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Identification and distribution of endoplasmic reticulum iPLA$_2$

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Abstract

Our laboratory demonstrated that endoplasmic reticulum iPLA$_2$ (ER-iPLA$_2$) activity protects renal cells from oxidant-induced cell death and lipid peroxidation. The goals of this study were to determine the PLA$_2$ isoform(s) responsible for ER-iPLA$_2$ activity in different species and tissues. ER-iPLA$_2$ activity was observed in microsomes from rabbit and rat kidney, heart, and brain as well as in human kidney (Caki-1 and HEK293) and glioblastoma (A172) cell lines. Reverse transcriptase-polymerase chain reaction results demonstrated the presence of iPLA$_2\gamma$ (group VIB PLA$_2$) message in all tissues tested. Immunoblot analysis and PLA$_2$ inhibitor studies with methyl arachidonyl fluorophosphonate and enantiomers of bromoenol lactone demonstrated that the ER-iPLA$_2$ in rabbit kidney and heart and rat kidney is iPLA$_2\gamma$. These results demonstrate the expression of ER-iPLA$_2\gamma$ (group VIB) across species and tissues, and suggest that iPLA$_2\gamma$ may play critical roles in oxidant-induced cell injury.

Keywords: iPLA$_2\gamma$; cPLA$_2\gamma$; Group VIB PLA$_2$; Group IVC PLA$_2$; Endoplasmic reticulum

Phospholipase A$_2$ (PLA$_2$) enzymes catalyze the hydrolysis of a fatty acid from the *sn*-2 position of glycerophospholipids generating biologically active free fatty acids and lysophospholipids. Numerous members of the PLA$_2$ family have been identified and can be classified according to their nucleotide sequences into 14 groups designated as I–XIV [1]. The Ca^{2+}-independent PLA$_2$ (iPLA$_2$) family members are iPLA$_2\gamma$ (group VIB), cPLA$_2\gamma$ (group IVC), and iPLA$_2\beta$ (group VIA). iPLA$_2\gamma$, cPLA$_2\gamma$, and splice variants of human and rat iPLA$_2\beta$ that contain exon 9 are membrane-associated [2–9]. iPLA$_2\gamma$ has been cloned and overexpressed in COS-7 [2] and Sf-9 [3] cells, and associates with crude membrane fractions in both models. Yang et al. [4] reported that iPLA$_2\gamma$ is located in rat liver peroxisomal membranes. cPLA$_2\gamma$ overexpression in HEK293, Sf-9, and CHO cells revealed perinuclear (endoplasmic reticulum and Golgi) membrane localization in each cell type [5–8]. One of the several splice variants of iPLA$_2\beta$ was detected in crude membrane preparations of COS-7 cells after transfection and in rat vascular smooth muscle cells [9]. While these studies demonstrate that iPLA$_2$ can be membrane associated, the identity of specific iPLA$_2$ in specific membrane locations has not been fully elucidated.

Endoplasmic reticulum-associated iPLA$_2$ (ER-iPLA$_2$) activity has been described in the heart [10,11], arteriolar endothelial cells [12], and kidney [13]. We recently demonstrated that rabbit renal proximal tubular cells (RPTC) contain an ER-iPLA$_2$ that is inhibited by low micromolar concentrations of bromoenol lactone (BEL) [13]. Inhibition of iPLA$_2$ in RPTC with BEL potentiated oxidant-induced lipid peroxidation and necrotic renal cell death, but had no effect on non-oxidant-induced necrosis [13].

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Further, doxorubicin-induced cardiomyocyte death, which is at least partially due to lipid peroxidation, is enhanced by inhibition of membrane-associated $iPLA_2$ activity with BEL [14]. Based on these studies, we suggested that ER-$iPLA_2$ protects renal and heart cells from oxidant toxicity by inhibiting lipid peroxidation.

To further study the role of ER-$iPLA_2$ in oxidant-induced injury, it is necessary to determine the PLA_2 isoform(s) responsible for this activity. One method to discriminate between PLA_2 isoforms is the use of different PLA_2 inhibitors, since each enzyme has a distinct inhibition profile. The enzymatic activity of $cPLA_2\gamma$ is efficiently inhibited by methyl arachidonyl fluorophosphonate (MAFP), but not the $iPLA_2$-specific inhibitor, bromoenol lactone (BEL), with IC_{50} values of <1 and >30 μM, respectively [7]. $iPLA_2\gamma$ and $iPLA_2\beta$, unlike $cPLA_2\gamma$, are inhibited by BEL (IC_{50} values, 3 and <1 μM, respectively [3,15]). Cytosolic $iPLA_2\beta$ is inhibited by MAFP with an IC_{50} of approximately 1 μM [16]. ER-$iPLA_2$ activity in the kidney and heart is sensitive to BEL, but insensitive to MAFP [11,13], suggesting that ER-$iPLA_2$ activity is not $cPLA_2\gamma$ or cytosolic $iPLA_2\beta$. Recently, Jenkins et al. [17] described enantioselective inhibition of $iPLA_2\beta$ and $iPLA_2\gamma$ by (*S*)-BEL and (*R*)-BEL, respectively. In a follow-up study, siRNA knock down of $iPLA_2\gamma$ and $iPLA_2\beta$ confirmed the effects of (*R*)- and (*S*)-BEL on $iPLA_2\gamma$- and $iPLA_2\beta$-mediated effects in 3T3-L1 preadipocytes [18].

Materials and methods

Materials. Female New Zealand White rabbits (1.5–2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). Male Sprague–Dawley rats (300–350 g) were purchased from Harlan (Indianapolis, IN). All other chemicals and materials were obtained from Sigma Chemical (St. Louis, MO) or reported previously [11–14,19].

Isolation of rabbit and rat tissue microsomes. Rabbits were euthanized by intravenous injection of 75 mg/kg pentobarbital sodium and rats were euthanized by intraperitoneal injection of sodium pentobarbital at 1 mg/kg. Rabbit and rat kidney cortex, heart and brain tissues were collected and placed on ice in either $iPLA_2$ activity buffer containing (mM): sucrose 250, KCl 10, imidazole 10, EDTA 5, and dithiothreitol 2 with 10% glycerol (pH 7.8), or $iPLA_2$ immunoblot buffer (activity buffer minus glycerol plus Sigma protease inhibitor cocktail, Catalog # P-8340). Cardiomyocytes were isolated from adult rabbits of either sex weighing 2–3 kg as described previously [19]. Microsomes were isolated by differential centrifugation as previously described [19,20].

Culture of Caki-1, A172, and HEK293 cell lines. The human cell lines Caki-1 (kidney carcinoma), A172 (glioblastoma), and HEK293 were purchased from ATCC (Manassas, VA). Caki-1 and A172 cells were grown under conditions recommended by ATCC. HEK293 cells were grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA), and 100 U/ml penicillin and 100 μg/ml streptomycin (ATCC). RNA and microsomes were all isolated from cells that were 80% confluent and at least 24 h after passage.

Isolation of rabbit RPTC, culture conditions, and inhibitor treatment. Rabbit RPTC were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved conditions as previously described [21,22]. Confluent monolayers were treated with (*R*)- or (*S*)-BEL or acetonitrile control for 30 min, and then microsomes were harvested as described previously [21] for activity assays.

Isolation of (R)- and (S)-enantiomers of BEL. (*R*)- and (*S*)-enantiomers of BEL were isolated from racemic BEL (Calbiochem) using a chirex 3,5-dinitrobenzoyl-(*R*)-phenylglycine chiral HPLC column (Phenomenex, Torrance, CA) using previously published methods [17]. The column was equilibrated with hexane/dichloroethane/ethanol (150:15:1) and the optical enantiomers were eluted isocratically at 2 ml/min. Elution of (*R*)- and (*S*)-BEL was monitored by UV absorbance at 280 nm. Under these conditions the retention times (R_t) for (*R*)- and (*S*)-BEL differ by almost 1 min with the R_t for (*S*)-BEL being 11.1 and 12.2 min for (*R*)-BEL [17]. Peaks corresponding to these R_t were collected, dried under N_2, and stored at −20 °C. The concentration of each enantiomer was determined spectrophotometrically based on its UV absorbance ($\varepsilon = 6130\ cm^{-1}\ M^{-1}$ in acetonitrile [17]).

Immunoblotting and $iPLA_2\gamma$ antibody. We contracted Sigma–Genosys (The Woodlands, TX) to generate the anti-rabbit $iPLA_2\gamma$ antibody. Briefly, a peptide corresponding to the internal sequence, CEELYRKLGSDIFSQ, was conjugated to keyhole limpet hemocyanin and injected into two hens. Resultant antisera were used as the primary antibody. Equal amounts of ER protein were separated by SDS–PAGE and transferred to PVDF membranes. Membranes were incubated with the $iPLA_2\gamma$ antisera at a dilution of 1:1000 and then with the goat–anti-chicken horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were visualized by chemiluminescence detection on a ChemiImager 5500 imager (Alpha Innotech, San Leandro, CA).

Reverse-transcriptase-polymerase chain reaction. RNA was isolated using Trizol (Gibco-BRL, Frederick, MD). RT-PCR using total RNA isolated from human cells or tissue extracts was performed using primers designed against the sequence of group VIB PLA_2 reported in Mancuso et al. [3] (sense: 5′-ATTGATGGTGGAGGAACAAGG-3′, anti-sense: 5′-ATGGCCTGCCACATTTTATAC-3′). The RT step was performed at 50 °C for 30 min followed by 2 min at 92 °C to inactivate the RT. PCR was then performed with 35 cycles of 30 s at 72 °C, 90 s at 55 °C, and 30 s at 92 °C followed by a final extension step of 2 min at 72 °C. RT-PCR products were analyzed by agarose gel electrophoresis.

Cloning and determination of rabbit $iPLA_2\gamma$ nucleotide sequences. Rabbit kidney $iPLA_2\gamma$ clones were generated from a rabbit kidney cDNA library (Stratagene, La Jolla, CA) by PCR analysis using primers designed against the 5′ and 3′-ends of human $iPLA_2\gamma$ (sense: 5′-GCATACTCGAGTCACAATTTTGAA-AAGAATGGAAGTCC-3′, anti-sense: 5′-CATTCCTCTCCCTTTCACTGGATCCACATAGC C-3′) purchased from Integrated DNA Technologies (Coralville, IL). Negative controls for PCR included the absence of polymerase or cDNA. PCR products were separated and visualized using agarose gel electrophoresis and ethidium bromide staining. PCR products were isolated using Ultrafree-DA gel extraction columns (Millipore, Bedford, MA) and directly sequenced by automated fluorescence sequencing at the University of Georgia. Once full-length rabbit kidney $iPLA_2\gamma$ cDNA sequences were isolated they were cloned between *Eco*RI sites using the TOP TA Cloning Kit from Invitrogen following the manufacturer's instructions.

Rabbit myocyte total RNA was isolated using the Versagene Cell Kit (Gentra Systems, Carlsbad, CA). 5′ and 3′ cDNA ends were cloned into the pCR4-TOPO vector using the GeneRacer kit (Invitrogen) according to manufacturer's instructions. Sequences were determined by automated DNA sequencing at the St. Louis University, Department of Biochemistry, DNA Sequencing Facility. Oligo(dT) primed cDNA was synthesized from rabbit total RNA and used as a template for RT-PCR amplification of full-length rabbit heart $iPLA_2\gamma$. PCR conditions and oligos used were as follows: 1 cycle (94 °C, 2 min), 35 cycles (94 °C, 1 min; 55 °C, 1 min; and 72 °C, 5 min), 1 cycle (72 °C, 10 min); FOR5′-GCCTTGCATTCCGGTAAAGAACATG-3′, REV5′-GGGAACAG CAGATGATAAGTCAGAGCTAG-3′. PCR products were cloned

into the pCR-XL-TOPO vector and transformed into TOP10 chemically competent *Escherichia coli* (Invitrogen). Five independent kanamycin resistant clones were isolated and subjected to automated sequencing. Following assembly, a consensus sequence was generated using Vector NTI Suite 9.0 (Invitrogen).

Measurement of iPLA$_2$ activity. PLA$_2$ activity was determined under linear reaction conditions in microsomes as described previously [19]. Activity was measured using synthetic (16:0, [^{3}H]18:1) plasmenylcholine (100 μM) in the absence of Ca^{2+} (presence of 4 mM EGTA). For PLA$_2$ activity inhibition studies, rabbit, rat, and human cell microsomal samples were incubated with either a solvent control [DMSO <0.1% (v/v)], BEL, or MAFP for 5 or 10 min prior to the addition of the phospholipid substrate to initiate the reaction.

Protein determination. Protein determination was performed using the bicinchoninic acid assay method as described by Sigma.

Statistical analysis. Microsomes or cytosol isolated from rabbit or rat tissues or from one passage of human cell cultures represented one experiment ($n = 1$). The appropriate analysis of variance (ANOVA) was performed for each data set using SigmaStat statistical software. Individual means were compared using Fisher's protected least significant difference test with $P \leqslant 0.05$ being considered indicative of a statistically significant difference between mean values.

Results and discussion

Microsomes from rabbit and rat tissues, and human cells possess iPLA$_2$ activity

While Northern blot analysis of human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas mRNA demonstrated that these tissues express the full length 3.4-kb iPLA$_2\gamma$ mRNA, activity assays nor immunoblot analysis were performed to support these data [3]. ER-iPLA$_2$ activities in rabbit and rat tissues, and three different human cell lines were determined by the hydrolysis of (16:0, [^{3}H]18:1) plasmenylcholine substrates in the absence of Ca^{2+} (presence of 4 mM EGTA). ER-iPLA$_2$ activity was detected in all tissues tested (Fig. 1). Rabbit heart and brain contained higher ER-iPLA$_2$ activity than rabbit kidney, and rat brain activity was greater than that of rat kidney or heart (Fig. 1A). The human embryonic kidney cell (HEK293) microsomes had significantly more iPLA$_2$ activity than that of either the glioblastoma (A172) or kidney carcinoma (Caki-1) microsomes (Fig. 1B). Importantly, all tissues tested displayed ER-iPLA$_2$ activity. The significance of the differences in activity among tissues and species, and differences between normal and cancer cells has not been investigated and is beyond the scope of this study.

Identification of iPLA$_2\gamma$ mRNA in rabbit, rat, and human tissues

The above data indicated that rabbit and rat tissues and human cell lines have ER-iPLA$_2$ activity. However, these data do not indicate what specific iPLA$_2$ isoforms are expressed in these tissues. RT-PCR using total RNA isolated from rabbit tissues and primers designed against

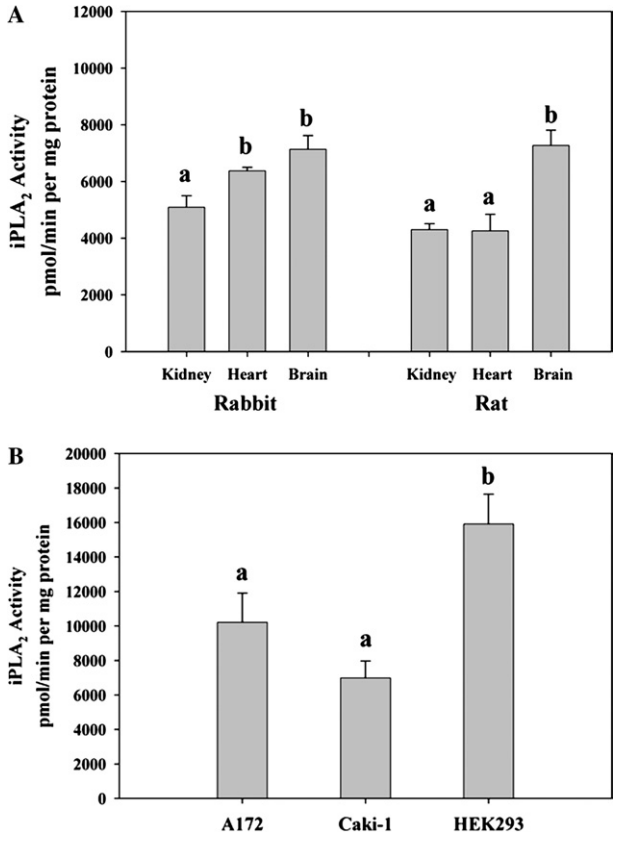

Fig. 1. Microsomal iPLA$_2$ activity in rabbit and rat tissues, and human cells. Microsomes were isolated from rabbits and rats and from human cells, and iPLA2 activity was measured using (16;0, [^{3}H]18:1) plasmenylcholine (100 mM) in the presence of 4mM EGTA. (A) Microsomal iPLA$_2$ activity in rabbit and rat tissues. (B) Microsomal iPLA$_2$ activity in human cells. Values are means ± SEM of at least three separate experiments. Means with different subscripts within groups are significantly different from each other, $P < 0.05$.

the human iPLA$_2\gamma$ sequence demonstrated the expression of a 475 bp cDNA product in kidney and heart (Fig. 2A). We consistently detected a faint band using RNA from rabbit brain. The 475 bp product also was detected in rat kidney, heart, and brain (Fig. 2B). The presence of two bands raises the possibility that splice variants of iPLA$_2\gamma$ are transcribed in rat. Each of the three human cell lines also expressed iPLA$_2\gamma$ (Fig. 2C). In conjunction with the data presented in Fig. 1, the RT-PCR results suggest that ER-iPLA$_2$ activity in each tissue tested is mediated by iPLA$_2\gamma$.

Rabbit iPLA$_2\gamma$ shares 88% homology with human iPLA$_2\gamma$

Using the iPLA$_2\gamma$ cDNA generated from total rabbit cardiomyocyte RNA (GenBank Accession No. AY738591) the amino acid sequence was determined. A comparison of the rabbit heart iPLA$_2\gamma$ and the human iPLA$_2\gamma$ (GenBank Accession No. NM015723) amino acid sequence is presented in Fig. 3. The conserved

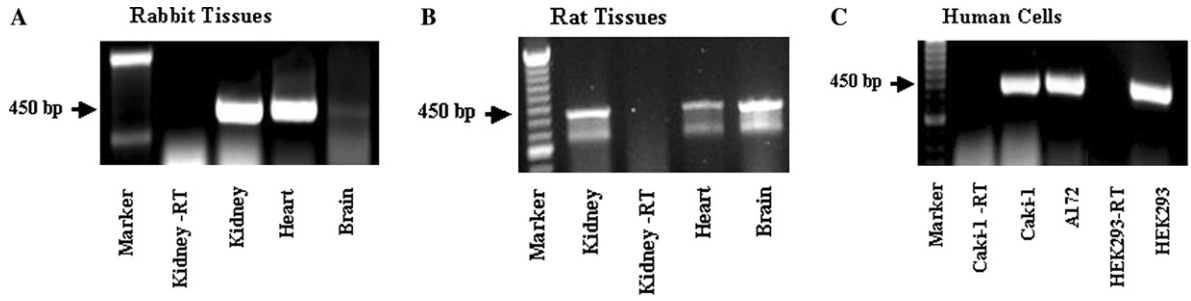

Fig. 2. Presence of iPLA$_2\gamma$ in rabbit and rat tissues, and human cells. Total RNA was isolated from rabbit and rat tissues, or human cell lines, and subjected to RT-PCR. Expression of iPLA$_2\gamma$ cDNA products in reactions performed using total RNA isolated from: (A) rabbit tissues, (B) rat tissues, and (C) human cell lines. Controls lacked RT (−RT). Results are typical of at least three separate RNA isolations from three separate rabbits or rats, or separate passages of human cells.

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Rabbit:   1   MSIWLTIDICIYLLSWARWLCGKWRSKQLWLVCSPWWCWKIRWVSLQRGLWPWKVRCKWT  60
Human:    1   MSIWLTVDIYIYLLSWARSVCGKQRSKQLYFLFSPKWYWRISWISLQRGFWTWIIRCKWT  60

Rabbit:  61   KSETWSCSKWYYSPSWWGLWIGILKLSTSAPKGLTKVSIRMSRIKSTLWSVSKAVFGSQW 120
Human:   61   KSEAWSCSKWCYSPSWWGLWIGILKLSTSAPKGLTKVWICMSRIKSTLWSVSKAVFGWQW 120

Rabbit:  121 EMISRLAQFKPSSRILRKVSDSGWLKQESIKQAIRSLKKYSDKSTEKSPVPEGRWWIIDK 180
Human:   121 EMISRLAQFKPSSQILRKVSDSGWLKQKWIKQAIKSLKKYSDKSAEKSPFPEEKSWIIDK 180

Rabbit:  181 EDDIGKQSLFWYTGWITTKFGESFYFLSWWINSYFKRAEKMSQDKEWSHFQEKSELEGKK 240
Human:   181 EEDIGKRSLFWYTSSITTKFGDSFYFLSWWINSYFKRKEKMSQQKEWEWFRDKSELEDKK 240

Rabbit:  241 VEEGKSSSLDPGILTSQADKPDPKSSAGTMDKATSPSGTPESLPISTKQSIAWFLSRPTE 300
Human:   241 VEEGKLRSPDPGIL---AYKPGSES-VWTVDKPTSPSAIPDVLQVSTKQSIAWFLSRPTE 296

Rabbit:  301 GVQALVGGYIGGLVPKLKYDSKSQAEEQEEPAKSEPAGSKDKTVEEKKHLSLQREKIIAR 360
Human:   297 GVQALVGGYIGGLVPKLKYDSKSQSEEQEEPAKTDQAVSKDRWAEEKKRLSLQREKIIAR 356

Rabbit:  361 VSIDWRTRALVQALRRTADPKLCITRVEELTFHLLEFPEGKGVAVKERLIPCLLRLRQMK 420
Human:   357 VSIDWRTRALVQALRRTTDPKLCITRVEELTFHLLEFPEGKGVAVKERIIPYLLRLRQIK 416

Rabbit:  421 DETLQAAVREILALIGYVDPVKGRGIRILTIDGGGTRGVVALQTLRKLVELTQKPVHQLF 480
Human:   417 DETLQAAVREILALIGYVDPVKGRGIRILSIDGGGTRGVVALQTLRKLVELTQKPVHQLF 476

Rabbit:  481 DYICGVSTGAILAFMLGLFHLPLDECEELYRKLGSDIFSQWVIVGTVKMSWSHAFYDSQT 540
Human:   477 DYICGVSTGAILAFMLGLFHMPLDECEELYRKLGSDVFSQWVIVGTVKMSWSHAFYDSQT 536

Rabbit:  541 WEKILKERMGSALMIETARMPMCPKVAAVSTIVWRGSTPKAFVFRNYGHFPGSQSHYLGG 600
Human:   537 WEWILKDRMGSALMIETARMPTCPKVAAVSTIVWRGITPKAFVFRNYGHFPGIWSHYLGG 596

Rabbit:  601 CQYKMWQAIRASSAAPGYFAEYALGWDLHQDGGLLLWNPSALAMHECKCLWPDAPLECIV 660
Human:   597 CQYKMWQAIRASSAAPGYFAEYALGWDLHQDGGLLLWNPSALAMHECKCLWPDVPLECIV 656

Rabbit:  661 SLGTGRYESDVRNWTTYTSLKTKLSWVIWSATDTEEVHIMLDGLLPPDTYFRFWPVMCEW 720
Human:   657 SLGTGRYESDVRNTVTYTSLKTKLSWVIWSATDTEEVHIMLDGLLPPDTYFRFWPVMCEW 716

Rabbit:  721 IPLDESRWEKLDQLQLEGSKYIERWEHKMKKVAKILSQEKTTLQKIWDWIKLKTDMYEGL 780
Human:   717 IPLDESRWEKLDQLQLEGLKYIERWEQKMKKVAKILSQEKTTLQKIWDWIKLKTDMYEGL 776

Rabbit:  781 PFFSKL 786
Human:   777 PFFSKL 782
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Fig. 3. Comparison of rabbit and human iPLA$_2\gamma$ amino acid sequences. NCBI Blast of the rabbit heart amino acid sequence (GenBank Accession No. AY738591) revealed 88% identity between rabbit heart and human (GenBank Accession No. NM015723) iPLA$_2\gamma$ sequences. The lipase site is boxed. The rabbit amino acid sequence used to develop the iPLA$_2\gamma$ antibody is underlined.

lipase site is boxed and the rabbit sequence used to develop a rabbit iPLA$_2\gamma$ anti-peptide antibody is underlined. The rabbit heart and rabbit kidney (GenBank Accession No. AY739721) iPLA$_2\gamma$ amino acid sequences display 88% and 80% homology with the human iPLA$_2\gamma$ sequence, respectively. The regions surrounding and including residues involved in the catalytic activity of human iPLA$_2\gamma$ (Ser-483, Asp-627, and Gly-Gly-Xaa-Arg-450–453 [23]) share 100% homology, suggesting that rabbit and human iPLA$_2\gamma$ possess similar lipase activity (Fig. 3).

iPLA$_2\gamma$ protein is expressed in rabbit microsomes

Using the rabbit iPLA$_2\gamma$ antibody, immunoreactive proteins of approximately 88 kDa were detected in microsomal fractions of rabbit kidney and heart (Fig. 4). Similar to RT-PCR results, a faint band of similar size was

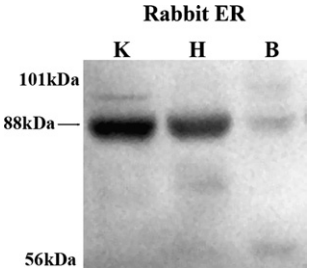

Fig. 4. Immunoblot analysis of iPLA$_2\gamma$ expression in rabbit tissues. Microsomes were isolated from rabbit kidney cortex (K), heart (H) or brain (B), separated by SDS–PAGE, and transferred to a PVDF membrane, and iPLA$_2$ expression was determined using a polyclonal anti-peptide antibody to rabbit iPLA$_2\gamma$. Each lane contained 60 μg. Blot is representative of at least three separate experiments.

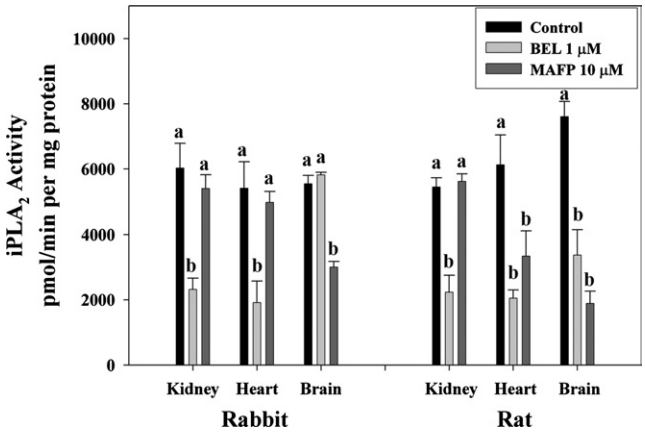

Fig. 5. Differential effect of racemic BEL and MAFP on ER-iPLA$_2$ activity in rabbit and rat tissues. Microsomes harvested from rabbits and rats were pretreated either with solvent control (DMSO), BEL or MAFP for 5 min, and iPLA$_2$ activity was measured using (16:0, [^{3}H]18:1) plasmenylcholine (100 μM) in the presence of 4 mM EGTA. Values are means ± SEM of at least three separate experiments. Means with different subscripts within each group are significantly different from each other, $P < 0.05$.

routinely detected in rabbit brain microsomes. The rabbit iPLA$_2\gamma$ antibody did not detect a similar sized protein in the rat or human tissues (data not shown). This is probably due to differences in the amino acid sequence between species. Based on the nucleotide sequence analysis of Mancuso et al. [3], iPLA$_2\gamma$ contains four possible translation initiation codons, which correspond to protein products of 88, 77, 74, and 63 kDa. The rabbit microsomal iPLA$_2\gamma$ is approximately 88 kDa, which is the long isoform of iPLA$_2\gamma$. In contrast, rat liver cells express the 63 kDa isoform of iPLA$_2\gamma$ [4]. Tissue- and/or species-specific promoters responsible for expression of different sized isoforms in the rat liver, rabbit kidney and heart, are currently under study. While similar sized immunoreactive proteins have been detected in these tissues with an iPLA$_2\beta$ antibody [11,13], the data presented in Fig. 4 and inhibition studies presented below demonstrate that the observed ER-iPLA$_2$ activity is due to iPLA$_2\gamma$ in rabbit kidney and heart.

Inhibition with BEL and MAFP demonstrates differential expression of iPLA$_2$ isoforms across species and tissues

As discussed above, each iPLA$_2$ has a distinct inhibition profile. BEL inhibits iPLA$_2\gamma$ and iPLA$_2\beta$ but not cPLA$_2\gamma$ [3,15,7]. MAFP inhibits cPLA$_2\gamma$ [7] and cytosolic iPLA$_2\beta$ [16], but the effects of MAFP on iPLA$_2\gamma$ and membrane-associated iPLA$_2\beta$ activity are not known. Rabbit kidney and heart microsomal iPLA$_2$ activity was significantly inhibited by BEL but not by MAFP (Fig. 5). Similar results have been previously reported [11,13]. Rat kidney microsomal iPLA$_2$ activity also was inhibited by BEL but not by MAFP (Fig. 5). In contrast, rabbit brain microsomal iPLA$_2$ activity was insensitive to BEL and sensitive to MAFP. iPLA$_2$ activity in rat heart and brain microsomes was significantly inhibited by both BEL and MAFP. These data illustrate the differential expression of ER-iPLA$_2$ isoforms across species and tissues, and suggest that rabbit and rat kidney and rabbit heart microsomes possess iPLA$_2\gamma$ and not cPLA$_2\gamma$. Further, we suggest that rabbit brain ER-iPLA$_2$ activity is mediated by cPLA$_2\gamma$ alone. Finally, rat heart and brain may contain iPLA$_2\beta$, iPLA$_2\gamma$, and/or cPLA$_2\gamma$. Additional studies are required to determine which isoforms are present and active in these tissues.

iPLA$_2\gamma$ is responsible for microsomal iPLA$_2$ activity in rabbit kidney and heart and HEK293 cells

(*R*)- and (*S*)-BEL were used to confirm that iPLA$_2\gamma$ is responsible for the observed activity in rabbit kidney and heart, and HEK293 cells. Primary cultures of rabbit RPTC were treated with solvent control or increasing concentrations of (*R*)-BEL or (*S*)-BEL prior to isolation of RPTC microsomes for iPLA$_2$ activity assays. Concentration dependent inhibition of RPTC microsomal iPLA$_2$ activity was observed with (*R*)-BEL, but not (*S*)-BEL, demonstrating that iPLA$_2\gamma$ is responsible for iPLA$_2$ activity in RPTC microsomes (Fig. 6A). A similar dose–response to (*R*)-BEL was observed for iPLA$_2$ activity in microsomes isolated from rabbit cardiomyocytes (Fig. 6B). While (*S*)-BEL inhibits approximately 40% of the activity at 10 μM, this effect is significantly less than (*R*)-BEL and similar to previously published results with (*S*)-BEL and iPLA$_2\gamma$ [17]. HEK293 microsomal iPLA$_2$ activity is sensitive to inhibition with racemic BEL (data not shown) and (*R*)-BEL, but not (*S*)-BEL (Fig. 6C). The selective inhibition of ER-iPLA$_2$ activity in rabbit kidney and heart and HEK293 cells by (*R*)-BEL and not (*S*)-BEL demonstrates that iPLA$_2\gamma$ is responsible for observed activity in these cells.

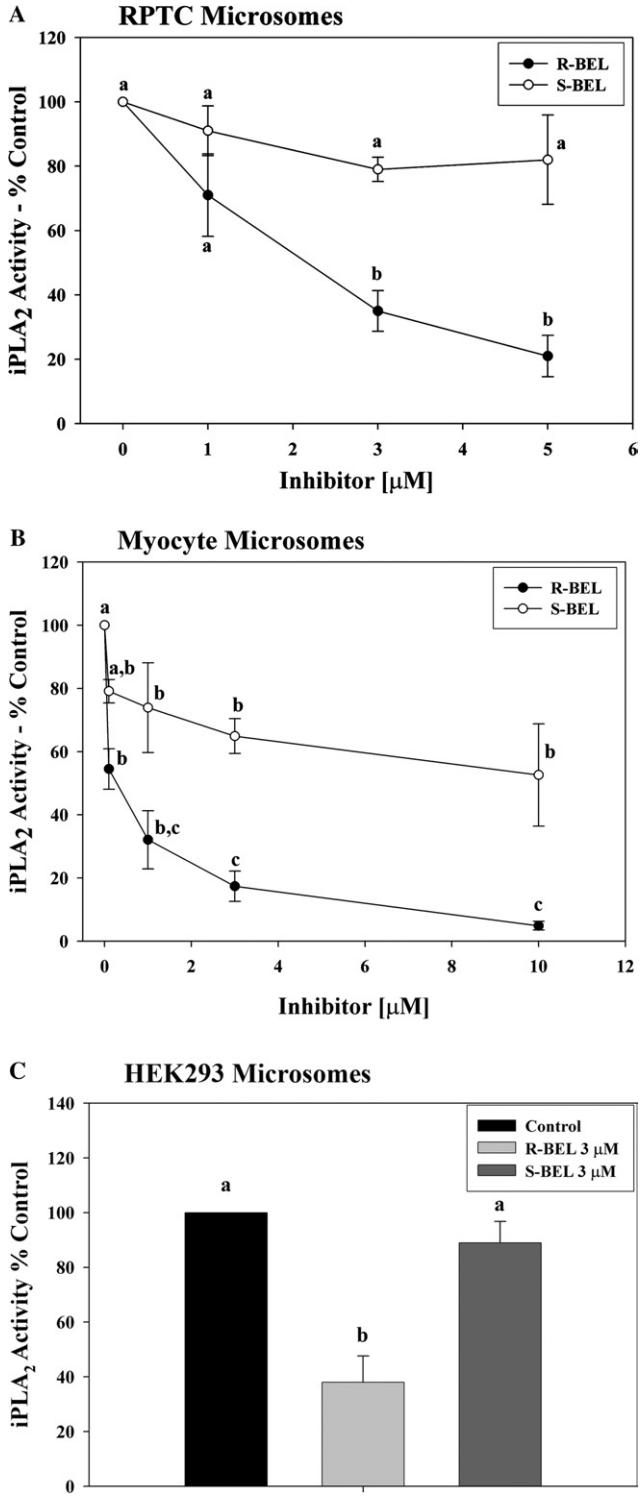

Fig. 6. Differential effect of *R* and *S* enantiomers of BEL on microsomal $iPLA_2$ activity in RPTC, ventricular myocyte microsomes or in microsomes isolated from HEK293 cells. RPTC (A) were treated for 30 min with either (acetonitrile), *R*-BEL or *S*-BEL. Rabbit cardiomyocyte (B) and HEK293 (C) microsomes were isolated and incubated with solvent control (acetonitrile), *R*-BEL or *S*-BEL for 10 min prior to activity assays. $iPLA_2$ activity was measured using plasmenylcholine (16:0, [^{3}H]18:1) or substrates in the presence of 4 mM EGTA. Values are means ± SEM of at least three separate experiments. Means with different subscripts are significantly different from each other, $P < 0.05$.

PLA_2 enzymes have been hypothesized to play a part in the repair of oxidized membranes [24]. In support of the hypothesis, glutathione peroxidase can effectively detoxify free fatty acids that are peroxidized, but only after they have been released from phospholipids by PLA_2 [25,26]. Further, in vitro studies have demonstrated that PLA_2 preferentially hydrolyze oxidized fatty acids from membranes [27,28]. The lysophospholipids generated by PLA_2 action can participate in the Lands cycle [29] and be reacylated to maintain membrane integrity. Thus, evidence exists that supports the hypothesis ER-$iPLA_2$ is involved in the protection of the ER membrane from lipid peroxidation induced by oxidative stress.

We have demonstrated ER-$iPLA_2$ activity across species and tissues, and detected the presence and activity of $iPLA_2\gamma$ in microsomes of rabbit kidney and heart, rat kidney, and HEK293 cells. Based on RT-PCR and inhibition assays $iPLA_2\gamma$ is also likely expressed in rat heart and brain along with other isoforms. In conjunction with our previous findings that demonstrate inhibition of microsomal $iPLA_2$ prior to oxidant treatment potentiates lipid peroxidation and cell death, these data suggest that $iPLA_2\gamma$ is the isoform of $iPLA_2$ involved in protecting renal and heart cells from oxidative stress.

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