Electrospray ionization mass spectrometry analyses of nuclear membrane phospholipid loss after reperfusion of ischemic myocardium

Scott D. Williams,* Fong-Fu Hsu,† and David A. Ford1,∗

Department of Biochemistry and Molecular Biology,* Saint Louis University Health Sciences Center, St. Louis, MO 63104; and Department of Medicine,† Washington University, St. Louis, MO 63110

Abstract The role of nuclear membrane phospholipids as targets of phospholipases resulting in the generation of nuclear signaling messengers has received attention. In the present study, we have exploited the utility of electrospray ionization mass spectrometry to determine the phospholipid content of nuclei isolated from perfused hearts. Rat heart nuclei contained choline glycerophospholipids composed of palmitoyl and stearoyl residues at the sn-1 position with oleoyl, linoleoyl, and arachidonoyl residues at the sn-2 position. Diacyl molecular species were the predominant molecular subclass in the choline glycerophospholipids, with the balance of the molecular species being plasmalogens. In the ethanolamine glycerophospholipid pool from rat heart nuclei approximately 50% of the molecular species were plasmalogens, which were enriched with arachidonic acid at the sn-2 position. A 50% loss of myoxygen nuclear choline and ethanolamine glycerophospholipids was observed in hearts rendered globally ischemic for 15 min followed by 90 min of reperfusion in comparisons with the content of these phospholipids in control perfused hearts. The loss of nuclear choline and ethanolamine glycerophospholipids during reperfusion of ischemic myocardium was partially reversed by the calcium-independent phospholipase A2 (iPLA2) inhibitor bromoenol lactone (BEL), suggesting that the loss of nuclear phospholipids during ischemia/reperfusion is mediated, in part, by iPLA2. Western blot analyses of isolated nuclei from ischemic hearts demonstrated that iPLA2 is translocated to the nucleus after myocardial ischemia. Taken together, these studies have demonstrated that nuclear phospholipid mass decreases after myocardial ischemia by a mechanism that involves, at least in part, phospholipolysis mediated by iPLA2.

Supplementary key words phospholipids • plasmalogen • phospholipase A2 • myocardial ischemia

In the past two decades the role of plasma membrane phospholipids as precursors of second messengers such as arachidonic acid, lysophospholipids, eicosanoids, diacylglycerols and inositol phosphates has received considerable attention (1–6). Cell regulatory mechanisms that involve phospholipolysis catalyzed by a plethora of phospholipases that target nuclear membrane phospholipids have been proposed that could potentially mediate nuclear function and thus gene regulation and programmed cell death (7–9). A role for phospholipases in nuclear signaling has been suggested by the observation that diacylglycerols derived from choline glycerophospholipid pools accumulate in the nuclear envelope of thrombin-stimulated fibroblasts by a biochemical mechanism that includes rhoA-dependent translocation of phospholipase D (PLD) to the nuclear envelope (8). In addition, cytosolic phospholipase A2 (cPLA2) is found in the nuclear membrane of histamine-stimulated endothelial cells (9). It is also likely that phospholipase A2 plays a major role in myocardial nuclear phospholipid signaling because transmission electron microscopic autoradiography has demonstrated rapid arachidonic acid turnover in the nuclear membranes of cardiac myocytes (10). However, a thorough evaluation of the myocardial nuclear membrane pool phospholipid content of these phospholipids in control perfused hearts.

Abbreviations: 14:0–14:0 (sn-1-sn-2, respectively), dietradecanoyl; 16:0–16:0, dihexadecanoyl; 16:0–18:1, 1-hexadecanoyl-2-octadec-9′-enoyl; 16:0–18:2, 1-hexadecanoyl-2-octadec-9′,12′-enoyl; 16:0–20:4, 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl; 16:0–22:6, 1-hexadecanoyl-2-docosahex-4′,7′,10′,13′,16′,19′-enoyl; 18:1–18:2, 1-octadec-9′-enoyl-2-octadec-9′,12′-enoyl; 18:1–20:4, 1-octadec-9′-enoyl-2-eicosatetra-5′,8′,11′,14′-enoyl; 18:0–18:2, 1-octadecanoyl-2-octadec-9′,12′-enoyl; 18:0–20:4, 1-octadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl; 20:0–20:4, 1-eicosanoyl-2-eicosatetra-5′,8′,11′-enoyl; BEL, bromoelactone; CAD, collision-activated dissociation; GPC, sn-glycerol-3-phosphocholine; GPE, sn-glycerol-3-phosphoethanolamine; ESI-MS, electrospray ionization mass spectrometry; HRP, horseradish peroxidase; PFK, phosphofructokinase; iPLA2, calcium-independent phospholipase A2; cPLA2, cytosolic phospholipase A2; sPLA2, secretory phospholipase A2; PLD, phospholipase D; PMSF, phenylmethylsulfonyl fluoride.

1 To whom correspondence should be addressed.
ids has not been forthcoming because of limitations in the detection levels of methods to analyze minute amounts of phospholipid.

Phospholipase A\(_2\) activity has been identified in the heart and implicated as a mediator of the pathophysiological sequelae of myocardial ischemia (11–14). Calcium-independent phospholipase A\(_2\) (iPLA\(_2\)) is a predominant phospholipase A\(_2\) activity present in myocardium (14, 15). In isolated perfused hearts rendered globally ischemic (i.e., zero-flow ischemia to the heart), iPLA\(_2\) is translocated to myocardial membranes within 2 min of the onset of ischemia (14). However, the role of iPLA\(_2\) in the ischemic heart remains to be fully elucidated because concomitant changes in cardiac myocyte phospholipid content have not been observed concomitant with ischemia-elicited increases in iPLA\(_2\) activity.

Because membrane-associated iPLA\(_2\) activity increases in a crude membrane fraction isolated from ischemic hearts (14, 15), the possibility that iPLA\(_2\) is translocated to the nucleus during myocardial ischemia was considered. Accordingly, in the current study, the unparalleled sensitivity of electrospray ionization mass spectrometry (ESI-MS) was exploited to characterize nuclear choline and ethanolamine glycerophospholipid molecular species and to determine the possible involvement of iPLA\(_2\) in nuclear phospholipid degradation. Herein, we report that rat myocardial nuclei contain a diverse array of both diacyl and plasmalogen molecular species and that the mass of these phospholipid pools is decreased after myocardial ischemia and reperfusion. Furthermore, the loss of nuclear choline and ethanolamine glycerophospholipids after ischemia and reperfusion is, at least in part, mediated by iPLA\(_2\), which is translocated to the nucleus during myocardial ischemia.

**MATERIALS AND METHODS**

**Materials**

Anti-iPLA\(_2\) was a generous gift from Genetics Institute (Cambridge, MA). Anti-cPLA\(_{2}\) (N-216), anti-secretory PLA\(_{2}\) (sPLA\(_{2}\)), and anti-lamin B\(_1\) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), Cayman Chemical (Ann Arbor, MI), and Zymed (South San Francisco, CA), respectively. Secondary antibodies including goat anti-rabbit horseradish peroxidase and goat anti-mouse horseradish peroxidase were purchased from Sigma (St. Louis, MO) and Bioc-Rad (Hercules, CA), respectively. Electrophoresis-grade reagents for polyacrylamide and agarose gel electrophoresis were purchased from ICN Pharmaceuticals (Costa Mesa, CA), Pharmacia (Piscataway, NJ), Bio-Rad, and Fisher (Pittsburgh, PA). Bromoenol lactone (BEL) was synthesized and prepared as previously described (16). Phospholipid internal standards, 1,2-ditetradecanoino-sn-glycero-3-phosphocholine (1,2-ditetradecanoino-sn-GPC), 1,2-ditetradecanoino-sn-glycero-3-phosphoethanolamine (1,2-ditetradecanoino-sn-GPE), and 1,2-dieicosanoino-sn-glycero-3-phosphocholine (1,2-dieicosanoino-sn-GPC), were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were of the highest grade available and were purchased from Sigma, Fisher, or VWR Scientific (San Francisco, CA).

**Preparation of myocardial nuclei from Langendorff perfused rat hearts subjected to myocardial ischemia and reperfusion**

Male Sprague-Dawley rats were utilized for the preparation of Langendorff perfused hearts as described previously (14, 17). In brief, male Sprague-Dawley rats (200–250 g body weight) were injected with heparin (200 U, intraperitoneal) 30 min before being anesthetized with pentobarbital sodium (25 mg, intraperitoneal). Hearts were rapidly removed and placed immediately in ice-cold saline solution before being perfused. Rat hearts were perfused retrograde through the aorta with a modified Krebs-Henseleit buffer (137 mm NaCl, 4.7 mm KCl, 3 mm CaCl\(_2\), 1.2 mm KH\(_2\)PO\(_4\), 1.2 mm MgSO\(_4\), 0.5 mm Na-EDTA, 15 mm NaHCO\(_3\), and 11 mm glucose, equilibrated with 95% O\(_2\), 5% CO\(_2\), pH 7.4) at 37°C for 15 min at a pressure of 60 mm Hg, followed by either control perfusions for 105 min, global zero-flow ischemia for 15 min, or global zero-flow ischemia for 15 min followed by 90 min of reperfusion. In selected experiments, 10 μM BEL was included in the perfusion buffer for 15 min after the initial 15-min equilibration period prior to ischemia and reperfusion protocols. Rat heart nuclei were prepared from isolated perfused hearts as previously described (18, 19). In brief, ventricles from perfused hearts were immediately minced in 10 volumes of homogenization buffer [10 mm Tris-HCl, 250 mm sucrose, 3 mm MgCl\(_2\), and 0.1 mm phenylmethylsulfonl fluoride, pH 7.4] and then homogenized with a Polytron (40% setting for 10 sec). The homogenate was centrifuged at 1,000 \(g_{\text{max}}\) for 10 min and the pellet was subsequently resuspended in 10 volumes of homogenization buffer and rehomogenized with six strokes of a Potter-Elvehjem homogenizer at a setting of 40%. The resulting homogenate was filtered through nylon sieve mesh (100 μm) and the filtrate was centrifuged at 1,000 \(g_{\text{max}}\) for 10 min. The pellet was resuspended in 10 volumes of homogenization buffer. Again, the resuspended pellet was centrifuged at 1,000 \(g_{\text{max}}\) for 10 min and subsequently resuspended in 20 volumes of homogenization buffer adjusted to 2.2 mm sucrose. The resuspended homogenate was underlaid with 5 ml of homogenization buffer containing 2.2 mm sucrose and then subjected to ultracentrifugation in a swinging bucket rotor (SW-28) at 113,000 \(g_{\text{max}}\) for 1 h. The nuclear pellet was washed in 10 mm Tris buffer (pH 7.4) containing 3 mm MgCl\(_2\) and centrifuged at 1,500 \(g_{\text{max}}\) for 20 min. The nuclear pellet was then resuspended in a small volume of the same buffer and microcentrifuged at 2,000 \(g_{\text{max}}\) for 15 min. The final nuclear pellet was resuspended in Tris buffer (10 mm, pH 7.4) containing 3 mm MgCl\(_2\) and subsequently was assayed either for iPLA\(_2\) activity or immediately frozen in liquid nitrogen prior to Western blot analysis. The purity of the nuclear preparations was verified by immunoblotting with anti-lamin B\(_1\) as well as by comparisons with other subcellular markers (18).

**Western blot analysis of iPLA\(_2\), cPLA\(_2\), and sPLA\(_2\) in isolated nuclei**

Nucleus-associated proteins were quantitated by the Lowry protein assay (20) and subsequently adjusted to equal protein concentrations prior to being subjected to Western blot analysis. For selected experiments, immunobLOTS were also performed on cytosolic proteins that were prepared from isolated perfused hearts as previously described (17). Primary antibodies were used at the indicated concentrations along with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit HRP, 1:7,000 dilution or goat anti-mouse HRP, 1:7,000 dilution). Immunoreactive bands were visualized by chemiluminescence detection on X-ray film (X-OMAT AR; Eastman Kodak, Rochester, NY), utilizing the ECL chemiluminescence system (Amersham, Arlington Heights, IL). Multiple exposures of
film to the blots were developed and the exposures that had
grain development within a linear range were used for quantita-
tion of band intensity, utilizing NIH Image software after scanning
and conversion of autoradiographic data to TIFF file formats.

**Calcium-independent phospholipase A₂ assay and
electrospray ionization mass spectrometry of nuclear membrane phospholipids**

Calcium-independent phospholipase A₂ activity in nuclei pre-
pared from isolated, perfused rat hearts was measured as de-
scribed previously by measuring the release of [14C]arachidonic
acid from 1-hexadecanoyl-2-[1-14C]eicosatetra-5,8,11,14-e n oyl-
n-GPE substrate (21). Lipids from isolated nuclei were ex-
tracted by the method of Bligh and Dyer (22) in the presence of
internal standards. Chloroform extracts from 200 μg of nuclear
protein were used for analysis of individual phospholipid molecu-
lar species, utilizing a Finnigan-MAT TSQ-7000 triple stage qua-
drupole mass spectrometer as described previously (23). Individ-
ual molecular species were quantitated by comparisons of the
individual ion peak intensities with that of either 1,2-ditetradec-
canoyl-sn-GPC (14:0–14:0-GPC) or 1,2-ditetradecanoyl-sn-GPE
(14:0–14:0-GPE) after correction for 13C isotope effects. Choline
glycerophospholipids in the extract were either directly quanti-
tated as their sodium adducts (M + Na⁺) or as their protonated
adducts (M⁺) after the addition of dilute acetic acid. Ethanol-
amine glycerophospholipids in the nuclear extract were directly
quantitated after the addition of 5 μmol of NaOH per milliliter.
Electrospray ionization tandem mass spectrometry was utilized
to substantiate individual molecular species. All values are pre-
sented as the mean ± standard deviation. Statistical evaluation
between two groups was by Student’s unpaired t-test. The values
were considered significant when P was less than 0.05.

**RESULTS**

**Ischemia/reperfusion-induced alterations in the mass of nuclear choline glycerophospholipid molecular species mediated by iPLA₂**

Analyses of nuclei isolated from control perfused rat
myocardium by ESI-MS in the positive-ion mode demon-
strated the preponderance of phosphatidylcholine molecu-
lar species predominantly containing either palmitic or
stearic acids at the sn-1 position and either oleic, linoleic, or
arachidonic acids at the sn-2 position [peaks at m/z 734
(16:0–16:0 phosphatidylcholine), 758 (16:0–18:2 phospha-
dylcholine), 760 (16:0–18:1 phosphatidylcholine), 782
(16:0–20:4 phosphatidylcholine), 784 (18:1–18:2 phos-
phatidylcholine), 786 (18:0–18:2 phosphatidylcholine), 788
(18:0–18:1 phosphatidylcholine), 806 (16:0–22:6 phospha-
dylcholine), 808 (18:1–20:4 phosphatidylcholine), 810
(18:0–20:4 phosphatidylcholine), and 834 (18:0–22:6 phos-
phatidylcholine)] (Fig. 1A and Table 1). Phosphatidylincho-
line molecular species constituted more than 90% of the
total choline glycerophospholipid mass in nuclei isolated
from control perfused rat myocardium. The balance of
choline glycerophospholipid molecular species was plas-
menylcholine molecular sub species [peaks at m/z 744
(16:0–18:1 plasm enylcholine), 766 (16:0–20:4 plasm enyl-
choline), 768 (18:1–18:2 plasm enylcholine), and 792
(18:1–20:4 plasm enylcholine)] (Fig. 1A and Table 1).

Comparisons of ESI mass spectra in the positive-ion
mode of extracts of phospholipids from nuclei isolated
from control perfused rat myocardium and phospholipids
from nuclei isolated from rat hearts that were rendered
 globally ischemic for 15 min demonstrated that global is-
chemia did not significantly alter any of the choline
glycerophospholipid molecular species (Fig. 1B and Ta-
ble 1). Furthermore, 15 min of global ischemia did not result
in a measurable change in total choline glycerophospho-
lipid mass (Fig. 2). In contrast, comparisons of ESI mass
spectra in the positive-ion mode of extracts of phospholip-
ids from nuclei isolated from control perfused rat myocard-
i um and phospholipids from nuclei isolated from rat
hearts that were rendered globally ischemic for 15 min
followed by 90 min of reperfusion demonstrated that re-
perfusion of ischemic myocardium induced the hydrolysis
of approximately 50% of the total choline glycerophos-
pholipid molecular species (Fig. 2). Statistically significant
reductions were observed in all choline glycerophospho-
lipid molecular species identified by ESI-MS with the ex-
ception of 18:1–20:4 phosphatidylcholine (Fig. 1C and Ta-
ble 1). It should also be appreciated that the ratio of
the mass of nuclear protein to the mass of nuclear DNA
was constant under the four conditions that were com-
pared and thus the same relative alterations of phospho-
lipid mass were also observed when data were normalized
to micrograms of nuclear DNA instead of micrograms of
nuclear protein.

Additional experiments were performed with the iPLA₂
specific inhibitor, BEL, in perfusions of isolated adult rat
hearts subsequently rendered globally ischemic for 15 min
followed by 90 min of reperfusion. Comparisons of ESI
mass spectra in the positive-ion mode of extracts of phos-
pholipids from nuclei isolated from control perfused rat
myocardium and phospholipids from nuclei isolated from
rat hearts that were pretreated with BEL followed by is-
chemia and reperfusion demonstrated a reduction in the
extent of hydrolysis of phosphatidylcholine molecular spe-
cies (Fig. 2). Statistically significant reductions in phos-
pholipid hydrolysis were observed in all choline glycer-
ophospholipid molecular species identified by ESI-MS (Fig.
1D and Table 1). The assignment of each molecular species
to each ion was confirmed by collision-activated dissociation
(CAD) tandem mass spectrometry of each precursor ion
(see below).

**Ischemia/reperfusion-induced alterations in the mass of nuclear ethanolamine glycerophospholipid molecular species**

Examination of nuclei isolated from control perfused
rat myocardium by ESI-MS in the negative-ion mode dem-
onstrated a preponderance of phosphatidylyethanolamine
molecular species predominantly containing either palmi-
tic or stearic acids at the sn-1 position and arachidonic
acid at the sn-2 position [peaks at m/z 738 (16:0–20:4 phospha-
dylyethanolamine), 742 (18:0–18:2 phosphatidylyethanol-
amine), 762 (16:0–22:6 phosphatidylyethanol-
amine), 764 (18:1–20:4 phosphatidylyethanolamine), 766
(18:0–20:4 phosphatidylyethanolamine), 790 (18:0–22:6 phosphatidylyethan-
olamine), and 794 (20:0–20:4 phosphatidylyetha-
nolamine)] (Table 2). Phosphatidylethanolamine molecular species constituted 67% of the total ethanolamine glycerophospholipid mass in nuclei isolated from control perfused rat myocardium. The balance of ethanolamine glycerophospholipid molecular species consisted of plasmeylenethanolamine molecular subspecies enriched with arachidonyl residues at the sn-2 position [peaks at m/z 722 (16:0–20:4 plasmeylenethanolamine), 748 (18:1–20:4 plasmeylenethanolamine), and 750 (18:0–20:4 plasmeylenethanolamine)] (Table 2).

Comparisons of ESI mass spectra in the negative-ion mode of extracts of phospholipids from nuclei isolated from control perfused rat myocardium and phospholipids from nuclei isolated from rat hearts that were rendered globally ischemic for 15 min again demonstrated that global ischemia did not significantly alter any of the ethanolamine glycerophospholipid molecular species (Table 2). Furthermore, 15 min of global ischemia did not result in a measurable change in total ethanolamine glycerophospholipid mass (Fig. 2). In contrast, comparisons of ESI mass spectra in the negative-ion mode of extracts of phospholipids from nuclei isolated from control perfused rat myocardium and phospholipids from nuclei isolated from rat hearts that were rendered globally ischemic for 15 min followed by 90 min of reperfusion demonstrated that reperfusion of ischemic myocardium induced the hydrolysis of approximately 50% of the total ethanolamine glycerophospholipid molecular species (Fig. 2). Statistically significant reductions were observed in all ethanolamine glycerophospholipid molecular species identified by ESI-MS with the exception of 18:1–20:4 plasmeylenethanolamine (m/z 748) (Table 2). Comparisons of ESI mass spectra in the negative-ion mode of extracts of phospholipids from nuclei isolated from control perfused rat myocardium and phospholipids from nuclei isolated from rat hearts that were pretreated with BEL followed by 15 min of ischemia and 90 min of reperfusion demonstrated a significant reduction in the extent of hydrolysis of only three of the ethanolamine glycerophospholipid molecular species (16:0–20:4, 18:0–20:4, and 18:0–22:6 phosphatidylethanolamine) (Table 2). The reduction of total ethanolamine glycerophospholipid hydrolysis was relatively small and not significant (Fig. 3). The assignment of each molecular species to each ion...
rather than the protonated species shown in Fig. 1, were
confirmed by CAD tandem mass spectrometry of each precursor ion (see below).

Confirmation of individual rat myocardial nuclear phospholipid molecular species, using ESI tandem mass spectrometry

The identification of individual molecular species of rat myocardial nuclear phospholipids was achieved by CAD tandem mass spectrometry (24). Selected examples of ESI tandem mass spectra of rat myocardial nuclei are shown in Fig. 3A and B. For these analyses, sodiated ions (M+23), rather than the protonated species shown in Fig. 1, were analyzed after the addition of 1 mM NaOH to the lipid extracts. Positive-ion tandem mass spectroscopy of sodiated phospholipid molecular species containing arachidonic acid (precursor ion scanning of m/z 489) confirmed the presence of arachidonic acid on precursor ions at m/z 788, 804, and 832 (Fig. 3A). Negative-ion tandem mass spectroscopy of deprotonated phospholipid molecular species containing arachidonic acid (precursor ion scanning of m/z 303) also confirmed the presence of arachidonic acid in precursor ions at m/z 722, 738, 748, 750, 764, 766, 778, and 794 (Fig. 3B).

In addition, positive-ion tandem mass spectra (precursor

<table>
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<tr>
<th>PC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>m/z</th>
<th>Control</th>
<th>15I</th>
<th>15I90R</th>
<th>BEL Treatment</th>
</tr>
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<tr>
<td>D16:0–16:0</td>
<td>734</td>
<td>2.50 ± 0.10</td>
<td>2.30 ± 0.10</td>
<td>1.52 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.11 ± 0.17&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>P16:0–18:1</td>
<td>744</td>
<td>0.50 ± 0.09</td>
<td>0.54 ± 0.02</td>
<td>0.31 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.44 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>D16:0–18:2</td>
<td>758</td>
<td>6.39 ± 0.64</td>
<td>6.31 ± 0.40</td>
<td>3.19 ± 0.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.03 ± 0.39&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>D16:0–18:1</td>
<td>760</td>
<td>5.09 ± 0.56</td>
<td>4.91 ± 0.31</td>
<td>2.02 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.57 ± 0.38&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>P16:0–20:4</td>
<td>766</td>
<td>0.96 ± 0.24</td>
<td>0.81 ± 0.05</td>
<td>0.48 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.78 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>P18:1–18:2</td>
<td>768</td>
<td>1.43 ± 0.30</td>
<td>1.22 ± 0.06</td>
<td>0.78 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.10 ± 0.15&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>D16:0–20:4</td>
<td>782</td>
<td>8.71 ± 1.12</td>
<td>8.15 ± 0.61</td>
<td>3.73 ± 0.18&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.05 ± 0.48&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>D18:1–18:2</td>
<td>784</td>
<td>3.45 ± 0.49</td>
<td>3.26 ± 0.23</td>
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<td>5.56 ± 0.40</td>
<td>2.75 ± 0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.34 ± 0.35&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>D18:0–18:1</td>
<td>788</td>
<td>2.15 ± 0.50</td>
<td>1.96 ± 0.17</td>
<td>1.03 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.50 ± 0.14&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>P18:1–20:4</td>
<td>792</td>
<td>0.82 ± 0.21</td>
<td>0.68 ± 0.04</td>
<td>0.42 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.66 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>D16:0–22:6</td>
<td>806</td>
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<td>2.01 ± 0.17</td>
<td>0.94 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>D18:1–20:4</td>
<td>808</td>
<td>2.78 ± 0.96</td>
<td>2.31 ± 0.25</td>
<td>1.04 ± 0.07</td>
<td>1.56 ± 0.11&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>9.95 ± 1.26</td>
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<td>5.45 ± 0.56&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>D18:0–22:6</td>
<td>834</td>
<td>1.35 ± 0.40</td>
<td>1.16 ± 0.10</td>
<td>0.55 ± 0.33&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.83 ± 0.06&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup>Nuclei were prepared from isolated and perfused hearts as described in Materials and Methods. Nuclear membrane phospholipids were extracted by the method of Bligh and Dyer (22) and directly analyzed by positive-ion ESI-MS. The results are expressed in pmol/μg nuclear protein and represent X ± SEM of six separate experiments. D (diacyl) and P (plasmenyl) indicate phosphatidylcholine and plasmenylcholine molecular species, respectively.

<sup>b</sup>Several minor species were also found that were of insufficient mass (<0.5% each) and have not been included in the table.

<sup>c</sup>An integer mass was used for all ions and the masses represent the protonated adduct ions.

<sup>d</sup>P < 0.05 for comparison between control perfused hearts and 15 min of global ischemia followed by 90 min of reperfusion (15I90R).

<sup>e</sup>P < 0.05 for comparison between 15 min of global ischemia followed by 90 min of reperfusion (15I90R) and BEL treatment.

was confirmed by CAD tandem mass spectrometry of each precursor ion (see below).
ion scan of m/z 465) of nuclear phospholipids from rat myocardium containing linoleic acid confirmed the presence of linoleoyl residues on precursor sodiated ions at m/z 780, 806, and 808 (Fig. 4A). Positive-ion ESI tandem mass spectrometry of the sodiated ion at m/z 806 displayed the loss of trimethylamine (loss of 59) at m/z 747 as well as the loss of the phosphocholine (loss of 183) at m/z 623 (Fig. 4B). Product ions at m/z 465 and 467 correspond to the neutral loss of the sn-1 oleic acid and the sn-2 linoleic acid, respectively, after the loss of trimethylamine. Positive-ion ESI tandem mass spectrometry of the sodiated ion at m/z 808 also displayed the loss of trimethylamine (m/z 749), loss of the phosphocholine (m/z 625), and ions at m/z 465 and 469 representing the neutral loss of the sn-1 stearic acid and sn-2 linoleic acid after the loss of trimethylamine (Fig. 4C).

**iPLA2 translocation to the nucleus during myocardial ischemia and reperfusion**

Because pretreatment of ischemic and reperfused rat hearts with the iPLA2-specific inhibitor BEL results in a reduction in the hydrolysis of nuclear phospholipids, and iPLA2 has been shown to be activated during myocardial ischemia (14, 15), the presence of iPLA2 in nuclear extracts from control, ischemic, and ischemic/reperfused heart was determined. Western blot analyses of nuclear protein isolated from control perfused, ischemic, and ischemic/reperfused hearts demonstrated that iPLA2 was present in both control and ischemia/reperfused hearts and that during ischemia iPLA2 was translocated to the nucleus within 5 min of global ischemia, which was reversible with reperfusion (Fig. 5A and C). Further increases in

**TABLE 2. Alterations in ethanolamine glycerophospholipid mass during ischemia and reperfusion conditions in rat myocardial nuclei**

<table>
<thead>
<tr>
<th>PE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>m/z&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Control</th>
<th>15I</th>
<th>15I90R</th>
<th>BEL Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16:0–20:4</td>
<td>722</td>
<td>2.11 ± 0.16</td>
<td>2.18 ± 0.25</td>
<td>0.91 ± 0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.19 ± 0.09</td>
</tr>
<tr>
<td>D16:0–20:4</td>
<td>738</td>
<td>0.78 ± 0.06</td>
<td>0.70 ± 0.05</td>
<td>0.42 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>D18:0–18:2</td>
<td>742</td>
<td>1.53 ± 0.24</td>
<td>1.38 ± 0.21</td>
<td>0.51 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>P18:1–20:4</td>
<td>748</td>
<td>1.91 ± 0.02</td>
<td>1.57 ± 0.04</td>
<td>1.67 ± 0.25</td>
<td>1.83 ± 0.11</td>
</tr>
<tr>
<td>D18:0–22:6</td>
<td>762</td>
<td>0.81 ± 0.05</td>
<td>0.69 ± 0.06</td>
<td>0.40 ± 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.60 ± 0.07&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>D18:1–20:4</td>
<td>764</td>
<td>0.90 ± 0.07</td>
<td>0.87 ± 0.05</td>
<td>0.42 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>D18:0–20:4</td>
<td>766</td>
<td>3.86 ± 0.37</td>
<td>3.50 ± 0.15</td>
<td>1.31 ± 0.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.87 ± 0.08&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>D18:0–22:6</td>
<td>790</td>
<td>2.03 ± 0.14</td>
<td>1.76 ± 0.01</td>
<td>1.02 ± 0.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.29 ± 0.05&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>D20:0–20:4</td>
<td>794</td>
<td>1.99 ± 0.37</td>
<td>1.71 ± 0.44</td>
<td>1.11 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.86 ± 0.07&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Nuclei were prepared from isolated and perfused hearts as described in Materials and Methods. Nuclear membrane phospholipids were extracted by the method of Bligh and Dyer (22) and directly analyzed by negative-ion ESI-MS. The results are expressed in pmol/μg nuclear protein and represent X ± SEM of six separate experiments. D (diacyl) and P (plasmenyl) indicate phosphatidylethanolamine and plasmenylethanolamine molecular species, respectively.

<sup>b</sup>Several minor species were also found that were of insufficient mass (<0.5% each) and have not been included in the table.

<sup>c</sup>An integer mass was used for all ions and the masses represent the deprotonated ions.

<sup>d</sup>P < 0.05 for comparison between control perfused hearts and 15 min of global ischemia followed by 90 min of reperfusion (15I90R).

<sup>e</sup>P < 0.05 for comparison between 15 min of global ischemia followed by 90 min of reperfusion (15I90R) and BEL treatment.

Fig. 3. CAD tandem mass spectra displaying phospholipid molecular species containing arachidonoyl residues. (A) Positive-ion spectrum (precursor ion scanning of m/z 489) of rat myocardial nuclear phospholipids displaying sodiated (M + 23) choline glycerophospholipid precursor ions containing arachidonic acid. (B) Negative-ion spectrum (precursor ion scanning of m/z 303) of rat myocardial nuclear phospholipids displaying ethanolamine glycerophospholipid precursor ions containing arachidonic acid.
nuclear iPLA₂ were observed with prolonged global ischemia (Fig. 5A and C). Western blot analysis of lamin B₁ was utilized as a control of constant nuclear protein loading (Fig. 5B). The recovery and purity of nuclei from individual hearts subjected to each experimental condition were similar as demonstrated by Western blot analyses of several nuclear and subcellular marker proteins (Fig. 6). The nuclear envelope protein lamin B₁ and the myocardial nuclear transcription factor GATA4 were both enriched in the nuclei fractions under control, ischemic, and ischemic and reperfused experimental conditions. It should be appreciated that the techniques used for the isolation of nuclei in this study have been reported to result in the isolation of nuclei derived from cardiac myocytes and not endothelial cells (25). The appearance of the myocyte (fibroblast)-specific nuclear marker, GATA-4 (26), in the nuclear preparations (Fig. 6) demonstrates that the nuclei isolated in the present study are predominantly derived from cardiac myocytes. Furthermore, there was a complete absence of contaminating subcellular markers of plasma membrane, sarcoplasmic reticulum (endoplasmic reticulum), and the myofibrillar apparatus in the purified nuclear fraction (Fig. 6). In contrast to iPLA₂, there was minimal or no translocation of either cPLA₂ or sPLA₂, respectively, to the nucleus during myocardial ischemia (Fig. 7A and B). In addition to the observed increase in iPLA₂ enzyme mass present in nuclei during myocardial ischemia, nuclear iPLA₂ was catalytically competent with measured enzyme activities of 8.41 ± 0.13 and 12.63 ± 0.04 pmol/min·mg nuclear protein in control and 15-min ischemic hearts, respectively. Nuclear phospholipase A₂ activity was calcium independent and totally ablated by the addition of the iPLA₂ inhibitor, BEL, to the assays (e.g., enzyme activity in BEL-treated hearts was 0.47 ± 0.01; 0.51 ± 0.01 pmol/min·mg nuclear protein for control perfused and 15-min ischemic hearts, respectively). Concomitant with the observed translocation of iPLA₂ to the nucleus during myocardial ischemia was a corresponding decrease in the presence of cytosolic iPLA₂ (Fig. 8). Similarly, the decrease in cytosolic iPLA₂ during myocardial ischemia was reversed with reperfusion of the ischemic myocardium.

**DISCUSSION**

The present results have characterized through electrospray ionization mass spectroscopic measurements the phospholipid molecular species present in the nuclei of cardiac cells in isolated perfused rat hearts. Mass-mass analysis of individual ions in these electrospray ionization mass spectroscopic analyses confirmed the assignment of molecular species to each ion measured. In the choline glycerophospholipid pools the primary sn-1 aliphatic chains were composed of palmitate and stearate residues while at the sn-2 position oleic and linoleic acid prevailed. In the ethanolamine glycerophospholipid pool the primary sn-1 aliphatic chains again were composed of palmitate and stearate residues while at the sn-2 position arachidonic acid was the major aliphatic group. An unusual
molecular species (1-eicosanoyl-2-eicosatetra-5,8,9,11,14-pentenoyl-sn-glycero-3-phosphoethanolamine) was observed that accounted for 11% of the total ethanolamine glycerophospholipid pool and again was confirmed by mass-mass analyses.

Although membrane-associated iPLA₂ activity is increased in ischemic myocardium, the translocation of this phospholipase to, as well as accelerated phospholipolysis within, specific subcellular membrane pools of the heart during myocardial ischemia have not been forthcoming.

Fig. 5. Translocation of iPLA₂ to the nucleus during myocardial ischemia. Nuclei were prepared from ventricular tissue of isolated adult rat hearts subjected to either control perfusion, 5 min of global ischemia, 15 min of global ischemia, 30 min of global ischemia, 60 min of global ischemia, 5 min of global ischemia followed by 10 min of reperfusion, 15 min of global ischemia followed by 10 min of reperfusion, 15 min of global ischemia followed by 30 min of reperfusion, or 15 min of global ischemia followed by 90 min of reperfusion as described in Materials and Methods. Nuclear proteins were subjected to SDS-PAGE and Western blot analyses using either anti-iPLA₂ [Genetics Institute; rabbit, 10 μg/ml; (A)] or anti-lamin B₁ [Zymed; mouse, 5 μl/10 ml; (B)] as the primary antibody. Denatured iPLA₂ (E. coli inclusion body) was used as a positive control in (A). The intensity of each band from multiple analyses with anti-iPLA₂ was quantitated with NIH Image software and expressed as a percentage of the intensity of the control sample (C). Values represent the means ± standard deviations from three individual independent experiments. *P < 0.05 for comparison between control perfused hearts and ischemic conditions. †P < 0.05 for comparison between 15 min of ischemia and 15 min of ischemia followed by reperfusion protocols.

Fig. 6. Subcellular marker analysis of nuclei prepared from isolated and perfused rat hearts. Cytosol, particulate, and nuclei were prepared from isolated perfused adult rat hearts that were either control perfused (C), subjected to 15 min of global ischemia (I), or subjected to 15 min of global ischemia followed by 90 min of reperfusion (I/R) as described in Materials and Methods. Cytosolic, particulate, and nuclear proteins were subjected to SDS-PAGE and Western blot analysis using either anti-lamin B₁ (Zymed; mouse, 5 μl/10 ml), anti-Na⁺/K⁺ ATPase β (Upstate Biotechnology; mouse, 9 μl/10 ml), anti-SERCA2 ATPase (Affinity Bioreagents; mouse, 5 μl/10 ml), anti-TnI (Chemicon; mouse, 5 μl/20 ml), or anti-GATA4 (Santa Cruz; mouse, 27 μl/10 ml) as the primary antibodies as described in Materials and Methods.
The present study now demonstrates that catalytically competent iPLA2 is translocated to the nucleus during myocardial ischemia. During ischemia and the beginning of reperfusion, significant levels of iPLA2 are present in the nuclei. Furthermore, iPLA2 remains detectable in the nucleus after 90 min of reperfusion. The localization of iPLA2 to the nucleus is accompanied by accelerated nuclear phospholipid catabolism during reperfusion of ischemic myocardium, which is partially mediated by iPLA2 based on the inhibition of phospholipolysis by the iPLA2 inhibitor BEL. It is likely that the remaining iPLA2 in the nucleus during reperfusion mediates the BEL-sensitive component of phospholipid degradation. In addition, it should be noted that iPLA2 is activated by ATP (27, 28) and it is likely that the recovery of cellular ATP levels to near normal levels during reperfusion (29) contributes significantly to the hydrolysis of phospholipid during reperfusion mediated by iPLA2. In contrast, it is possible that the absence of nuclear phospholipid degradation during ischemia despite the appearance of iPLA2 in the nucleus is due to depressed levels of ATP during ischemia (29). Another component of the decrease in nuclear phospholipid content after ischemia/reperfusion may also result from a decrease in phospholipid biosynthesis or lysophospholipid reacylation under these conditions as well as the activity of other phospholipases (e.g., cPLA2).

Similar to measurements of whole rat heart phospholipid composition (30–32), rat heart nuclear choline glycerophospholipid pools were not enriched with plasmalogen molecular species. However, the rat heart nuclear ethanolamine glycerophospholipid pool contained substantial amounts of plasmalogen molecular species. After reperfusion of ischemic hearts, there is a BEL-sensitive component to both diacyl and plasmalogen molecular species loss in both the ethanolamine and choline glycerophospholipid pools. The lack of specificity for phospholipid molecular species degradation suggests that, in intact myocardium, iPLA2 is promiscuous as compared with that described in in vitro assay systems where iPLA2 has been shown to prefer plasmalogen and diacyl species containing arachidonic acid (33, 34). It should be noted, however, that these previously reported studies (33, 34) did report iPLA2 activity with other molecular species and other studies have demonstrated that brain iPLA2 utilizes phospholipid molecular species containing linoleic, palmitic, and oleic acid residues at the sn-2 position (35). Taken together, as suggested by several in vitro assay systems of iPLA2 activity, the present study utilizing intact tissue suggests that iPLA2 is promiscuous in the ischemic/
reper fus ed heart. Furthermore, although BEL only statistically altered the loss of diacyl ethanol amine gly cerophos pholip id molecular species and not plasm alogen loss, it should be appreciated that this enzyme can readily hydro lyze arachi donylated di acyl molecular species.

Although PLD and cPLA2 have previously been shown to be translocated to the nuclei of stimulated cells (8, 9), the present studies are the first to demonstrate that iPLA2 is translocated to the nucleus in response to a pathophysiological stimulus (i.e., myocardial ischemia). In addition, the present results demonstrate that cPLA2 is present in the myocytic nuclei but does not translocate to the nucleus with the same magnitude as iPLA2 during myocardial ischemia. Although iPLA2 possesses no nuclear localization sequence, it is possible that its translocation is mediated via its cotranslocation with protein complexes possessing proteins with nuclear localization sequences that occurs during myocardial ischemia. It is possible that the nuclear translocation of iPLA2 during myocardial ischemia is mediated through its association with phospho¬fructokinase (PFK) because iPLA2 has been shown to exist in a tight complex with a specific 85-kDa isof orm of PFK and PFK has been shown to translocate to the membrane fraction during myocardial ischemia (36, 37). In addition, a human isof orm of iPLA2 has been shown to have a consensus motif that is shared with the proline-rich middle linker domains of the Smad protein DAF-3 (38) from Caenorhabditis elegans and the mammalian protein Smad4 (39). The Smad proteins are products of tumor suppressor genes, which form hetero-oligomers with signaling proteins through the proline-rich middle linker domain (39). One intriguing possibility for iPLA2 translocation to the nucleus during ischemia would involve its association with proteins that may bind to the Smad domain. Alternatively during ischemia, iPLA2 may undergo a conformational change that results in surface amino acids providing a contact nuclear localization sequence site.

It is likely that nuclear membrane phospholipids are targets of phospholipases resulting in the generation of nuclear signaling messengers. In the present study, we have exploited the utility of electrospray ionization mass spectrometry to determine the phospholipid content of nuclei isolated from the cardiac myocytes of isolated perfused hearts and have revealed a 50% loss of myocytic nuclear choline and ethanolamine glycerophospholipids in hearts subjected to ischemia followed by reperfusion. The loss of nuclear choline and ethanolamine glycerophospholipids during reperfusion of ischemic myocardium is mediated, at least in part, by iPLA2. The role of accelerated nuclear membrane phospholipid hydrolysis in the ischemic/reper¬fused heart remains to be resolved. However, these changes in myocytic nuclear membrane phospholipid content during ischemia and reperfusion likely lead to the production of lipidic second messengers that may have profound effects on nuclear signaling that potentially lead to changes in the genetic programming of the postischemic heart.

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