Molecular Vision 2014; 20:511-521 <http://www.molvis.org/molvis/v20/511>
Received 10 October 2013 | Accepted 23 April 2014 | Published 25 April 2014

Calcium-independent phospholipase A_2, group VIA, is critical for RPE cell survival

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Purpose: To investigate the significance of calcium-independent phospholipase A_2, group VIA (iPLA$_2$-VIA), in RPE cell survival following responses to sodium iodate (SI) in cell cultures.
Methods: The human retinal pigment epithelium (RPE) cell line (ARPE-19) cells and primary mouse-RPE cultures were treated with SI to induce cell death. Cells were transfected with an iPLA$_2$-VIA promoter-luciferase construct to evaluate the regulation of iPLA$_2$-VIA after exposure to SI. PCR analysis, western blot analysis, and activity assays were performed to evaluate the mRNA level, protein level, and activity levels of iPLA$_2$-VIA after SI exposure. Inhibitors of iPLA$_2$-VIA were used to explore a potential protective role in cells exposed to SI. Primary RPE cell cultures were grown from iPLA$_2$-VIA knockout mice and wild-type mice. The cultures were exposed to SI to investigate a possible increased protection against SI in iPLA$_2$-VIA knockout mice compared to wild-type mice.
Results: The study revealed upregulation of iPLA$_2$-VIA expression (promoter activity, iPLA$_2$-VIA mRNA, iPLA$_2$-VIA protein, and iPLA$_2$-VIA protein activity) in ARPE-19 cells exposed to SI. SI-induced cell death was shown to be inhibited by iPLA$_2$-VIA-specific inhibitors in ARPE-19 cell cultures. RPE cultures from iPLA$_2$-VIA knockout mice were less vulnerable to SI-induced cell death compared to RPE cultures from wild-type mice.
Conclusions: SI -induced RPE cell death involves iPLA$_2$-VIA upregulation and activation, and amelioration of SI-induced RPE cell death can be facilitated by inhibitors of iPLA$_2$-VIA. Thus, we suggest iPLA$_2$-VIA as a possible pharmaceutical target to treat RPE-related retinal diseases.

The RPE is a monolayer of nondividing cuboidal cells that are critically important for the nourishment and overall integrity of photoreceptor cells [1]. Thus, RPE cells are a primary target of studies that aim to understand the fundamental mechanisms of cell survival. Failure in sustaining RPE cell viability is a key event in the early pathophysiology of age-related macular degeneration and in the expression of mutations that lead to retinitis pigmentosa [2,3]. Moreover, there are still numerous voids in our knowledge regarding endogenous events that sustain RPE cell survival.

Several models attempt to investigate degeneration of RPE cells, including the model of intravenous injection of sodium iodate (SI) [4]. While it has been shown that SI exerts toxic effects on RPE cells [5-8], the mechanisms by which the damage occurs are poorly understood.

The complexity of cell survival is obvious and the understanding limited by the multiple pathways being involved. However, some pathways are increasingly being recognized as important in the maintenance of cells. One of these involves phospholipases A_2 (PLA$_2$), which have been shown to participate in cell survival and death [9-13].

Generally, PLA$_2$ consists of a superfamily of enzymes with the shared ability to catalyze hydrolysis of the *sn*-2 fatty acyl ester bond of glycerophospholipids, resulting in the production and release of both lysophospholipids and free fatty acids. PLA$_2$ is commonly divided into four groups: 1) calcium-independent (i)PLA$_2$ including mammalian groups VIA1(iPLA$_2$), VIA2(iPLA$_2\beta$), VIB(iPLA$_2\gamma$), VIC(iPLA$_2\delta$), VID(iPLA$_2\varepsilon$), VIE(iPLA$_2\zeta$), VIF(iPLA$_2\eta$); 2) cytosolic (c) PLA$_2$ including mammalian groups IVA (cPLA$_2\alpha$), IVB (cPLA$_2\beta$), IVC(cPLA$_2\gamma$), IVE(cPLA$_2\varepsilon$), IVF(cPLA$_2\zeta$); 3) calcium-dependent secreted (s)PLA$_2$ [14-24];and 4) platelet activation factor-specific PLA$_2$ (PAF) also known as PAF-acetylhydrolases [25-29].

Previously, we identified various PLA$_2$ in RPE cells and suggested that iPLA$_2$-VIA participates in RPE homeostasis [30-33]. Our studies have revealed the highest concentration of iPLA$_2$-VIA in RPE cells and have shown a role of iPLA$_2$-VIA in RPE phagocytosis of photoreceptor outer segments [32,33] and in RPE cell migration [30]. Strokin et al. demonstrated an increased expression and activity of iPLA$_2$-VIA

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in triggering a damaging neuroinflammatory response [34], and other studies have demonstrated a role of $iPLA_2$ in cell death [9,10].

In most cases, the proliferative phenotype of RPE is seen during pathological conditions in which proliferation is enhanced and cell survival is at risk. In line with this, we demonstrated an increased expression and activity of $iPLA_2$-VIA in proliferating RPE [30], and it is plausible that $iPLA_2$-VIA tilts the balance from RPE survival to cell death. The present study examines the toxicity of SI on RPE cells and uncovers a potential role of $iPLA_2$-VIA and $cPLA_2$-IVA in RPE cell survival.

METHODS

Human ARPE-19 cultures: All materials used and methods performed in the present study are in compliance with the Declaration of Helsinki and the ARVO animal declaration. The human cell line ARPE-19, an immortal RPE cell line from a 19 year-old donor (purchased from ATCC, LGC Promochem AB, Borås, Sweden) was maintained at 37 °C in a humidified chamber of 5% CO_2 in Dulbecco's modified Eagle's medium-F12 (DMEM; Product number 31966, Life Technologies, Paisley, UK) containing 5 mM glucose, supplemented with 15% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Product number 15140-122, GIBCO, Life Technologies). Experiments were performed using ARPE-19 cells from passages 20 through 24. Cells were grown in 96-well or six-well plates, and the culture medium was replaced with fresh medium twice a week. Cells were harvested after 5 days (nonconfluent) or after 3 weeks (confluent) and kept at –80 °C until use.

Primary mouse RPE cultures: Primary mouse RPE cultures were set up from eyes of 12- to 14-week-old mice as briefly described below [35]. The $iPLA_2$-VIA transfection studies were performed with B6 wild-type mice (WT; Taconic Europe, Ejby, Denmark), whereas the knockout studies were done with $iPLA_2$-VIA$^{-/-}$ knockout (KO) 129/SvJ x C57BL/6 mice. WT and KO mice were identified through genotyping before inclusion in the $iPLA_2$-VIA$^{-/-}$ knockout studies.

Primary RPE cultures were grown as previosuly described [33]. Briefly, mouse eyes were enucleated and left in HEPES Hanks Balanced Salt Solution (HBSS, Product number BE10-527F, Lonza, BioWhittaker, Vallensbæk, Denmark) buffered (pH 7.4) with Ca^{2+} and Mg^{2+} for 3 h on ice to facilitate separation of the neural retina from the RPE layer. Eyes were cut circumferentially along the limbus, and the anterior segments and the lens were removed. The remaining eye cups were incubated in a 0.2% trypsin solution without EDTA (pH 8.0; Product number T4799, Sigma, Copenhagen, Denmark) for >1 h at 37 °C, after which they were transferred to HEPES buffered HBSS for the isolation of RPE cells. RPE cells were carefully scraped off using a needle, and the cells were collected into an attached syringe. Cells were then transferred to a tube and centrifuged at 560 ×*g*. Finally, the cells were resuspended in DMEM containing 0.5 mM L-arginine and supplemented with 20% heat-inactivated fetal calf serum, L-glutamine (Product number 25030-024, Lifetechnologies, Nærum, Denmark) 2 mM, MEM nonessential amino acid solution 0.1 mM, and gentamycin (Product number 15710-049, Lifetechnologies) sulfate 10 µg/ml. Cells were grown in 96-well plates and incubated at 37 °C in a humidified chamber with 10% CO_2, and two-thirds of the culture medium was replaced with fresh medium twice a week.

Luciferase assay: Eighty percent confluent ARPE-19 cells in 96-well plates were transfected with the pGL3-basic vector (a luciferase reporter vector; Promega, Roskilde, Denmark) containing a human $iPLA_2$-VIA promoter driving luciferase, using the ExGen 500 (Product number R051; Fermentas, Copenhagen, Denmark) transfection kit. In short, a mixture of the vector-DNA and ExGen 500 was prepared of which 20 µl was added to each well containing the adherent ARPE-19 cells and 200 µl culture medium with serum. The plates were centrifuged at 280 ×g for 5 min. to enhance the transfection process and subsequently incubated for 48 h at 37 °C in a humidified chamber with 10% CO_2. Finally, the cell were exposed to 1 mM SI for 24 h before analysis.

Quantitative reverse transcriptase-PCR: Nonconfluent and confluent ARPE-19 cells were harvested, and RNA was converted into cDNA using a RevertAid First Strand Synthesis kit (Fermentas) according to the manufacturer's instructions. $iPLA_2$ (forward: 5′-GCA ATG CTC GGT GCA ACA T-3′, reverse: 5′-ACA CCC CTT CTG AGA GAA CTT CA-3′) and $cPLA_2$ (forward: 5′-ACT GCA CAA TGC CCT TTA CC-3′, reverse: 5′-GAG CCT CTG CTT TGT GAA CC-3′) primers were purchased from MWG Biotech. Quantitative PCR (qPCR) was run in 96-well plates using the Brilliant SYBR Green qPCR Master Mix (Product number 600882, Stratagene, Hørsholm, Denmark) in a Stratagene Mx3000P qPCR system. The PCR program was: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. A dissociation curve was generated for each gene, and a standard curve for both the test gene and the housekeeping gene (GAPDH) was run to monitor PCR efficiency. Data were analyzed using MxPro software version 3.20 (Stratagene) and the ΔCt method, by which an algoritm (Qty=10-ΔCt/slope) is used to calculate relative changes in the gene expression.

Western-blot analysis: Nonconfluent and confluent ARPE-19 cells were collected and homogenized in 500 µl lysis buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM Na-ortho-vanadate, protease inhibitor cocktail; Sigma Aldrich, St Louis, MO). Samples containing 25 µg protein were loaded onto the gels, and blotting was performed according to the manufacturer's instructions (Invitrogen). Blots were blocked over night with Tris buffered saline (TBS; 0.2 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat dry milk and incubated with iPLA$_2$-VIA (CAY-160507, 1:500; Cayman Chem.,), cPLA$_2$-IVA (SC-438, 1:500; Santa Cruz), and iPLA$_2$-VIB (1:100) in TBS-1% skimmed milk and 25 µl sodium azide over night at 4 °C. Blots were washed in TBS and incubated with goat anti-rabbit immunoglobulin G alkaline phosphatase secondary antibody (TriChem ApS; Interkemi, Skanderborg, Denmark) followed by visualization using 5-bromo-4-chloro-3-indoyl phosphate-nitroblue tetrazolium substrate (VWR International ApS, Product number 8118, VWR, Herlev, Denmark).

Measurement of PLA$_2$ activity: Protein samples were purified using a Cayman cPLA$_2$ assay kit. Briefly, nonconfluent and confluent ARPE-19 cells were collected and homogenized in 150 µl lysis buffer (50 mM HEPES, 1 mM EDTA, 1 mM Na-ortho-vanadate, protease inhibitor cocktail 1:100; Sigma). Samples were centrifuged at 2,000 ×*g* for 30 min at –4 °C. Supernatants were collected and subsequently spun through 30 kDa cut-off filters (Microcon YM-30; Millipore) for 12 min at 14,000 ×*g*.

PLA$_2$ activity was determined in the supernatants using a cPLA$_2$ assay kit (Cayman Chem., Ann Arbor, MI) in the presence and absence of bromoenol lactone, a specific inhibitor of Ca^{2+}-iPLA$_2$. Activity was calculated by measuring the absorbance at 405 nm, using the 5,5′-dithiobis(2-dinitrobenzoic acid) extinction coefficient of 10.66 per mm and reported as nmoles per minute per gram cytosolic protein.

Cytotoxicity and cell viability assays: Lactate dehydrogenase (LDH) release was used to quantitatively access cell injury in cells exposed to SI. Inhibitors of PLA$_2$ were used to identify their influence on RPE cell survival. The iPLA$_2$-VIA-specific inhibitors included bromoenol lactone (BEL; BioNordika, Herlev, Denmark, Cayman Chemicals, Product number 13179) and 1, 1, 1, 2, 2- pentafluoro- 7- phenyl- 3- heptanone (FKGK; Cayman). Additionally, the cPLA$_2$-IVA inhibitor CAY 10502 (Cayman) as well as the combined cPLA$_2$-IVA and iPLA$_2$-VIA inhibitor arachidonyl trifluoromethylketone (AACOCF3; Sigma) were used. LDH-release was assayed using a MWG Discovery HT-R spectrophotometer. In addition, cell viability was determined by the colorimetric assay 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) according to the manufacturer's instructions.

Statistics: Quantitative results are expressed as the mean ± standard deviation. One-way analysis of variance and Tukey's multiple comparison post-test were used to evaluate the statistical significance of differences between other experimental groups. Paired Student *t* test was used to evaluate the statistical significance of differences between some experimental groups. $p<0.05$ was considered statistically significant.

RESULTS

Sodium iodate inhibits retinal pigment epithelium cell survival in a dose-dependent manner: ARPE-19 cell death was induced gradually by SI in a dose-dependent manner. Hence, after 24 h of SI exposure in nonconfluent cells, 0.5 mM of SI induced 34% cell death ±9% (n = 5), 0.75 mM induced 39% cell death ±8% (n = 3), 1 mM induced 46% cell death ±12% (n = 5), 2 mM induced 50% cell death ±11% (n = 3), and 5 mM induced 99% cell death ±57% (n = 2). In confluent cells exposed to SI for 24 h, cell death was generally less prominent. Hence, 0.5 mM of SI induced 31% cell death ±6% (n = 5), 0.75 mM induced 29% cell death ±6% (n = 2), 1 mM induced 26% cell death ±4 (n = 5), 2 mM induced 39% cell death ±16% (n = 5), and 5 mM induced 86% cell death ±9% (n = 2; Figure 1A).

Nonconfluent ARPE-19 cells were more vulnerable to SI treatment compared to confluent ARPE-19 cells when exposed from 2 to 48 h. A significant difference between nonconfluent cells (n = 3) and confluent cells (n = 3; p = 0.05) was found when ARPE-19 cells were exposed to 1 mM of SI for 24 h (Figure 1A,B). The same tendency was found after SI exposure for 2 and 48 h. Moreover, a significant increase in SI-induced toxicity was found at longer exposure periods. In nonconfluent cells, 20±12% cell death was seen after 2 h of exposure to SI (n = 3), whereas a 47±11% cell death and a 56±11% cell death were seen after 24 (n = 3) and 48 (n = 3) hours, respectively. In confluent cells the degree of cell death was consistently lower compared to nonconfluent cells. Hence, 3±19% cell death was seen after 2 h of SI exposure (n = 3), and 19±19% cell death and 29±18% cell death were seen after 24 h (n = 3) and 48 h (n = 3), respectively (Figure 1B). In primary mice RPE cultures a dose-dependent toxicity of SI exposure was found. Cells were exposed to 0.5, 1.0, 2.0, 5.0, and 10.0 mM SI, which resulted in 7±1%, 17±2%, 32±11%, 46±7%, and 54±3%, cell death, respectively (n = 3; Figure 1C).

Sodium iodate enhances iPLA2-VIA promoter activation and upregulates iPLA$_2$-VIA expression: To investigate the significance of iPLA2-VIA activation in RPE cell survival,

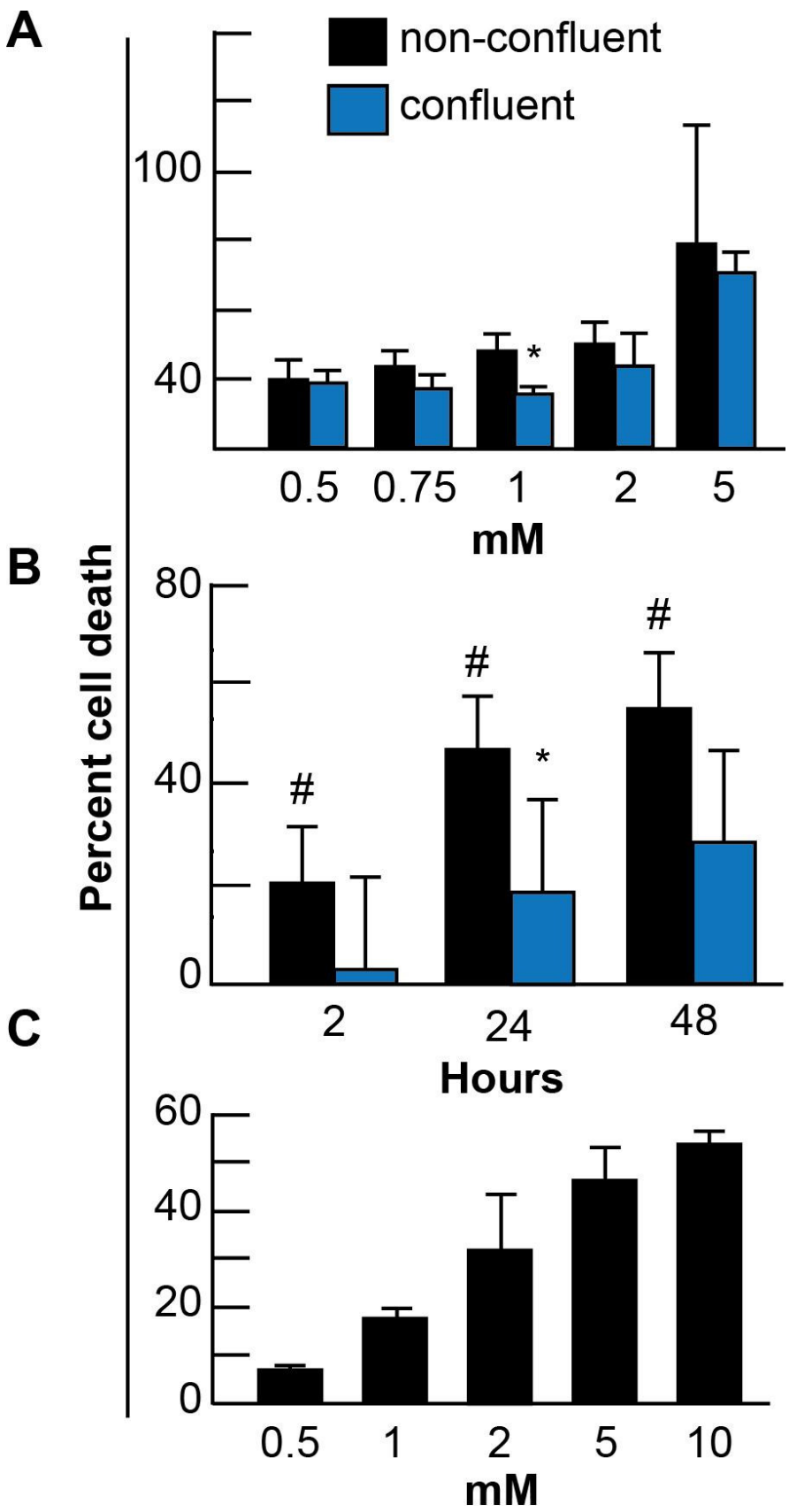

Figure 1. Sodium iodate (SI) induces retinal pigment epithelium cell death in a dose- and time-dependent manner. **A:** Percent ARPE-19 cell death after 24 h of exposure to different doses of SI. Black bars indicate nonconfluent cells, and blue bars indicate confluent cells. * indicates $p<0.05$ (0.5 mM SI, n=5; 0.75 mM SI, n=3; 1 mM SI, n=5; 2 mM SI, n=3; 5 mM SI, n=2) when cell death is compared between nonconfluent and confluent ARPE-19 cells. **B:** Percent cell death of ARPE-19 cells after exposure to 1 mM SI for 2 h, 24 h, and 48 h in nonconfluent and confluent cells. Black bars indicate nonconfluent cells, and blue bars indicate confluent cells. * indicates $p<0.05 \pm$ SD (n=3) when cell death is compared between nonconfluent and confluent ARPE-19 cells. # indicates $p<0.01\pm$ SD (n=3) when cell death is compared between 2 h SI treatment compared to 24 and 48 h. **C:** Percent cell death in mouse primary RPE cells after exposure to different doses of SI. Cells were exposed to SI for 24 h.

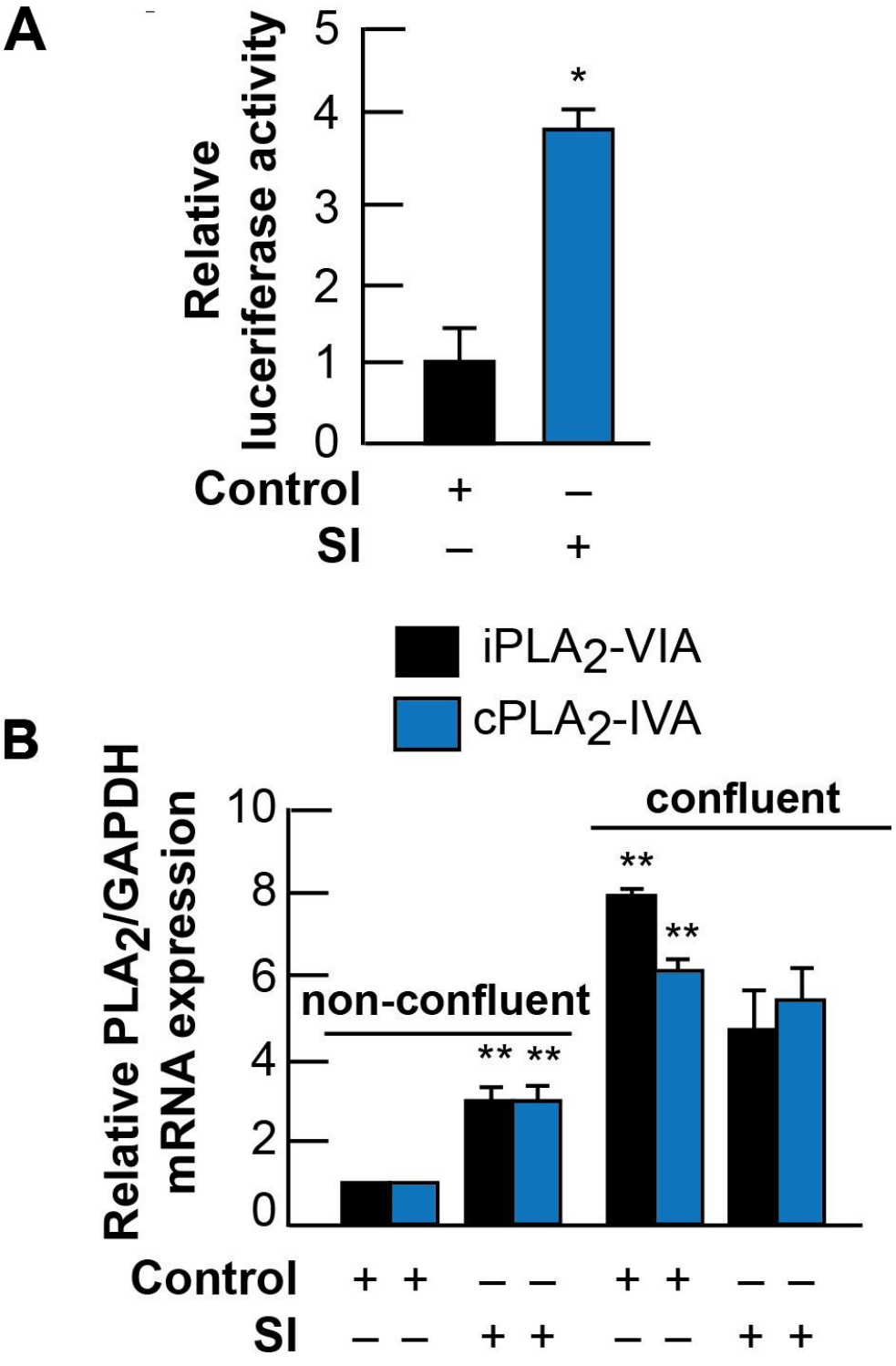

Figure 2. Sodium iodate (SI) induces promoter activity and mRNA upregulation of calcium-independent phospholipase A^2, group VIA. **A:** Luciferase activity in ARPE-19 cell cultures is increased in response to 1 mM SI exposure for 24 h. * indicates $p<0.05 \pm$ SD (n=3). **B:** $iPLA_2$-VIA and $cPLA_2$-IVA mRNA expression in nonconfluent and confluent ARPE-19 cells after exposure to 1 mM SI for 24 h. A threefold induction of both $iPLA_2$-VIA and $cPLA_2$-IVA mRNA was found in nonconfluent ARPE-19 cells. A tendency for downregulation of $iPLA_2$-VIA was found when confluent ARPE-19 cells were exposed to SI ($p = 0.08 \pm$ SEM; n=3). However, no change was found in $iPLA_2$-VIA or $cPLA_2$-IVA expression when confluent cells were exposed to SI. ** indicates $p<0.01\pm$ SEM (n=3) when mRNA levels from nonconfluent ARPE-19 cells are compared with mRNA levels from SI-treated nonconfluent ARPE-19 cells.

ARPE-19 cells were transfected with pGL3-basic vector containing the human iPLA2-VIA promoter luciferase and subjected to 1 mM SI. After 24 h of exposure, luciferase activity was detected using the Luciferase Assay Freezer Pack (Promega). The results showed an increase in luciferase activity for cells exposed to SI when compared to controls ($p<0.05$, n = 3; Figure 2A) reflecting transcriptional activation by SI.

Furthermore, quantification of $iPLA_2$-VIA mRNA expression revealed a threefold induction after exposure to 1 mM SI for 24 h in nonconfluent ARPE-19 cells ($p = 0.01$, n = 3). Similarly, 1 mM of SI exposure induced a threefold induction of $cPLA_2$-IVA mRNA ($p<0.0001$, n = 3; Figure 2B). The control levels of $iPLA_2$-VIA were sixfold higher in confluent cells compared to nonconfluent cells ($p = 0.003$, n = 3). A tendency toward downregulation of $iPLA_2$-VIA mRNA was observed when confluent ARPE-19 cells were exposed to SI ($p = 0.08$, n = 3; Figure 2B). No change was found in either $iPLA_2$-VIA nor $cPLA_2$-IVA expression when confluent cells were exposed to SI (n = 3; Figure 2B).

Sodium iodate triggers increased protein expression and activity of $iPLA_2$-VIA: Upregulation of the 85-kDa $iPLA_2$-VIA protein was 1.6 fold ($p = 0.01$) in nonconfluent ARPE-19 cells exposed to 1 mM of SI (Figure 3A,B). No upregulation was found in confluent cells exposed to SI (Figure 3A). A similar upregulation was seen for $cPLA_2$-IVA after exposure to 1 mM of SI. Hence, $cPLA_2$-IVA was upregulated 2.6 fold ($p<0.01$). Furthermore, $iPLA_2$-VIA-specific activity was found to be significantly higher in nonconfluent ARPE-19 cells after exposure to 1 mM of SI for 24 h compared to its respective control. The results revealed a 2.5-fold enhancement in activity after SI exposure in nonconfluent cells ($p<0.05$; Figure 3C). The same tendency was seen for confluent RPE cells (1.89-fold induction); however this was not significant ($p = 0.06$).

RPE cells from $iPLA_2$-VIA knockout mice show increased RPE cell survival upon exposure to sodium iodate: Cell death of mice RPE cells was increased in WT mice compared to KO mice. Exposing WT RPE cells to 2 mM of SI for 24 h revealed a toxic effect of SI resulting in 56±13% cell death compared to 15±9% cell death in $iPLA_2$-VIA KO mice RPE cells ($p<0.001$, n = 8; Figure 4).

Inhibition of $iPLA_2$ and $cPLA_2$ mediates enhancement of RPE cell survival after exposure to sodium iodate: To investigate the effect of $iPLA_2$ and $cPLA_2$ inhibition on RPE cell death, more $iPLA_2$-, and $cPLA_2$-specific inhibitors were added, respectively, to nonconfluent and confluent cells exposed to SI. To determine the concentrations of the inhibitors, a preliminary experiment was conducted to measure the toxic effect of different concentrations of inhibitors on nonconfluent ARPE-19 cells. The inhibitors were not significantly toxic until reaching concentrations of 10 µM or higher (data not shown).

Treament with both iPLA2-VIA- and cPLA2-IVA-inhibitors generally increased cell survival in ARPE-19 cells exposed to SI. Over all, the protective effect of the inhibitors was more pronounced in nonconfluent cells compared to confluent ARPE-19 cells. Hence, BEL (10 µM) and FKGK (10 µM) treatment resulted in increased survival of 40% ($p<0.0001$, n = 3) and 8% ($p<0.05$, n = 3), respectively. In confluent cells, BEL and FKGK inhibition did not protect against SI-induced cell death. The $cPLA_2$-inhibitor CAY10502 (10 µM) inhibited SI-induced cell death in nonconfluent ARPE-19 cells by 45% ($p<0.0001$, n = 3) and 7% ($p<0.01$, n = 3) in confluent ARPE-19 cells. After treating ARPE-19 cells with AACOCF3 (20 µM) to inhibit $iPLA_2$ and $cPLA_2$, an 80% increase in survival was found in nonconfluent cells ($p<0.0001$, n = 3), whereas a 20% increase in survival was observed after SI exposure in confluent cells ($p<0.0001$, n = 3; Figure 5).

DISCUSSION

Retinal degenerations compromise RPE cell survival, thus an improved understanding of the mechanisms involved is needed to help increase our knowledge of these conditions and lead to improved clinical therapies. SI is toxic to RPE cells [5-8], and doses below 20 mg/kg have little effect on the RPE cells in vivo, but doses of 25 mg/kg and above result in toxic effects to the retina [36,37]. In the present study a similar dose and time dependent SI toxicity was seen in ARPE-19 cell cultures. Furthermore, a general tendency towards more vulnerability in nonconfluent ARPE-19 cells compared to confluent cells was found (Figure 1B). Since RPE cells are known to proliferate early in diseased stages [30,31], it is tempting to suggest that their regenerative proliferative stage may be more vulnerable compared to their nonproliferative stable stage. In line with this, Kiilgaard and colleagues have shown that older confluent ARPE-19 cells were more resistant toward indocyanin green compared to nonconfluent cells [38].

We previously showed that increased amounts of $iPLA_2$-VIA are present in proliferating ARPE-19 cells [31]. Furthermore, other studies have identified the roles of $iPLA_2$-VIA and $cPLA_2$-IVA in RPE cell death [11-13]. Based on the previous evidence regarding the involvement of PLA_2 in cell death and the findings that $iPLA_2$-VIA induction occurs in the more vulnerable proliferating RPE cells, the present study

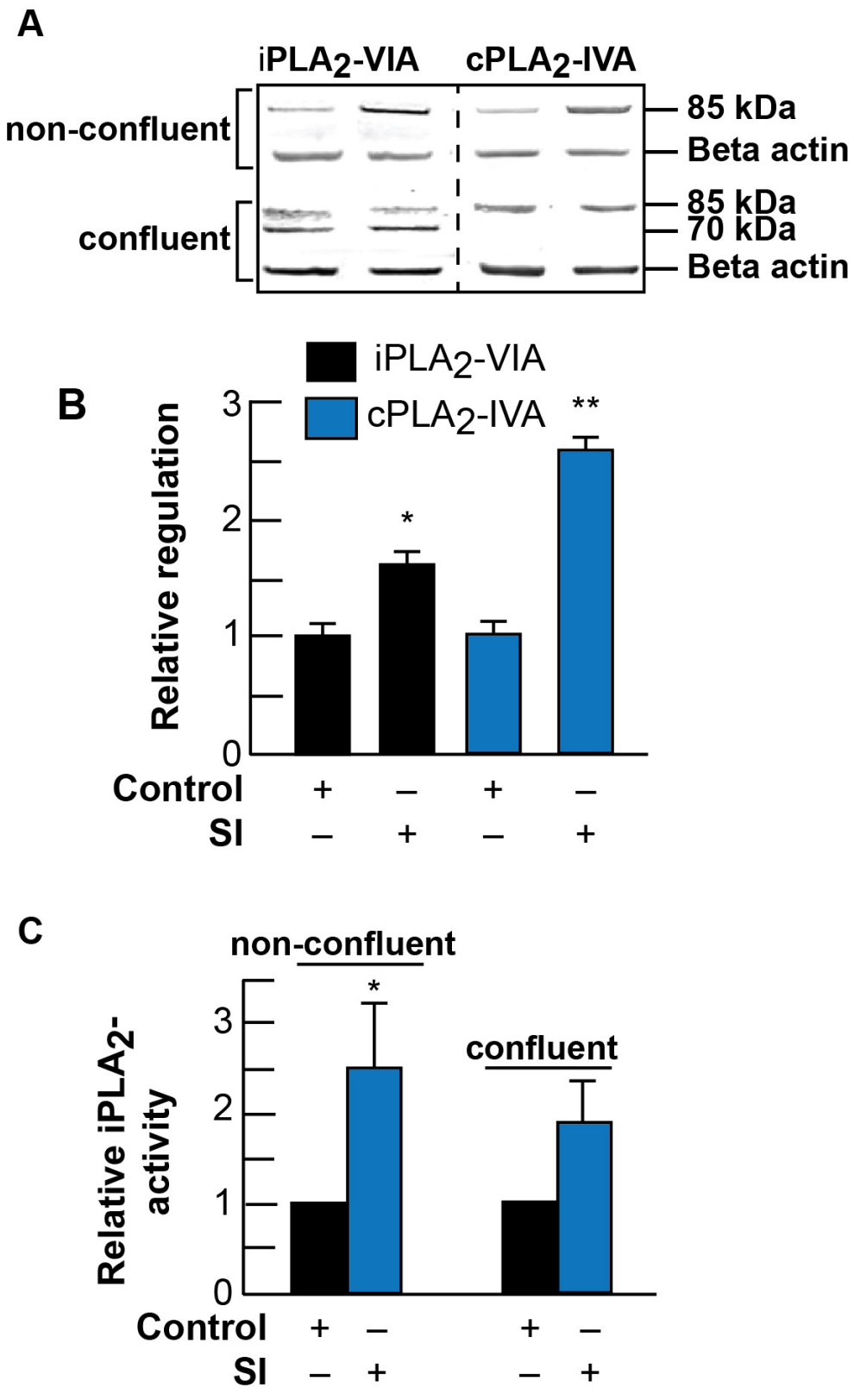

Figure 3. Calcium-independent phospholipase A^2, group VIA ($iPLA_2$-VIA) expression and activity are upregulated in ARPE-19 cells exposed to sodium iodate. **A:** Western blot analysis was performed to detect the expression pattern of $iPLA_2$-VIA and $cPLA_2$-IVA in nonconfluent and confluent ARPE-19 cells exposed to 1mM SI for 24 h. A representative blot is shown (n = 3). **B:** Bars represent the ratio of the 85 kDa $iPLA_2$-VIA splice variant and $cPLA_2$-IVA protein expression normalized to beta actin expression in nonconfluent ARPE-19 cells. * indicates $p < 0.05 \pm$ SD (n=3); ** indicates $p<0.01$. **C:** $iPLA_2$-VIA activity in ARPE-19 cells exposed to SI. Bars represent the relative $iPLA_2$-VIA activity in confluent and nonconfluent cells. A tendency for upregulation was found in confluent cells $p=0.06 \pm$ SEM (n=3). * indicates significant difference from its respective control ($p<0.05$).

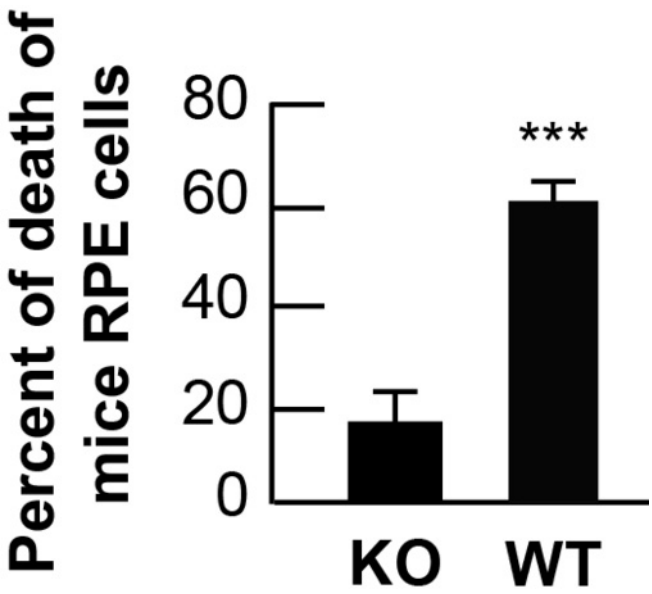

Figure 4. Sodium iodate (SI) exposure causes less cell death in retinal pigment epithelium cells isolated from calcium-indepent phospholipase A^2 ($iPLA_2$-VIA) KO mice compared to wild-type mice. Primary RPE cultures were exposed to 2 mM of SI for 24 h. A significant discrepancy was seen in SI-induced cell death in KO compared to WT mice. Hence, 2 mM SI induced 56% cell death, whereas only 15% cell death was seen in the KO mice. WT, $iPLA_2$-VIA wild-type mice; KO, $iPLA_2$-VIA KO mice; *** indicates $p<0.001$ ± SD (n=8).

elucidated a possible role of $iPLA_2$-VIA and $cPLA_2$-IVA in RPE cell survival.

To study RPE cell death, ARPE-19 cells were exposed to SI and the levels of $iPLA_2$-VIA and $cPLA_2$-IVA were explored. Overall, the general mRNA and protein levels of $iPLA_2$-VIA and $cPLA_2$-IVA were increased in confluent ARPE-19 cells and induction due to SI exposure was evident in nonconfluent ARPE-19 cells. Upregulation of $iPLA_2$-VIA after SI exposure was found in the high molecular 85-kDa band, which is comparable to the higher band expressed in proliferating ARPE-19 cells [31] (Figure 3A). Expression of the low-molecular 70-kDa $iPLA_2$-VIA was confirmed to be higher in confluent cells (Figure 3A), which may explain the increased total mRNA expression of $iPLA_2$-VIA in confluent cells compared to nonconfluent ARPE-19 cells (Figure 3A).

To confirm the role of $iPLA_2$-VIA in RPE cell death, primary RPE cells were exposed to SI. Consistent with ARPE-19 cell death, mouse RPE cell death was dose dependent (Figure 1C). Primary RPE cultures from $iPLA_2$-VIA KO mice were exposed to SI, and the induced cell death was compared to RPE cultures isolated from WT mice. Primary RPE cells from the KO mice were more resistant to SI exposure compared to RPE cells isolated from WT mice, thus confirming the role of $iPLA_2$-VIA in RPE cell death (Figure 4).

By means of commercially available inhibitors against $iPLA_2$ and $cPLA_2$, we finally elucidated their ability to decrease SI-induced ARPE-19 cell death. When nonconfluent ARPE-19 cells were treated with the inhibitors against

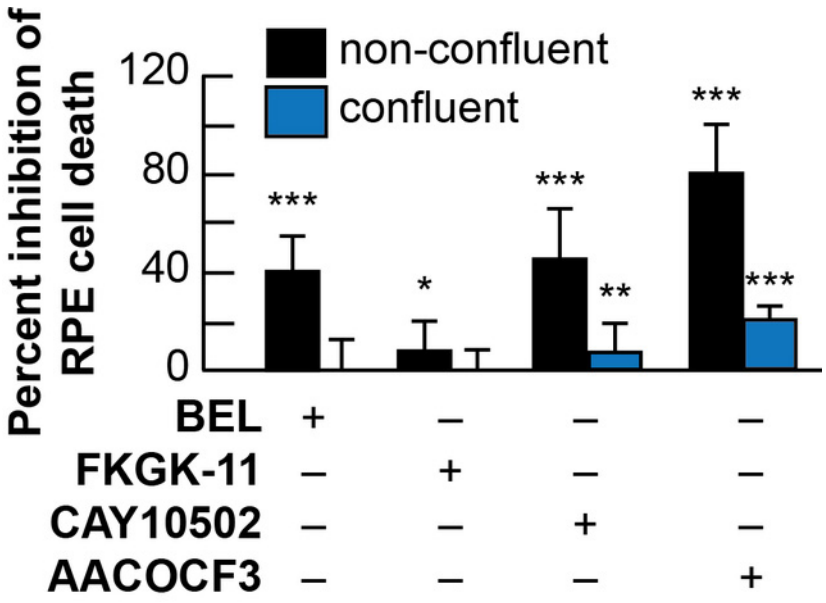

Figure 5. Inhibitors of phospholipases A^2 ($iPLA_2$) decreased sodium iodate-induced cell death more significantly in nonconfluent retinal pigment epithelium cells compared to confluent cells. Inhibitors of $iPLA_2$-VIA (BEL, FKGK-11, AACOCF3) and $cPLA_2$-IVA (CAY10502, AACOCF3) were added to ARPE-19 cells exposed to 1 mM for 24 h. All inhibitors protected against cell death in nonconfluent cells, whereas only CAY10502 and AACOCF3 elicited protection in confluent cells. * indicates $p<0.05$; ** indicates $p<0.01$; *** indicates $p<0.001$. Error bars are presented as ± SEM (n=3 for all conditions).

iPLA$_2$ (BEL and FKGK), SI-induced cell death was decreased accordingly. Moreover, an apparent reduction in nonconfluent ARPE-19 cell death was seen by inhibiting cPLA$_2$ through CAY10502. Selective inhibitors of iPLA$_2$ did not prevent SI-induced cell death in confluent ARPE-19 cells (Figure 5).

The inhibitor studies indicated a dominant involvement of iPLA$_2$ and cPLA$_2$ in RPE homeostasis in nonconfluent cells compared to confluent cells. Since RPE proliferation is limited in the normal retina and since RPE proliferation occurs during pathological conditions in the retina, our findings suggests a potential role of PLA$_2$ inhibition in RPE-related diseases. In line with the inhibitor studies, our present results showed decreased RPE death in mice deficient in iPLA$_2$, further implying a role of PLA$_2$ in RPE death. Future studies are needed to confirm if iPLA$_2$ and cPLA$_2$ inhibition impedes RPE death in primary mice RPE and to further evaluate the importance of PLA$_2$ in RPE homeostasis.

The involvement of PLA$_2$ in cell death has been reported in several studies [12,13,39]. The present findings identify a potential pathway in RPE cell death involving induction of iPLA$_2$ and cPLA$_2$. As mentioned previously, the function of RPE is essential for visual functioning, and various retinal diseases lead to RPE differentiation and proliferation; however, there is insufficient understanding regarding the underlying mechanisms that lead to RPE cell death. The present data suggest that iPLA$_2$ and cPLA$_2$ may be targets to consider in future treatments of RPE-related diseases. However, the involvement of iPLA$_2$ is complex since it also has been shown to play a role in RPE phagocytosis of photoreceptor outer segments [32]. This process is essential for the normal retina. Hence, iPLA$_2$ may be an important molecule in normal RPE, whereas iPLA$_2$-induction may cause RPE damage in diseased proliferating RPE cells. The role of cPLA$_2$ may be more important in diseased RPE cells since the present study found a pronounced induction of cPLA$_2$, indicating that it may take part in the cascade reactions that lead to RPE cell death.

In conclusion, the data presented here provides evidence for the involvement of iPLA$_2$-VIA and cPLA$_2$-IVA in SI-induced RPE cell survival. These studies could lead to future pharmaceutical targets involving PLA$_2$ to help protect RPE cells from retinal degenerative diseases.

ACKNOWLEDGMENTS

The authors thank technician Charlotte Taul Brændstrup, Department of Neuroscience and Pharmacology, University of Copenhagen, Denmark for her skillful assistance in this study. The KO mice were kindly provided by Professor John Turk (Washington University, School of Medicine, St. Louis, MO. The study was supported by The Danish Council for Independent Research|Medical Sciences, The Lundbeck foundation, Fight for Sight Denmark, The Danish Eye Foundation and The A.P Møller Foundation. **Grant information:** The Danish Council for Independent Research|Medical Sciences (Ref no.: 09–066351/FSS)**;** The Lundbeck Foundation (Ref. no.: R17A1764)**;** Fight for Sight Denmark (Ref. no.: 28214)**;** The Danish Eye Foundation (Ref. no.:26982)**;** The A.P Møller Foundation (Ref. no.: 070123)

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 25 April 2014. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.