18.3 ± 2.4% in Intralipid [n = 9] vs. 54.9 ± 2.9% in the control group [n = 10], P < 0.01, fig. 2E-J). These data suggest that Intralipid protects both mice and rats from ischemia/reperfusion injury.

**Administration of Intralipid during the First Few Minutes of Reperfusion is Sufficient to Induce Protection and the Protection is Maintained after Removal of Intralipid**

Because the first few minutes of reperfusion are critical in myocardial protection, we investigated whether administration of Intralipid for durations shorter than 40 min could protect the heart and whether the functional recovery is maintained upon removal of Intralipid. The isolated mouse hearts were therefore treated with Intralipid for only the first 5, 10, and 20 min of reperfusion followed by KH for the remainder of 40 min as shown in figure 2A. When Intralipid was administered for only 5 min (ILP-5), the hemodynamic indices at the end of 40 min reperfusion were all significantly higher than those of the control group that did not receive any Intralipid at reflow (fig. 2B–D, Table 1). It was also interesting to note that hemodynamic indices were maintained when Intralipid was switched to KH at 5 min reperfusion, as these values were not significantly different between 5, 10, and 20 min in the Intralipid group. When Intralipid was applied for longer times of 10 min (ILP-10) or 20 min (ILP-20), the hemodynamic indices at the end of 40 min reperfusion were not significantly higher than ILP-5: RPP = 8,058 ± 1,297 in ILP-5 (n = 6); RPP = 9,468 ±
1,272 in ILP-10 (n = 6); and 10,571 ± 797 mmHg·beats/min in ILP-20 (n = 6).

Consistent with the higher functional recovery in Intralipid-treated groups, the infarct sizes were also significantly smaller compared with the control group (33.1 ± 1.4% in ILP-5 [n = 6]; 26.8 ± 2.1% in ILP-10 [n = 6]; 25.7 ± 1.8% in ILP-20 [n = 6]; and 18.3 ± 2.4% in ILP-40 [n = 9] vs. 54.9 ± 2.9% in the control group [n = 10]; P < 0.01, fig. 2E-I). In fact, administration of Intralipid during the first 5 min of reperfusion was sufficient to reduce the myocardial infarct size by approximately 50%. The infarct sizes between ILP-5, ILP-10, and ILP-20 were similar but were significantly larger than ILP-40 (P < 0.05). These data strongly support the view that administration of Intralipid

Fig. 2. Administration of Intralipid at reperfusion improves heart functional recovery and reduces infarct size against reperfusion injury. (A) Experimental protocol. The isolated mouse hearts were reperfused with Krebs Henseleit (KH, control group, CTRL), or 1% Intralipid for 5 min (ILP-5), 10 min (ILP-10), 20 min (ILP-20), or 40 min (ILP-40), followed by reperfusion with KH for the remainder of 40 min. Rate pressure product (RPP, B), the maximum rate of left ventricle (LV) pressure rise (dP/dt_{max}) and decline (−dP/dt_{min}, C) and left ventricular developed pressure (LVDP, D) as a function of time in CTRL (black, n = 6), ILP-5 (purple, n = 6), ILP-10 (blue, n = 6), ILP-20 (gray, n = 6) and ILP-40 (red, n = 6). Four slices of the same heart after 2,3,5-triphenyltetrazolium chloride (TTC) staining in CTRL (E), ILP-5 (F), ILP-10 (G), ILP-20 (H), and ILP-40 (I). The white area represents the infarct zone and the red shows the viable area. (J) The area of necrosis as the percentage of total left ventricular (LV) area in CTRL (black, n = 10), ILP-5 (purple, n = 6), ILP-10 (blue, n = 6), ILP-20 (gray, n = 6), and ILP-40 (red, n = 9). The individual measurements in each group are shown in open circles whereas the averages (mean ± SEM) are shown in filled circles. * * P < 0.01 versus CTRL, # P < 0.05 versus ILP-40.
Intralipid-induced cardioprotection was fully abolished when Intralipid + LY294002 (ILP + LY) was applied, as RPP was significantly lower compared with the group treated with Intralipid alone, but still significantly higher than control (fig. 3D and E). The reduction in Intralipid-induced cardioprotection in the presence of PD98059 demonstrates that ERK signaling is also participating in the protection offered by Intralipid against ischemia/reperfusion injury.

**Intralipid Induces Akt, ERK, and GSK-3β Phosphorylation**

To further confirm the involvement of reperfusion injury salvage kinase pathway in Intralipid-induced cardioprotection against ischemia/reperfusion injury, Western blot analysis of whole heart lysates (ex vivo) that were reperfused for 10 min with KH (control), Intralipid, ILP + LY, or ILP + PD was performed. Intralipid-induced cardioprotection was associated with significant increase in phosphorylation of Akt (approximately eightfold) that was reversed when Intralipid was applied together with LY294002 but not with PD (in arbitrary units normalized to control group [n = 6]: 8.3 ± 1.2 in Intralipid [n = 4], vs. 3.2 ± 0.8 in ILP + LY [n = 4] and 8.5 ± 2.2 in ILP + PD [n = 4]), (figure 5A, B). These data further
confirm our findings that the protection by Intralipid is mediated via the prosurvival PI3K-Akt pathway. Phospho-ERK1 levels were also significantly increased in the Intralipid group (approximately threefold) compared with the control group. PD completely abolished phosphorylation of ERK induced by Intralipid (in arbitrary units normalized to control group \([n = 6]\): 2.5 \(\pm\) 0.3 in Intralipid \([n = 4]\) vs 2.9 \(\pm\) 1.1 in ILP+LY \([n = 4]\) and 3.3 \(\pm\) 0.7 in ILP+PD \([n = 4]\), fig. 5C and D). These data clearly demonstrate that in addition to PI3K/Akt pathway, the protection by Intralipid is also mediated via the ERK pathway. GSK-3\(\beta\) phosphorylation was also significantly increased (approximately ninefold) by Intralipid and the administration of both LY294002 and PD significantly reduced the level of phosphorylated GSK-3\(\beta\) in the myocardium treated with Intralipid (in arbitrary units normalized to control group \([n = 6]\): 8.5 \(\pm\) 1.5 \([n = 4]\) vs 2.9 \(\pm\) 1.1 in ILP+LY \([n = 4]\) and 3.3 \(\pm\) 0.7 in ILP+PD \([n = 4]\), fig. 5E and F). These data strongly support the role of Akt/ERK/GSK-3\(\beta\) in Intralipid-induced cardioprotection against ischemia/reperfusion injury.

**Intralipid Inhibits the Opening of the mPTP and This Inhibition is Abolished by PI3K Inhibitor**

Because the inhibition of the mPTP opening during reperfusion has been shown to induce cardioprotection,\(^{19}\) we investigated whether Intralipid-induced cardioprotection is mediated by inhibition of the mPTP opening. We compared...
the threshold for opening of mPTP in response to Ca\(^{2+}\) overload in isolated mitochondria from hearts reperfused with KH (control) or Intralipid for 10 min (fig. 6A). An example of the time course of Ca\(^{2+}\) concentration in the mitochondrial external medium is shown in figure 6B. In the control group, seven pulses of 20 nM Ca\(^{2+}\) were sufficient to trigger the opening of mPTP. Interestingly, the Ca\(^{2+}\) load significantly increased in mitochondria isolated from the Intralipid group as the number of Ca\(^{2+}\) pulses required for opening of mPTP was increased to 14 pulses. The bar plot in figure 6C summarizes the CRC; CRC was significantly higher in the Intralipid group compared with the control group (274.3 ± 8.4 in Intralipid [n = 7] vs. 168.6 ± 9.6 nM/mg mitochondrial protein in the control group [n = 7], P < 0.01). These data strongly suggest that inhibition of mPTP opening by Intralipid is one of the key events in the Intralipid-induced cardioprotection against ischemia/reperfusion injury. In the presence of LY (45 μM), Intralipid effect was prevented as the CRC was reduced to 170.0 ± 15.3 nM/mg mitochondrial protein (n = 6), which was not significantly different from the control group. These data clearly demonstrate that Intralipid inhibits the opening of mPTP and this inhibition is abolished in the presence of LY.

**Discussion**

We show here that administration of Intralipid right before the onset of reperfusion results in approximately 70% reduction in infarct size in the *in vivo* rat model. Intralipid appli-
Table 2. ERK Inhibitor PD98059 Partially Abolishes Intralipid-induced Cardioprotection against Ischemia/Reperfusion Injury

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Cardiac functional recovery parameters of left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), and heart rate (HR) before ischemia (baseline) and at different times of reperfusion in control group (CTRL), 1% Intralipid (ILP), 1% Intralipid + PD98059 (ILP+PD), and PD98059 alone (PD). Values are mean ± SEM. * P < 0.01 vs. CTRL; † P < 0.01 vs. ILP (n = 6).

Reperfusion at reperfusion also improves the functional recovery of isolated Langendorff-perfused mouse hearts by approximately fourfold and significantly reduces the infarct size. Administration of Intralipid during even the first 5 min of reperfusion is sufficient to induce protection, and the protection is maintained after removal of Intralipid. These data strongly indicate that Intralipid treatment has a cardioprotective effect against ischemia/reperfusion injury both in mice and rats. In this study, we provided the underlying mechanism of Intralipid-induced cardioprotection, which is mediated by inhibition of the mPTP and the recruitment of the reperfusion injury salvage kinase pathway leading to phosphorylation of GSK-3β.

The Ovize group was the first to demonstrate a direct link between PI3K activation and opening of mPTP during post-conditioning. Here we show that the cardioprotection provided by Intralipid at reperfusion is associated with inhibition of the mPTP opening, as the mitochondrial Ca\(^{2+}\) uptake required for the opening of the mPTP was significantly higher in Intralipid-treated hearts in comparison with the control group (fig. 6). We propose that Intralipid enhances the homeostasis of cardiomyocytes to better regulate Ca\(^{2+}\) overload and therefore increase the threshold for opening of the mPTP. To determine whether the Intralipid-induced inhibition of the mPTP opening was through the PI3K pathway, LY294002 (ILP+LY), top panels, or CTRL, ILP, and 1% Intralipid+PD98059 (ILP+PD), bottom panels. (B, D, F) Western blot quantification (mean±SEM) of pAkt protein to total Akt (pAkt/Akt) ratios in CTRL, ILP, and ILP+LY, and pGSK-3β (pGSK-3β/Akt) ratios in CTRL, ILP, and ILP+LY, and pERK1/2 (pERK1/2/Akt) ratios in CTRL, ILP, and ILP+LY.

Fig. 5. Involvement of PI3K-Akt and ERK pathways and their downstream target GSK-3β in Intralipid-induced protection. (A, C, E) Representative immunoblots of pAkt and total Akt (bottom panels. (B) Western blot quantification of (mean±SEM) pAkt protein to total Akt (pAkt/Akt) ratios in CTRL+N, ILP, and ILP+LY, top panels, or CTRL, ILP, and 1% Intralipid+PD98059 (ILP+PD), bottom panels. (B, D, F) Western blot quantification of pAkt protein to total Akt (pAkt/Akt) ratios in CTRL+N, ILP, and ILP+LY, and pGSK-3β (pGSK-3β/Akt) ratios in CTRL+N, ILP, and ILP+LY, and pERK1/2 (pERK1/2/Akt) ratios in CTRL+N, ILP, and ILP+LY.

Cardiac functional recovery parameters of left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), and heart rate (HR) before ischemia (baseline) and at different times of reperfusion in control group (CTRL), 1% Intralipid (ILP), 1% Intralipid + PD98059 (ILP+PD), and PD98059 alone (PD). Values are mean ± SEM. * P < 0.01 vs. CTRL; † P < 0.01 vs. ILP (n = 6).
Intralipid inhibits the opening of the mitochondrial permeability transition pore and this inhibition is abolished by PI3K inhibitor. (A) Experimental protocol for measuring calcium retention capacity (CRC). At the onset of reperfusion, isolated hearts are perfused with Krebs Henseleit (KH, control group, CTRL), 1% Intralipid (ILP), or 1% Intralipid+LY294002 (ILP+LY) for 10 min. (B) Typical recording of the mitochondria permeability transition pore (mPTP) opening in isolated mitochondria from control (black trace), ILP (light gray trace), and ILP+LY (dark gray trace). Fourteen pulses (gray arrows) of 20 nM calcium were required to trigger the opening of the mPTP in the ILP group compared with seven pulses (black arrows) in CTRL and eight pulses in ILP+LY. (C) CRC in CTRL (circles, n = 7), ILP (squares, n = 7) and ILP+LY (triangles, n = 6). The individual measurements in each group are shown in open shapes whereas the averages (mean±SEM) are shown in filled shapes. **P < 0.01 versus CTRL and ILP+LY.

Fig. 6. Intralipid inhibits the opening of the mitochondrial permeability transition pore and this inhibition is abolished by PI3K inhibitor. (A) Experimental protocol for measuring calcium retention capacity (CRC). At the onset of reperfusion, isolated hearts are perfused with Krebs Henseleit (KH, control group, CTRL), 1% Intralipid (ILP), or 1% Intralipid+LY294002 (ILP+LY) for 10 min. (B) Typical recording of the mitochondria permeability transition pore (mPTP) opening in isolated mitochondria from control (black trace), ILP (light gray trace), and ILP+LY (dark gray trace). Fourteen pulses (gray arrows) of 20 nM calcium were required to trigger the opening of the mPTP in the ILP group compared with seven pulses (black arrows) in CTRL and eight pulses in ILP+LY. (C) CRC in CTRL (circles, n = 7), ILP (squares, n = 7) and ILP+LY (triangles, n = 6). The individual measurements in each group are shown in open shapes whereas the averages (mean±SEM) are shown in filled shapes. **P < 0.01 versus CTRL and ILP+LY.

prosurvival PI3K/Akt as well as ERK pathway in Intralipid-induced cardioprotection and are in agreement with previous work showing that activation of these two pathways have a cardioprotective effect in ischemia/reperfusion experiments in rodents.20 In pigs, however, the activation of the reperfusion injury salvage kinase pathway does not seem to be crucial for postconditioning.21 GSK-3β phosphorylation has emerged as an end effector step where multiple protective signaling pathways converge. Here we show that GSK-3β mediates Intralipid-induced cardioprotection via PI3/Akt and mitogen-activated protein kinases kinase/ERK pathways. These are the two major pathways that have been demonstrated to be involved in cardioprotection against ischemia/reperfusion injury. However, other upstream kinases such as protein kinases C, A, G, and p70S6 could also phosphorylate GSK-3β to induce cardioprotection.22 Whether Intralipid-induced cardioprotection is mediated through these kinases remain to be seen in future studies. Using genetic manipulation and pharmacologic agents, GSK-3β phosphorylation has also been shown to lead to inhibition of the mPTP opening and therefore inducing cardioprotection.13,23 Here we found that Intralipid treatment increased phosphorylation of GSK-3β (fig. 5). These observations are in agreement with other studies that have also implicated the increased phosphorylation of GSK-3β to be a common feature of different cardioprotective agents,22,24 although the requirement of GSK-3β inactivation to induce the inhibition of mPTP opening has been challenged recently.13,25

Figure 7 summarizes our hypothetical scheme of the mechanism of protection by Intralipid against ischemia/reperfusion injury. Activation of the reperfusion injury salvage kinase pathway by Intralipid increases the phosphorylated levels of Akt and ERK. These two pathways converge to shift the equilibrium of GSK-3β from active form (not phosphorylated) toward the GSK-3β inactive form (phosphorylated). Once GSK-3β is phosphorylated, it inhibits the opening of the mPTP, which results in cardioprotection.

Our data strongly indicate that Intralipid is a very powerful postischemic pharmacologic agent. The role of Intralipid in preconditioning, however, seems to be controversial.10,26 Hu et al. reported that Intralipid has no effect on the infarct size in the in vivo model of I/R injury26 whereas in another work from the same group, Intralipid was shown to reduce the infarct size in the ex vivo model of I/R in rats.10 These conflicting results could be due to the fact that only male rats were used in one study,10 whereas in the other study both male and females rats were used.26 It is now well accepted that female rats are better protected against ischemia/reperfusion injury compared with their male counterparts.27,28 It was also not clear whether an equal number of males and females were used in each group.26 Therefore, the fact that Intralipid administration before ischemia failed to reduce the infarct size in the in vivo model of ischemia/reperfusion injury could simply be due to the higher numbers of males in the Intralipid group.26

Intralipid has also been proposed as a rescue therapy for severe local anesthetic-induced cardiovascular collapse and cardiotoxicity of certain drugs.29–33 Several case reports demonstrate the effectiveness of Intralipid in treating amiodipine poisoning7 and diltiazem poisoning,34 but the only recommended therapeutic use for Intralipid as a rescue
Further studies are required to clarify the role of Intralipid on vasculature in health and in various disease models.

**Conclusions**

We show here that only one bolus of Intralipid (20%) right before reperfusion is sufficient to protect the heart against ischemia/reperfusion injury in vivo. Intralipid application at the reperfusion also improves the functional recovery of isolated Langendorff-perfused mouse hearts approximately fourfold and significantly reduces the infarct size. Postischemic administration of Intralipid inhibits the opening of the mPTP. Phosphorylation of GSK-3β, which has emerged as a new target for cardioprotection, is involved in the cardioprotective action of Intralipid against ischemia/reperfusion injury. Intralipid has already been in clinical use for almost four decades for patients who need total parenteral nutrition, and it has been shown to be safe and well tolerated. Here we propose that Intralipid could be a clinically safe compound for targeting GSK-3β at the time of reperfusion to protect the myocardium against ischemia/reperfusion injury and certainly warrant further investigation in human heart.

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**References**


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