# Inhibition of mitochondrial calcium-independent phospholipase $A_2$ ($iPLA_2$) attenuates mitochondrial phospholipid loss and is cardioprotective

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Calcium-independent phospholipase $A_2$ ($iPLA_2$) is the predominant phospholipase $A_2$ present in myocardium, and its pathophysiological role in acute myocardial infarction has been suggested by the rapid increase in membrane-associated $iPLA_2$ activity during myocardial ischaemia and reperfusion (I/R). We therefore examined $iPLA_2$ in mitochondrial fractions prepared from Langendorff-perfused adult rabbit hearts. Our studies indicate that $iPLA_2\beta$ is present in rabbit heart mitochondrial inner membranes with no apparent translocation during ischaemia, I/R or preconditioning. Mitochondrion-associated $iPLA_2$ was catalytically competent and exhibited 2-, 3- and 2.5-fold increases in measured $iPLA_2$ activity following ischaemia, I/R and preconditioning, respectively, when compared with the activity of $iPLA_2$ measured in mitochondria from control hearts. Mitochondrial phospholipids are essential for maintaining the ordered structure and function of the organelle. I/R resulted in a rapid overall decrease in phosphatidylcholine and phosphatidylethanolamine glycerophospholipid species, as determined by electrospray ionization MS, that was partially alleviated by pretreatment of hearts with the $iPLA_2$-specific inhibitor, bromoenol lactone (BEL). Pretreatment of I/R hearts with 10 $\mu$M BEL significantly reduced the infarct size almost to that of continuously perfused hearts and was cardioprotective only when administered prior to ischaemia. Cardioprotection by BEL was reversed by the simultaneous perfusion of 100 $\mu$M 5-hydroxydecanoate, implicating the mitochondrial $K_{ATP}$ channel in BEL-mediated protection from I/R. Preconditioning also significantly reduced the infarct size in response to I/R but protection was lost by concurrent perfusion of 10 $\mu$M arachidonic acid. Taken together, these data strongly implicate mitochondria-associated $iPLA_2$ in the signal transduction of myocardial I/R injury.

Key words: bromoenol lactone, cardioprotection, mitochondria, myocardial ischaemia.

## INTRODUCTION

Phospholipase $A_2$ activity has been identified in the heart and implicated as a mediator of ischaemia and reperfusion (I/R) injury [1–4]. Calcium-independent phospholipase $A_2$ ($iPLA_2$) is the predominant phospholipase $A_2$ activity present in myocardium, and a role in acute myocardial infarction has been demonstrated by the rapid increase in membrane-associated $iPLA_2$ activity [2], the nuclear translocation of catalytically competent $iPLA_2$ [5] and bromoenol lactone (BEL)-sensitive accelerated nuclear-membrane phospholipid catabolism during myocardial I/R [5]. The myocardium contains two distinct forms of $iPLA_2$, $iPLA_2\beta$ and $iPLA_2\gamma$ [6,7]. The $\beta$-form exists as multiple isoforms of $\approx$ 80 kDa which possess different specific activities [6], whereas the $\gamma$-form was determined to have apparent molecular masses of 77 and 63 kDa [7]. Both of these forms are calcium independent and inhibited by BEL [6,7]. The $\approx$ 80 kDa phospholipase ($iPLA2\beta$) exists as a multimeric complex of $\approx$ 300 kDa, contains eight ankyrin motifs [8], and is selective for arachidonylated plasmalogen substrates [2,9]. Plasmalogens are the predominant subclass of the choline and ethanolamine glycerophospholipid pools present in myocardium and possess a vinyl-ether bond between their *sn*-1 aliphatic group and glycerol backbone [10]. Accelerated plasmalogen catabolism and lysophospholipid accumulation accompany myocardial ischaemia and are probably mediated by the activation of one or more phospholipases [2,9,11].

The death of cardiomyocytes has been implicated in both animal models of myocardial ischaemia and human patients with acute myocardial infarction [12–15]. The induction of cell death by myocardial I/R injury facilitates the loss of a significant proportion of cardiomyocytes and contributes to mortality. Although it has become increasingly clear that myocardial ischaemia and prolonged reperfusion result in the death of myocytes through apoptosis or necrosis, the molecular basis of this process remains to be defined [12,16]. Several studies have suggested the involvement of phospholipase $A_2$, arachidonic acid release and eicosanoid production in the induction of cell death [17–19]. The cytosolic phospholipase $A_2$ ($cPLA_2$) has been implicated in arachidonic acid release during tumour necrosis factor $\alpha$-induced apoptosis [20–22] and in hydroperoxide-induced cytotoxicity [23].

Mitochondrial dysfunction from I/R leads to myocardial injury, and the immediate recovery of mitochondrial function is imperative for myocardial contractility and survival [24]. The effects of I/R on mitochondrial function include a transient increase followed by a steady decrease in oxidative phosphorylation, the loss of pyridine nucleotides, impaired respiration through complex I, increased superoxide production, opening of the permeability transition pore, loss of calcium homoeostasis and the release of cytochrome *c* [24–28]. Mitochondrial phospholipids are essential for maintaining the ordered structure of the organelle and are necessary for the proper localization and function of key mitochondrial enzymes. The myocardial mito-

Abbreviations used: $iPLA_2$, calcium-independent phospholipase $A_2$; $cPLA_2$, cytosolic phospholipase $A_2$; BEL, bromoenol lactone; ESI-MS, electrospray ionization MS; I/R, ischaemia and reperfusion; $AACOCF_3$, arachidonyltrifluoromethyl ketone; BPE, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine; TTC, 2,3,5-triphenyltetrazolium chloride; 5-HD, 5-hydroxydecanoic acid; EF, elongation factor.

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chondrial membrane phospholipid pool has not been characterized thoroughly. Previous studies have indicated that ischaemia and I/R reduce the amount of total mitochondrial phospholipids [29]. Alterations have been reported in the mitochondrial content of phosphatidylcholine and phosphatidylethanolamine, with the most pronounced changes occurring in the cardiolipin content [29–31].

The evaluation of rat myocardial nuclear-membrane phospholipids in response to I/R was recently accomplished by utilizing electrospray ionization MS (ESI-MS) [5]. These studies reported a loss of nuclear choline and ethanolamine glycerophospholipids after I/R that was mediated by $iPLA_2$, which translocated to the nucleus [5]. In the present study, we used ESI-MS to investigate the dynamic alterations in mitochondrial phospholipids that occur during myocardial I/R.

Preconditioning of ischaemic myocardium is a phenomenon in which a series of brief periods of ischaemia followed by reperfusion confers protection to the heart against subsequent extended periods of ischaemia [32]. The mitochondrial $K_{ATP}$ channel has been proposed to mediate the effects of ischaemic preconditioning [33]. The pharmacological opening of the mitochondrial $K_{ATP}$ channel through the use of diazoxide before ischaemia has been shown to significantly reduce the extent of the resulting infarction [34,35]. Additionally, phospholipase $A_2$ activity and the release of arachidonic acid have been implicated as antagonists of mitochondrial $K_{ATP}$ channel activity [36–38]. Accordingly, we used the mitochondrial $K_{ATP}$ channel antagonist, 5-hydroxydecanoic acid (5-HD), in studies of BEL-sensitive cardioprotection during I/R. The involvement of $iPLA_2$ and arachidonic acid release in preconditioning through opening of the mitochondrial $K_{ATP}$ channel was also examined.

Since membrane-associated $iPLA_2$ activity increases in ischaemic hearts [2] we investigated the role of $iPLA_2$ in the signal transduction of myocardial I/R injury. We report that catalytically competent $iPLA_2$ is localized to the outer face of the inner mitochondrial membrane and contributes to a decrease in mitochondrial phospholipid content during I/R. Additionally, our results indicate that inhibition of $iPLA_2$ by BEL is cardioprotective, as reflected by a reduction in infarct size after global I/R. We concluded that $iPLA_2$ activity is detrimental to the heart through the release of arachidonic acid and closure of the mitochondrial $K_{ATP}$ channels. Therefore, the specific inhibition of myocardial $iPLA_2$ may be a point of intervention to prevent post-ischaemic myocardial tissue loss.

## MATERIALS AND METHODS

### Materials

New Zealand White rabbits (< 2.5 kg, male) were obtained from Western Oregon Animal (Philomath, OR, U.S.A.). Anti-$iPLA_2$ and anti-Hsp60 were purchased from Cayman Chemicals (Ann Arbor, MI, U.S.A.) and Stressgen (Victoria, BC, Canada), respectively. Anti-EF-Tu/Ts antibody (where EF corresponds to elongation factor; raised against the complex of EF-Tu–EF-Ts, recognizes EF-Tu in inner membrane and matrix and EF-Ts in matrix; we used detection of EF-Ts as a marker of matrix) and anti-Rieske FeS antibody (raised against the Rieske iron–sulphur protein of the inner mitochondrial membrane) were gifts from Dr Linda Spremulli (University of North Carolina, Chapel Hill, NC, U.S.A.) and Dr Akemi Matsuno-Yagi (The Scripps Research Institute, La Jolla, CA, U.S.A.), respectively. Goat anti-rabbit IgG and goat anti-mouse IgG antibodies (both alkaline phosphatase-conjugated) were purchased from Caltag Laboratories (Burlingame, CA, U.S.A.). Electrophoresis-grade reagents for PAGE and prepackaged gels were purchased from Sigma (St Louis, MO, U.S.A.) and Invitrogen (Carlsbad, CA, U.S.A.), respectively. BEL and arachidonyltrifluoromethyl ketone ($AACOCF_3$) were purchased from Calbiochem (La Jolla, CA, U.S.A.). The $iPLA_2$ substrate, 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine (BPE), was purchased from Molecular Probes (Eugene, OR, U.S.A.). Silica gel 60 TLC plates were obtained from Whatman (Clifton, NJ, U.S.A.). Dimyristoylphosphatidylcholine and dimyristoylphosphatidylethanolamine were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.) and arachidonic acid was purchased from Sigma. 2,3,5-Triphenyltetrazolium chloride (TTC) and 5-HD were purchased from Sigma. All other reagents were of the highest grade available and were purchased from Sigma or Fisher (Pittsburgh, PA, U.S.A.).

### Langendorff perfusion of rabbit hearts subjected to myocardial I/R and infarct size studies

Rabbits were utilized for the preparation of Langendorff-perfused hearts as described previously [39]. The experiments were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and all experimental procedures were approved by the Scripps Research Institute Animal Care and Use Committee. In brief, rabbits were anaesthetized with ketamine and xylazine (45 and 4 mg/kg, respectively; subcutaneously) followed by heparin (1000 units; intravenously), before being killed with pentobarbital sodium (100 mg; intravenously). Hearts were removed rapidly and perfused in a retrograde fashion through the aorta with a modified Krebs–Ringer buffer (118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18 mM $KH_2PO_4$, 1.18 mM $MgSO_4$, 25 mM $NaHCO_3$ and 11.1 mM glucose; equilibrated with 95 % $O_2$/5 % $CO_2$, pH 7.35) at 37 °C. Following an initial equilibration time of 15 min, hearts were subjected to a non-infarcted control perfusion for 30, 90 or 120 min, 30 min of global zero-flow ischaemia, or 30 or 45 min of global zero-flow ischaemia followed by reperfusion for 120 min. In selected experiments, either 10 $\mu$M BEL or 10 $\mu$M $AACOCF_3$ was included in the perfusion buffer for 15 min following the initial equilibration period and before global ischaemia. Arachidonic acid (10 $\mu$M) or 100 $\mu$M 5-HD was perfused either during preconditioning or simultaneously with BEL treatment, respectively. Ischaemic preconditioning was induced by three cycles of 5 min of global ischaemia and 5 min of reperfusion immediately preceding the regular I/R protocols.

After 120 min of reperfusion, infarct size was determined as described previously [40,41]. In brief, the heart was frozen, cut into rings of 2 mm thickness, and incubated in 1 % TTC in PBS (pH 7.0) at 37 °C for 20 min. Following the TTC reaction, tissue slices were fixed with 3.7 % formaldehyde in PBS for 20 min and washed twice with PBS. Heart slices were scanned, and the images converted into TIFF files and analysed in Adobe PhotoShop 5.5. The images were manipulated digitally in an identical manner to obtain histograms containing only red and white, corresponding to non-infarcted and infarcted regions, respectively. The infarct size was calculated as 'white' counts divided by the sum of 'red' plus 'white' counts.

### Preparation of myocardial mitochondria and cytosol

Mitochondria were prepared as described previously [42] and all procedures were performed at 4 °C. Ventricles were minced in 20 ml of MSE buffer (225 mM mannitol, 75 mM sucrose, 1 mM

EGTA and 20 mM Hepes/KOH, pH 7.4) and homogenized further by a Polytron homogenizer for 5 s at maximum power. The homogenate was centrifuged for 10 min at 600 $g_{max}$, nuclear and cytoskeletal fractions were discarded, and the centrifugation was repeated. The supernatant was centrifuged for 10 min at 10000 $g_{max}$ to pellet mitochondria. The supernatant (crude cytosol) was centrifuged for a further 30 min at 100000 $g_{max}$ to obtain cytosol. The mitochondrial pellet was washed twice in 10 ml of MSE buffer and the final pellet was subjected to a hybrid Percoll/metrizamide discontinuous gradient purification consisting of 6% Percoll and 17% and 35% metrizamide, prepared in 250 mM sucrose [42]. The pellet was washed twice in MSE buffer and resuspended in MSE buffer for $iPLA_2$ assays or Western-blot analysis. For ESI-MS analyses, mitochondria were resuspended in PBS and extracted immediately (see below). For experiments involving digitonin and limited proteinase K digestion, mitochondria were incubated with 50 $\mu$g/ml proteinase K, treated with 0.1% digitonin, or treated with proteinase K and digitonin in the presence or absence of 1 mM PMSF for 30 min at 4 °C.

### Suborganellar fractionation of mitochondria

Isolated and purified mitochondria were fractionated by a modification of Comte and Gautheron [43]. In brief, purified mitochondria were subjected to hypotonic swelling by incubation in 10 mM $KH_2PO_4$, pH 7.4, for 20 min at 4 °C. The mitochondria were centrifuged for 15 min at 10000 $g_{max}$ to pellet mitoplasts (inner membrane and matrix). The supernatant consisting of the outer membrane and contents of the intermembrane space was centrifuged for 30 min at 100000 $g_{max}$ to separate the outer membrane (pellet) and intermembrane-space contents (supernatant). The mitoplast pellet was resuspended in MC buffer (300 mM sucrose, 1 mM EGTA and 20 mM Mops, pH 7.4) and sonicated on ice for 5 cycles of 20 s bursts with 30 s rest intervals at 8–10 W. The sonicated mitoplasts were centrifuged for 10 min at 10000 $g_{max}$ to clear any remaining intact mitoplasts and the supernatant was centrifuged for 30 min at 100000 $g_{max}$ to separate the inner membrane (pellet) and matrix (supernatant). For experiments involving the alkali treatment of inner membranes, inner membranes were incubated with 0.1 mM $Na_2CO_3$, pH 11.5, for 30 min at 4 °C followed by centrifugation at 100000 $g_{max}$ for 30 min.

### Western-blot analysis of $iPLA_2$

Mitochondrial proteins were quantified by the Coomassie Blue binding assay (Pierce Chemical Co.) with BSA standards, adjusted subsequently to equal protein concentrations and solubilized in SDS sample buffer prior to Western-blot analysis. Anti-$iPLA_2$ antibody was used at a 1:1000 dilution followed by alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG, 1:7000 dilution). Anti-Hsp60, anti-Rieske FeS and anti-EF-Tu/Ts were used at 1:1000 dilutions followed by alkaline phosphatase-conjugated secondary antibodies. For anti-Hsp60 and anti-Rieske FeS, goat anti-rabbit IgG was used at a dilution of 1:7000; for anti-EF-Tu/Ts, goat anti-mouse IgG was used (1:7000 dilution).

### $iPLA_2$ assay and ESI-MS of mitochondrial membrane phospholipids

The $iPLA_2$ activity in 50 $\mu$g of mitochondria was measured *in vitro* as described previously by measuring the release of BODIPY®-$C_{12}$ (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) from BPE [5,44]. TLC plates were photographed under UV light using Polaroid film and quantified by densitometry. Fluorescence in a single band was quantified as a percentage of total fluorescence observed for each reaction (an entire lane of the TLC plate) to correct for any variations in sample application. This value was then converted into pmol/min per mg of mitochondrial protein. BEL was included at a final concentration of 10 $\mu$M in selected assays to confirm $iPLA_2$ activity. Lipids from isolated mitochondria were extracted by the method of Bligh and Dyer [45] in the presence of internal standards.

Chloroform extracts from 5 mg of mitochondrial protein were used for analysis of individual phospholipid molecular species utilizing a Hewlett Packard 1100 MSD single-quadrupole mass spectrometer as described previously [5,46]. Individual molecular species were quantified by comparisons of the individual ion peak intensities with that of either dimyristoylphosphatidylcholine or dimyristoylphosphatidylethanolamine after correction for $^{13}C$ isotope effects. The assignment of individual molecular species was as described previously [5].

### Statistical analyses

Data are presented as means ± S.E.M. For comparisons between two groups, ANOVA was performed. A $P$ value of less than 0.05 was considered significant.

## RESULTS

### Submitochondrial localization of $iPLA_2$ during myocardial I/R

We investigated $iPLA_2$ in mitochondria from control, ischaemic, I/R and preconditioned rabbit hearts. Western-blot analyses of mitochondria demonstrated that $iPLA_2\beta$ was present in mitochondria from control perfused hearts with no apparent translocation to, or from, the mitochondria in response to ischaemia, I/R or preconditioning (Figure 1A). A small amount of $iPLA_2\beta$ was detected in cytosolic fractions from ischaemic, I/R and preconditioned hearts and may have increased following I/R and preconditioning (Figure 1A). This is possibly due to mitochondrial leakage during processing of the mitochondria; however, $iPLA_2$ activity has been demonstrated in the cytosolic compartment [5,8,47]. Additionally, another dominant band of lower molecular mass is clearly detected in the cytosolic fraction and may represent a different isoform of $iPLA_2$. In contrast with $iPLA_2$, neither $cPLA_2$ nor secreted $PLA_2$ was detected in mitochondrial fractions from any hearts (results not shown). The mitochondrial localization of $iPLA_2$ was confirmed visually by immunohistochemical analysis in rat neonatal cardiac myocytes by the co-localization of $iPLA_2$ with Mitotracker Red (results not shown).

The subcellular localization of $iPLA_2$ to the mitochondria was determined further to be localized specifically to the inner mitochondrial membrane. Metrizamide-purified mitochondria from control perfused rabbit hearts were osmotically shocked to yield mitoplasts (inner membrane and matrix) and the combination of the outer membrane and intermembrane space. Western-blot analysis detected the absence of $iPLA_2$ in the outer membranes and intermembrane space (Figure 1B). In contrast, $iPLA_2$ was abundant in mitoplasts (Figure 1B). Further fractionation of mitoplasts into inner membranes and matrix indicated that $iPLA_2$ was restricted to the inner membranes (Figure 1B).

The isolation and purification of the submitochondrial fractions was demonstrated by Western-blot analyses of several mitochondrial marker proteins (Figure 1C). The purity of the mitoplast (inner membrane + matrix) was confirmed by the

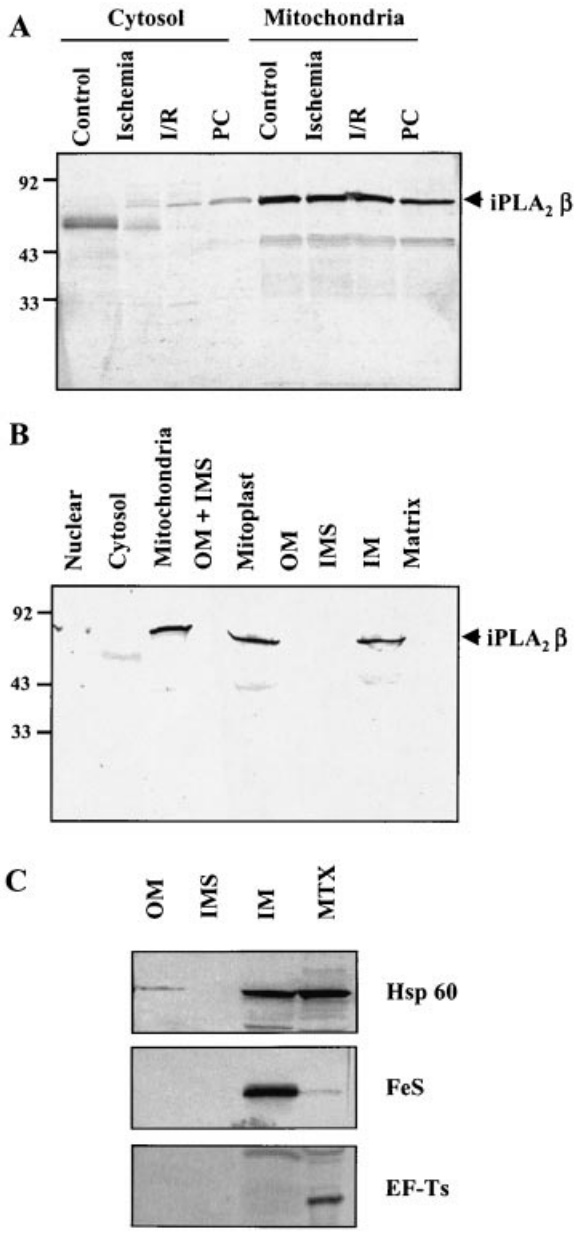


**Figure 1 Submitochondrial localization of iPLA$_2$ prepared from isolated perfused rabbit hearts**

(**A**) Mitochondria and cytosolic fractions were prepared and subjected to Western-blot analysis as described in the Materials and methods section. PC, preconditioning. (**B**) Submitochondrial fractions were prepared and subjected to Western-blot analysis as described in the Materials and methods section. The iPLA$_2$ was detected with anti-iPLA$_2$ antibody and visualized by anti-rabbit antibody conjugated to alkaline phosphatase, as described in the Materials and methods section. (**C**) Submitochondrial marker analysis of mitochondrial fractions prepared from isolated an perfused rabbit hearts. Outer membrane (OM), intermembrane space (IMS), inner membrane (IM) and matrix (MTX) were prepared from isolated perfused adult rabbit hearts that were control perfused as described in the Materials and methods section. Submitochondrial proteins were subjected to SDS/PAGE and Western-blot analysis as described in the Materials and methods section.

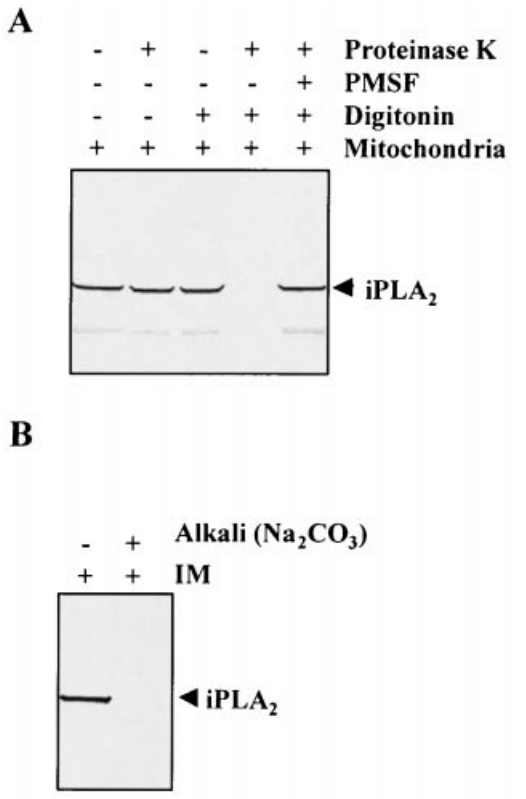


**Figure 2 Western-blot analysis of mitochondria isolated from control perfused rabbit heart**

Mitochondria were prepared from control perfused rabbits hearts and either not treated (control), treated with 50 $\mu$g/ml proteinase K, 0.1 % digitonin or both in the presence or absence of PMSF for 30 min at 4 °C (**A**). Inner mitochondrial (IM) membranes were prepared as described in the Materials and methods section and incubated with an equal volume of 0.1 M $Na_2CO_3$, pH 11.5, for 30 min at 4 °C (**B**). Mitochondria and inner membranes were subjected to Western-blot analysis with anti-iPLA$_2$ and visualized by alkaline phosphatase-conjugated secondary antibody.

presence of Hsp60 in both the inner membrane and matrix fractions (Figure 1C). Hsp60 is predominantly a matrix protein, but does associate with the inner mitochondrial membrane. To demonstrate the purity of the inner membrane and matrix submitochondrial fractions further, the Rieske FeS protein, an inner mitochondrial membrane protein, was demonstrated to be enriched only in the inner membrane fraction (Figure 1C). Additionally, the soluble subunit of the mitochondrial elongation factor (EF-Ts), which is specifically a mitochondrial matrix protein, was enriched in the matrix fraction (Figure 1C).

### Catalytically competent iPLA$_2$ is localized to the outer face of the inner mitochondrial membrane

Confirmation of the localization of iPLA$_2$ to the inner mitochondrial membrane was obtained in experiments utilizing digitonin and limited proteinase K digestion. Western-blot analysis of mitochondria isolated from control perfused rabbit heart treated with 50 $\mu$g/ml proteinase K did not result in the proteolysis of iPLA$_2$ (Figure 2A), confirming that iPLA$_2$ was not exposed on the outer mitochondrial membrane and verifying that the isolated mitochondria had intact outer membranes. Permeabilization of mitochondria with 0.1 % digitonin followed by proteinase K digestion led to proteolysis of iPLA$_2$ (Figure 2A), indicating that iPLA$_2$ was exposed to the intermembrane space. Since iPLA$_2$ localized with mitoplasts, and specifically with the inner mitochondrial membrane, we conclude that iPLA$_2$ is located on the outer face of the inner mitochondrial membrane. This assessment

**Table 1 Mitochondrially associated iPLA$_2$ activity from isolated and perfused rabbit hearts**

Activity data are expressed as pmol/min per mg of mitochondrial protein and represent means ± S.E.M. from three separate experiments.

| | iPLA$_2$ activity | |
|---|---|---|
| Condition | Without BEL | With BEL |
| Control | 195.2 ± 27.2 | 85.6 ± 43.2* |
| Ischaemia | 360.5 ± 40.9† | 85.2 ± 21.2* |
| I/R | 627.5 ± 52.9† | 183.5 ± 29.7* |
| Preconditioning | 521.6 ± 96.9† | 76.6 ± 10.2* |

* $P < 0.01$ for all comparisons between conditions with and without BEL.
† $P < 0.05$ for comparisons between the control and all other conditions.

was confirmed by treatment of mitochondrial inner membranes with 0.1 M sodium carbonate, pH 11.5. Alkali treatment of mitochondrial inner membranes completely dissociated iPLA$_2$ from the inner membranes (Figure 2B), revealing that iPLA$_2$ is a peripheral membrane protein and not integrated into the lipid bilayer.

In addition to the submitochondrial localization of iPLA$_2$, mitochondrial iPLA$_2$ was found to be catalytically competent (Table 1). Myocardial ischaemia and I/R resulted in 2- and 3-fold increases in measured mitochondrial iPLA$_2$ activity, respectively. Additionally, preconditioning did not significantly reduce mitochondrially associated iPLA$_2$ activity (Table 1). Mitochondrial iPLA$_2$ activity was calcium-independent and was inhibited totally by the addition of the iPLA$_2$ inhibitor, BEL, to the assays (Table 1). The difficulties inherent in assaying endogenous membrane-associated phospholipase activity with exogenous labelled phospholipids should be appreciated. Problems such as the appropriate substrate access, interfacial kinetics and surface dilution complicate the interpretation of the results. Therefore, the specific activity of iPLA$_2$ measured in intact mitochondria may not be an accurate representation of the endogenous activity of the enzyme during the indicated experimental conditions.

## Alterations in the mass of mitochondrial choline glycerophospholipid molecular species induced by myocardial I/R

Analyses of mitochondria isolated from control perfused rabbit hearts by ESI-MS in the positive-ion mode demonstrated the presence of phosphatidylcholine and plasmenylcholine molecular species (Table 2). Phosphatidylcholine molecular species constituted 42 % of the total choline glycerophospholipid mass in mitochondria isolated from control perfused rabbit hearts (Table 2). Plasmalogen molecular species were in the majority, constituting 58 % of the choline glycerophospholipids (Table 2). The predominant fatty acid in the *sn*-1 position of choline glycerophospholipids was palmitic acid, with stearic and oleic acids comprising the remaining fatty acids detected. The fatty acids in the *sn*-2 position were equally distributed between oleic, linoleic and arachidonic acids (Table 2).

Comparisons of positive-ion ESI mass spectra of mitochondrial phospholipids from hearts subjected to control and I/R conditions demonstrated that I/R induced the hydrolysis of 47 % of the total choline glycerophospholipid molecular species (Figure 3). Only two choline glycerophospholipid molecular species identified by ESI-MS, 16 : 0–18 : 2 and 18 : 0–18 : 1 phosphatidylcholine, did not decrease significantly in mass (Table 2). Positive-ion ESI mass spectra of mitochondrial phospholipids from preconditioned or BEL-treated hearts followed by I/R revealed significant reductions in the extent of choline glycerophospholipid hydrolysis when compared with choline glycerophospholipid hydrolysis from I/R only (Figure 3 and Table 2). The reduction of hydrolysis was greater for preconditioned hearts than the reduction of hydrolysis for BEL-treated hearts. Preconditioning hearts before I/R resulted in the hydrolysis of 23 % of the total choline glycerophospholipid molecular species, which represented a reduction in hydrolysis of ≈ 50 % when compared with I/R (Figure 3). BEL treatment of hearts before I/R resulted in the hydrolysis of 38 % of the total choline glycerophospholipid molecular species, a reduction of ≈ 20 % compared with I/R (Figure 3). Preconditioning reduced phospholipid hydrolysis in all choline glycerophospholipid

**Table 2 Alterations in choline glycerophospholipid mass during I/R conditions in rabbit myocardial mitochondria**

Mitochondria were prepared from isolated and perfused rabbit hearts as described in the Materials and methods section. Mitochondrial membrane phospholipids were extracted by the method of Bligh and Dyer [45] and analysed directly by positive-ion ESI-MS. The results are expressed in pmol/$\mu$g of mitochondrial protein and represent means ± S.E.M. from three separate experiments. D (diacyl) and P (plasmenyl) indicate phosphatidylcholine and plasmenylcholine molecular species, respectively. Several minor species were also found, which were of insufficient mass (< 0.5% each) and have not been included. An integer mass was used for all ions and the masses represent the sodiated ions ($M + Na^+$).

| Choline | *m/z* | Control | I/R | Preconditioned | BEL-treated |
|---|---|---|---|---|---|
| $P_{16:0–18:2}$ | 764 | 47.7 ± 2.38 | 13.26 ± 0.95* | 22.03 ± 4.02† | 22.45 ± 7.43† |
| $P_{16:0–18:1}$ | 766 | 46.64 ± 0.69 | 13.74 ± 0.82* | 21.71 ± 2.59† | 18.88 ± 4.61† |
| $D_{16:0–18:2}$ | 780 | 43.95 ± 8.88 | 34.9 ± 0.28 | 40.9 ± 6.01 | 30.66 ± 6.72 |
| $D_{16:0–18:1}$ | 782 | 38.64 ± 0.97 | 26.16 ± 1.33* | 35.7 ± 5.0† | 26.08 ± 3.34 |
| $P_{16:0–20:4}$ | 788 | 35.72 ± 2.03 | 17.44 ± 1.4* | 23.96 ± 2.78† | 21.42 ± 2.56† |
| $P_{18:1–18:2}$ | 790 | 37.78 ± 2.48 | 19.58 ± 0.36* | 32.18 ± 3.80† | 23.41 ± 4.6 |
| $P_{18:0–18:1}$ | 794 | 5.23 ± 0.65 | 3.08 ± 0.49* | 5.77 ± 1.01† | 4.29 ± 0.72† |
| $D_{16:0–20:4}$ | 804 | 16.41 ± 1.02 | 9.89 ± 0.58* | 15.5 ± 1.69† | 12.0 ± 1.83† |
| $D_{18:1–18:2}$ | 806 | 11.53 ± 0.78 | 7.75 ± 0.63* | 12.25 ± 1.33† | 9.64 ± 1.31† |
| $D_{18:0–18:2}$ | 808 | 13.0 ± 0.86 | 9.35 ± 0.73* | 14.7 ± 1.4† | 10.94 ± 1.19† |
| $D_{18:0–18:1}$ | 810 | 5.03 ± 0.68 | 4.82 ± 0.06 | 6.02 ± 0.7† | 4.53 ± 0.59 |
| $P_{18:1–20:4}$ | 814 | 13.01 ± 0.92 | 7.46 ± 0.33* | 10.42 ± 1.75† | 9.96 ± 1.58† |
| $D_{18:1–20:4}$ | 830 | 5.26 ± 0.8 | 2.85 ± 0.23* | 5.03 ± 1.0† | 4.42 ± 0.72† |

* $P < 0.05$ for comparisons between control-perfused hearts and I/R.
† $P < 0.05$ for comparisons between I/R and preconditioning or BEL treatments.

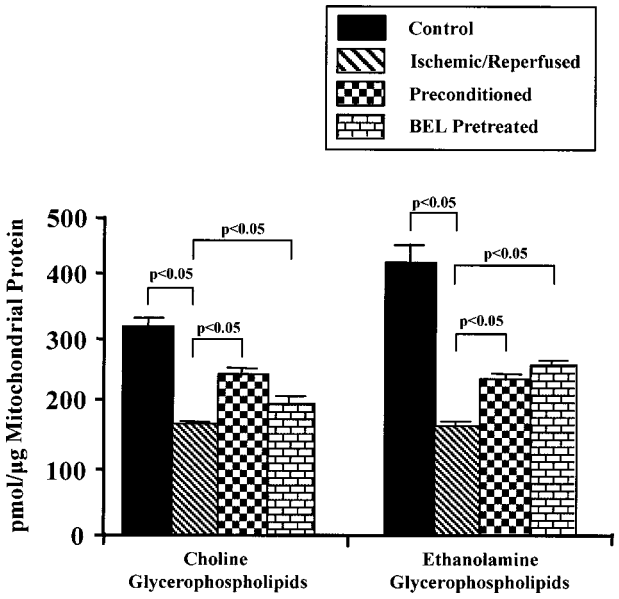


**Figure 3 Total choline and ethanolamine glycerophospholipid mass in rabbit myocardial mitochondria as determined by ESI-MS**

Mitochondria were prepared from isolated perfused adult rabbit hearts that were control perfused, subjected to 30 min of global ischaemia followed by 90 min of reperfusion, pretreated with 10 $\mu$M BEL for 15 min followed by 30 min of ischaemia and 90 min of reperfusion, or subjected to preconditioning followed by 30 min of ischaemia and 90 min of reperfusion, as described in the Materials and methods section. Mitochondrial membrane phospholipids were extracted and analysed directly by ESI-MS as described in the Materials and methods section. Values represent the means $\pm$ S.E.M. from at least three individual independent experiments.

molecular species with the exception of 16:0–18:2 phosphatidylcholine. BEL pretreatment reduced the hydrolysis of all choline glycerophospholipid molecular species, with the exceptions of 16:0–18:2, 16:0–18:1 and 18:0–18:1 phosphatidylcholine and 18:1–18:2 plasmenylcholine (Table 2).

### Myocardial I/R induces alterations in the mass of mitochondrial ethanolamine glycerophospholipid molecular species mediated by iPLA$_2$

Examination of mitochondrial phospholipids prepared from control perfused rabbit heart by ESI-MS in the negative-ion mode demonstrated the presence of phosphatidylethanolamine and plasmenylethanolamine molecular species, with the former species representing the majority of ethanolamine glycerophospholipids detected. Phosphatidylethanolamine molecular species constituted 53% of the total ethanolamine glycerophospholipid mass in mitochondria isolated from control perfused rabbit hearts, whereas plasmalogen molecular species constituted 47% (Table 3). Ethanolamine glycerophospholipids predominantly contained stearic acid in the *sn*-1 position and arachidonic acid in the *sn*-2 position (Table 3). Plasmenylethanolamine glycerophospholipid molecular subspecies were particularly enriched with arachidonyl residues at the *sn*-2 position.

Comparisons of negative-ion ESI mass spectra of mitochondrial phospholipids from control and I/R hearts demonstrated that I/R induced the hydrolysis of 58% of the total ethanolamine glycerophospholipid molecular species (Figure 3). Only one ethanolamine glycerophospholipid molecular species identified by ESI-MS, 18:0–20:4 plasmenylethanolamine, did not decrease significantly in mass (Table 3). Additional experiments involving preconditioning or BEL treatment in rabbit hearts subjected to I/R demonstrated significant reductions in the extent of ethanolamine glycerophospholipid hydrolysis when compared with that resulting from I/R alone (Table 3 and Figure 3). In contrast with the results observed for the choline glycerophospholipid pool, the reduction of hydrolysis was greater for BEL-treated hearts than that observed for preconditioned hearts (Figure 3). BEL treatment of hearts prior to I/R resulted in the hydrolysis of 35% of the total ethanolamine glycerophospholipid molecular species, a reduction of $\approx$ 60% (Figure 3). Preconditioning of hearts prior to I/R resulted in the hydrolysis of 40% of the total ethanolamine glycerophospholipid molecular species and represented a reduction in hydrolysis of $\approx$ 31% (Figure 3). Preconditioning reduced phospholipid hydrolysis in all ethanolamine glycerophospholipids, with the exceptions of

**Table 3 Alterations in ethanolamine glycerophospholipid mass during I/R conditions in rabbit myocardial mitochondria**

Mitochondria were prepared from isolated and perfused rabbit hearts as described in the Materials and methods section. Mitochondrial membrane phospholipids were extracted by the method of Bligh and Dyer [45] and analysed directly by negative-ion ESI-MS. The results are expressed in pmol/$\mu$g of mitochondrial protein and represent means $\pm$ S.E.M. from three separate experiments. D (diacyl) and P (plasmenyl) indicate phosphatidylethanolamine and plasmenylethanolamine molecular species, respectively. Several minor species were also found that were of insufficient mass ($< 0.5\%$ each) and have not been included. An integer mass was used for all ions and the masses represent the deprotonated ions.

| Ethanolamine | *m/z* | Control | I/R | Preconditioned | BEL-treated |
|---|---|---|---|---|---|
| $P_{16:0-20:4}$ | 722 | 41.47 $\pm$ 4.55 | 17.39 $\pm$ 0.86* | 23.38 $\pm$ 1.86† | 25.3 $\pm$ 1.35† |
| $D_{16:0-20:4}$ | 738 | 9.68 $\pm$ 1.16 | 2.5 $\pm$ 0.24* | 2.28 $\pm$ 0.46 | 5.02 $\pm$ 0.66 |
| $D_{18:0-18:2}$ | 742 | 21.48 $\pm$ 4.19 | 7.18 $\pm$ 0.16* | 13.21 $\pm$ 0.19† | 13.93 $\pm$ 1.22† |
| $D_{18:0-18:1}$ | 744 | 9.16 $\pm$ 2.14 | 3.62 $\pm$ 0.25* | 3.82 $\pm$ 0.59 | 3.82 $\pm$ 0.60 |
| $P_{18:1-20:4}$ | 748 | 64.16 $\pm$ 10.04 | 31.41 $\pm$ 3.73* | 44.51 $\pm$ 3.42† | 46.8 $\pm$ 3.16† |
| $P_{18:0-20:4}$ | 750 | 36.67 $\pm$ 9.43 | 23.62 $\pm$ 2.82 | 34.18 $\pm$ 3.67† | 33.06 $\pm$ 0.15† |
| $D_{18:1-20:4}$ | 764 | 14.34 $\pm$ 1.99 | 4.75 $\pm$ 0.44* | 7.88 $\pm$ 0.43† | 6.85 $\pm$ 0.70† |
| $D_{18:0-20:4}$ | 766 | 111.3 $\pm$ 12.52 | 45.66 $\pm$ 1.70* | 73.78 $\pm$ 5.53† | 63.03 $\pm$ 6.44† |
| $P_{18:0-22:6}$ | 774 | 20.43 $\pm$ 6.32 | 6.64 $\pm$ 1.63* | 10.65 $\pm$ 0.72 | 10.32 $\pm$ 0.34 |
| $P_{18:1-22:4}$ | 776 | 31.52 $\pm$ 5.12 | 8.8 $\pm$ 0.89* | 10.99 $\pm$ 0.48† | 14.47 $\pm$ 1.40† |
| $D_{18:0-22:6}$ | 790 | 7.54 $\pm$ 1.15 | 3.66 $\pm$ 0.32* | 2.79 $\pm$ 0.16† | 6.9 $\pm$ 0.02† |
| $D_{18:1-22:4}$ | 792 | 24.1 $\pm$ 6.8 | 5.55 $\pm$ 0.75* | 4.2 $\pm$ 0.58† | 14.96 $\pm$ 0.69† |
| $D_{18:0-22:4}$ | 794 | 24.99 $\pm$ 6.17 | 5.5 $\pm$ 0.78* | 6.2 $\pm$ 0.86 | 14.59 $\pm$ 1.75† |

* $P < 0.05$ for comparisons between control-perfused hearts and I/R.

† $P < 0.05$ for comparisons between I/R and preconditioning or BEL treatments.

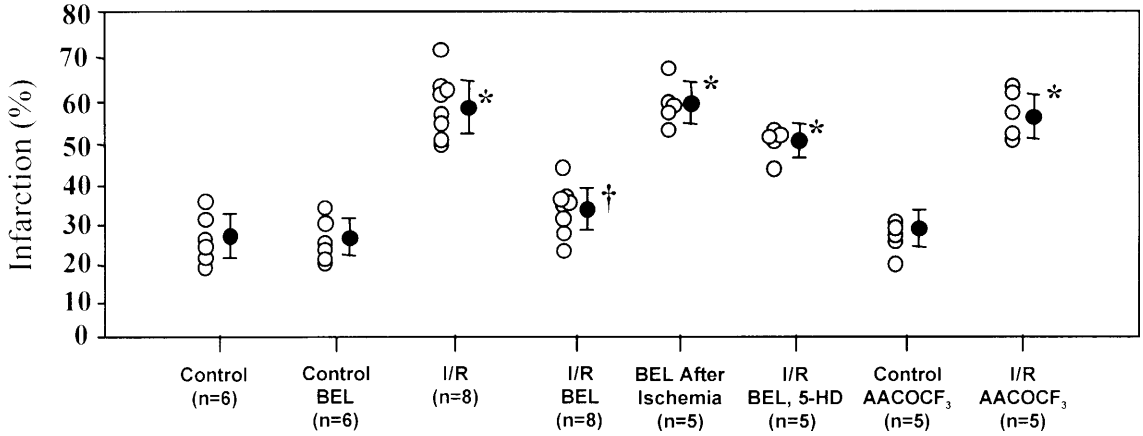


**Figure 4 Inhibition of iPLA$_2$ by BEL reduces infarct size**

Isolated perfused rabbit hearts were subjected to either control perfusion or 30 min of global ischaemia followed by 2 h of reperfusion (I/R) in the presence or absence of the selective phospholipase inhibitors BEL or AACOCF$_3$ (both 10 $\mu$M) as described in the Materials and methods section. Infarct size was determined by TTC staining and is reported as a percentage of the risk zone. BEL protected the ischaemic heart only when administered prior to ischaemia. 5-HD (a $K_{ATP}$ channel closer) blocked the cardioprotective effects of BEL treatment. ○, Individual experiments; ●, mean ± S.E.M. *$P < 0.05$ for comparisons between control and I/R conditions. †$P < 0.05$ for comparisons between I/R and BEL treatment.

16:0–20:4, 18:0–18:1 and 18:0–22:4 phosphatidylethanolamine and 18:0–22:6 plasmenylethanolamine (Table 3). Pretreatment of hearts with BEL prior to I/R reduced the hydrolysis of all ethanolamine glycerophospholipid molecular species with the exceptions of 16:0–20:4 and 18:0–18:1 phosphatidylethanolamine and 18:0–22:6 plasmenylethanolamine (Table 3).

### Inhibition of iPLA$_2$ reduces infarct size and is cardioprotective

Mitochondrial function is essential for myocardial recovery from I/R injury. Since we have identified catalytically competent iPLA$_2$ localized to the inner mitochondrial membranes, and since we have demonstrated the BEL-sensitive hydrolysis of mitochondrial membrane phospholipids during I/R, the protective effect of BEL on infarct size was determined. In control perfused hearts or hearts perfused with 10 $\mu$M BEL for 15 min, measured infarct sizes were 27.7 ± 5.6 % and 27.5 ± 4.3 %, respectively (Figure 4). It should be appreciated that these experimental conditions were not subjected to ischaemia and the modest baseline infarct sizes obtained in control perfused hearts were due to the Langendorff perfusion model of the isolated, perfused rabbit heart and are within normal acceptable levels for these types of experiment. Isolated and perfused rabbit hearts subjected to 30 min of ischaemia and 90 min of reperfusion (I/R) resulted in an infarct size of 59.2 ± 6.4 % (Figure 4). Pretreatment of hearts with 10 $\mu$M BEL followed by I/R was cardioprotective and reduced infarct size to near continuously perfused (control) levels (34.5 ± 5.1 %), which represented a near complete salvage (Figure 4). The inhibition of iPLA$_2$ by BEL was only cardioprotective when administered prior to the onset of global ischaemia. Experiments in which 10 $\mu$M BEL was included only in the reperfusion buffer did not result in a reduction in infarct size (60.3 ± 5.0 %, compared with 59.2 ± 6.4 % for I/R alone; Figure 4). To confirm that the specific inhibition of iPLA$_2$ by BEL was cardioprotective, hearts were perfused with the cPLA$_2$ inhibitor AACOCF$_3$. Perfusion of hearts with 10 $\mu$M AACOCF$_3$ prior to I/R was not cardioprotective (58.7 ± 5.5 %, compared with 59.2 ± 6.4 % for I/R alone; Figure 4). Control hearts perfused with 10 $\mu$M AACOCF$_3$ for 15 min resulted in a measured infarct size that was comparable with those observed in control perfused or control hearts perfused with BEL (Figure 4).

### Inhibition of iPLA$_2$ reduces infarct size through a $K_{ATP}$ channel-sensitive mechanism

The activation of iPLA$_2$ results in the production of free fatty acids, namely arachidonic acid, and lysophospholipids. Lysophospholipids have been identified as having the ability to modulate selected ion channels by altering the surrounding lipid bilayer [48–50]. More importantly, arachidonic acid is a precursor for the production of prostaglandins and leukotrienes. It has been suggested that arachidonic acid and the production of prostaglandins by cyclo-oxygenases 1 and 2 may also directly affect ion channels, specifically the mitochondrial $K_{ATP}$ channel, through currently unknown mechanisms [36–38,48,51]. Since iPLA$_2$ is intricately involved in lipid signalling and mediates mitochondrial phospholipid hydrolysis, and since the mitochondrial $K_{ATP}$ channel has been suggested to be modulated by lipid metabolites, we determined the involvement of the mitochondrial $K_{ATP}$ channel in the cardioprotective effects of iPLA$_2$ inhibition by BEL in myocardial I/R. Administration of 100 $\mu$M 5-HD, a selective mitochondrial $K_{ATP}$ channel closer, simultaneously with 10 $\mu$M BEL for 15 min prior to I/R protocols abolished protection, resulting in a measured infarct size of 51.0 ± 3.7 % (compared with 59.2 ± 6.4 for I/R alone; Figure 4). The closing of the mitochondrial $K_{ATP}$ channel has been implicated in myocardial ischaemic injury, and it has been shown that mitochondrial $K_{ATP}$ channel agonists are cardioprotective [52,53]. This suggests that iPLA$_2$ activity is detrimental to the heart and indicates that the products of iPLA$_2$ activity may act through a mechanism to close the $K_{ATP}$ channel. We tested this hypothesis by perfusing hearts with arachidonic acid, a product of iPLA$_2$ activity on membrane phospholipids. Administration of 10 $\mu$M arachidonic acid to control hearts for 2 h did not alter infarct size (23.8 ± 6.0 %; Figure 5). The opening of the mitochondrial $K_{ATP}$ channel has also been implicated in preconditioning [52,54]. We hypothesized that if preconditioning is cardioprotective through opening of the mitochondrial $K_{ATP}$ channel, and if iPLA$_2$ activity is detrimental through closure of the mitochondrial $K_{ATP}$ channel by lipid metabolites, then lipid metabolites should reverse the cardioprotective effects of preconditioning. Hearts that were perfused with 10 $\mu$M arachidonic acid for 15 min followed by preconditioning protocols and I/R resulted in the complete

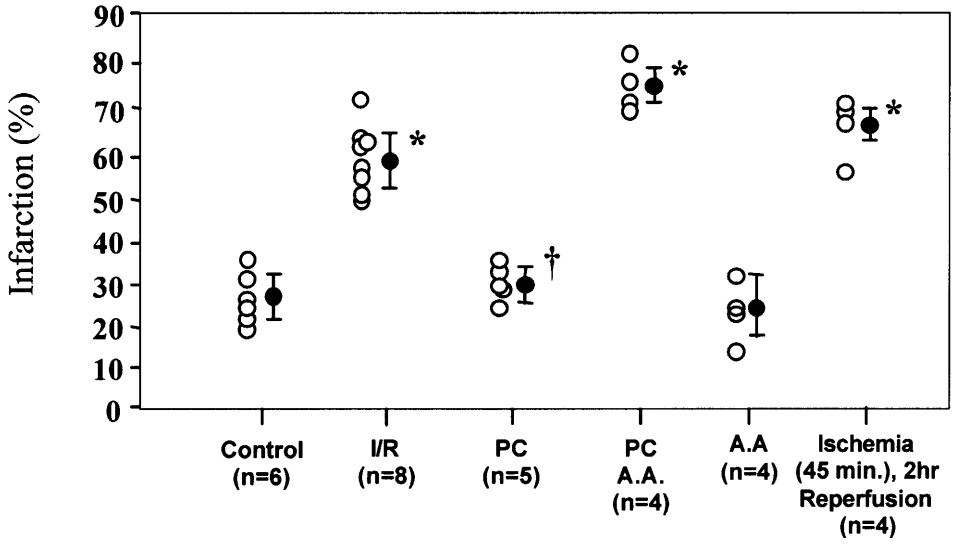


**Figure 5 Alleviation of the cardioprotective effects of preconditioning by arachidonic acid**

Isolated perfused rabbit hearts were subjected to control perfusion, 30 or 45 min of global ischaemia followed by 2 h of reperfusion (second and sixth columns from the left, respectively), preconditioning (PC) followed by 30 min of ischaemia and 2 h of reperfusion, perfusion with 10 $\mu$M arachidonic acid (A.A.) for 2 h, or perfusion with 10 $\mu$M arachidonic acid for 15 min followed by preconditioning including 10 $\mu$M arachidonic acid followed by 30 min of ischaemia and 2 h of reperfusion. Infarct size was determined by TTC staining and is reported as a percentage of the risk zone. ○ and ● represent individual experiments and means ± S.E.M., respectively. *$P < 0.05$ for comparisons between control and I/R, preconditioning + arachidonic acid, or 45 min of ischaemia and 2 h of reperfusion. †$P < 0.05$ for comparisons between I/R and preconditioning.

ablation of the cardioprotective effect of preconditioning (75.0 ± 4.71 %; Figure 5) and produced an infarct size similar to that observed with a 45 min period of ischaemia followed by 2 h of reperfusion (66.0 ± 4.97 %; Figure 5).

## DISCUSSION

Although membrane-associated $iPLA_2$ activity is increased in ischaemic myocardium, a role for the activation of this phospholipase in myocardial I/R injury has not been defined adequately [2,9]. The present study now demonstrates for the first time that catalytically competent $iPLA_2$ is localized on the outer face of the inner mitochondrial membrane. The localization of $iPLA_2$ to the inner mitochondrial membrane is accompanied by accelerated mitochondrial phospholipid catabolism mediated, at least in part, by $iPLA_2$ during myocardial I/R and during preconditioning. A component of the decrease in mitochondrial phospholipid content following I/R may also result from a decrease in phospholipid biosynthesis as well as the activity of other phospholipases (e.g. $cPLA_2$ and phospholipase D).

The work presented herein has utilized ESI-MS to characterize the phospholipid molecular species present in myocardial mitochondria in isolated and perfused rabbit hearts. Analyses indicated abundant choline and ethanolamine glycerophospholipid molecular species consisting of diacyl and plasmalogen molecular subspecies. The primary *sn*-1 aliphatic chains of choline glycerophospholipids were composed of palmitate residues, whereas oleic, linoleic and arachidonic acids were evenly distributed at the *sn*-2 position. Ethanolamine glycerophospholipids predominantly contained stearic acid at the *sn*-1 position whereas the *sn*-2 position was enriched in arachidonyl residues.

We observed significant decreases in both choline- and ethanolamine-containing glycerophospholipid molecular species from mitochondria isolated from I/R hearts. Our findings are in accordance with a previous study that showed significant decreases in mitochondrial phosphatidylcholine and phosphatidylethanolamine and also cardiolipin from isolated and perfused rat hearts subjected to ischaemia or I/R [29]. Significant decreases in mitochondrial phosphatidylcholine and phosphatidylethanolamine were also observed in ischaemic small intestine, with more prominent decreases resulting from I/R [30]. The content of phosphatidylethanolamine molecular species in liver mitochondria, quantified by reversed-phase HPLC–ESI-MS, has been reported recently [55]. The most abundant phosphatidylethanolamine molecular species in liver mitochondria was 16:0–22:6; however, this phospholipid was not detected in heart mitochondria in the present study. The most abundant ethanolamine glycerophospholipid detected in heart mitochondria consisted of 18:0–20:4, which was the second most abundant phosphatidylethanolamine molecular species detected in liver mitochondria [55]. Other abundant ethanolamine glycerophospholipid species in heart mitochondria consist of plasmalogen molecular subspecies which are not present in liver mitochondria. The comparisons between heart and liver mitochondrial phospholipids are complicated by the enrichment of plasmalogen molecular subspecies in the myocardium [10]. Therefore, direct comparisons are difficult to establish.

Although membrane-associated $iPLA_2$ activity increases during myocardial ischaemia, only one previous study has attempted to localize this phospholipase to a specific subcellular membrane pool. This study reported the nuclear translocation of catalytically competent $iPLA_2$ during myocardial I/R in isolated and perfused rat hearts, with concomitant nuclear-membrane catabolism [5]. The present study now demonstrates that catalytically competent $iPLA_2$ is localized constitutively to the outer face of the inner mitochondrial membrane. The localization of $iPLA_2$ to rabbit heart mitochondria is also accompanied by accelerated mitochondrial-membrane catabolism, which is due in part to $iPLA_2$, based on studies using the $iPLA_2$-specific inhibitor, BEL. It should be appreciated that the concentration of BEL

(10 $\mu$M) used in these studies is a potent inhibitor of $iPLA_2$ activity *in vitro* and has been shown previously to result in the inhibition of phospholipid hydrolysis in isolated perfused rat hearts [5]. These results indicate that $iPLA_2$ contributes to the hydrolysis of mitochondrial phosphatidylcholine and phosphatidylethanolamine phospholipid species. Furthermore, we have defined several critical mitochondrial lipid components that are altered during myocardial I/R. We hypothesize that such alterations in mitochondrial membrane phospholipids probably make the membranes more fluid and may be involved in the loss of calcium homoeostasis, permeability transition and release of cytochrome *c* that occur in mitochondria during apoptosis. In fact, it is well known that ischaemia induces the disruption of the inner mitochondrial membrane and increases its permeability to protons and inhibits the respiratory chain [56].

Since $iPLA_2$-mediated mitochondrial membrane catabolism has been demonstrated, and since mitochondrial membrane integrity is central to the recovery of ischaemic myocardium, studies were performed to determine the cardioprotective effects of $iPLA_2$ inhibition in isolated perfused rabbit hearts. Perfusion of hearts with BEL was cardioprotective when administered prior to the ischaemic episode and significantly reduced the infarct size. In contrast, since the specific $cPLA_2$ inhibitor $AACOCF_3$ was not cardioprotective, it is likely that $cPLA_2$ is not involved in I/R injury. It should be noted that the effects of BEL on the reduction of phospholipid hydrolysis are not as dramatic as the effects of the inhibitor on infarct size. Therefore, the preservation of mitochondrial membrane phospholipids may not be responsible for the cardioprotective effects observed in this study. The possibility exists that BEL may affect other unidentified signalling pathways independent of the inhibition of $iPLA_2$.

The mitochondrial $K_{ATP}$ channel has been implicated in the cardioprotection of hearts during myocardial I/R. It has been reported that arachidonic acid, a product of the enzymic action of $iPLA_2$ on phospholipids, directly modulates myocardial $K_{ATP}$ channels by inhibiting $K_{ATP}$ activity [36,38]. It has also been suggested that $iPLA_2$ can modulate $K_{ATP}$ channels in insulin-secreting cell lines, also through arachidonic acid release and the inhibition of $K_{ATP}$ channel activity [37]. Perfusion of isolated rabbit hearts with the $K_{ATP}$ channel blocker 5-HD simultaneously with BEL reversed the cardioprotective effects of BEL. These results suggest that the cardioprotective effect of BEL is due to $K_{ATP}$ channel opening. Therefore, it can be inferred that mitochondrially associated $iPLA_2$ activated during myocardial I/R results in the closing of the mitochondrial $K_{ATP}$ channels and precipitates damage to the mitochondria. It should be appreciated that mitochondrial $K_{ATP}$ channels are also located on the inner mitochondrial membrane. The closing of $K_{ATP}$ channels by activated $iPLA_2$ is most likely mediated either through the production of free fatty acids, such as arachidonic acid, or through the production of lysophospholipid second messengers. We tested this hypothesis by perfusing hearts with arachidonic acid. Whereas arachidonic acid alone did not induce an infarction, hearts perfused with arachidonic acid prior to preconditioning followed by I/R reversed the cardioprotective effects of preconditioning. Since preconditioning is generally believed to open the mitochondrial $K_{ATP}$ channel, the effect of arachidonic acid on preconditioning can be interpreted to result in the closure of the $K_{ATP}$ channel.

The closing of mitochondrial $K_{ATP}$ channels could impair mitochondrial volume homoeostasis, leading to opening of the mitochondrial permeability transition pore with subsequent induction of cell death. In insulin-secreting HIT cells the addition of low concentrations of lysophospholipids or arachidonic acid reduced $K_{ATP}$ channel activity [48]. Additionally, arachidonic acid could modulate $K_{ATP}$ channel activity through its conversion into prostaglandins by cyclo-oxygenase. Perfusion of rat hearts with prostaglandins I2, E2 and D2 has been shown to activate $K_{ATP}$ channels [38]. Cyclo-oxygenase has also been shown to mediate the cardioprotective effects of the late phase of preconditioning in rabbit hearts [51]. These protective effects of cyclo-oxygenase and prostaglandin synthesis from arachidonic acid may indicate the dual importance of arachidonic acid in the ischaemic heart. Free arachidonic acid may be detrimental and act to close $K_{ATP}$ channels, whereas arachidonic acid that is converted into prostaglandins may open $K_{ATP}$ channels and be cardioprotective. Whatever the outcome, the unifying theme underlying the role of arachidonic acid in $K_{ATP}$ channel modulation is the initial production of the free fatty acid, through the activity of $iPLA_2$. It should also be appreciated that $iPLA_2$ may affect $K_{ATP}$ channels independent of arachidonic acid production through alterations in the lipid composition of the surrounding membrane. The direct action of $iPLA_2$ activity on the membrane bilayer has been shown to alter $K^+$ channels in the absence of supramicellar concentrations of fatty acids [57].

In conclusion, the results presented in this study demonstrate the localization of catalytically competent $iPLA_2$ to the outer face of the inner membrane of rabbit heart mitochondria. Accelerated mitochondrial membrane phospholipid catabolism mediated in part by $iPLA_2$ was observed during I/R of isolated perfused rabbit hearts. Finally, the inhibition of $iPLA_2$ by BEL was shown to be cardioprotective through a mechanism that involved the opening of the mitochondrial $K_{ATP}$ channel. These results establish $iPLA_2$ and accelerated phospholipid catabolism as key mediators of the pathophysiology of myocardial I/R injury and suggest that $iPLA_2$ may represent an important therapeutic target for the amelioration of ischaemic injury.

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## REFERENCES

1 Corr, P. B., Gross, R. W. and Sobel, B. E. (1984) Amphipathic metabolites and membrane dysfunction in ischemic myocardium. Circ. Res. **55**, 135–154
2 Ford, D. A., Hazen, S. L., Saffitz, J. E. and Gross, R. W. (1991) The rapid and reversible activation of a calcium-independent plasmalogen-selective phospholipase $A_2$ during myocardial ischemia. J. Clin. Invest. **88**, 331–335
3 Katz, A. M. and Messineo, F. C. (1981) Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. Circ. Res. **48**, 1–16
4 Prasad, M. R., Popescu, L. M., Moraru, I. I., Liu, X. K., Maity, S., Engelman, R. M. and Das, D. K. (1991) Role of phospholipase $A_2$ and C in myocardial ischemic reperfusion injury. Am. J. Physiol. Heart Circ. Physiol. **260**, H877–H883
5 Williams, S. D., Hsu, F. F. and Ford, D. A. (2000) Electrospray ionization mass spectrometry analyses of nuclear membrane phospholipid loss after reperfusion of ischemic myocardium. J. Lipid Res. **41**, 1585–1595
6 Wolf, M. J. and Gross, R. W. (1996) Expression, purification, and kinetic characterization of a recombinant 80-kDa intracellular calcium-independent phospholipase $A_2$. J. Biol. Chem. **271**, 30879–30885
7 Mancuso, D. J., Jenkins, C. M. and Gross, R. W. (2000) The genomic organization, complete mRNA sequence, cloning, and expression of a novel human intracellular membrane-associated calcium-independent phospholipase $A_2$. J. Biol. Chem. **275**, 9937–9945
8 Tang, J., Kriz, R. W., Wolfman, N., Shaffer, M., Seehra, J. and Jones, S. S. (1997) A novel cytosolic calcium-independent phospholipase $A_2$ contains eight ankyrin motifs. J. Biol. Chem. **272**, 8567–8575
9 Hazen, S. L., Ford, D. A. and Gross, R. W. (1991) Activation of a membrane-associated phospholipase $A_2$ during rabbit myocardial ischemia which is highly selective for plasmalogen substrate. J. Biol. Chem. **266**, 5629–5633
10 Gross, R. W. (1984) High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: a fast atom bombardment mass spectroscopic and gas chromatography-mass spectroscopic characterization. Biochemistry **23**, 158–165

11 Ford, D. A. and Gross, R. W. (1989) Differential accumulation of diacyl and plasmalogenic diglycerides during myocardial ischemia. Circ. Res. **64**, 173–177
12 Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M. and Engler, R. L. (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J. Clin. Invest. **94**, 1621–1628
13 Kajstura, J., Cheng, W., Reiss, K., Clark, W. A., Sonnenblick, E. H., Krajewski, S., Reed, J. C., Olivetti, G. and Anversa, P. (1996) Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. Lab. Invest. **74**, 86–107
14 Olivetti, G., Quaini, F., Sala, R., Lagrasta, C., Corradi, D., Bonacina, E., Gambert, S. R., Cigola, E. and Anversa, P. (1996) Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. J. Mol. Cell Cardiol. **28**, 2005–2016
15 Saraste, A., Pulkki, K., Kallajoki, M., Henriksen, K., Parvinen, M. and Voipio-Pulkki, L. M. (1997) Apoptosis in human acute myocardial infarction. Circulation **95**, 320–323
16 Fliss, H. and Gattinger, D. (1996) Apoptosis in ischemic and reperfused rat myocardium. Circ. Res. **79**, 949–956
17 Agarwal, M. L., Larkin, H. E., Zaidi, S. I., Mukhtar, H. and and Oleinick, N. L. (1993) Phospholipase activation triggers apoptosis in photosensitizes mouse lymphoma cells. Cancer Res. **53**, 5897–5902
18 Atsumi, G., Tajima, M., Hadano, A., Nakatani, Y., Murakami, M. and Kudo, I. (1998) Fas-induced arachidonic acid release is mediated by $Ca^{2+}$-independent phospholipase $A_2$ but not cytosolic phospholipase $A_2$, which undergoes proteolytic inactivation. J. Biol. Chem. **273**, 13870–13877
19 Korystov, Y. N., Dobrovinskaya, O. R., Shaposhnikova, V. V. and Eidus, L. (1996) Role of arachidonic acid metabolism in thymocyte apoptosis after irradiation. FEBS Lett. **388**, 238–241
20 Hayakawa, M., Ishida, N., Takeuchi, K., Shibamoto, S., Hori, T., Oku, N., Ito, G. and Tsujimoto, M. (1993) Arachidonic acid-selective cytosolic phospholipase $A_2$ is crucial in the cytotoxic action of tumor necrosis factor. J. Biol. Chem. **268**, 11290–11295
21 Voelkel-Johnson, C., Thorne, T. E. and Laster, S. M. (1996) Susceptibility to TNF in the presence of inhibitors of transcription or translation is dependent on the activity of cytosolic phospholipase $A_2$ in human melanoma tumor cells. J. Immunol. **156**, 201–207
22 Wissing, D., Mouritzen, H., Egeblad, M., Poirier, G. G. and Jaattela, M. (1997) Involvement of caspase-dependent activation of cytosolic phospholipase $A_2$ in tumor necrosis factor-induced apoptosis. Proc. Natl. Acad. Sci. U.S.A. **94**, 5073–5077
23 Sapirstein, A., Spech, R. A., Witzgall, R. and Bonventre, J. V. (1996) Cytosolic phospholipase $A_2$ ($PLA_2$), but not secretory $PLA_2$, potentiates hydrogen peroxide cytotoxicity in kidney epithelial cells. J. Biol. Chem. **271**, 21505–21513
24 Borutaite, V., Mildaziene, V., Brown, B. C. and Brand, M. D. (1995) Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia? Biochim. Biophys. Acta **1272**, 154–158
25 Di Lisa, F. and Bernardi, P. (1998) Mitochondrial function as a determinant of recovery or death in cell response to injury. Mol. Cell. Biochem. **184**, 379–391
26 Duan, J. and Karmazyn, M. (1989) Relationship between oxidative phosphorylation and adenine nucleotide translocase activity of two populations of cardiac mitochondria and mechanical recovery of ischemic hearts following reperfusion. Can. J. Physiol. Pharmacol. **67**, 704–709
27 Halestrap, A. P., Kerr, P. M., Javadov, S. and Woodfield, K. Y. (1998) Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. Biochim. Biophys. Acta **1366**, 79–94
28 Piper, H. M., Sezer, O., Schleyer, M., Hutter, J. F. and Spieckermann, P. G. (1985) Development of ischemia-induced damage in defined mitochondrial subpopulations. J. Mol. Cell Cardiol. **17**, 885–896
29 Paradies, G., Petrosillo, G., Pistolese, M., Venosa, N. D., Serena, D. and Ruggiero, F. M. (1999) Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. Free Radical Biol. Med. **27**, 42–50
30 Madesh, M., Ramachandran, A., Pulimood, A., Vadranam, M. and Balasubramanian, K. A. (2000) Attenuation of intestinal ischemia/reperfusion injury with sodium nitroprusside: studies on mitochondrial function and lipid changes. Biochim. Biophys. Acta **1500**, 204–216
31 Lesnefsky, E. J., Slabe, T. J., Stoll, M. S. K., Minkler, P. E. and Hoppel, C. L. (2001) Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. Am. J. Physiol. Heart Circ. Physiol. **280**, H2770–H2778
32 Murry, C. E., Jennings, R. B. and Reimer, K. A. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation **74**, 1124–1136
33 Cohen, M. V., Baines, C. P. and Downey, J. M. (2000) Ischemic preconditioning: from adenosine receptor to $K_{ATP}$ channel. Annu. Rev. Physiol. **62**, 79–109
34 Pain, T., Yang, X. M., Critz, S. D., Yue, Y., Nakano, A., Liu, G. S., Heusch, G., Cohen, M. V. and Downey, J. M. (2000) Opening of mitochondrial $K_{ATP}$ channels triggers the preconditioned state by generating free radicals. Circ. Res. **87**, 460–466
35 Wang, S., Cone, J. and Liu, Y. (2001) Dual roles of mitochondrial $K_{ATP}$ channels in diazoxide-mediated protection in isolated rabbit hearts. Am. J. Physiol. Heart Circ. Physiol. **280**, H246–H255
36 Kim, D. and Duff, R. A. (1990) Regulation of $K^+$ channels in cardiac myocytes by free fatty acids. Circ. Res. **67**, 1040–1046
37 Turk, J., Gross, R. W. and Ramanadham, S. (1993) Amplification of insulin secretion by lipid messengers. Diabetes **42**, 367–374
38 Bouchard, J. F., Dumont, E. and Lamontagne, D. (1994) Evidence that prostaglandins I2, E2, and D2 may activate ATP sensitive potassium channels in the isolated rat heart. Cardiovasc. Res. **28**, 901–905
39 Tsuchida, A., Liu, Y., Liu, G. S., Cohen, M. V. and Downey, J. M. (1994) Alpha 1-adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. Circ. Res. **75**, 576–585
40 Klein, H. H., Puschmann, S., Schaper, J. and Schaper, W. (1981) The mechanism of the tetrazolium reaction in identifying experimental myocardial infarction. Virchows Arch. **393**, 287–297
41 Ytrehus, K., Liu, Y., Tsuchida, A., Miura, T., Liu, G. S., Yang, X., Herbert, D., Cohen, M. V. and Downey, J. M. (1994) Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing. Am. J. Physiol. **267**, H2383–H2390
42 Storrie, B. and Madden, E. A. (1990) Isolation of subcellular organelles. Methods Enzymol. **182**, 203–225
43 Comte, J. and Gautheron, D. C. (1979) Preparation of outer membrane from pig heart mitochondria. Methods Enzymol. **55**, 98–104
44 Ella, K. M., Meier, G. P., Bradshaw, C. D., Huffman, K. M., Spivey, E. C. and Meier, K. E. (1994) A fluorescent assay for agonist-activated phospholipase D in mammalian cell extracts. Anal. Biochem. **218**, 136–142
45 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917
46 Han, X. L., Zupan, L. A., Hazen, S. L. and Gross, R. W. (1992) Semisynthesis and purification of homogeneous plasmenylcholine molecular species. Anal. Biochem. **200**, 119–124
47 Liu, S. J. and McHowat, J. (1998) Stimulation of different phospholipase $A_2$ isoforms by TNF-alpha and IL-1beta in adult rat ventricular myocytes. Am. J. Physiol. **275**, H1462–H1472
48 Eddlestone, G. T. (1995) ATP-sensitive K channel modulation by products of $PLA_2$ action in the insulin-secreting HIT cell line. Am. J. Physiol. **268**, C181–C190
49 Caldwell, R. A. and Baumgarten, C. M. (1998) Plasmalogen-derived lysolipid induces a depolarizing cation current in rabbit ventricular myocytes. Circ. Res. **83**, 533–540
50 Corr, P. B. and Yamada, K. A. (1995) Selected metabolic alterations in the ischemic heart and their contributions to arrhythmogenesis. Herz **20**, 156–168
51 Shinmura, K., Tang, X. L., Wang, Y., Xuan, Y. T., Liu, S. Q., Takano, H., Bhatnagar, A. and Bolli, R. (2000) Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. Proc. Natl. Acad. Sci. U.S.A. **97**, 10197–10202
52 Garlid, K. D., Paucek, P., Yarov-Yarovoy, V., Murray, H. N., Darbenzio, R. B., D'Alonzo, A. J., Lodge, N. J., Smith, M. A. and Grover, G. J. (1997) Cardioprotective affect of diazoxide and its interaction with mitochondrial ATP-sensitive $K^+$ channels: possible mechanism of cardioprotection. Circ. Res. **81**, 1072–1082
53 Wong, S., Cone, J. and Liu, Y. (2001) Dual roles of mitochondrial $K_{ATP}$ channels in diazoxide-mediated protection in isolated rabbit hearts. Am. J. Physiol. Heart Circ. Physiol. **280**, H246–H255
54 Gross, G. J. and Fryer, R. M. (1999) Sarcolemmal versus mitochondrial ATP-sensitive $K^+$ channels and myocardial preconditioning. Circ. Res. **84**, 973–979
55 Kevala, J. H. and Kim, H. Y. (2001) Determination of substrate preference in phosphatidylserine decarboxylation by liquid chromatography-electrospray ionization mass spectrometry. Anal. Biochem. **292**, 130–138
56 Farber, J. L., Chien, K. R. and Mittnacht, S. (1981) Myocardial ischemia: the pathogenesis of irreversible cell injury in ischemia. Am. J. Pathol. **102**, 271–281
57 Gubitosi-Klug, R. A., Yu, S. P., Choi, D. W. and Gross, R. W. (1995) Concomitant acceleration of the activation and inactivation kinetics of the human delayed rectifier $K^+$ channel (Kv1.1) by $Ca^{2+}$-independent phospholipase $A_2$. J. Biol. Chem. **270**, 2885–2888


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