SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
PREDICTIVE MARKERS
BIOMARKER RESEARCH

Received
29 May 2014

Accepted
29 September 2014

Published
22 October 2014

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sPLA2 IB induces human podocyte apoptosis via the M-type phospholipase A2 receptor

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The M-type phospholipase A2 receptor (PLA2R) is expressed in podocytes in human glomeruli. Group IB secretory phospholipase A2 (sPLA2 IB), which is one of the ligands of the PLA2R, is more highly expressed in chronic renal failure patients than in controls. However, the roles of the PLA2R and sPLA2 IB in the pathogenesis of glomerular diseases are unknown. In the present study, we found that more podocyte apoptosis occurs in the kidneys of patients with higher PLA2R and serum sPLA2 IB levels. In vitro, we demonstrated that human podocyte cells expressed the PLA2R in the cell membrane. After binding with the PLA2R, sPLA2 IB induced podocyte apoptosis in a time- and concentration-dependent manner. sPLA2 IB-induced podocyte PLA2R upregulation was not only associated with increased ERK1/2 and cPLA2α phosphorylation but also displayed enhanced apoptosis. In contrast, PLA2R-silenced human podocytes displayed attenuated apoptosis. sPLA2 IB enhanced podocyte arachidonic acid (AA) content in a dose-dependent manner. These data indicate that sPLA2 IB has the potential to induce human podocyte apoptosis via binding to the PLA2R. The sPLA2 IB-PLA2R interaction stimulated podocyte apoptosis through activating ERK1/2 and cPLA2α and through increasing the podocyte AA content.

The M-type phospholipase A2 receptor (PLA2R), which is a transmembrane receptor belonging to the mannose receptor family, is a receptor for secretory phospholipase A2 (sPLA2)[1]. The PLA2R is detected in the lung, placenta and kidney in humans[2]. Podocytes are terminally differentiated epithelial cells that are crucial components of the glomerular filtration barrier. Injury to or depletion of podocytes plays a vital role in proteinuria onset and glomerular disease progression[3–6]. Podocytes express the PLA2R in normal human glomeruli[7,8]. However, PLA2R expression is accentuated in the glomeruli of some patients with idiopathic membranous nephropathy (IMN)[9,10]. Despite this strong association, the role of the PLA2R in glomerular disease pathogenesis is unclear.

sPLA2 IB, which is a type of sPLA2, has long been believed to be a digestive enzyme because of its abundance in the pancreas. However, the discovery of the PLA2R has further enhanced our understanding of sPLA2 IB[2]. Several studies have demonstrated that sPLA2 IB mediates cell proliferation, cell migration, hormone release and eicosanoid production in peripheral tissues via its receptor[11–13]. Additionally, sPLA2 IB has been reported to induce apoptosis in neuronal cells and in macrophages[14–16]. Interestingly, sPLA2 IB levels were also found to be 10-fold higher in patients with chronic renal failure when compared with controls[17]. However, the contributory role of sPLA2 IB in maintaining glomerular homeostasis under physiological or pathological conditions remains unclear.

The phospholipase A2 (PLA2) reaction is the primary pathway through which arachidonic acid (AA), which is the precursor of the eicosanoid signalling molecules, is liberated from membrane phospholipids[18]. Cytosolic PLA2α (cPLA2α) plays a key role in initiating AA metabolism. cPLA2α-induced AA accumulation is an event that has been shown to trigger apoptosis[19]. However, no data concerning the involvement of sPLA2s in human podocyte apoptosis exist. In the present study, we tested whether sPLA2 IB and the PLA2R correlated with podocyte apoptosis in the human kidney. Moreover, we examined whether podocytes express the PLA2R in vitro and, if so, whether the PLA2R plays a role in sPLA2 IB-induced apoptosis. To explore the involved mechanisms, we studied the effects of sPLA2 IB on the interaction of cPLA2α with the ERK MAPK signalling pathway and the relation between sPLA2 IB and AA.

Table 1 | Clinical feature of each group patients with IMN

	Group I (n = 9)	Group II (n = 16)	P
Age(years)	51.1 ± 7.2	54.2 ± 11.3	NS
Gender(male/female)	(5/4)	(9/7)	NS
SBP(mmHg)	136.0 ± 10.6	140.6 ± 14.5	NS
DBP(mmHg)	81.8 ± 7.1	85.1 ± 6.8	NS
Leukocyte(x10⁹/l)	7.4 ± 1.3	7.5 ± 1.2	NS
Hemoglobin(g/l)	121.1 ± 16.4	118.0 ± 17.8	NS
Serum creatinine on diagnosis(μmol/l)	93.6 ± 19.1	98.5 ± 27.5	NS
Cholesterol(mmol/l)	6.3 ± 1.2	7.4 ± 1.5	NS
Triglyceride(mmol/l)	3.5 ± 0.7	4.0 ± 0.8	NS
Serum albumin(g/l)	31.4 ± 2.8	27.8 ± 4.4	<0.05
Urinary protein(g/24h)	3.4 ± 1.1	4.9 ± 1.7	<0.05
sPLA2 IB(ng/ml)	7.9 ± 1.7	13.5 ± 2.4	<0.01

Group I: group with low level of serum sPLA2 IB.
Group II: group with high level of serum sPLA2 IB.
NS: no statistical significance.

Results

Clinical data from two IMN patient groups. Renal biopsies were performed on all of the patients, and the pathological diagnoses were performed independently by two renal pathologists. Twenty-five IMN patients at I and II stage were enrolled in this study. Nine of the patients had weak PLA2R expression in their kidneys, the other sixteen patients had enhanced PLA2R expression in their kidneys. No differences were observed between two groups, including age, gender, BP, leukocyte, hemoglobin, serum creatinine, cholesterol and triglyceride values. However, compared with the patients with weak PLA2R expression, the accentuated PLA2R expression group presented a significantly lower level of serum albumin (31.4 ± 2.8 vs. 27.8 ± 4.4 g/l, $P < 0.05$) and a higher level of urinary protein excretion (3.4 ± 1.1 vs. 4.9 ± 1.7 g/24 h, $P < 0.05$) (Table 1).

The levels of podocyte apoptosis and PLA2R expression in the kidney and of serum sPLA2 IB of IMN patients. M-type PLA2R expression in human podocytes in vivo has been reported previously[7]. To investigate the relation among podocyte apoptosis and PLA2R expression in the kidney and serum sPLA2 IB of IMN patients, kidney podocyte apoptosis was detected using a TUNEL assay, PLA2R expression in the kidney was detected using an immunohistochemical assay, and serum sPLA2 IB was measured using an enzyme-linked immunosorbent assay (ELISA), respectively. As shown in Fig. 1A, with the PLA2R level accentuated in group II, podocyte apoptosis in the glomerulus clearly increased compared with group I, which had weak PLA2R expression. Consistent with the PLA2R expression and with podocyte apoptosis changes, the level of serum sPLA2 IB in group II was higher than that in group I (7.9 ± 1.7 vs. 13.5 ± 2.4 ng/ml, $P < 0.01$) (Table 1). Moreover, PLA2R and serum sPLA2 IB level correlated positively to kidney podocyte apoptosis ($r = 0.418$, $P < 0.05$ and $r = 0.736$, $P < 0.01$ respectively, Table 2 and Fig. S1).

Human podocytes expressed the M-type PLA2R in vitro. To confirm PLA2R expression in human podocytes, we performed immunofluorescence imaging and Western blotting. As shown in Fig. 2A, human podocytes expressed the M-type PLA2R in the cell membrane. However, M-type PLA2R expression in mouse

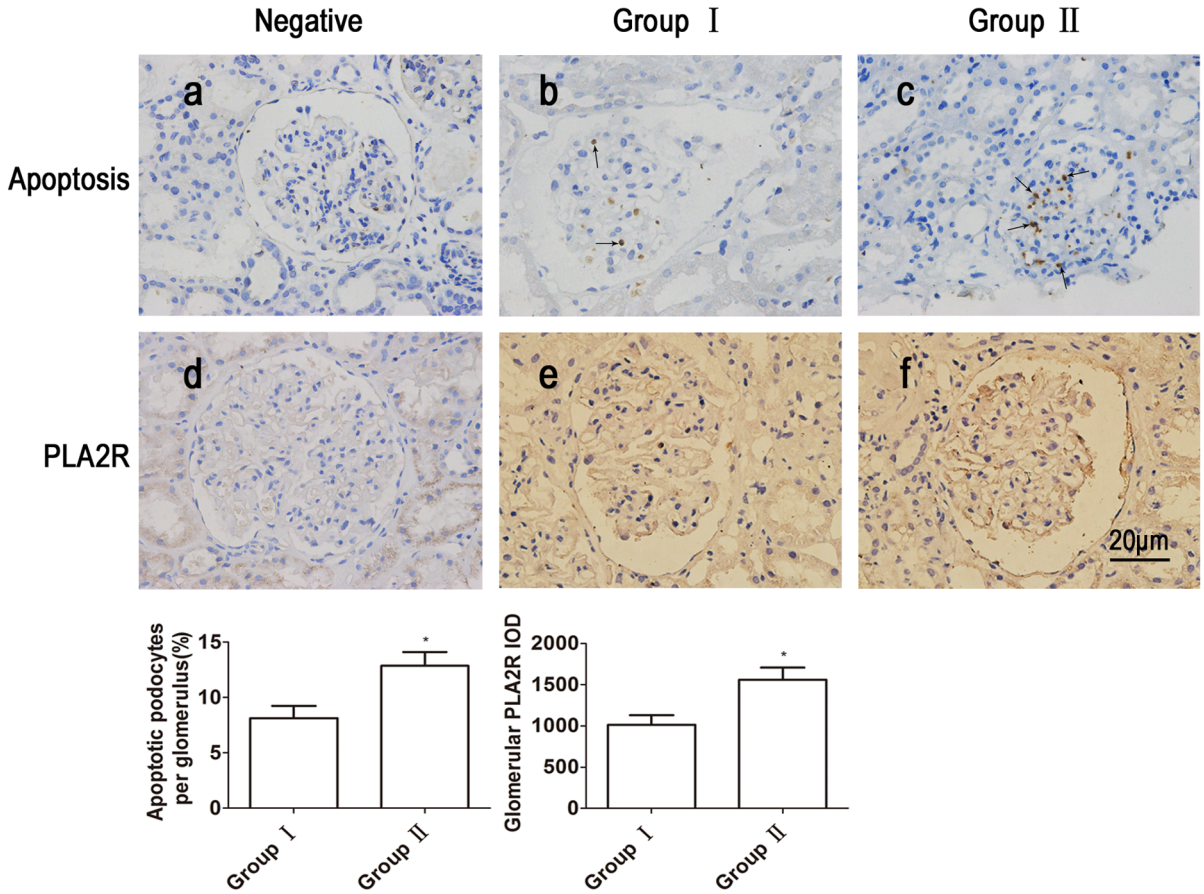


Figure 1 | The levels of podocyte apoptosis and PLA2R expression in kidneys of different IMN patient groups. *a,d* Negative control group; *b,e* Group I: the group with low levels of serum sPLA2 IB; *c,f* Group II: the group with high levels of serum sPLA2 IB (original magnification, ×400). * $p < 0.05$ vs. Group I.

Table 2 | Correlation analysis results

		Serum sPLA2 IB level	Kidney PLA2R IOD
Podocyte apoptosis	Pearson correlation	0.736	0.418
	Sig. (two-tailed)	0.001*	0.033#

* Correlation is significant at the 0.01 level (two-tailed).
# Correlation is significant at the 0.05 level (two-tailed).

podocytes was relatively weak when compared with that in human podocytes. The same results were obtained in the Western blotting analysis (Fig. 2B).

Effects of sPLA2 IB on human podocyte apoptosis and on the cPLA2α, ERK1/2 and PLA2R levels in vitro. To determine the effects of sPLA2 IB on cultured human podocytes, the cells were treated with sPLA2 IB (10^{-6} mol/L) at variable time points (0, 0.5, 1, 2 and 6 h). Subsequently, cells were prepared for apoptosis assays (Hoechst 33342 and flow cytometry), or protein blots were probed for phos-cPLA2α and for phos-ERK1/2. Then, the same blots were reprobed for total cPLA2α, total ERK1/2, and actin. Representative gels are shown in Fig. 3A. sPLA2 IB stimulated podocyte cPLA2α and ERK1/2 phosphorylation. cPLA2α and ERK1/2 phosphorylation peaked at 1 h (Fig. 3A); in contrast, sPLA2 IB did not alter podocyte PLA2R expression (Fig. 3A). The effects of sPLA2 IB on podocyte apoptosis are displayed in representative microfluorograms (Fig. 3B) and in flow cytograms (Fig. 3D). sPLA2 IB promoted podocyte apoptosis in a time-dependent manner.

To determine the dose-response effect of sPLA2 IB, human podocytes were incubated in media containing variable concentrations of sPLA2 IB (0, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mol/L) for 2 h; subsequently, cells were assayed for apoptosis and prepared for Western blotting studies. Representative microfluorograms and flow cytograms are shown in Fig. 4B and in Fig. 4D, respectively. Cumulative data are shown as bar graphs (Fig. 4D). sPLA2 IB enhanced podocyte apoptosis in a dose-dependent manner. Protein blots were probed for phos-cPLA2α and for phos-ERK1/2; then, the same blots were reprobed for total cPLA2α, total ERK1/2, and actin. Representative gels are shown in Fig. 4A. Cumulative data are shown in bar graphs in Fig. 4A. sPLA2 IB enhanced podocyte cPLA2α and ERK1/2 phosphorylation in a dose-dependent manner; however, sPLA2 IB did not modulate podocyte PLA2R expression (Fig. 4A).

Effects of PLA2R upregulation and downregulation on sPLA2 IB-induced human podocyte apoptosis. To evaluate the effects of the M-type PLA2R on sPLA2 IB-induced human podocyte apoptosis, we upregulated podocyte PLA2R expression through M-type PLA2R plasmid transfection and downregulated podocyte PLA2R expression through M-type PLA2R siRNA transfection. Podocytes transfected with either control plasmid or control siRNA were used as controls. Subsequently, control and experimental cells were treated with sPLA2 IB (10^{-6} mol/L) for 2 h. Protein blots were probed for PLA2R, phos-cPLA2α, and phos-ERK1/2; then, the same blots were reprobed for total cPLA2α, total ERK1/2, and actin. Additionally, PLA2R and sPLA2 IB expression was evaluated using immunofluorescence assays. The effect of PLA2R downregulation or upregulation on podocyte apoptosis was evaluated by flow cytometry. Representative gels displaying PLA2R, phos-cPLA2α, phos-ERK1/2, cPLA2α, ERK1/2, and actin expression are displayed in Fig. 5A. Representative microfluorographs displaying podocyte PLA2R and sPLA2 IB expression are shown in Fig. 5B. Podocytes transfected with the PLA2R plasmid displayed PLA2R expression upregulated by 30%, and podocytes transfected with PLA2R siRNA displayed PLA2R expression downregulated by 35%. Podocyte phos-cPLA2α and phos-ERK1/2 expression correlated with podocyte PLA2R expression (Fig. 5A). Podocytes transfected with the PLA2R plasmid displayed higher numbers of apoptotic cells when compared with

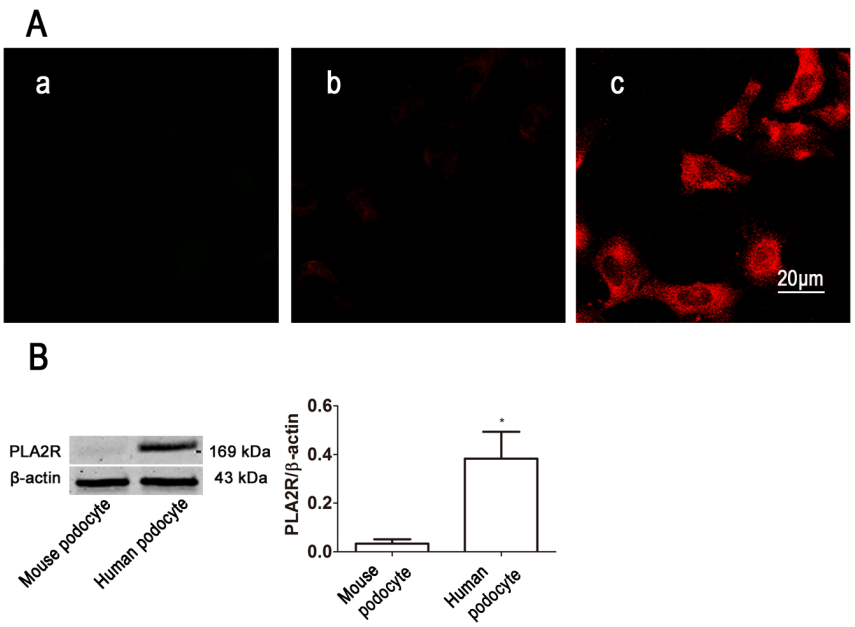


Figure 2 | The levels of PLA2R expression in mouse podocytes and in human podocytes. A. Immunofluorescence staining of mouse podocyte and human podocyte PLA2R expression (original magnification, ×400). *a* Negative control group: PBS instead of the PLA2R antibody. *b* Mouse podocytes. *c* Human podocytes. B. Representative Western blotting of the levels of PLA2R in mouse podocytes and in human podocytes. * $p < 0.05$ vs. mouse podocytes.

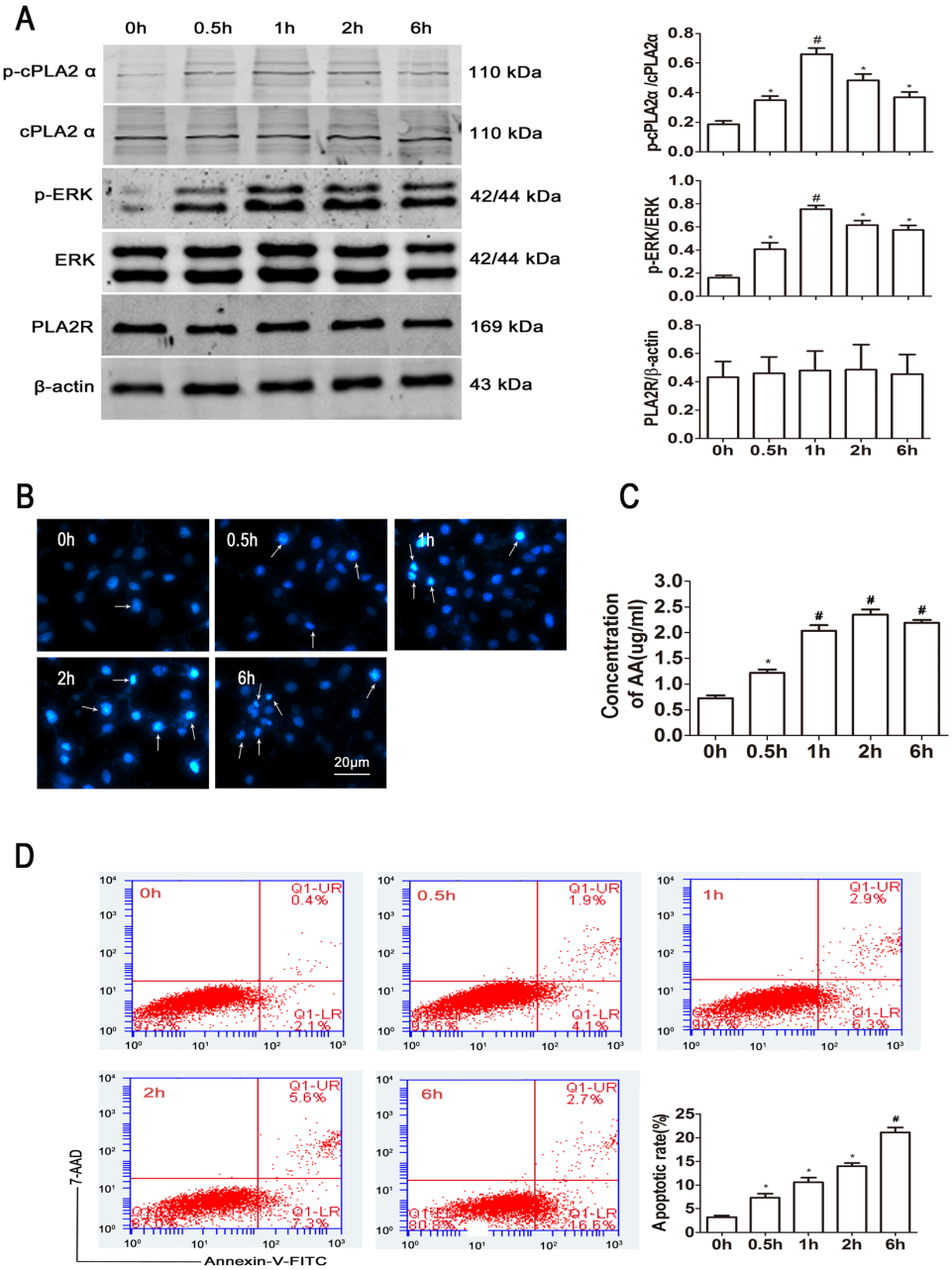


Figure 3 | sPLA2 IB induced podocyte apoptosis in a time-dependent manner. A. Representative Western blotting results for cPLA2α, ERK1/2 and their phosphorylation and for the PLA2R at different time points (n = 3). All of the groups were treated with a 10^{-6} M concentration of sPLA2 IB. B. Representative Hoechst 33342 staining of apoptotic podocytes stimulated by 10^{-6} M sPLA2 IB at various time points (original magnification, ×400). C. HPLC of podocyte AA production at different time points (n = 3). All of the groups were treated with a 10^{-6} M concentration of sPLA2 IB. D. Flow cytometry analysis of apoptosis in differentiated podocytes at different time points (n = 3). All of the groups were treated with a 10^{-6} M concentration of sPLA2 IB. * $p < 0.05$ vs. 0 h group, # $p < 0.01$ vs. 0 h group.

control cells, whereas podocytes transfected with PLA2R siRNA displayed lower numbers of apoptotic cells (Fig. 5D and Fig. S5).

Effects of cPLA2α and ERK1/2 on sPLA2 IB-induced AA accumulation levels in human podocytes. As shown in Figs. 3C, 4C and 5C, the AA levels in human podocytes correlated with the cPLA2α and ERK1/2 phosphorylation levels. Following sPLA2 IB treatment, the podocyte AA content increased and reached a maximum at 2 h (Fig. 3C and Fig. S2). sPLA2 IB enhanced podocyte AA content in a dose-dependent manner (Fig. 4C and Fig. S3). Moreover, the PLA2R plasmid transfection enhanced the podocyte AA content, whereas PLA2R siRNA transfection attenuated the podocyte AA content (Fig. 5C and Fig. S4).

Effects of sPLA2 IB on the interaction between ERK1/2 and cPLA2α and on podocyte apoptosis. To characterise the role of ERK1/2 in the signalling cascade of sPLA2 IB-induced human podocyte apoptosis, co-immunoprecipitation experiments were performed. As shown in Fig. 6B, sPLA2 IB increased the levels of cPLA2α that co-immunoprecipitated with ERK1/2 in human podocytes, and this response was attenuated by U0126. To confirm the interaction between cPLA2α and ERK1/2, human podocytes were pretreated with MAFP or with U0126 (selective inhibitors of cPLA2α and ERK1/2, respectively). Subsequently, sPLA2 IB (10^{-6} mol/L) was used to stimulate the human podocytes for 2 h. The results indicated that U0126 decreased phos-cPLA2α and phos-ERK1/2 expression. However, MAFP only decreased cPLA2α

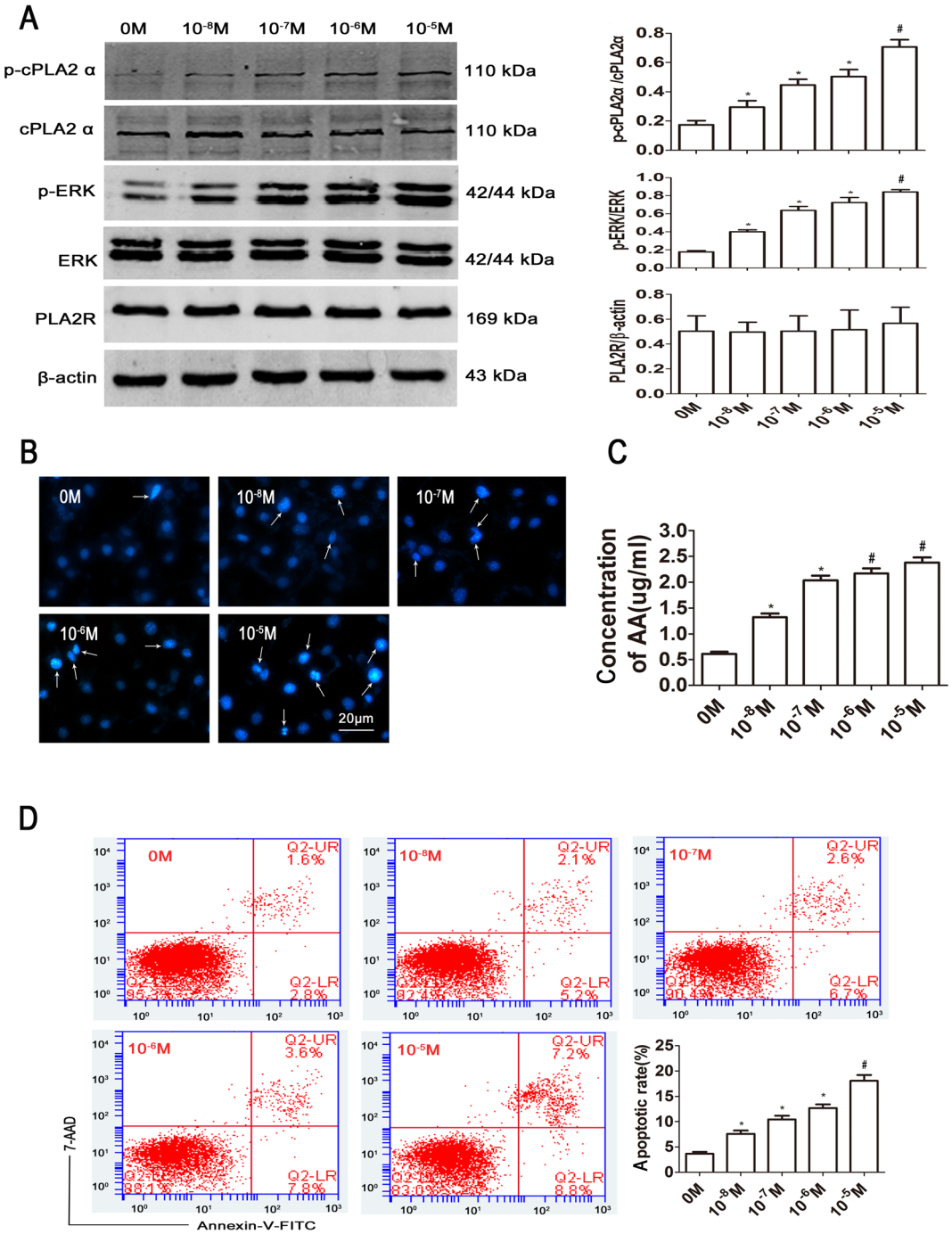


Figure 4 | sPLA2 IB induced podocyte apoptosis in a dose-dependent manner. A. Representative Western blotting results for cPLA2α, ERK1/2 and their phosphorylation and for the PLA2R at different concentrations of sPLA2 IB (n = 3). All of the groups were treated with sPLA2 IB for 2 h. B. Representative Hoechst 33342 staining of apoptotic podocytes stimulated by different concentrations of sPLA2 IB for 2 h (original magnification, ×400). C. HPLC of podocyte AA production at different concentrations of sPLA2 IB (n = 3). All of the groups were treated with sPLA2 IB for 2 h. D. Flow cytometry analysis of apoptosis in differentiated podocytes stimulated by different concentrations of sPLA2 IB (n = 3). All of the groups were treated with sPLA2 IB for 2 h. * $p < 0.05$ vs. 0 M group, # $p < 0.01$ vs. 0 M group.

phosphorylation (Fig. 6A). The effects of sPLA2 IB on podocyte apoptosis are displayed in representative microfluorograms (Fig. 6C) and in flow cytograms (Fig. 6D). The effects of sPLA2 IB on podocyte apoptosis were reduced by MAFP and by U0126.

Effects of AA on human podocyte apoptosis in vitro. To further confirm the role of AA in human podocyte apoptosis, human podocytes were treated with AA (10^{-5} mol/L) for different time points (0, 0.5, 1, 2 and 6 h). As shown in Fig. 7A, nuclear p53 expression increased at 0.5, 1, 2 and 6 h. In addition, AA promoted podocyte apoptosis in a time-dependent manner (Fig. 7B and 7C).

Discussion

sPLA2 IB not only mediates cell migration, cell proliferation, hormone release and eicosanoid production via its receptor but also mediates apoptosis[14]. The in vivo investigation showed that human podocytes apoptosis significant increased with higher levels of sera sPLA2 IB and PLA2R expression in the kidneys of IMN patients. The

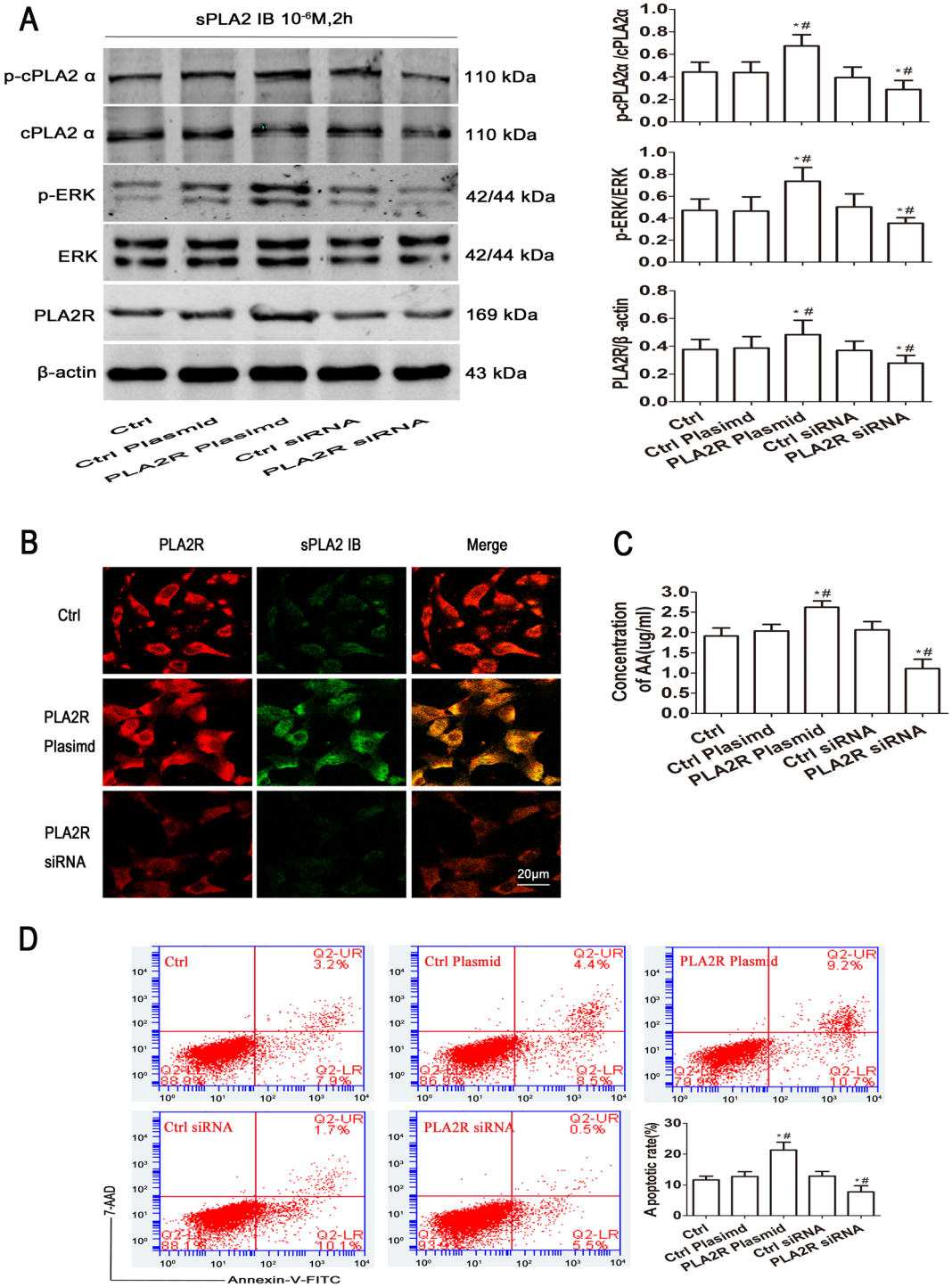
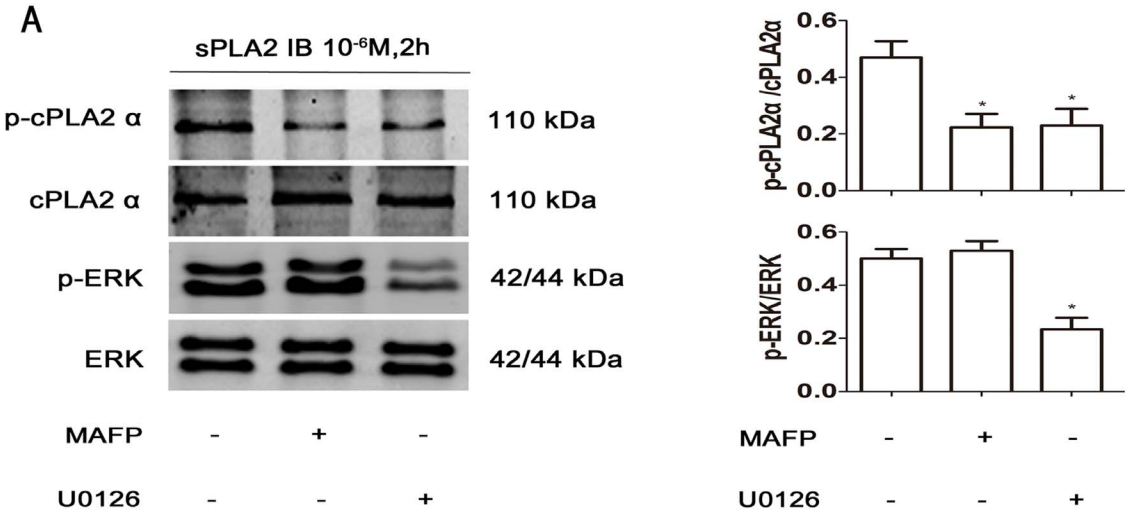
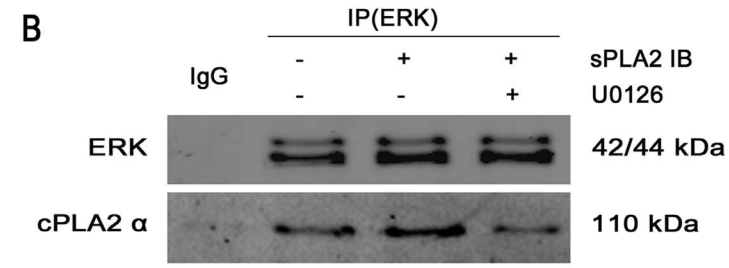


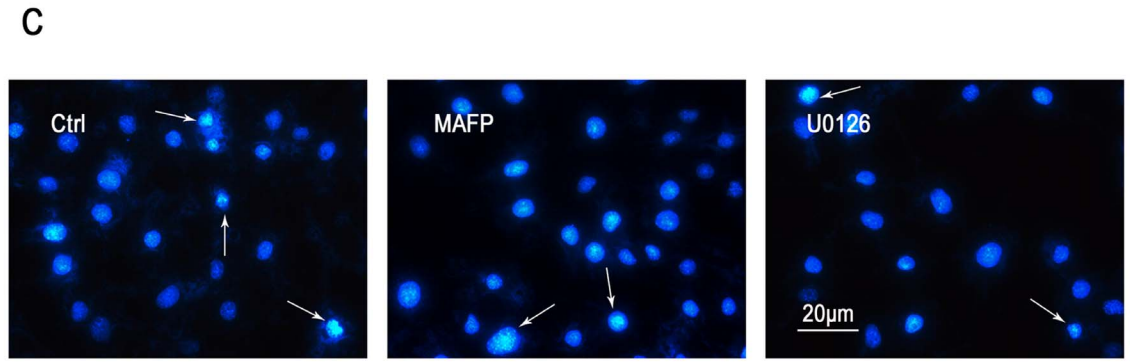
Figure 5 | Effects of the PLA2R plasmid and PLA2R siRNA on sPLA2 IB-induced podocyte apoptosis. A. Western blotting analysis of cPLA2α, ERK and their phosphorylation and for the PLA2R in cultured podocytes stimulated by 10^{-6} M sPLA2 IB for 2 h and treated with no plasmid, scrambled plasmid, PLA2R plasmid, scrambled siRNA or PLA2R siRNA (n = 3). B. Representative immunofluorescence staining of PLA2R and sPLA2 IB expression in cultured podocytes stimulated by 10^{-6} M sPLA2 IB for 2 h and treated with no plasmid, PLA2R plasmid or PLA2R siRNA (original magnification, ×400). C. HPLC of podocyte AA production in cultured podocytes stimulated with 10^{-6} M sPLA2 IB for 2 h and treated with no plasmid, scrambled plasmid, PLA2R plasmid, scrambled siRNA or PLA2R siRNA (n = 3). D. Flow cytometry analysis of apoptosis in differentiated podocytes stimulated by 10^{-6} M sPLA2 IB for 2 h and treated with no plasmid, scrambled plasmid, PLA2R plasmid, scrambled siRNA or PLA2R siRNA (n = 3). * $p < 0.05$ vs. control group, # $p < 0.05$ vs. scrambled plasmid group or scrambled siRNA group.

in vitro studies demonstrated that sPLA2 IB induced apoptosis in human podocytes. However, methyl arachidonyl fluorophosphonate (MAFP, an inhibitor of cPLA2α)-treated podocytes or PLA2R-silenced podocytes displayed attenuated apoptosis in response to sPLA2 IB. Conversely, podocytes transfected with the PLA2R plasmid displayed accentuated podocyte apoptosis in response to sPLA2 IB. These results indicate that sPLA2 IB-induced podocyte apoptosis is mediated by the PLA2R.

The present study indicated that relatively high sPLA2 IB levels might induce human podocyte apoptosis in IMN patients with







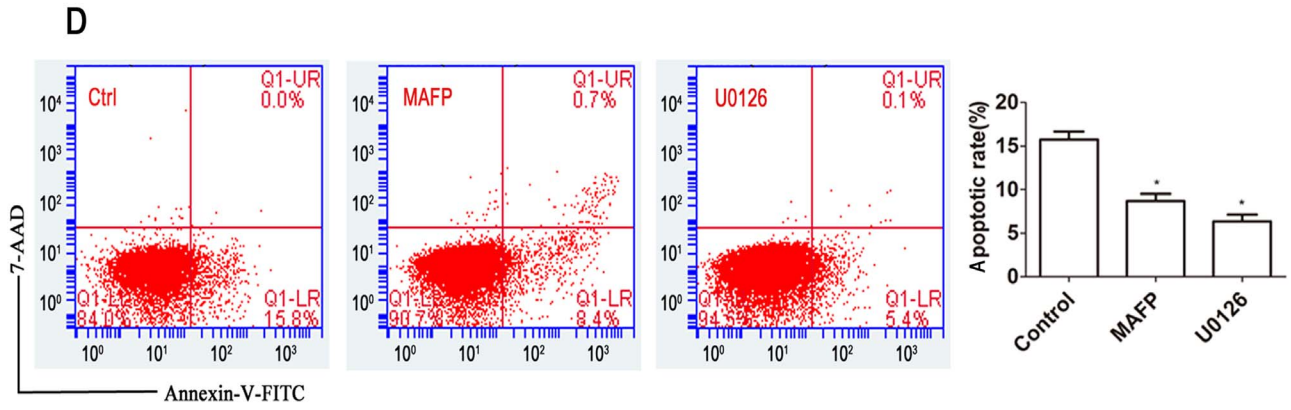


Figure 6 | The cPLA2α and ERK interaction in podocytes and its effect on podocyte apoptosis. A. Western blotting analysis of cPLA2α, ERK and their phosphorylation in cultured podocytes stimulated by 10^{-6} M sPLA2 IB for 2 h and pretreated with 10 μM MAFP or 10 μM U0126 for 1 h (specific inhibitors of cPLA2α and ERK, respectively; n = 3). B. cPLA2α was immunoprecipitated from normal podocytes and from sPLA2 IB-treated podocytes for 2 h (10^{-6} M), which were pretreated with 10 μM U0126 for 1 h. Then, the immunoprecipitates were analysed by Western blotting with ERK and cPLA2α antibodies (n = 3). C. Representative Hoechst 33342 staining of apoptotic podocytes stimulated by 10^{-6} M sPLA2 IB for 2 h and pretreated with 10 μM MAFP or 10 μM U0126 for 1 h (original magnification, ×400). D. Flow cytometry analysis of apoptosis in differentiated podocytes stimulated by 10^{-6} M sPLA2 IB for 2 h and pretreated with 10 μM MAFP or 10 μM U0126 for 1 h (n = 3). * $p < 0.05$ vs. control group.

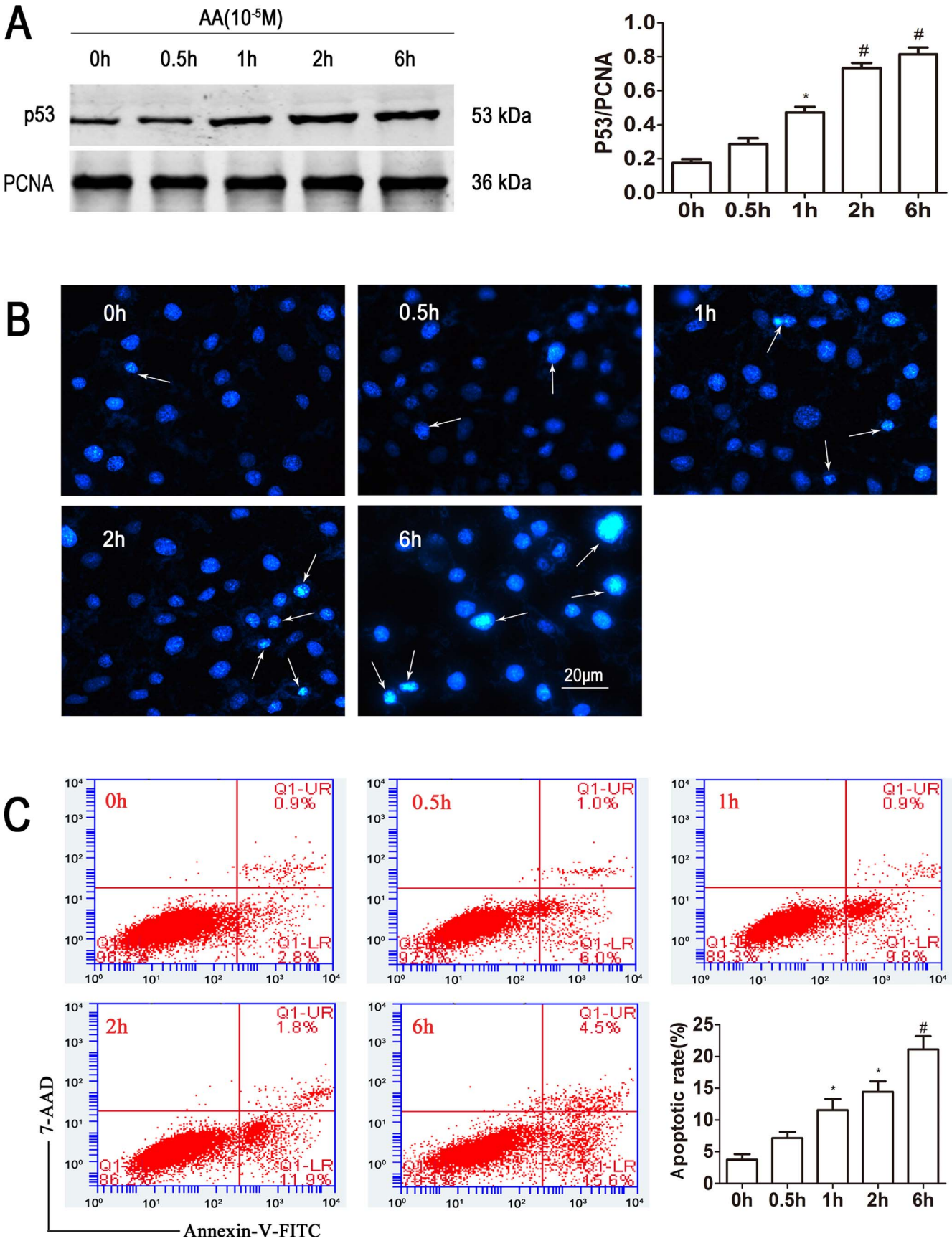


Figure 7 | Effects of AA on podocyte apoptosis. A. Representative Western blotting results of nuclear P53 at different time points (n = 3). All of the groups were treated with a 10^{-5} M concentration of AA. B. Representative Hoechst 33342 staining of apoptotic podocytes at different time points. (original magnification, ×400). C. Flow cytometry analysis of apoptosis in differentiated podocytes at different time points (n = 3). All of the groups were treated with a 10^{-5} M concentration of AA. * $p < 0.05$ vs. 0 h group, # $p < 0.01$ vs. 0 h group.

enhanced PLA2R expression in their kidneys. Previous studies clearly detected the PLA2R in sub-epithelial deposits along glomerular capillary loops in 69% of IMN patients[9]. However, approximately 30% of IMN patients still weakly expressed the PLA2R. These two groups may have different podocyte apoptosis in their kidneys. Therefore, our data demonstrated that the enhanced PLA2R expression group with a higher serum sPLA2 IB level had an increased podocyte apoptosis rate. Moreover, serum albumin level was lower and the level of urinary protein was higher in the enhanced PLA2R expression group than in the other group. Although only twenty-five patients with IMN were involved, these results revealed that at least higher sPLA2 IB and PLA2R levels correlate with podocyte apoptosis. In contrast, sPLA2 IB-induced podocyte apoptosis should be demonstrated by large clinical trials in the future.

In the present study, human podocytes displayed robust PLA2R expression in vitro. sPLA2 IB induced human podocyte apoptosis through the PLA2R. Up- and down-regulated PLA2R expression correlated with the degree of podocyte apoptosis. Moreover, sPLA2 IB induced podocyte apoptosis in a time- and concentration-dependent manner. These findings are consistent with the observations of Yagami T and Augert A[14,20]. Analyses of the full set of sPLA2s have demonstrated that up to seven sPLA2s, including sPLA2 IB, can bind to the M-type PLA2R and, thus, can be regarded as natural ligands of this receptor[21,22]. In contrast, a common functional feature of PLA2Rs is their ability to undergo endocytosis, thus indicating their involvement in the internalisation of extracellular ligands. PLA2R internalisation, coupled with the binding of sPLA2 to its specific receptor, could be an important initial component of the signal transduction pathway[23]. The interaction of sPLA2 IB with the PLA2R triggers cellular signalling events that contribute to the biological effects of sPLA2[24]. Previous studies demonstrated that Meindoxam, which is a competitive PLA2R inhibitor, dramatically decreased the affinity of various sPLA2s for their receptors[25]. In the present study, we demonstrated that sPLA2 IB was bound to PLA2R and internalised by its receptor in human podocytes.

sPLA2 IB-induced apoptosis is not mediated via inflammatory cytokines[14]. PLA2s are generally considered the key enzymes that control the release of lipid mediator precursors. However, these enzymes also behave as ligands for receptors, and their physiological function is not confined to their catalytic activity[26]. The PLA2R was reported to promote replicative senescence in human fibroblasts, partly by causing reactive oxygen species production and DNA damage[20]. Our results were consistent with the findings of these investigators. In contrast, both cPLA2α and sPLA2 IB have been associated with various physiological and pathological conditions[27–29]. The relation between cPLA2α and sPLA2 IB is unclear. As reported previously, cPLA2 is a downstream effector of the MAPK pathway. Shibata N demonstrated that cPLA2 was activated by p38[30]. However, other investigators have suggested that the PLA2R does not have a crucial effect on ERK1/2 or p38 in experimental settings[20]. In the present study, we used an inhibitor of ERK (U0126) to ensure the participation of ERK1/2 signalling in cPLA2 activation. In our studies, sPLA2 IB increased podocyte cPLA2α phosphorylation. However, U0126 suppressed cPLA2α activation via reducing podocyte ERK1/2 phosphorylation. Moreover, our co-immunoprecipitation studies demonstrated that sPLA2 IB treatment promoted the interaction between ERK1/2 and cPLA2α and that this interaction was abolished by U0126. Thus, the present study at least partly indicates that cPLA2α was activated by ERK1/2.

Previous studies have also suggested that AA can be produced in a PLA2R-dependent manner[31]. cPLA2-induced AA accumulation is an event that has been associated with apoptosis induction[32]. Therefore, studies were conducted to determine whether sPLA2 IB-treated human podocytes underwent apoptosis through AA. The present study suggests that cPLA2α activation contributed to the increased levels of AA in a time- and dose-dependent manner. Moreover, AA participated in the pathogenesis of sPLA2 IB-induced damage in human podocytes. In the present study, the human podocyte apoptotic rate decreased in tandem with decreases in cPLA2α phosphorylation and AA content after U0126 and MAFP treatments, respectively. These results also indicate that sPLA2 IB did not directly induce human podocyte apoptosis. In nucleated cells, high AA concentrations contributed to mitochondrial dysfunction and to cell apoptosis[19]. Because sPLA2 IB-induced podocyte apoptosis directly correlated with cPLA2α activation and with AA generation, we tested whether AA itself was involved in apoptosis. Our results confirm that AA induced human podocyte apoptosis in a time-dependent manner at a concentration of 10^{-5} M. Furthermore, our data suggest that AA-induced apoptosis partially occurred in a p53-dependent manner. However, the mechanism by which the AA and p53 interaction induced podocyte apoptosis remains to be determined.

In summary, the present study demonstrates an important role for the PLA2R in sPLA2 IB-induced podocyte apoptosis. sPLA2 IB stimulates podocyte cPLA2α phosphorylation via the ERK1/2 pathway and increases AA generation with the upregulation of PLA2R expression. This study also indicates that sPLA2 IB-induced podocyte apoptosis may occur via the ERK1/2-cPLA2α-AA-p53 signalling pathway. These findings provide a framework to investigate new therapeutic targets for sPLA2 IB-associated human podocyte apoptosis.

Methods

Patients and sera. Twenty-five patients with IMN, who were diagnosed in Division of Nephrology, Renmin Hospital of Wuhan University, Wuhan, PR China and Division of Nephrology, First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, China from 2012 to 2014, were enrolled in this study. All of the patients showed MN characteristics on renal biopsy. IMN was diagnosed after the exclusion of secondary causes. Twenty-five patients were divided into two groups according to the sPLA2 IB level in their sera and PLA2R expression in kidney. We defined the serum sPLA2 IB level less than 10 ng/ml with weak PLA2R expression as the low sPLA2 IB expression group (Group I) and more than 10 ng/ml with enhanced PLA2R expression as the high sPLA2 IB expression group (Group II). Nine patients with IMN were included in Group I, and sixteen patients with IMN were included in Group II. Clinical data of each group of patients with IMN were collected at the time of diagnosis. The clinical data are shown in Table 1. Serum from all of the patients were collected before immunosuppressive treatment and preserved at −70°C until use.

Study protocol was approved by the Ethics Committee of Renmin Hospital of Wuhan University and First Affiliated Hospital of Fujian Medical University. All experiments were performed in accordance with approved guidelines of Wuhan University and Fujian Medical University. The research complied with the Declaration of Helsinki. Written informed consent was obtained from the patients for publication of this study and any accompanying images.

Cell culture. Conditionally immortalised human podocytes were obtained from Dr. Moin A. Saleem (University of Bristol, Southmead Hospital, Bristol, UK). Briefly, human podocytes were conditionally immortalised by introducing a temperature-sensitive SV40-T antigen by transfection[33]. Additionally, the cells were transfected with a human telomerase construct[34]. Conditionally immortalised murine podocytes were kindly provided by Dr. Peter Mundel (Department of Medicine, Massachusetts General Hospital, Charlestown, MA, USA). These cells proliferate at a permissive temperature (33°C) and enter growth arrest after transfer to a nonpermissive temperature (37°C). When the podocytes reached approximately 70–80% confluence at 33°C, the podocytes were transferred to 37°C for differentiation in a medium without insulin, transferrin or selenium (ITS) for approximately 7 days.

The growth medium for human podocytes consisted of RPMI 1640 medium (HyClone, USA) supplemented with 10% foetal calf serum (Gibco, USA), 1× penicillin-streptomycin, 1 mM L-glutamine and 1× ITS (Invitrogen, Grand Island, NY, USA) at a permissive temperature (33°C). The growth medium for murine podocytes consisted of RPMI 1640 (HyClone, USA) supplemented with 10% foetal calf serum (Gibco, USA), 10 units/ml recombinant mouse interferon-c (Peprotech, USA), 100 U/ml penicillin G and 100 g/ml streptomycin (Invitrogen, USA).

Immunohistochemistry. Immunostaining for PLA2R expression was performed on paraffin-embedded sections. Slides were deparaffinised and treated with 3% H_2O_2 for 30 min at room temperature. Antigen retrieval for PLA2R was performed in high pressure citrate buffer (0.01 mol/L, pH 6.0) for 15 min. Endogenous peroxidase was blocked with 5% bovine serum albumin in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) for 30 min, and sections were incubated with a rabbit anti-PLA2R antibody (1 : 200; Abcam, UK) overnight at 4°C. Sections were washed in PBS, followed by incubation with a biotinylated anti-rabbit secondary antibody and an avidin–biotin peroxidase complex (Dako) for 30 min. After rinsing, the peroxidase activity was visualised by DAB (Dako), and sections were counter-stained with

haematoxylin. Negative controls were performed by omitting the primary antibody and by replacing the primary antibody with normal rabbit IgG. Glomeruli were analysed using Image-Pro Plus 5.10 software (Media Cybernetics) at 400× magnification, and integrated optical density (IOD) was used as the relative amount of glomerular positive staining.

Enzyme-linked immunosorbent assay (ELISA). The sera sPLA2 IB level was measured by ELISA according to the manufacturer's instructions. Briefly, all samples and the standard protein (100 μl each) were prepared in TBS containing 0.1% BSA and added to the treated wells. The incubation was performed using a microplate agitator at room temperature for 90 min. Subsequently, the wells were washed 4 times with wash buffer, followed by the addition of rabbit anti-human sPLA2 IB anti-serum (100 μl/well of a 1 : 2000 dilution). After a 60 min incubation, the wells were washed again, and alkaline phosphatase-conjugated goat anti-rabbit IgG (100 μl of a 1 : 1000 dilution) was added to the wells. Final washing was performed 60 min later, and 50 μl of 1 mg/ml p-nitrophenylphosphate was added to each well. Absorbance was measured at 450 nm after 30 min incubation at 37°C using a microplate absorbance spectrophotometer (MiniReader, Thermo Scientific, USA). All of the assays were performed in duplicate. Purified human sPLA2 IB was used to generate a standard calibration curve.

Western blotting and Co-immunoprecipitation. The podocyte proteins were extracted and lysed in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, and 50 mM Tris, pH 8.0) with protease/phosphatase inhibitors and then were centrifuged at 13,000× *g* for 20 min at 4°C. The protein samples were mixed with 5× lane marker sample buffer to create a 1× final solution loading buffer, which was boiled for 5 min at 95°C. Then, proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to nitrocellulose membranes (GE Healthcare, USA) by semidry blotting. A PLA2R rabbit polyclonal antibody (1 : 1,000; Abcam, UK), a phospholipase A2 IB rabbit polyclonal antibody (1 : 500; Sigma, USA), a phospholipase A2 rabbit polyclonal antibody (1 : 500; Abcam, UK), a phospholipase A2-phospho S505 rabbit polyclonal antibody (1 : 500; Abcam), an ERK1/2 mouse monoclonal antibody (1 : 500; Santa Cruz Biotechnology, USA), an ERK1/2-phospho PT202/PY204 mouse monoclonal antibody (1 : 2,000; Santa Cruz Biotechnology), a p53 mouse monoclonal antibody (1 : 200; Santa Cruz Biotechnology), a proliferating cell nuclear antigen (PCNA) mouse monoclonal antibody (1 : 1,000; Thermo, USA) and a β-actin mouse monoclonal antibody (1 : 5,000; Santa Cruz Biotechnology) were used as primary antibodies. Western blots were probed with a goat anti-rabbit or anti-mouse secondary antibody conjugated with IRDye 800 (1 : 10,000; LI-COR Biotechnology, USA). Blotted proteins were detected and quantified using an Odyssey infrared imaging system (LI-COR Biotechnology, USA).

Co-immunoprecipitation was performed using a co-immunoprecipitation kit (Beyotime, China) according to the manufacturer's instructions. Briefly, the ERK1/2 mouse monoclonal antibody (1 : 50) was added to the protein sample and was rotated overnight at 4°C. Then, the samples were mixed with 30 μl of IgG + IgA agarose beads and were centrifuged at 4°C for 3 h, followed by centrifuging the beads at 2,500× *g* for 5 min at 4°C and washing the beads 3 times with IP lysis buffer. The samples were mixed with 40 μl of 1× lane marker sample buffer and were heated at 95°C for 5 min. Subsequently, the samples were analysed by Western blotting for ERK1/2 and cPLA2α, as described above.

Immunofluorescence assay. The podocyte climbing film was fixed in 4% paraformaldehyde for 30 min at 4°C. The cells were incubated with the PLA2R mouse polyclonal antibody (1 : 200) and the phospholipase A2 IB rabbit polyclonal antibody (1 : 200) overnight at 4°C. Then, the cells were incubated with the TRITC-conjugated IgG (1 : 100) and FITC-conjugated IgG (1 : 100) secondary antibodies at 37°C for 90 min in darkness. The cells were observed under a laser scanning confocal microscope (FV1000, Olympus, Japan).

High-performance liquid chromatography (HPLC). The AA concentration in the podocytes was measured by HPLC, as previously described[35]. A HPLC 1100 series instrument (Agilent, Germany) was used for the measurements. HPLC separation was performed using a 250 mm × 4.6 mm internal diameter (ID) stainless-steel column packed with 6 μm Kovasil C_{18} end-capped particles (Chemie Uetikon, Switzerland). An acetonitrile-water mixture (90 : 10), also containing 0.3% formic acid, was used as the mobile phase at a flow rate of 1.0 mL/min^{-1}. Each fraction was collected for 1 min (1.5 mL), with a column temperature of 26°C. Dilutions of prepared 10 μg/L AA (Sigma, USA) standard solution with 50% acetonitrile were used as the internal standards for AA concentration determination.

Transfection. The human PLA2R plasmid and empty vectors were purchased from Origene Company (USA). The PLA2R plasmid or empty vectors were transfected into podocytes using Lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions.

Small interfering RNAs (siRNA) for gene-specific silencing of the PLA2R were designed and synthesised by QIAGEN (Germany). PLA2R siRNA transfection was performed according to the HiPerFect transfection reagent handbook (QIAGEN). Briefly, 3 × 10^5 cells were seeded in 6-well plates and transfected with complexes consisting of 5 nM PLA2R siRNA or a negative control scrambled siRNA. The podocytes were incubated with the transfection complexes under normal growth conditions for 48 h.

Apoptosis studies. Podocyte apoptosis in kidney tissue was determined by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labelling (TUNEL) assay according to the manufacturer's instructions (C1098, Beyotime). In brief, dewaxed sections (3 μm thick) were incubated with protease K for 30 min at 37°C, followed by 3% H_2O_2 for 15 min at room temperature. Then, sections were incubated with a mixture of terminal deoxynucleotidyl transferase (TdT) and biotin-dUTP in a humidified chamber for 1 h at 37°C, and then were subjected to streptavidin-horseradish peroxidase for 30 min at room temperature. The slides were stained with DAB, with haematoxylin as a counterstain. The negative control omitted TdT. Apoptotic podocytes from single cross-sections (through the glomerulus) were counted using the Weibel-Gomez method[36].

Flow cytometry was used to evaluate cultured human podocyte apoptosis. FITC-conjugated Annexin V and 7-AAD were used to identify apoptotic cells in different cell cycle stages according to the manufacturer's instructions (Biolegend, USA). Cells in the lower right quadrant were classified as exhibiting early apoptosis (labelled by FITC-conjugated Annexin V), and cells in the upper right quadrant were identified as exhibiting late-stage apoptosis (labelled by 7-AAD).

For detecting apoptosis by flow cytometry, cells were stained using an Annexin V-FITC kit (R&D Systems) according to the manufacturer's instructions. Briefly, the cells were cultured in a 6-well plate (5.0 × 10^5/well). After overnight incubation, the cells were treated with or without various concentrations of sPLA2 IB for different amounts of time. Both floating and adherent (released by trypsin) cells were collected and washed with ice-cold phosphate-buffered saline (PBS). The cells were resuspended in 100 μl of binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl, and 2 mM $CaCl_2$) containing 0.25 μg/ml Annexin V-FITC and 5 μg/ml 7-AAD. After 15 min of incubation at room temperature in the dark, 400 μl of binding buffer was added, and the samples were processed by flow cytometry. In total, 10,000 cells were acquired. Unlabelled cells suspended in PBS were used as a negative control and for determining the gates to be employed in the apoptosis assays. Data acquisition and analysis were performed with a FACScan flow cytometer using Cell Quest software (BD Biosciences).

Apoptosis in cultured human podocytes was also assessed using Hoechst 33342 (Sigma) staining. The cells were stained with Hoechst 33342 dye at room temperature for 5 min after being fixed in 4% paraformaldehyde for 10 min at room temperature and then washed 3 times with ice-cold PBS. Podocytes were observed under fluorescence microscopy, and all of the microscopic images were obtained with a 12.8-megapixel camera (DP72, Olympus, Japan).

Statistical analysis. All of the experiments were performed at least three times, with similar results obtained between experiments. The data are presented as the mean ± SDs. Statistically significant differences in mean values were assessed using Student's *t*-test or a one-way analysis of variance (ANOVA) for multiple comparisons. Correlation analysis was performed by Pearson's correlation test. The data were analysed using SPSS software, version 17.0. A value of $P < 0.05$ was considered statistically significant.

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Acknowledgments

This study was supported by grants from the National Science Foundation of China (81270762 to G. Ding and 81100478 to W. Liang) and National Institutes of Health Grant. RO1DK084910 (PCS).

Author contributions

Y.P., J.W., Y.L., Q.Y. and W.L. performed experiments and data analysis. Y.P. designed the experiments and wrote the manuscript; P.C.S. and M.A.S. provided conceptual input. G.D. designed the experiments, supervised the research and revised manuscripts.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pan, Y. *et al.* sPLA2 IB induces human podocyte apoptosis via the M-type phospholipase A2 receptor. *Sci. Rep.* **4**, 6660; DOI:10.1038/srep06660 (2014).