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The effect of inhibition of Ca^{2+}-independent phospholipase A_2 on chemotherapeutic-induced death and phospholipid profiles in renal cells

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Abstract

We demonstrate that cells derived from primary cultures of rabbit proximal tubules (RPTC), human embryonic kidney (HEK293) and human kidney carcinomas (Caki-1) express microsomal Ca^{2+}-independent phospholipase A_2 ($iPLA_2\gamma$) and cytosolic Ca^{2+}-independent phospholipase A_2 ($iPLA_2\beta$). Inhibition of $iPLA_2$ activity in these cells using the $iPLA_2$ inhibitor bromoenol lactone (BEL) (0–5.0 μM) for 24 h did not induce cell death as determined by annexin V and propidium iodide (PI) staining. However, BEL treatment prior to cisplatin (50 μM) or vincristine (2 μM) exposure reduced apoptosis 30–50% in all cells tested (RPTC, HEK293 and Caki-1 cells). To identify the phospholipids altered during cell death electrospray ionization-mass spectrometry and lipidomic analysis of HEK293 and Caki-1 cells was performed. Cisplatin treatment reduced 14:0–16:0 and 16:0–16:0 phosphatidylcholine (PtdCho) 50% and 35%, respectively, in both cell lines, 16:0–18:2 PtdCho in Caki-1 cells and increased 16:1–22:6 plasmenylcholine (PlsCho). BEL treatment prior to cisplatin exposure further decreased 14:0–16:0 PtdCho, 16:0–16:1 PlsCho and 16:0–18:1 PlsCho in HEK293 cells, and inhibited cisplatin-induced increases in 16:1–22:6 PlsCho in Caki-1 cells. Treatment of cells with BEL prior to cisplatin exposure also increased the levels of several arachidonic containing phospholipids including 16:0–20:4, 18:1–20:4, and 18:0–20:4 PtdCho, compared to cisplatin only treated cells. These data demonstrate that inhibition of $iPLA_2$ protects against chemotherapeutic-induced cell death in multiple human renal cell models, identifies specific phospholipids whose levels are altered during cell death, and demonstrates that alterations in these phospholipids correlate to the protection against cell death in the presence of $iPLA_2$ inhibitors.

Keywords: Phospholipase A_2; Apoptosis; Phospholipids; Electron ionization-mass spectrometry

1. Introduction

Phospholipase A_2 (PLA_2s) are esterases that cleave glycerophospholipids at the *sn*-2 position, releasing a fatty acid and a lysophospholipid [1]. Historically, PLA_2 were classified into three groups: secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), and Ca^{2+}-independent PLA_2 ($iPLA_2$). Since 1997, PLA_2 have been categorized according to their nucleotide sequence into at least 14 different groups encompassing over 22 different PLA_2 [2–4]. Several members of these groups mediate numerous cellular functions. However, the cellular functions of newer PLA_2 are not well characterized, especially those belonging to $iPLA_2$.

Group VI PLA_2 are referred to as $iPLA_2$, or those that do not require Ca^{2+} for their activity or translocation to membranes. Group VI PLA_2 includes Group VIA ($iPLA_2\beta$) and Group VIB ($iPLA_2\gamma$). $iPLA_2\beta$ is predominately expressed in the cytosol of cells in which it is found and can exist in two different splice variants, denoted Group VIA-1 and VIA-2 [5]. In contrast to $iPLA_2\beta$, $iPLA_2\gamma$ has only been identified bound to membranes, including those of the peroxisomes [6], mitochondria [7] and the endoplasmic reticulum [8–10].

$iPLA_2\beta$ is expressed in several tissues from several species including those from rat, rabbit and human brain, heart, lung and kidney [2,5,11–14]. In contrast, the expression of $iPLA_2\gamma$ is not as well characterized. Endoplasmic reticulum-bound $iPLA_2$ activity has been reported in heart,

Abbreviations: PLA_2, phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; BEL, bromoenol lactone; RPTC, rabbit renal proximal tubule cell(s); PtdCho, phosphatidylcholine; PlsCho, plasmenylcholine

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arteriole endothelial cells and primary cultures of rabbit renal proximal tubular cells [9,15,16]. We recently demonstrated that iPLA$_2\gamma$ is expressed in rabbit and rat kidney heart and brain as well as in human glioblastomas [17]. We also demonstrated that (E)-6-(bromoethylene)-3-(1-naphthaleny)-2*H*-tetrahydropyran-2-one (bromoenol lactone or BEL), a well characterized iPLA$_2$ inhibitor [9,18], decreased iPLA$_2$ activity in rabbit kidney and heart microsomes, rat kidney, heart and brain microsomes, and RPTC and HEK293 cells with a IC_{50} of 2–3 μM.

Little is known about the role of iPLA$_2$ in kidney and cancer cell death, and even less is known about the role of microsomal bound-iPLA$_2\gamma$. Our previous studies suggest that inhibition of rabbit RPTC iPLA$_2\gamma$ using BEL, decreased cisplatin-induced apoptosis via a mechanism that involved a decrease in caspase 3 activation downstream of p53 nuclear translocation [19]. However, the expression of iPLA$_2\gamma$ and the ability of iPLA$_2$ inhibitors to alter chemotherapeutic-induced cell death in human renal cell models has not been determined.

Recent studies from our laboratory demonstrate that oxidant-induced necrotic cell death in rabbit renal cells correlates to the loss of specific phospholipids [20]. These studies also demonstrated that inhibition of iPLA$_2$ with BEL further decreased these phospholipids. However, the ability of iPLA$_2$ inhibition to alter the phospholipid profile in human renal cells, under both normal (non-stressed) and cell death conditions, have not been determined. Further, the exact phospholipids altered during apoptosis in renal cells have never been determined. This study addresses the hypothesis that inhibition of iPLA$_2$ alters chemotherapeutic-induced apoptosis in two different models of human renal cells (HEK293 and Caki-1 cells) and uses lipidomics to identify the phospholipids involved.

2. Materials and methods

2.1. Reagents

Female New Zealand white rabbits (1.5–2.0 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). HEK293 (human embryonic kidney 293) and Caki-1 (kidney carcinoma), cell growth medium, and fetal bovine serum were purchased from American Type Culture Collection (Mannasas, VA). Penicillin and streptomycin were obtained from GibcoBRL. Annexin-V FITC was obtained from R&D Systems (San Diego, CA). Cellstripper was obtained from Mediatech, Inc. (Herndon, VA). Propidium iodide (PI) and bromoenol lactone (BEL) were obtained from Cayman Chemical Co. (Ann Arbor, MI). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

2.2. Primary culture of RPTC and renal cell lines

RPTC were isolated from New Zealand white rabbits by the iron oxide perfusion method as previously described [21,22]. HEK293 and Caki-1 were purchased from ATCC

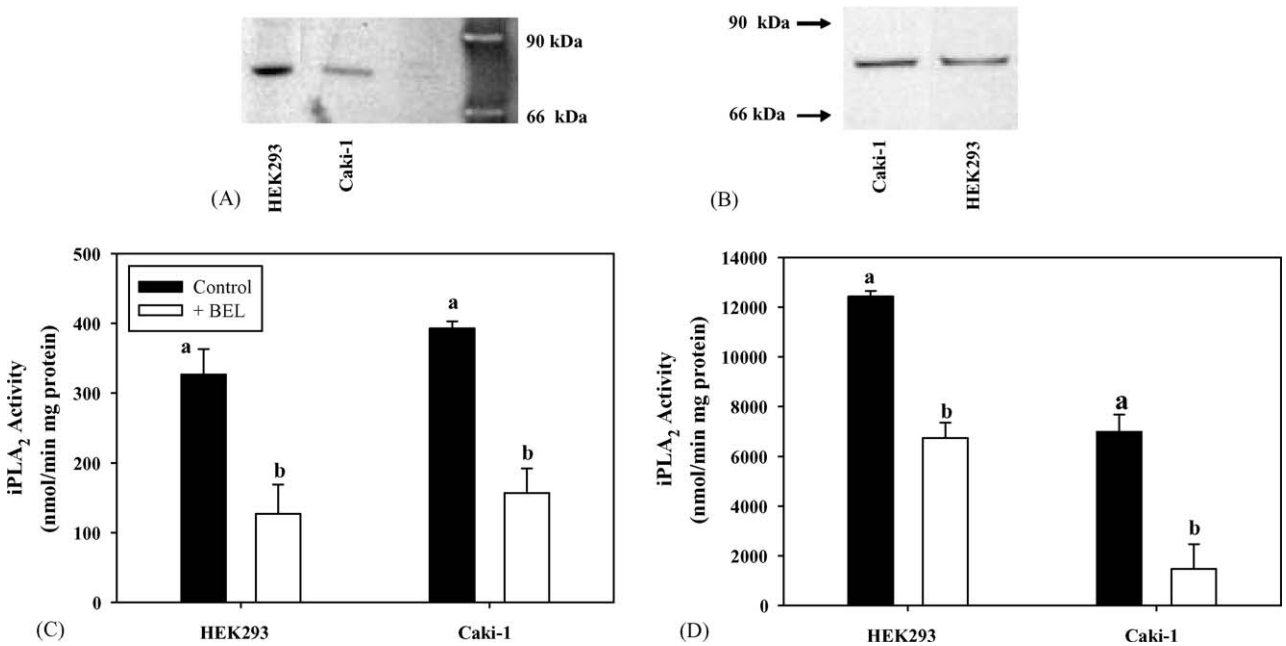

Fig. 1. Expression and activity of iPLA$_2$ isoforms in renal cells. (A) Immunoblot analysis of microsomes isolated from the indicated cells using a peptide anti-iPLA$_2\gamma$ antibody. (B) Immunoblot analysis of cytosol from the indicated cells using a polyclonal anti-iPLA$_2$((antibody). (C) Cytosolic and (D) microsomal iPLA$_2$ activity in renal cells as based on plasmenylcholine and phosphatidylcholine cleavage in the presence of EGTA (4 mM). Data in (C) and (D) are represented as the average ± the S.E. of at least three separate experiments. Means with different subscripts are significantly different from each other ($P < 0.05$).

(Manassas, VA) and grown under the recommended conditions. These cells were chosen for study as they represent two different human renal cell lines that are epithelial in origin. Further, previous studies demonstrate that $iPLA_2$ isoforms are expressed in HEK293 cells [17], and have determined the time- and concentration-dependence of cisplatin and vincristine-induced apoptosis [23]. Experiments using RPTC were performed on confluent monolayers (day 6 after isolation). Experiments using other cell lines were performed when cells were 80% confluent and 24 h after passage. All cells were treated with BEL for 30 min prior to treatment with chemotherapeutics or solvent control.

2.3. Immunoblot analysis

Samples (20 μg) were mixed with SDS-sample buffer, heated to 70 °C for 10 min, separated under reducing conditions on a 12% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane with 3% (w/v) bovine serum albumin in TBS buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) overnight at room temperature. Membranes were then incubated with a polyclonal rabbit anti-$iPLA_2\beta$ antibody (Cayman Chemical Co.) or an peptide antibody against $iPLA_2\gamma$ (CZSKYIERNEHKMKKVAK) for 2 h. Goat anti-rabbit IgG conjugated with horse-radish peroxidase was used as a secondary antibody for $iPLA_2\beta$, while an human anti-chicken secondary antibody was used for $iPLA_2\gamma$ [17]. Bands were detected by enhanced chemiluminescence (Amersham Biosciences, Inc. Piscataway, NJ).

2.4. Measurement of $iPLA_2$ activity

PLA_2 activity was determined under linear conditions in microsomes and cytosol as previously described [24]. Activity was measured using synthetic (16:0, [^{3}H]18:1) plasmenylcholine and phosphatidylcholine substrates (100 μM) in the absence of Ca^{2+} (presence of 4 mM EGTA). These radiolabeled substrates were synthesized as previously described [25].

2.5. Measurement of annexin V and PI staining

Annexin V and PI staining were determined using flow cytometry as previously described [26–28] with modifications [29]. Briefly, media was removed, cells washed twice with PBS and incubated in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) containing annexin V-FITC (2.5 μg/ml) and PI (25 μg/ml) for 10 min. Cells were washed three times in binding buffer, released from the monolayers using a rubber policeman and staining quantified using a Becton Dickinson *Facs*Calibur flow cytometer. For each measurement 10,000 events were counted.

2.6. Characterization and quantitation of cellular phospholipids using electrospray ionization-mass spectrometry (ESI-MS)

Cellular phospholipids were extracted using chloroform and methanol according to the method of Bligh and Dyer [30] at 4 °C. Lipid extracts were dried under argon, and lipid phosphorus was quantified using malachite green [31]. Mass spectrometry analysis was performed essentially as previously described [32]. Lipid extracts from cells were analyzed using a LCT Premier time of flight mass spectrometer (Waters, Milford, MA) equipped with an electrospray ion source. 5 μl of sample (500 pmol/ml) dissolved in chloroform: methanol (2:1, v/v) was introduced, by means of a flow injector, into the ESI chamber at a flow rate of 2 ml/min. The elution solvent was acetonitrile: methanol: water (2:3:1, v/v/v) containing 0.1% (w/v) ammonium formate

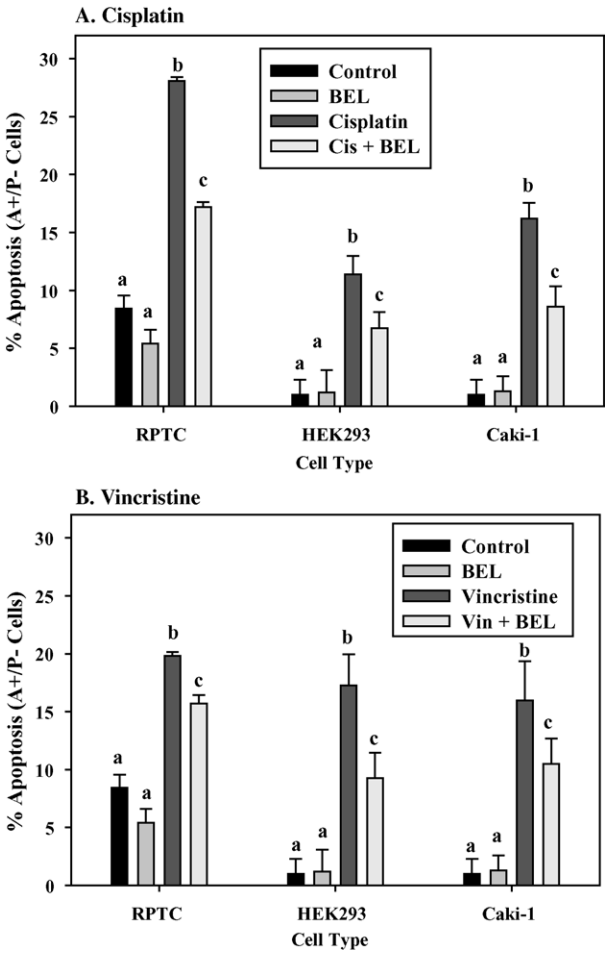

Fig. 2. Effect of $iPLA_2$ inactivation on chemotherapeutic-induced renal cell death. Confluent RTPC or cell lines were treated with 2.5 μM BEL for 30 min prior to exposure to 50 μM cisplatin (A) or 2 μM vincristine (B) for 24 h. After treatment, cells were isolated and prepared for measurement of annexin V and PI staining using flow cytometry. Data are represented as the mean ± the S.E. of at least three separate experiments. Means with different subscripts are significantly different from each other ($P \leq 0.05$).

(pH 6.4). The mass spectrometer was operated in the positive ion scan mode. The nitrogen drying gas flow rate was 10 l/min, and its temperature was 80 °C. The capillary voltage was set at 2.5 kV, and the cone voltage at 30 V. Identification of individual molecular species was based on theoretical monoisotopic mass values as given in previous publications in similar cell lines [32,33]. In accordance with other studies [32,33], phospholipids comparisons are relative to the expression of the most abundant phospholipid in each sample, which corresponded to a *m/z* ratio of 760 ((34:1 (16:0–18:1)) PtdCho.

2.7. Protein determination

Protein concentrations were determined using the bicinchonic acid assay method as described by Sigma.

2.8. Statistical analysis

RPTC isolated from one rabbit represented one experiment ($n = 1$). Cells isolated from a distinct passage of HEK293 or Caki-1 cells represented one experiment ($n = 1$). Data are represented as the average ± the S.E.M. of at least three separate experiments ($n = 3$). The appropriate analysis of variance (ANOVA) was performed for each data set using SAS software. Individual means were compared with $P < 0.05$ being considered indicative of a statistically significant difference between mean values.

3. Results

3.1. Expression of iPLA$_2\beta$ and iPLA$_2\gamma$ in human renal cell lines

Immunoblot analysis performed using a peptide antibody against iPLA$_2\gamma$ demonstrated the presence of a 85 kDa protein in the microsomal fractions of all cells tested (Fig. 1A). Immunoblot analysis using a polyclonal antibody to iPLA$_2\beta$ resulted in the detection of a ~80-kDa band in cytosolic fractions of all the cell lines tested (Fig. 1B). Both cytosolic and microsomal iPLA$_2$ activity were detected in both cultures and treatment of cells with 2.5 μM BEL for 30 min significantly decreased this activity (Fig. 1C and D). These data demonstrate that both iPLA$_2\beta$ and iPLA$_2\gamma$ are expressed in multiple human kidney cells.

3.2. Effect of iPLA$_2$ inactivation on chemotherapeutic-induced apoptosis in renal cells

Previous time- and concentration-dependent studies demonstrated that exposure of RTPC to 50 μM cisplatin or 2 μM vincristine for 24 h resulted in maximal RPTC apoptosis [19,23,29]. Previous studies also determined the time- and concentration-dependency of cisplatin and vincristine-induced cell death in the currently used cell models [23]. Based on these studies, 50 μM cisplatin and 2 μM vincristine were used to study the effect of iPLA$_2$ inhibition on chemotherapeutic-induced renal cell death. As previously

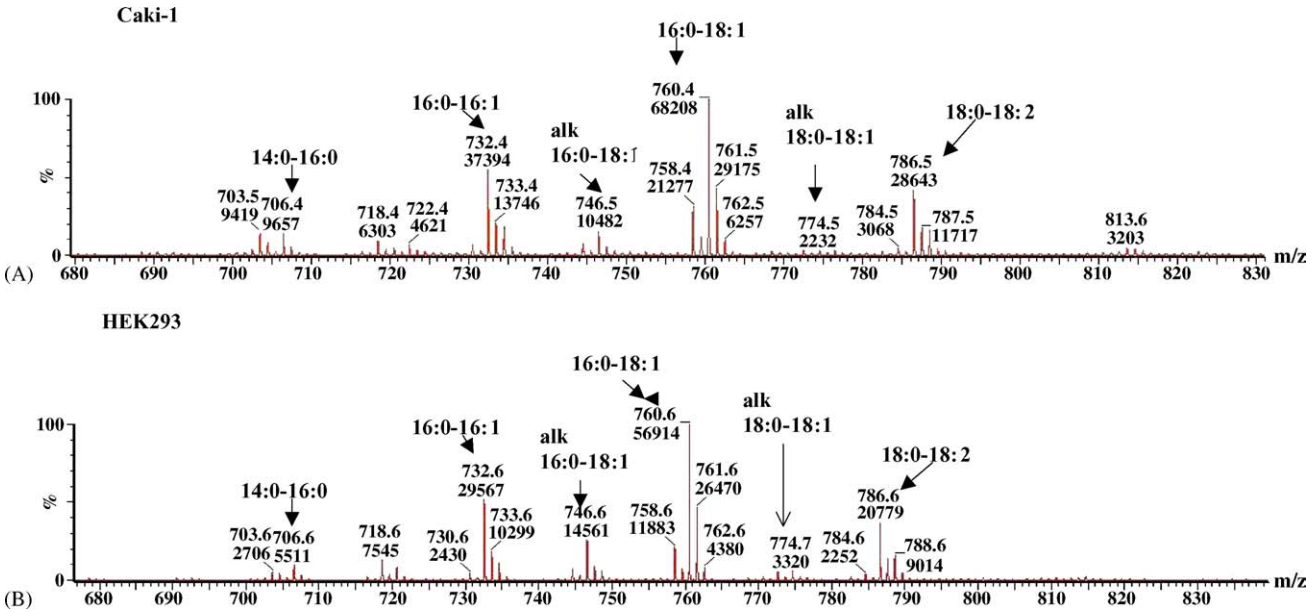

Fig. 3. ESI-MS analysis of phospholipids in HEK293 cells and Caki-1 cells. Total lipids from confluent Caki-1 (A) and HEK293 (B) cells were extracted by the Bligh–Dyer method, and individual phospholipids separated and quantified as described in the methods. For each experiment 5 μl of sample (500 pmol/μl) was analyzed. Above spectra are representative of data collected in the positive ion scan mode. Alk represents plasmenylcholine while all other lipids are phosphatidylcholine. In both cell types the most abundant phospholipid was 16:0–18:1 PtdCho.

Table 1
Select phospholipids detected in HEK293 and Caki-1 cells[a]

Phospholipid	*m/z*	Phospholipid	*m/z*
14:0–16:0[b] PtdCho	706.6	16:0–18:0 PtdCho	762.7
16:0–16:1 PlsCho	718.7	18:0–18:1 PtdCho	774.6
16:0–16:1 PtdCho	732.6	16:1–20:4 PtdCho	780.6
16:0–16:0 PtdCho	734.6	18:1–18:2 PtdCho	786.7
16:0–18:1 PlsCho	746.7	16:0–20:1 PlsCho	788.7
16:0–18:0 PlsCho	748.6	18:0–18:0 PtdCho	790.7
16:0–18:2 PtdCho	758.7	20:1–22:4 PlsCho	850.8
16:0–18:1 PtdCho	760.7	22:0–22:6 Plscho	876.7[c]

[a] Identification of individual molecular species was based on theoretical monoisotopic mass values as shown in this table.
[b] Designates carbon length and number of double bonds in the *sn*-1 and *sn*-2 fatty acids, respectively.
[c] May also represent 16:1–22:7 PtdCho.

described, treatment of RPTC with BEL (2.5 μM) for 30 min prior to cisplatin exposure for 24 h significantly decreased annexin V staining (Fig. 2A). Decreases in annexin V staining were not accompanied by increases in PI staining and BEL alone did not alter annexin V or PI staining at similar concentrations (Fig. 2). Pretreatment of RPTC with BEL also decreased vincristine-induced annexin V staining (Fig. 2B). Similar results were seen in HEK293 and Caki-1 cells (Fig. 2A and B).

3.3. Lipidomic analysis of the effect of $iPLA_2$ inactivation on chemotherapeutic-induced cell death

We previously identified specific phospholipids targeted by oxidants during renal oncotic/necrotic cell death using HPLC analysis [20]. However, the exact phospholipids altered during chemotherapeutic-induced renal cell death and the effect of $iPLA_2$ inactivation on these phospholipids has never been determined. It is well established that apoptosis in numerous cells is mediated by the release of arachidonic acid from phospholipids via the action of PLA_2 [34–38], but the source of this arachidonic acid and the role of $iPLA_2$ in these processes are not known. Hence, lipidomic analysis was carried out in HEK293 and Caki-1 cells, in the presence and absence of chemotherapeutics and BEL, using electrospray-ionization mass-spectrometry (ESI-MS). ESI-MS offers several advantages over HPLC, including the fact that a lower amount of sample is needed for analysis and multiple head groups can be analyzed in one run. Further, the increased sensitivity of ESI-MS allows for the analysis of phospholipids whose abundance is as low as 1% of total cellular phospholipids [32]. The typical ESI-MS spectra for phospholipids extracted from HEK293 and Caki-1 cells are presented in Fig. 3. Phospholipid species detected in both cell types are listed in Table 1 along with their respective *m/z* ratio. Comparison of phospholipid profiles demonstrated that the expression of at least 7 phospholipids differed between HEK293 and Caki-1 cells (Fig. 4). Of these phospholipids four were plasmenylcholines (PlsCho) (16:0–18:1 PlsCho, 16:0–18:2 PlsCho, 20:1–22:4 PlsCho, and 22:0–22:6 PlsCho) and two were phosphatidylcholines (PtdCho) (18:0–18:2 PtdCho and 16:0–20:1 PtdCho). There were no phospholipids found exclusively in either cell type. HEK293 cells expressed higher levels of 16:0–18:1 PlsCho and 16:0–20:1 PtdCho, compared to Caki-1 cells. In contrast, the relative abundances of 16:1–18:2 PlsCho, 18:0–18:2 PtdCho, 20:1–22:4 PlsCho and 22:0–22:6 PlsCho were greater in Caki-1 cells.

Treatment of HEK293 and Caki-1 cells with BEL alone only decreased the abundance of 14:0–16:0 PtdCho, compared to control cells (Fig. 5A and C). Treatment of HEK293 with cisplatin (50 μM) alone for 12 h decreased the relative abundance of *m/z* ratios that correspond to 14:0–16:0 PtdCho and 16:0–16:0 PtdCho (Fig. 5A). In contrast, treatment of Caki-1 cells with cisplatin alone decreased those peaks that correspond to 14:0–16:0 PtdCho, 16:0–16:0 PtdCho and 16:0–18:2 PtdCho, compared to controls (Fig. 5C). Treatment of HEK293 cells with BEL prior to cisplatin exposure further decreased the

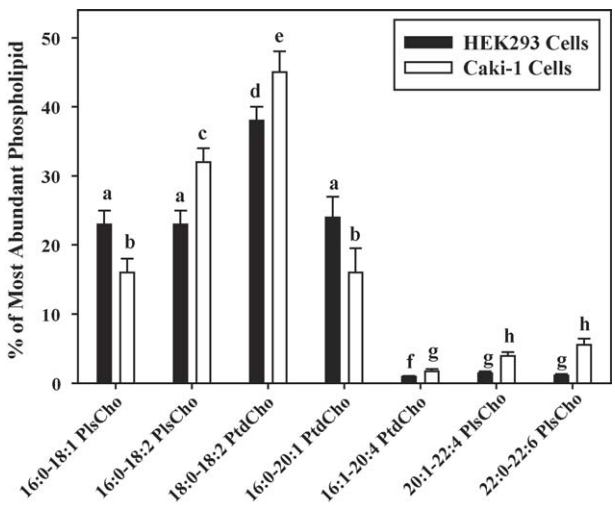

Fig. 4. Differences in the phospholipid profiles of HEK293 and Caki-1 cells. Total lipids from confluent HEK293 and Caki-1 cells were extracted by the Bligh–Dyer method and individual phospholipids separated and quantified as described in the methods. PtdCho represent phosphatidylcholine and PlsCho represents plasmenylcholine. In both cell types the most abundant phospholipid was 16:0–18:1 PtdCho. Data are represented as the mean ± S.E. of at least three separate experiments. Means with different subscripts are significantly different from each other ($P \leq 0.05$).

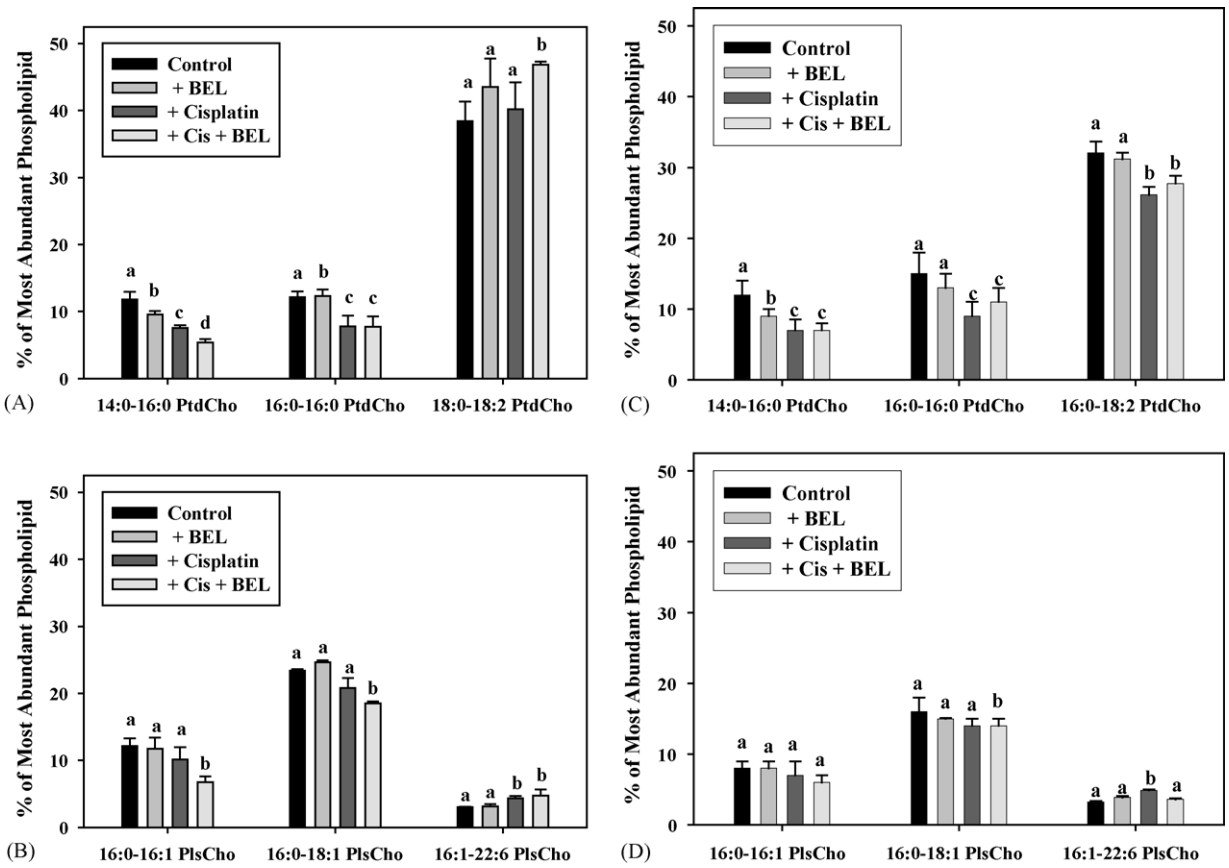

Fig. 5. Effect of $iPLA_2$ inactivation on cisplatin-induced alterations in individual phosphatidylcholine and plasmenylcholine phospholipids. Confluent HEK293 (A and B) and Caki-1 (C and D) cells were exposed to 2.5 μM BEL or diluent control for 30 min prior to exposure to 50 μM cisplatin for 12 h. Individual phospholipids were then separated and quantified as described in the methods. The legend in Panel A is the same for Panels B–D. (A) Effect of BEL and cisplatin on HEK293 phosphatidylcholines; (B) Effect of BEL and cisplatin on HEK293 plasmenylcholines; (C) Effect of BEL and cisplatin on Caki-1 phosphatidylcholines; (D) Effect of BEL and cisplatin on Caki-1 plasmenylcholines. Data are represented as the mean ± S.E. of at least three separate experiments. Means with different subscripts are significantly different from each other ($P \leq 0.05$).

levels of 14:0–16:0 PtdCho, 16:0–16:1 PlsCho and 16:0–18:1–PlsCho, compared to BEL or cisplatin only treated controls (Fig. 5A and B). In Caki-1 cells BEL treatment did not further decrease any phosphatidylcholine phospholipid compared to cells treated with cisplatin alone (Fig. 5C and D). Interestingly, treatment of both HEK293 and Caki-1 cells with cisplatin alone significantly increased the levels of 16:1–22:6 PlsCho (Fig. 5B and D). Inhibition of $iPLA_2$ had no effect on 16:1–22:6 PlsCho in HEK293 cells, but decreased the abundance of this

Table 2
Effect of $iPLA_2$ inhibition on cisplatin-induced alterations in renal phospholipids profiles

Phospholipid	m/z[a]	Carbons/bonds[b]	Cisplatin[c]	Cis + BEL[d]
14:0–16:0 PtdCho	706	30:0	↓	↓
16:0–16:1 PlsCho	718	32:1	↔	↓
16:0–16:0 PtdCho	734	32:0	↓	↔
18:0–18:2 PtdCho	736	36:2	↔	↑
16:0–20:4 PtdCho	782	36:4	↔	↔
16:1–22:6 PlsCho	790	38:7	↑	↔
18:1–20:4 PtdCho	808	38:5	↔	↑
18:0–20:4 PtdCho	810	38:4	↔	↑
20:1–22:4 PlsCho	850	42:5	↑	↔
22:0–22:6 PlsCho	876	44:6	↑	↔

[a] As determined by ESI-MS analysis of lipids extracts from HEK293 and Caki-1 cells.
[b] Identification of individual molecular species is based on theoretical monoisotopic mass values.
[c] Cells were dosed with 50 μM cisplatin for 12 h and changes are based on comparison to control (untreated cells).
[d] Changes based on comparison to cisplatin only treated cells.

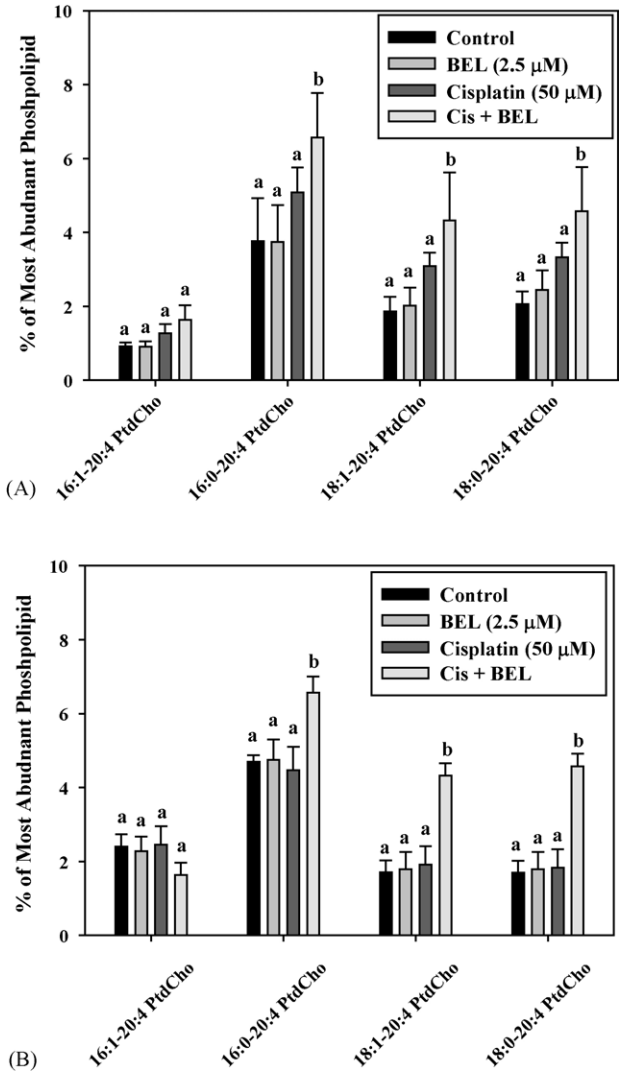

Fig. 6. Effect of $iPLA_2$ inactivation on cisplatin-induced alterations in arachidonic acid containing phospholipids. HEK293 (A) and Caki-1 (B) cells were exposed to 2.5 μM BEL or diluent control for 30 min prior to exposure to 50 μM cisplatin for 12 h. Individual phospholipids were then separated and quantified as described in the methods. Data are represented as the mean ± S.E. of at least three separate experiments. Means with different subscripts are significantly different from each other ($P \leq 0.05$).

phospholipid to control levels in Caki-1 cells (Fig. 5D). Further, treatment of HEK293 with BEL prior to cisplatin exposure increased the relative abundance of 18:0–18:2 PtdCho (Fig. 5A). These results are summarized in Table 2.

As mentioned above, the release of arachidonic acid during chemotherapeutic-exposure is believed to be a major pathway for the activation of apoptosis. Because of this we treated HEK293 and Caki-1 cells with either solvent control or BEL (2.5 μM) prior to exposure to cisplatin (50 μM) for 12 h. We then isolated phospholipids, performed ESI-MS and focused on phospholipids containing arachidonic acid (20:4) at the *sn*-2 position. Fig. 6 demonstrates that BEL or cisplatin alone did not alter the levels of 16:1–20:4, 16:0–20:4, 18:1–20:4 and 18–20:4 PtdCho. However, treatment of both cell types with BEL prior to cisplatin exposure significantly increased these arachidonic acid-containing phospholipids, with the exception of 16:1–20:4 PtdCho. These data demonstrate that inhibition of cisplatin-induced apoptosis in HEK293 and Caki-1 cells during $iPLA_2$ inhibition correlates to the retention of arachidonic acid-containing phospholipids in the membranes.

4. Discussion

$iPLA_2$ is a rapidly expanding class of enzymes whose members include both $iPLA_2\beta$ and $iPLA_2\gamma$. The expression of these isoforms in human kidney cells has not been thoroughly examined. In this study we demonstrated that $iPLA_2\beta$ is expressed in two different models of human renal cells. $iPLA_2\beta$ was not previously detected in renal cells isolated from rabbit kidney but was detected in rat kidney cells [9,17,19,39]. Thus, $iPLA_2\beta$ expression in the kidney appears to be species-dependent. Data from HEK293 and Caki-1 cells also suggests that cytosolic $iPLA_2\beta$ expression is higher in human renal cells than those isolated from the rabbit and rat [9,17].

Immunoblot analysis demonstrated the presence of $iPLA_2\gamma$ in both HEK293 and Caki-1 cells. Previous studies demonstrated that $iPLA_2\gamma$ is expressed in HEK293 cells, but Caki-1 cells were not studied [17]. Thus, $iPLA_2\gamma$ appears to be expressed in multiple models of human renal cells. Assessment of microsomal $iPLA_2$ activity (an indirect measurement of $iPLA_2\gamma$ expression) supports this hypothesis. Comparison of these data with previously published results [9,10,17] suggest that kidney cells isolated from rat, rabbit and human kidneys express relatively high levels of $iPLA_2\gamma$ compared to $iPLA_2\beta$.

Renal cell death can be induced after the treatment of cancer patients with chemotherapeutics and can lead to acute renal failure [40]. In fact, it is estimated that 20% of acute renal failure occurrences in hospitalized patients result from exposure to anti-cancer agents including cisplatin, carboplatin, ifosfamide, 5-fluorouracil, mithramycin, interleukins, and in some cases, vincristine [23,40–42]. Thus, treatments are needed in the clinic that reduce chemotherapeutic-induced renal cell death. Inhibition of $iPLA_2$ activity decreased chemotherapeutic-induced apoptosis in all kidney cells tested. These data agree with previously published studies in RPTC and extend these findings to vincristine and to human renal cells. These data also support the hypothesis that inhibitors of $iPLA_2$ may protect against chemotherapeutic-induced acute renal failure. In RPTC [9] inhibition of $iPLA_2$ decreased caspase 3 activation downstream of p53 nuclear translocation [19]. The determination if similar mechanisms are occurring in the above cells is the subject of future studies, including those investigating alterations in $iPLA_2$ activity and expression during renal cell apoptosis.

Data from this study, and others, demonstrate that BEL at the concentration used above, inhibits $iPLA_2$ activity in multiple cellular models [9,17]. It is unlikely that the effects of BEL are due to the inhibition of phosphatidic acid phosphohydrolase-1 (PAPH-1) because PAPH-1 is only inhibited 50% by 25 μM BEL [43], which is 10-fold higher than the concentrations used in this study. Further, it is unlikely that inhibition of cell death is a result of inhibition of $cPLA_2\gamma$ (Group IVC) as BEL, at the concentration used, does not significantly decrease the activity of this protein [44]. BEL at a concentration of 25 μM induces cell death in several models including U937, THP-1, and MonoMac (human phagocyte), RAW264.7 (murine macrophage), Jurkat (human T lymphocyte), and GH3 (human pituitary) cell lines [45]. Further, a strong correlation exists between the ability of BEL to induce cell death and inhibit PAPH-1 [45]. However, concentrations of BEL below 5 μM do not effectively inhibit PAPH-1, and, similar to data presented above, did not induce cell death [45]. Thus, as previously hypothesized, the toxic effects of BEL appear to be mediated by PAPH-1, while its protective effect on renal cell apoptosis appear to be mediated by $iPLA_2$.

Lipidomics can identify how alterations in lipid metabolism alter cellular homeostasis and disease. The use of lipidomics to assess renal cell apoptosis or the role of $iPLA_2$ in these processes have not received much attention. We focused on the role of $iPLA_2$ on the expression of choline-containing phospholipids and identified several phospholipids whose relative abundance differed between two different renal cell lines. We also identified several specific plasmenyl- and phosphatidylcholine whose abundance decreased prior to the advent of cisplatin-induced renal cell apoptosis (Table 2). These data suggest that these lipids are being lost, or degraded, and released to the cytosol. This hypothesis is supported by the ability of BEL to alter the loss of these phospholipids. The increased loss of these phospholipids during $iPLA_2$ inhibition may result from an inability of $iPLA_2$ to cleave and release the *sn*-2 fatty acid. Such cleavage readies the resulting lipids for reacylation and re-insertion into the membrane. In the absence of $iPLA_2$ activity no re-insertion would occur, resulting in an overall decline in the affected phospholipid. This hypothesis is supported by our previous studies in RPTC demonstrating that inhibition of $iPLA_2$ alters the loss of several choline-containing phospholipids during oxidative stress [20]. However, data in this study are among the first to demonstrate similar findings for chemotherapeutics during apoptosis.

The ability of cisplatin to increase phospholipids containing 22:6 fatty acids at the *sn*-2 position is intriguing. Fatty acids pertaining to 22:6 are docasahexanoic acids (DHA), which are potent signaling fatty acids that have been implicated in apoptosis in a number of cellular models [46,47]. The role of DHA in renal cell apoptosis has not been extensively studied, but increases in DHA containing phospholipids during cisplatin exposure suggest that this fatty acid may play a critical role in chemotherapeutic-induced apoptosis. DHA is released into the cytosol by the action of PLA_2 [48], but the role of $iPLA_2$ in DHA release has not been determined. The ability of BEL to inhibit cisplatin-induced increases of 16:1–22:6 PlsCho suggests that $iPLA_2$ mediate the maintenance of DHA containing phospholipids during apoptosis in renal cells.

Apoptosis in several cellular models, including renal cells [36,38,39], is mediated by arachidonic acid (20:4) release. Further, arachidonic acid is known to be a key mediator of several pathological events in the kidney including inflammation, vasodilatation, and oxidant-induced cell death, and hypertension [49–52]. Yet despite these findings, few studies have identified that actual phospholipids from which arachidonic acid is released. Data in this study demonstrate that inhibition of $iPLA_2$ in two different human renal cell models resulted in the retention of select arachidonic acid-containing phospholipids during cisplatin exposure. Retention of these phospholipids correlated to the inhibition of apoptosis and suggest the hypothesis that one mechanism by which apoptosis is prevented in these cells is by inhibition of metabolism of these phospholipids. This hypothesis is also supported by the fact that changes in phospholipid abundances occurred at 12 h, which is prior to first detectable increases in cell death. Further, decreases in these phospholipids occurred selectively, in the absence of changes in overall lipid phosphate levels. Thus, it is unlikely that these changes are the result of over all cell death. However, further studies are needed to directly implicate these phospholipids as key mediators of renal cell apoptosis.

Neither BEL, nor cisplatin, alone caused alterations in arachidonic acid phospholipids after 12 h. It is not surprising that cisplatin alone does not alter arachidonic acid levels at 12 h as this time point is relatively early in the genesis of apoptosis in renal cells [19,29]. The inability of BEL alone to alter arachidonic acid-containing phospholipids may be due to the fact that multiple enzymes are used in cells to maintain these critical phospholipids in non-stressed cells, including PAPH-1, other PLA_2, and acyltransferases during physiological conditions. Thus, inhibition of $iPLA_2$ may only become a factor in controlling these phospholipids during pathological events.

In summary, both $iPLA_2\beta$ and $iPLA_2\gamma$ were detected in multiple models of human renal cells. Inhibition of $iPLA_2$ protected against chemotherapeutic-induced apoptosis in all kidney cells tested supporting the hypothesis that $iPLA_2$ mediates chemotherapeutic-induced renal cell apoptosis. The phospholipid profiles of two different renal cell models were altered by cisplatin, and inhibition of $iPLA_2$ significantly altered the affect of cisplatin on these profiles. Further, the protection afforded by $iPLA_2$ inhibitors against chemotherapeutic-induced renal cell apoptosis correlated to the alteration of potent signaling phospholipids such as those containing docasahexanoic acids and arachidonic

acid-containing phospholipids. Collectively, these data demonstrate that inhibition of $iPLA_2$ protects against chemotherapeutic-induced cell death in multiple human renal cell models, identifies specific phospholipids whose levels are altered during cell death, and demonstrates that alterations in these phospholipid correlate to the protection against cell death in the presence of $iPLA_2$ inhibitors.

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