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Shear stress induces cell apoptosis via a c-Src-phospholipase D-mTOR signaling pathway in cultured podocytes

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Abstract

The glomerular capillary wall, composed of endothelial cells, the glomerular basement membrane and the podocytes, is continually subjected to hemodynamic force arising from tractional stress due to blood pressure and shear stress due to blood flow. Exposure of glomeruli to abnormal hemodynamic force such as hyperfiltration is associated with glomerular injury and progressive renal disease, and the conversion of mechanical stimuli to chemical signals in the regulation of the process is poorly understood in podocytes. By examining DNA fragmentation, apoptotic nuclear changes and cytochrome c release, we found that shear stress induced cell apoptosis in cultured podocytes. Meanwhile, podocytes exposed to shear stress also stimulated c-Src phosphorylation, phospholipase D (PLD) activation and mammalian target of rapamycin (mTOR) signaling. Using the antibodies against c-Src, PLD_1, and PLD_2 to perform reciprocal co-immunoprecipitations and *in vitro* PLD activity assay, our data indicated that c-Src interacted with and activated PLD_1 but not PLD_2. The inhibition of shear stress-induced c-Src phosphorylation by PP_2 (a specific inhibitor of c-Src kinase) resulted in reduced PLD activity. Phosphatidic acid, produced by shear stress-induced PLD activation, stimulated mTOR signaling, and caused podocyte hypertrophy and apoptosis.

Keywords

Podocytes; shear stress; apoptosis; c-Src; phospholipase D; mTOR

1. Introduction

Glomerular podocytes, terminally differentiated epithelial cells, support glomerular capillary structure and consist of a cell body, major processes and foot processes that are enriched in cytoskeletal elements and that form the slit diaphragm (1, 2). The glomerular capillary wall consists of three layers: endothelial cells, the glomerular basement membrane, and the outer

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most epithelial or podocyte layer. Under normal conditions, the glomerular capillary wall is continually subjected to hemodynamic force arising from capillary blood pressure and fluid flow in the form of tractional and shear stresses (2). Through an actin-based contractile apparatus, podocytes counterbalance the pressure within the underlying capillary to prevent outward ballooning of the vessel and to preserve the normal architecture of the capillary (3, 4). Cell responses to mechanical force lead to increased accumulation of filamentous actin at focal adhesions, increased rigidity of the cell membrane, and the activation of a large number of enzymes which can produce second messengers to regulate a variety of signaling pathways, gene expression and biological functions (5–7). Exposure of glomeruli to abnormal hemodynamic force such as hyperfiltration leads to glomerular injury and progressive renal disease. Understanding the behavior of podocytes in response to mechanical force is indispensable for elucidating the molecular mechanisms of hyperfiltration-induced podocyte injury and progressive renal disease.

Exploring the conversion of mechanical stimuli to biochemical signals has become an important area of biomedical research. An increasing number of signaling systems and proteins are involved in responses to mechanical force. In podocytes, mechanical force induces angiotensin II secretion and AT_1 receptor expression (8), leads to downregulation of nephrin expression (9), stimulates extracellular signal-regulated kinases, c-Jun N-terminal protein kinases, Src, and focal adhesion kinase (10, 11). These kinases are further involved in many other signaling pathways that regulate cell processes including growth, proliferation, differentiation and apoptosis. Mechanical force has also been documented to affect phospholipid metabolism through activation of phospholipase A_2 (12), phospholipase C (13), phospholipase D (PLD) (14), and phosphatidylinositol 3-kinase (11) as well as phosphatidylcholine (PC) biosynthesis (15). Phospholipids are major structural components in plasma membranes. Cell proliferation and apoptosis through signaling pathways regulate membrane phospholipid metabolism. Cell proliferation which needs new plasma membranes for daughter cells significantly stimulates phospholipid synthesis, while cell apoptosis in which cells are degraded leads to the breakdown of membrane phospholipids. The products of phospholipid hydrolysis are also a major source of lipid second messengers which play important roles in complex intracellular signaling network to regulate cellular functions. (16–18).

In glomerular hyperfiltration, podocytes may experience mechanical force in a number of ways; such as lateral stretch as a capillary dilates and shear stress from increased filtrate flow. By exposure of podocytes with surface fibronectin-coated ferric beads to magnetic force as a stretch force, we recently found that mechanical force leads to Rho translocation, actin cytoskeleton reorganization, and PLD activation via a $G\alpha_{12/13}$/Rho/F-actin-dependent signaling pathway (14). PLD is a primary enzyme in cell signaling to produce phosphatidic acid (PA) which is a critical activator of mammalian target of rapamycin (mTOR) signaling (19–21), a process linked to increase cell mass associated with glomerular hypertrophy (22, 23). To understand how abnormal hemodynamic force such as hyperfiltration induces glomerular injury and leads to progressive renal disease, we created fluid flow on the surface of cultured podocytes by placing the culture dishes on a rotating platform for varying periods of time. Using this system to define the signaling pathway, we found that shear stress-enhanced podocyte apoptosis is associated with the phosphorylation of c-Src and the activation of PLD and mTOR signaling. To test the role of PLD in this potential signaling pathway, we blocked c-Src-PLD signaling, and demonstrated that shear stress induces podocyte apoptosis via a c-Src-PLD-mTOR dependent signaling pathway in cultured podocytes.

2. Materials and Methods

2.1. Materials

All chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless specified otherwise. Cell culture reagents were purchased from Mediatech, Inc. (Herndon, VA). [^{3}H]Choline chloride (86 Ci/mmol) and [^{3}H]palmitic acid (43 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Phosphatidylethanol (PEt) was supplied by Avanti Polar Lipids, Inc. (Alabaster, AL). The polyclonal anti-phospho-c-Src (Tyr416) antibody was purchased from Cell Signaling Technology (Cambridge, MA). The polyclonal anti-c-Src, anti-PLD_1 and anti-PLD_2 antibodies, and monoclonal anti-c-Src, anti-cytochrome c, anti-tubulin, and anti-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-synaptopodin antibody was purchased from Fitzgerald Industries International, Inc. (Concord, MA). The rabbit monoclonal anti-phospho-S6 kinase antibody (T389) was purchased from Abcam Inc. (Cambridge, MA). PP_2 and PP_3 were obtained from Calbiochem (LaJolla, CA). Dynabead Protein A, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-Rabbit IgG were supplied by Invitrogen (Carlsbad, CA). Phosphatidyl-[^{3}H]choline was prepared from [^{3}H]choline prelabeled podocytes as previously described (24).

2.2. Application of shear stress

Conditionally immortalized mouse podocytes were cultured as previously described (25). Briefly, podocytes were propagated at 33°C in RPMI 1640 medium with supplements. To induce differentiation, podocytes were seeded on Type I collagen-coated plates and the cultures were maintained at 37°C without γ-interferon for 10–14 days.

Fluid flow (shear stress) was introduced by placing monolayer podocyte cultures on an orbital shaker in a standard 37°C tissue culture incubator (26, 27). This technique does not produce unidirectional laminar shear stress, and the flow regimen within a shaker dish is too complex for precise quantitative analysis, but the maximal shear stress at the bottom of the dish (cell surface) can be estimated using the equation $\tau_{max} = \alpha[\rho\eta(2\pi f)^3]^{1/2}$, where α is the radius of orbit rotation (5 cm for 100 mm dish), ρ is the density of the culture medium (1.0 g/ml), η is the viscosity of the medium (0.0075 dynes/s/cm^2), and f is the frequency of rotation (rotate per sec). At a shaking frequency of 75 cycles/min, the calculated value for τ_{max} is approximately 10 dynes/cm^2 which are a value compatible with that found by other investigators for podocytes and foot process (28, 29).

2.3. Measurement of DNA fragmentation

Differentiated podocytes were serum-deprived overnight, and then exposed to shear stress for 0 to 2 hr. The cells (both floating and adherent) were harvested and centrifuged at 1,500 *g* for 2 min, and the pellets were resuspended in 0.5 ml of lysis buffer containing 5 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.5% Triton X-100 and placed on ice for 15 min. The samples were then centrifuged at 15,000 *g* for 20 min, and the supernatant containing DNA cleavage products in equal amount of cellular proteins was precipitated overnight using isopropyl alcohol. The samples were centrifuged at 15,000 g for 20 min. Pellets were resuspended in 20 μl Tris-EDTA buffer and digested with 1 μl of 0.2 mg/ml proteinase K and 1 μl of 1 mg/ml RNase A for 60 min at 48°C. DNA fragments were separated on a 1.5% agarose gel, visualized with ethidium bromide, and photographed using the Bio-Rad image system. To identify the apoptotic cells, Tunel staining (Click-iT TUNEL Alexa Fluor Imaging Assay) was performed using the in situ cell apoptosis detection kit according to the manufactrurer's instructions (Invitrogen).

2.4. Immunoblotting, immunocytochemistry, immunoprecipitation and *in vitro* PLD activity assay

Differentiated podocytes were exposed to shear force for different time periods. The cells were harvested and the homogenized samples were centrifuged at 200,000 g for 60 min to yield pellets (membrane and nuclei) and cytosol. The cytosol was precipitated with 0.015% deoxycholate and 10% trichloroacetic acid and washed with acetone. Equal amounts of cellular proteins from cell lysates or cellular fractions were subjected to 6% or 11% SDS-PAGE, and processed for immunoblotting with the appropriate antibodies. In some experiments cells were pretreated with vehicle or the inhibitors during last 1 hr and shear force-stimulation period, and the samples were processed for immunoblotting.

Differentiated podocytes in 100 mm dishes with two glass cover-slips per dish were exposed to shear stress for 0 to 2 hr, the cover-slips were picked up, fixed with cold 4% paraformaldehyde for 20 min, and further processed for double immunofluorescence using a monoclonal anti-synaptopodin antibody and a polyclonal anti-phospho-c-Src antibody as the primary antibodies, and Alexa Fluor 488 goat anti-mouse IgG (green) and Alexa Fluor 594 goat anti-Rabbit IgG (red) as secondary antibodies. The cover-slips were also stained with 200 nM 4,6-diamino-phenylindole (DAPI) during PBS washing period, and then observed using fluorescent microscopy (Zeiss, Model LSM-5 Pascal) and images were collected using the Axiovert 200 program (Zeiss).

The remaining cells in the dishes were lysed on ice with 1 × RIPA buffer for 30 min, and the lysates were centrifuged at 15,000 g for 1 hr at 4°C. The lysates (200 g/assay) were used for co-immunoprecipitation as described previously (30). Briefly, the polyclonal anti-c-Src, anti-PLD_1 or anti-PLD_2 antibodies were loaded onto the Dynabead-protein A, and slowly rotated for 2 hr. The antibody-loaded Dynabead-protein A complex was rinsed twice and the beads were mixed with the lysates and rotated in the cold room overnight. The samples were placed in Dynal-MPC, the supernatants were discarded, and the Dynabead-protein A complex was washed once with 1×PBS, and eluted by the loading buffer. The samples were subjected to SDS-PAGE for immunoblotting using the antibodies indicated.

The immunoprecipitation pellets were also used for *in vitro* PLD activity assay. In brief, the assay mixture containing 150 μl of buffer (400,000 dpm phosphatidyl-[^{3}H]choline/assay, 20 mM Hepes, pH7.5, 0.5 mM $CaCl_2$, and 0.05% Triton X-100) was added into the tubes with immunoprecipitation pellet. The samples were vortexed and incubated at 30°C in water bath with shaker for 30 min, the reaction was stopped by adding cold methanol, and the samples were extracted by chloroform/methanol/water (5: 5: 4.5, v/v). The [^{3}H]choline in aqueous phase was analyzed as an index of PLD activity (24).

2.5. Cell radiolabeling and measurement of PLD activity

Differentiated podocytes were prelabeled with 1 μCi/ml of [^{3}H]choline chloride or [^{3}H]palimitic acid in 5 ml of 1% FBS-RPMI 1640 overnight, and equilibrated with serum-free RPMI 1640 for 1hr. In some experiments, the equilibrated media contained vehicle or the inhibitors at the concentrations indicated. The cells prelabelled with [^{3}H]choline chloride were incubated in 5 ml of the same medium and exposed to shear stress for defined time periods. At defined time points, one tenth of medium was collected, and centrifuged at 15,000 g for 5 min. The radiolabeled metabolic products in the media were evaporated under the hood. The samples were resolved in 50% ethanol, separated by thin layer chromatography as earlier described (14). The podocytes prelabeled with 1 μCi/ml of [^{3}H]palimitic acid were exposed to shear stress in the medium containing 2% ethanol. Total cellular lipids were extracted and [^{3}H]phosphatidylethanol (PEt) was separated (24). The standard was visualized with iodine vapor, and the areas corresponding to choline, PEt and

others were scraped into scintillation vials and quantitated with liquid scintillation spectrometry.

2.6. Data analysis

The data were analyzed for significance using one-way repeated measures of ANOVA followed by Tukey's test for comparisons between the experimental groups shown in the figures. The data represent means of three or more experiments performed with duplicate or triplicate samples.

3. Results

3.1 Shear stress induces cell apoptosis

To assess the biologic significance of shear stress in cultured podocytes, we investigated the effects of shear stress on apoptosis by analyzing DNA fragmentation, TUNEL assay, DAPI staining, and mitochondrial cytochrome c release. Podocytes were exposed to shear stress for 0–120 min, and then analyzed for DNA fragmentation. Fig. 1A shows that shear stress induced a typical apoptotic DNA ladder in a time-dependent manner. DNA fragmentation in shear-stress stimulated podocytes was also detected by TUNEL assay. TUNEL-positive cells were increased from 5% to 15% when podocytes were exposed to shear stress for 2 hr (Fig. 1B and 1C). Shear stress-induced nuclear changes were further evaluated using DAPI staining. Fig. 1D-1F illustrated that the number of apoptotic nuclei (highly fluorescent and condensed chromatin) were increased about three folds in podocytes for 2 hr shear stress (Fig. 1G). Finally, we determined the release of mitochrondial cytochrome c. Podocytes exposed to shear stress for 0–120 min were separated into pellet and cytosol, and the samples were processed for immunoblotting using an antibody against cytochrome c. As shown in Fig. 1H and 1I, a cytochrome c specific band was significantly increased in the cytosol and decreased in pellet when podocytes were exposed to shear stress.

3.2. Shear force stimulates renal PLD activation

To investigate the role of PLD in podocyte injury, we determine shear stress-induced PLD activity. Podocytes were prelabeled with [^{3}H]choline to synthesize phosphatidyl[^{3}H]choline, and then exposed to shear force for different periods of time to induce PLD activation which catalyzes the hydrolysis of phosphatidyl[^{3}H]choline. The release of [^{3}H]choline as an index of PLD activity was analyzed. Figure 2A shows a time-dependent increase in [^{3}H]choline in podocytes in response to shear stress. To confirm that shear stress activates PLD activity, we also assessed PLD activity by the transphosphatidylation reaction in which the phosphatidyl group of PC is transferred to ethanol and PEt is generated. As shown in Fig. 2B, shear stress stimulates a similar time-depended manner of [^{3}H]PEt formation in cultured podocytes.

3.3. Shear stress induces c-Src phosphorylation

Fluid shear stress can simultaneously regulate multiple cell signaling pathways (5–7). To assess whether shear stress induces c-Src mediated signaling, we exposed cultured podocytes to shear stress for varying time periods, and then fractionated the cells to yield pellet and cytosol. The samples were processed for immunoblotting using antibodies against phospho-c-Src and c-Src. Fig 3A showed that c-Src phosphorylation rose to a peak at 1 hr (3.65±0.32-fold of control, Fig. 3B) and was sustained through at least two hours. However, there was no significant change in c-Src protein level in response to shear stress (Fig. 3A, lower panel). To confirm this, we performed immunostaining to determine phosphorylated c-Src protein in shear stress-induced podocytes. Figure 3D shows that shear stress increased phosphorylated c-Src staining and that phosphorylated c-Src was concentrated at plasma membrane loci in a punctate pattern. Staining with an anti-synaptopodin antibody as a

differentiation marker shows no difference in the pattern and level of synaptopodin in response to shear stress (Fig. 3E and 3F). However, the co-localization of phosphorylated c-Src and synaptopodin in plasma membrane loci and the perinuclear area were evident. Our data from both immunoblotting (Fig. 3A) and immunofluorescence (Fig. 3D) demonstrate that shear stress induces c-Src phosphorylation in cultured podocytes.

3.4. Reciprocal immunoprecipitation of c-Src with PLD_1 but not PLD_2

Recent reports demonstrated that c-Src acts as a kinase of PLD in several cell lines (31, 32). To test whether c-Src directly interacts with PLD in a manner that would allow it to regulate PLD activity, we performed reciprocal immunoprecipition using the cell lysates from podocytes and the antibodies against c-Src, PLD_1 or PLD_2. The data show that a specific band representing PLD_1 (Fig. 4A, lower left) but not PLD_2 (Fig. 4B, lower left) was co-immunoprecipitated with c-Src using the anti-c-Src antibody for immunoprecipitation, while c-Src was only co-immunoprecipiated with PLD_1 (Fig. 4A, upper right) but not PLD_2 (Fig. 4B, upper right) using anti-PLD_1 and anti-PLD_2 antibodies as immunoprecipitation antibodies. These reciprocal immunoprecipitation data demonstrate that c-Src interacts with PLD_1 and not PLD_2 in podocytes. To confirm whether the anti-PLD_1 and anti-PLD_2 antibodies pull down PLD_1 or PLD_2, and whether shear stress stimulates PLD_1 activity, we performed *in vitro* PLD activity assay using the immunoprecipitation pellets as a source of enzymes and other cofactors. As shown in Fig. 4C, the activity of PLD is significantly increased in the pellets pulled down by anti-PLD_1 or anti-PLD_2 antibody compared to no antibody. The data further showed that the activity of PLD_1 but not PLD_2 is enhanced with shear stress stimulation.

3.5. Effect of c-Src inhibitor on shear stress-stimulated PLD activity and apoptosis

To assess the role of c-Src on shear stress-induced PLD activation and podocyte apoptosis, we used chemical inhibitors to block c-Src activation and to determine the effect of c-Src on shear stress-induced PLD activity and podocyte apoptosis. PP_2 is a specific inhibitor of c-Src kinase, while PP_3 is an inactive analog. Podocytes were pretreated with vehicle, 10 μM PP_2 or PP_3 for 1 hr, and then exposed to shear force for 2 hr. As shown in Fig. 5A, PP_2 completely abolished c-Src phosphorylation, while PP_3 had no effect. When we measured total c-Src protein in these samples, the treatments did not alter the level of c-Src expression. To test that c-Src phosphorylation induces PLD activation, we prelabeled podocytes with 1μCi/ml [^{3}H]choline or [^{3}H]palmitic acid overnight, pretreated with vehicle, 10 μM PP_2 or PP_3 for 1 hr, and then exposed to shear force for 2 hr. Pretreatment with PP_2 significantly diminished shear stress-induced PLD activity (~50% inhibition), while PP_3 treatment had no effect (Fig. 5B). To investigate if c-Src-PLD signaling pathway plays a role in shear stress-induced apoptosis of podocyte, we pretreated podocytes with or without PP_2 or PP_3, and then exposed to shear force. Fig. 5C shows that PP_2 inhibited the release of cytochrome c into the cytosol. The results indicate that shear stress-induced c-Src phosphorylation leads to PLD activation and apoptosis in podocytes.

3.6. Effect of c-Src inhibitor on shear stress-stimulated mTOR signaling

PLD takes center stage in mTOR signaling by which PA, a PLD product, physically associates with FKBP12-rapamycin-binding domain of mTOR (20, 21). Increased mTOR activity enhances Thr^{389} phosphorylation of p70S6 kinase (S6K) as an indicative of activation of mTOR signaling, and leads to glomerular hypertrophy in diabetic nephropathy (22, 23). To address whether mTOR signaling is involved in shear stress-induced podocyte apoptosis, we exposed cultured podocytes to shear stress for varying time periods, and then the cell lysates were processed for immunoblotting using antibodies against hosphor-S6K and tubulin as a loading control. As shown in Fig 6A and 6B, shear-stress induces a time-dependent increase in the phosphorylation of S6K. Fig. 5 showed that PP_2, a specific

inhibitor of c-Src kinase, blocks c-Src phosphorylation, PLD activation and podocyte apoptosis. Podocytes were also pretreated with vehicle, 10 μM PP_2 or PP_3 for 1 hr, and then exposed to shear force for 2 hr. Fig. 6C and 6D illustrated that PP_2 significantly inhibited the phosphorylation of S6K, while PP_3 had no effect. The results demonstrate that mTOR activation is associated with shear stress-induced c-Src phosphorylation, PLD activation, and apoptosis in podocytes.

4. Discussion

Glomerular hypertension due to elevated blood pressure and increased glomerular filtration rate results in compensatory changes in the glomerulus such as glomerular podocyte injury and pathology in the form of glomerulsclerosis (33–35). Mechanical stresses by stretch or shear forces drives podocytes towards cell cycle arrest, hypertrophy, detachment and apoptosis (11). By loading fibronectin-coated ferric beads onto mouse embryonic fibroblasts and exposing them to magnetic force, Mak et al. reported that this tensile force induced caspase 3 cleavage and DNA fragmentation (36). Mechanical strain generated by high intracapillary pressures induces podocyte hypertrophy (37), and apoptosis via up-regulation of angiotensin II receptor expression and increase in angiotensin II production (8). By placing podocytes grown in monolayer cultures on a rotating platform to model filtrate flow over podocytes (shear stress), we reported here that shear stress induced DNA fragmentation, apoptotic nuclear changes and cytochrome c release in the cultured podocytes. Although mechanotransduction is a complex process, it is clear that mechanical forces can stimulate podocyte apoptosis.

The c-Src family tyrosine kinases play an important role in PLD-mediated signaling (38). In rat vascular smooth muscle cells angiotensin II induces PLD activation via $G\alpha_{12}$ and c-Src-dependent mechanisms (39). By co-expression of v-Src and PLD_1 in COS-7 cells, v-Src interacts with the N- and C-terminal halves of PLD_1 and increases its activity and phosphorylation which occurs on more than one site. (40). In A431 cell co-transfection experiments, highly tyrosine-phosphorylated PLD_2 is strongly associated with c-Src, and c-Src binds to a region of PLD_2 containing PH domain, which is known to be involved in protein-protein interactions as well as binding of phospholipids (32). The components of this signaling cascade such as c-Src, PLD_1 and PLD_2 are expressed in podocytes (Figs 3 and 4), and Src signaling is associated with mechanical stretch induced podocyte osteopontin expression (11). Using anti-c-Src, anti-PLD_1 and anti-PLD_2 antibodies to perform reciprocal immunoprecipitation, our data show that endogenous c-Src only interacts with endogenous PLD_1 in podocytes. The results were further confirmed by the *in vitro* PLD activity assay, in which PLD activity significantly increases in the pellet immunoprecipitated by an anti-PLD_1 antibody when podocytes were exposed to shear stress. Treatment of podocytes with PP_2, a c-Src specific inhibitor, inhibited c-Src phosphorylation and PLD activation in response to shear force (Fig. 5). On the other hand, increasing evidence indicates that Src has been implicated in the coordination and facilitation of cell-signaling pathways controlling a wide range of cellular functions, including growth, survival, invasion, adhesion, and migration, and is one of the best-studied targets for cancer therapy that deregulation and increased activity of Src has been observed in multiple human malignancies (41). Recently, Cuadrado et al. reported that Aplidin-induced apoptosis in MDA-MB-231 cells is partly the result of Src activation (42), and Webb et al. showed that in v-Src transformed Rat-2 fibroblasts apoptosis induced by v-Src is accompanied by a loss of mitochondrial membrane potential and release of cytochrome *c* (43). These data indicate that Src tyrosine kinases also induce apoptosis. Using shear stress to stimulate podocytes, we found that shear force stimulated c-Src phosphorylation and PLD activation and then promoted podocyte apoptosis. This novel signaling pathway explains the conversion of mechanical stimuli to chemical signals and

also bridges hemodynamic force stimulation to podocyte hypertrophy, detachment and apoptosis.

Recently, the role of PLD and PA in the mechanical activation of mTOR signaling has been reported in skeletal muscle cells (44). mTOR governs a myriad of biological and cellular processes, including cell growth, proliferation, survival, autophagy, differentiation and metabolism (20, 21). Activation of mTOR increases cell mass which plays a major role in mediating the glomerular hypertrophy associated with the loss of nephrons in chronic kidney disease and acute kidney injury, while mTOR activation also induces podocyte dedifferentiation and glomerular cell proliferation in HIV-associated nephropathy, autosomal dominant polycystic kidney disease and renal carcinoma (45, 46). Here, we precisely defined the molecular mechanism of how mechanical force activates c-Src-PLD-mTOR signaling in the cultured podocytes and leads to apoptosis.

Choline released by PLD-catalyzed PC hydrolysis is a biomarker of cardiovascular damage (47). By injecting recombinant wild type and inactive mutant PLD protein (brown spider toxin) into mice, Kusma et al. (48) recently compared their histopathological changes in kidneys, and found that only wild type PLD caused glomerular foot process effacement and cell detachment. The data provide a piece of direct evidence that PLD activation play a crucial role in renal injury. Some clinic reports indicate that the levels of serum free choline are significantly increased in chronic hemodialysis patients (49) and in the patients with end stage renal disease compared to age-matched controls (50). Vazquez et al. found a significant reduction (25–30%) in the content of PC isolated from renal cortex brush border membranes of spontaneously hypertensive rats compared to Sprague-Dawley rats (51), and the levels of serum PC are also significantly decreased in the patients with end stage renal disease (52). In the differentiated cells such as hematopoietic cells, Jurkat T cells, A20 B cells, and HL60 cells, PLD activation induced by actinomycin D, anti-Fas antibody, and TNF-a promotes cell death (53). Taken together, our data and those earlier studies support that the degradation of PC by PLD action is one of the key biochemical events in podocyte apoptosis and glomerular injury which leads to renal diseases.

5. Conclusions

A schematic model for mechanical force-induced glomerular podocyte apoptosis is outlined in Figure 7. We found that shear stress induced cell apoptosis, enhanced c-Src phosphorylation, and stimulated the activation of PLD and mTOR in cultured podocytes. By performing reciprocal co-immunoprecipitations of c-Src and PLD_1 or PLD_2, our data demonstrated that c-Src interacted with and activated PLD_1 but not PLD_2. The inhibition of shear stress-induced c-Src phosphorylation by PP_2 also attenuated the activation of PLD and mTOR. These data demonstrtae that c-Src-PLD-mTOR signaling plays an important role in hypertension-associated glomerular injury.

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Abbreviations

PA	phosphatidic acid
PC	phosphatidylcholine
PEt	phosphatidylethanol

PLD	phospholipase D
mTOR	mammalian target of rapamycin
S6K	p70S6 kinase

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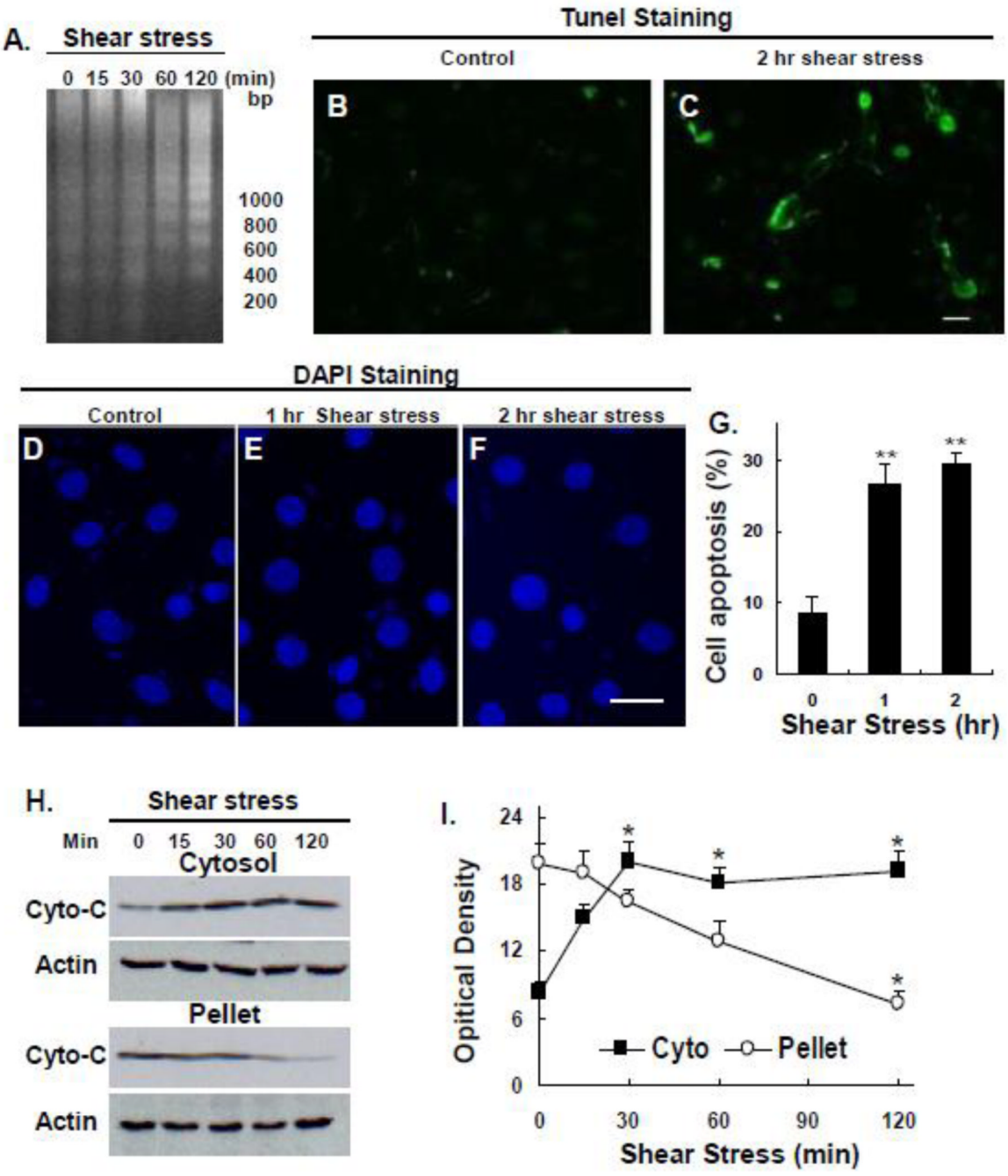

Fig. 1. Shear stress induces apoptosis of cultured podocytes

Podocytes were exposed to shear force for different periods of time. After exposure to shear stress, A) the cells (both floating and adherent) were collected and lysed for DNA preparation. The DNA samples were separated on a 1.5% agarose gel. B to F) After exposure to shear stress, the cells on glass coverslips were used for Tunel imaging assay (B and C) or DAPI staining (D, E and F). G) Statistical data, apoptotic nuclei/total nuclei: control, 8/93 (8.6%); 1 hr shear stress, 37/139 (26.6%) and 2 hr shear stress, 53/179 (29.6%). H) The remained cells were fractionated and processed for immunoblotting using antibodies against cytochrome C (Cyto-C) and actin, and I) the cytochrome C specific bands on the immunoblot films taken from three individual experiments were scanned and the

optical density was measured using Scion Image software. The data represent means of three separate experiments performed with at least duplicate samples. The values with shear force were statistically different from those without force. * $P<0.05$; ** $p<0.01$.

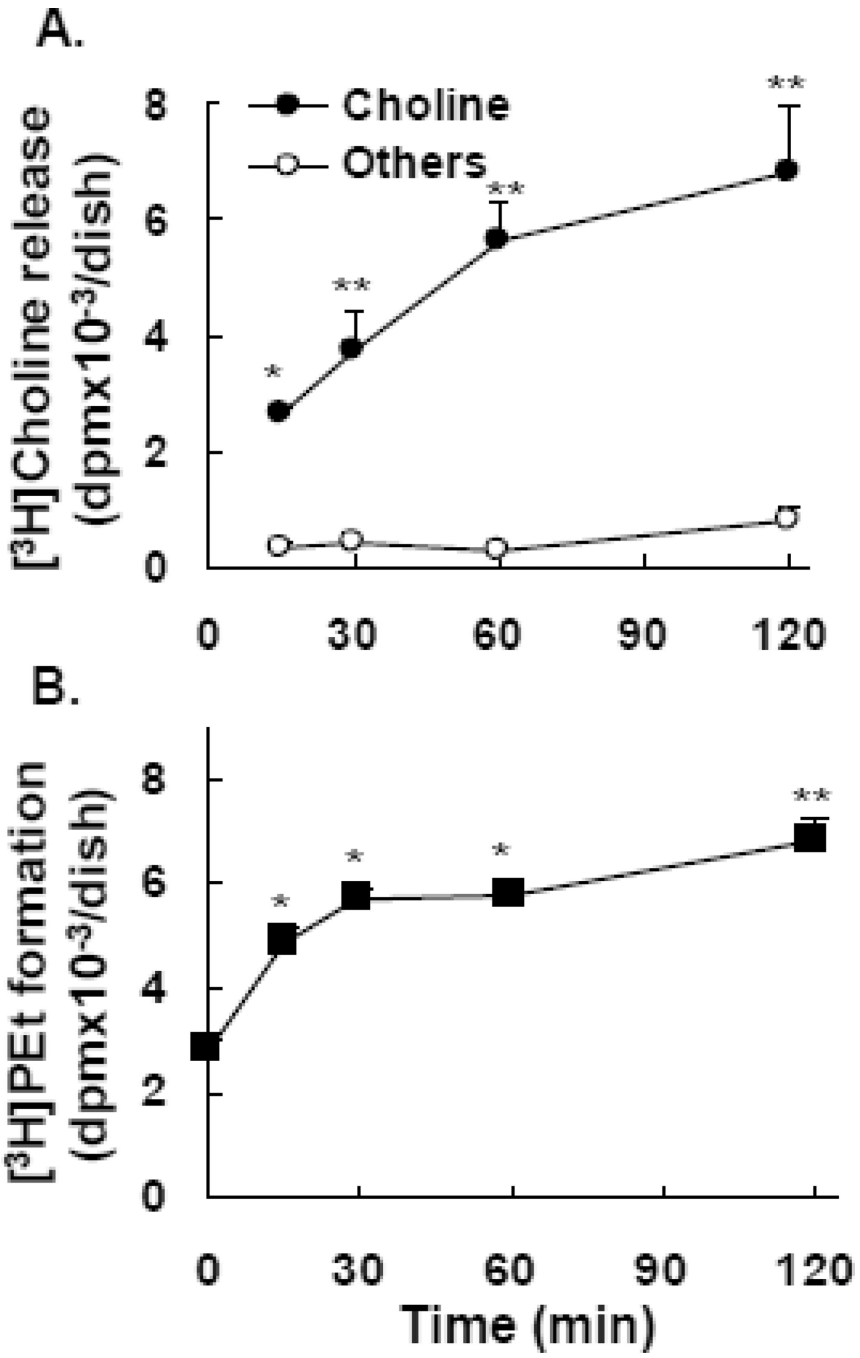

Fig. 2. Time course of [^{3}H]choline release and [^{3}H]PEt formation in podocytes in response to shear stress

Podocytes were prelabeled with 1 μCi/ml of [^{3}H]choline or [^{3}H]palmitic acid overnight, and then subjected to shear stress for the time periods indicated. The medium for [^{3}H]palmitate-labeled podocytes contained 2% ethanol during shear stress stimulation. [^{3}H]choline (A) and [^{3}H]PEt (B) as an index of PLD activation were analyzed by thin layer chromatography. The data represent means of three separate experiments performed with duplicate samples. The values with shear force were statistically different from those without force. * $P<0.05$; ** $P<0.01$.

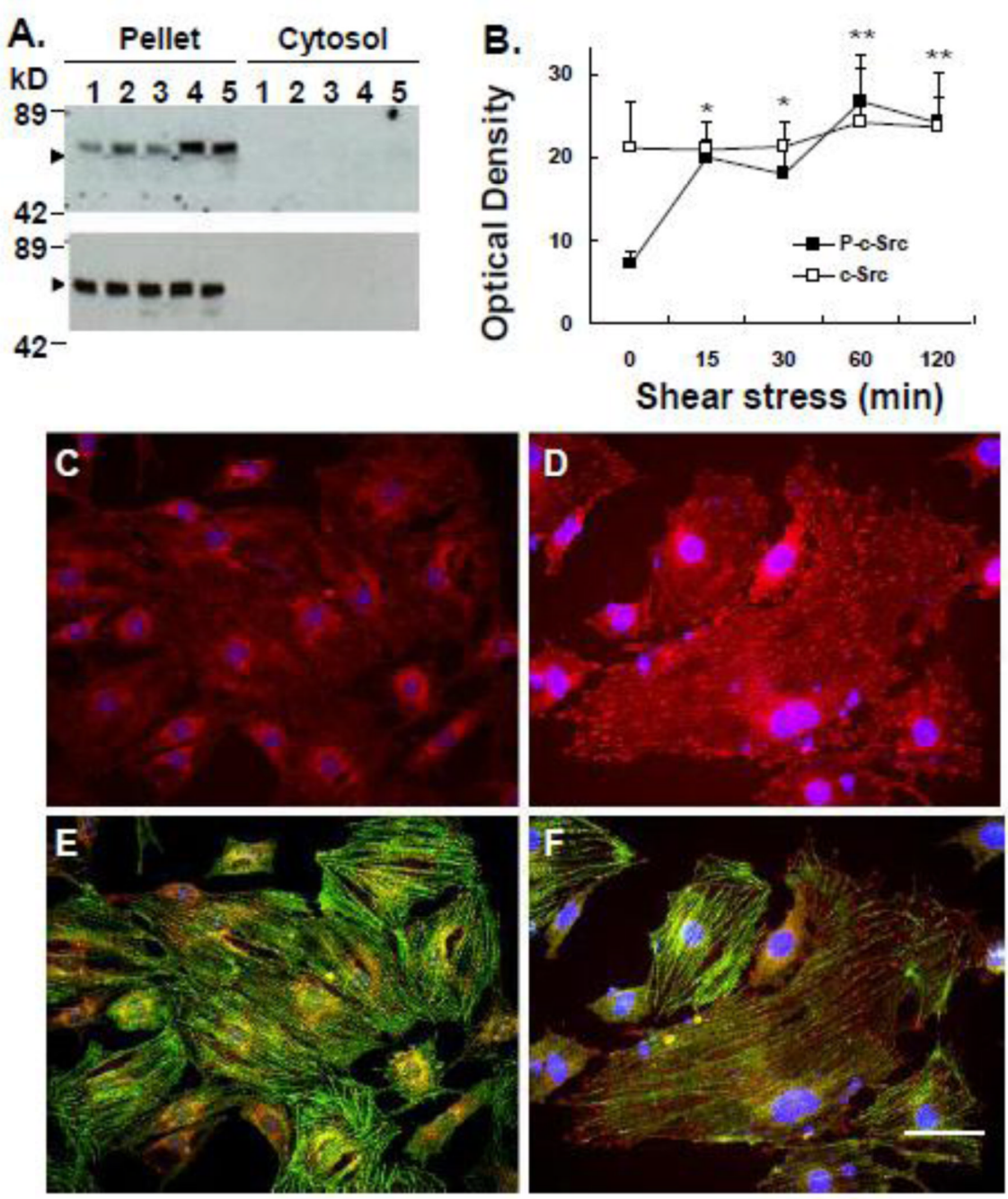

Fig. 3. Shear stress induces c-Src phosphorylation in podocytes

A) Podocytes were serum-deprived and exposed to shear force for the time periods indicated (lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 60 min and lane 5, 120 min), and then were fractionated and processed for immunoblotting using antibodies against c-Src or phosphorylated c-Src. B) Specific bands representing c-Src and phosphorylated c-Src on the immunoblot films were scanned and the optical density was measured using Scion Image software. The data represent means of three separate experiments performed with duplicate samples. The values with shear stress were statistically different from those without shear stress. * $P<0.05$; ** $P<0.01$. After serum-deprivation and exposed to shear stress for 0 or 1 hr, the cells cultured on glass coverslips were fixed and doubly stained with polyclonal anti-

phospho-c-Src (red) and monoclonal anti-synptopodin (green) antibodies and the appropriate secondary antibodies. C) and E) no shear stress; D) and F) 1 hr shear stress. Images are representative of four independent experiments. Bar = 10 μm.

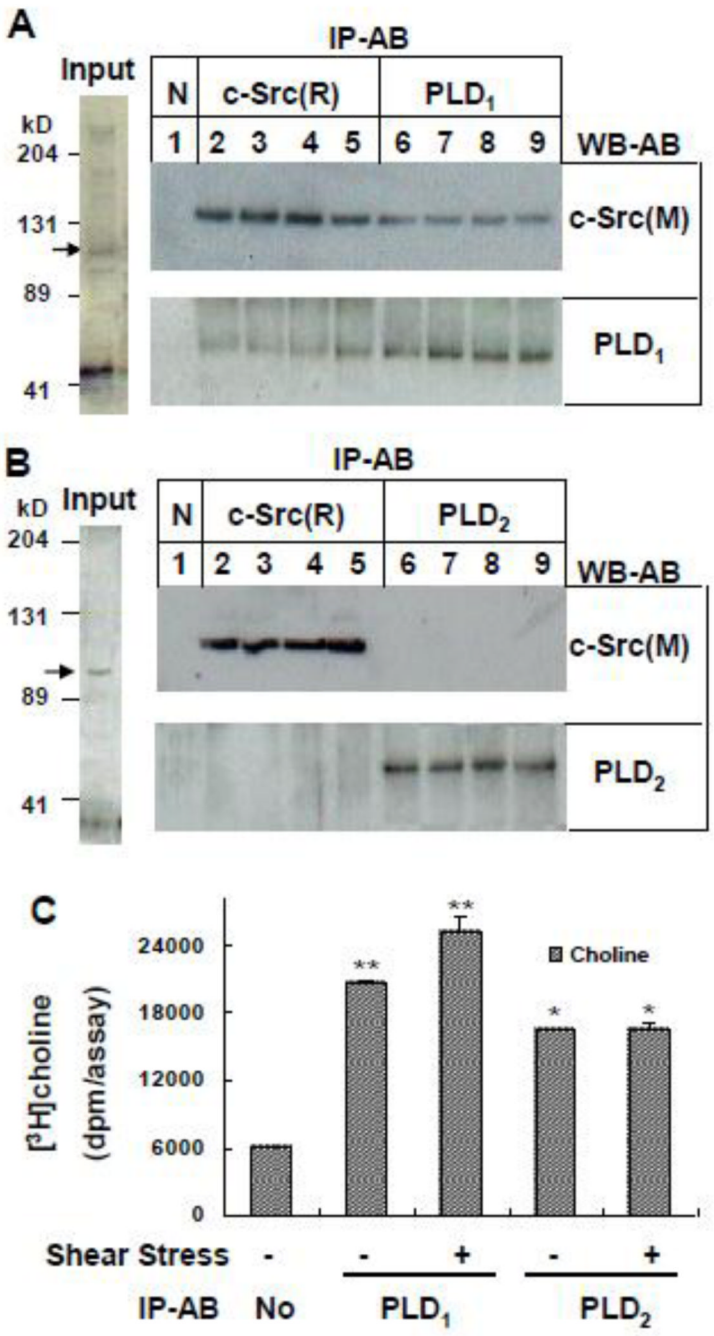

Fig. 4. Reciprocal co-immunoprecipitation of c-Src and PLD_1 but not PLD_2

A) and B) Podocytes were serum-deprived overnight and then exposed to shear force for 0 or 1 hr. The cell lysates were processed for reciprocal co-immunoprecipitation using polyclonal antibodies against c-Src (R), PLD_1 or PLD_2. The immunoprecipitated complexes were immunoblotted with polyclonal anti-PLD_1 and anti-PLD_2 antibodies as well as monoclonal anti-c-Src (M) antibodies. Lane 1, no immunoprecipitating antibody (N), lane 2, 3, 6, and 7, no shear stress, lane 4, 5, 8, and 9, shear stress. C) The immunoprecipitation samples using either anti-PLD_1 or anti-PLD_2 antibody as immunoprecipitation antibodies were analyzed for *in vitro* PLD activity as described in materials and methods. The data

shown are representative of three experiments. The values with IP-AB were statistically different from those without IP-AB. * $P<0.05$; ** $P<0.01$.

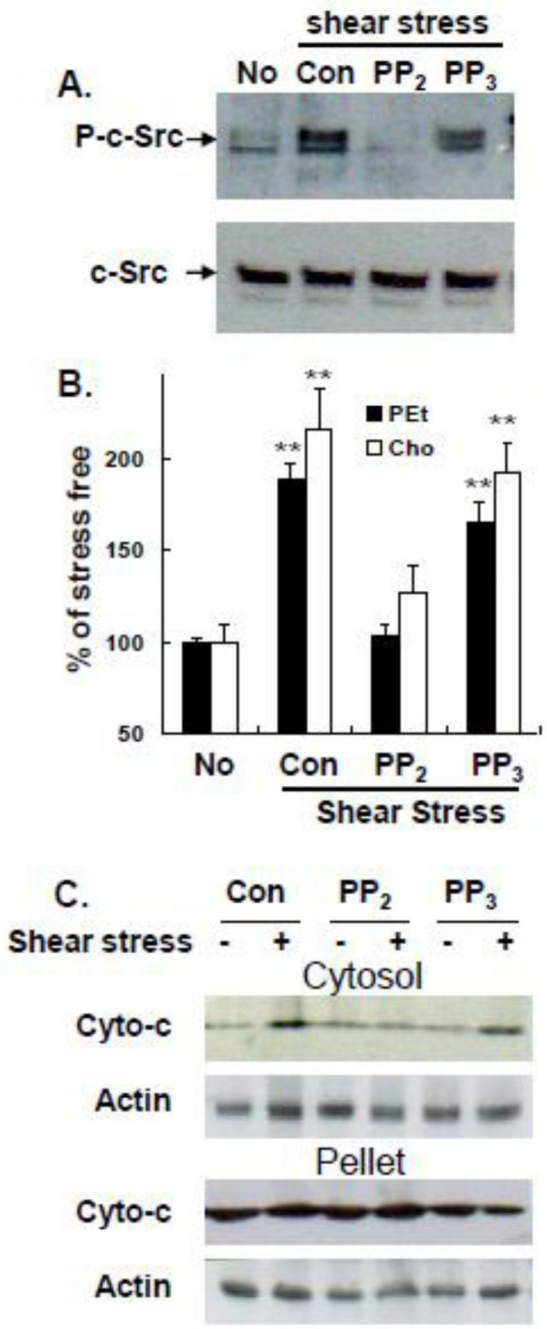

Fig. 5. Effect of c-Src inhibitor on shear stress-induced PLD activation, c-Src phosphorylation and cytochrome c release in podoctyes

Podocytes with or without radioactivity labeling were incubated in the presence or absence of 10 μM PP_2 or PP_3 for 1hr, and then exposed to shear force for 2 hr. The cells without radioactivity labeling were fractionated and processed for immunoblotting using appropriate antibodies. A) anti-phospho-c-Src (P-c-Src) and c-Src antibodies and C) anti-cytochrome c (Cyto-c) and actin antibodies. [^{3}H]choline or [^{3}H]PEt from the cells that were prelabeled with [^{3}H]choline or [^{3}H]palmitic acid were analyzed (B) as described under “Materials and Methods”. The data represent means of three experiments with duplicate samples. No, no

shear stress; Con, control. The values with inhibitors were statistically different from those without inhibitors. * $P<0.05$; ** $P<0.01$.

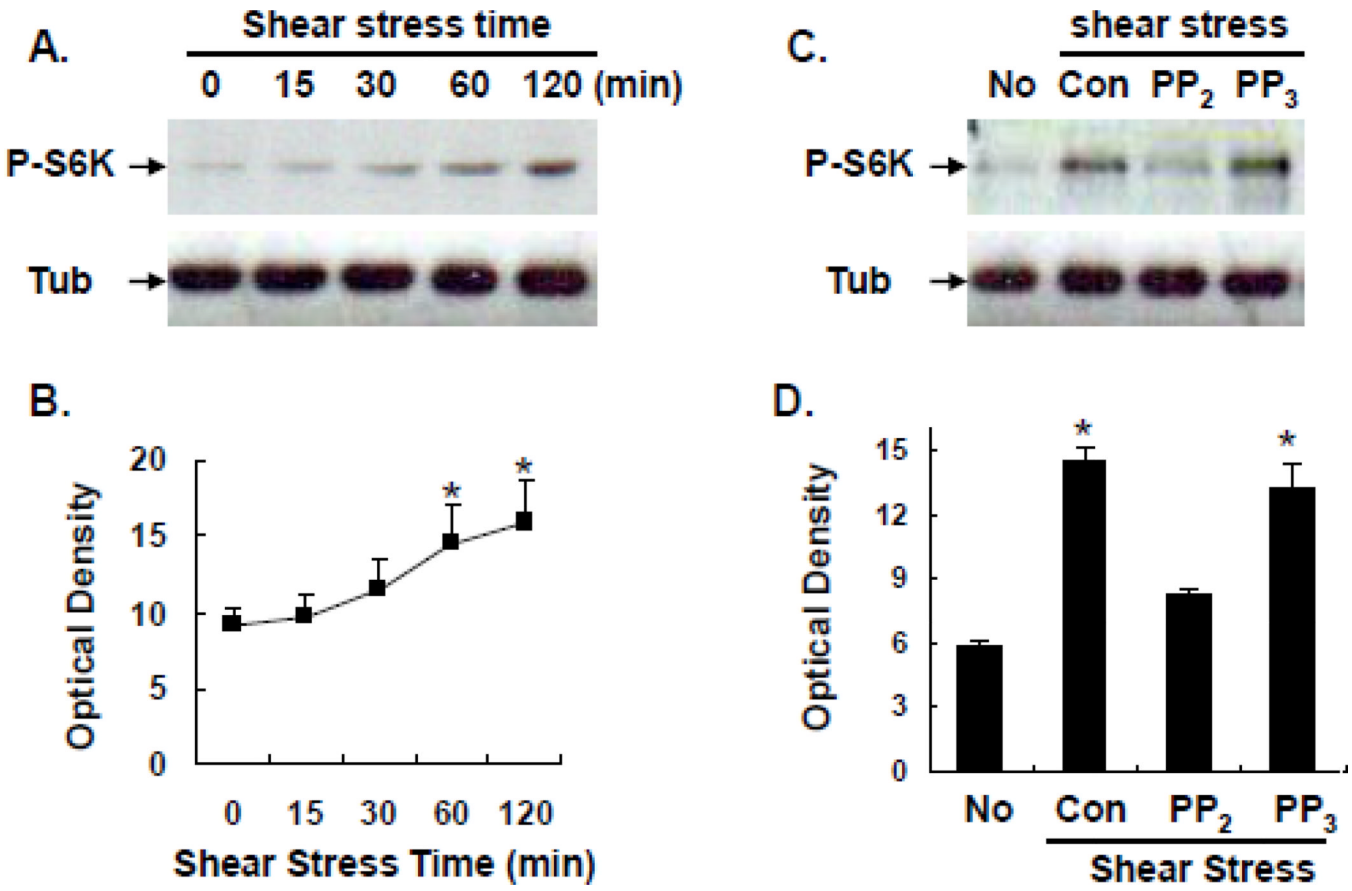

Fig. 6. Shear stress induces the phosphorylation of S6K in podocytes

A) Podocytes were exposed to shear force for the time periods indicated (lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 60 min and lane 5, 120 min). C) Podocytes were incubated in the presence or absence of 10 μM PP_2 or PP_3 during last 1 hr, and then exposed to shear force for 2 hr. The cells were lysed and processed for immunoblotting using antibodies against phosphor-S6K and tubulin. B) and D) Specific bands representing phospho-S6K on the immunoblot films were scanned and the optical density was measured using Scion Image software. The data represent means of three separate experiments performed with duplicate samples. The values with shear stress were statistically different from those without shear stress. * $P<0.05$.

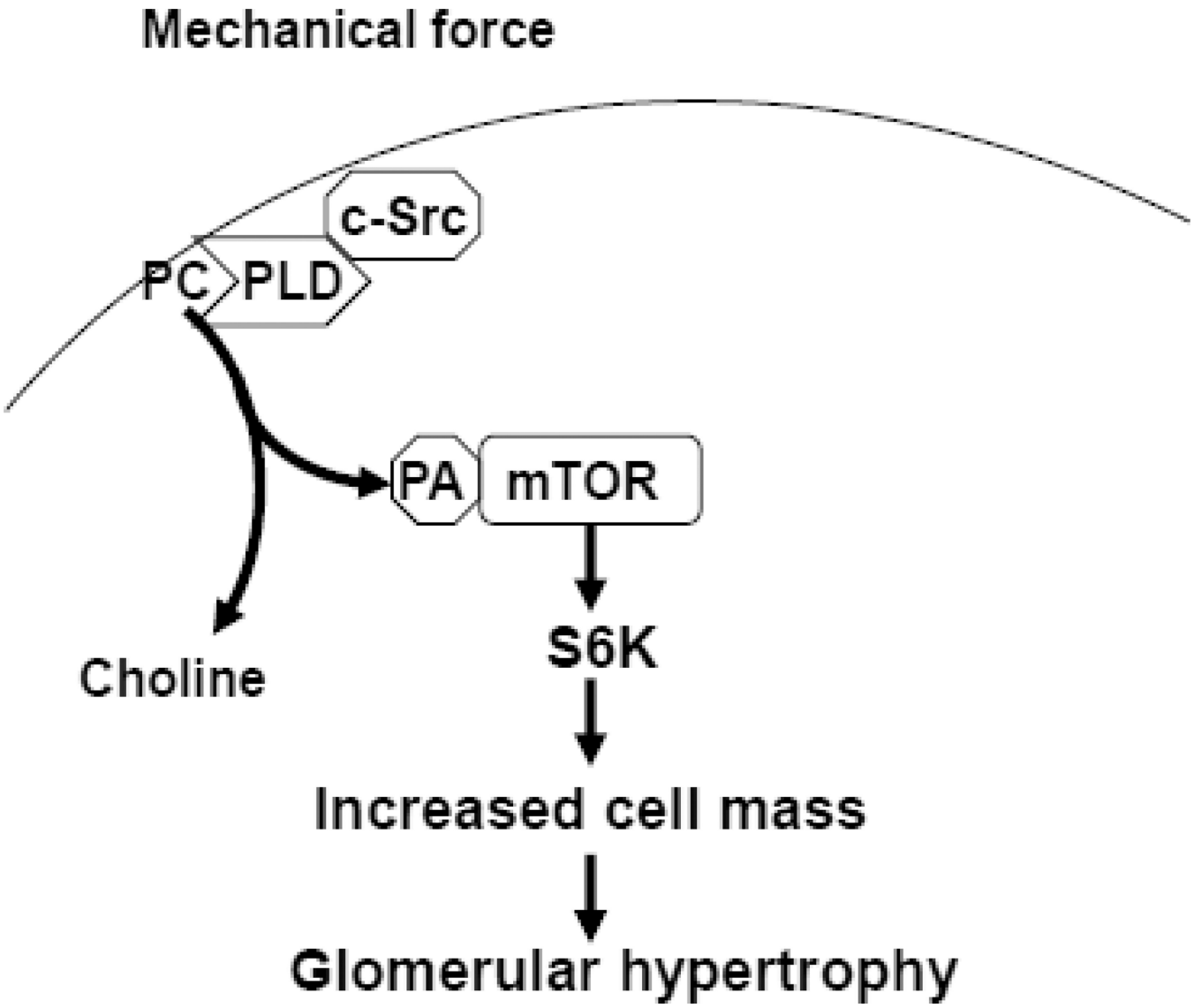

Fig. 7.

A schematic model for mechanical force-induced glomerular podocyte apoptosis. Shear stress induces c-Src phosphorylation, which stimulates PLD activity and PA formation. PA further activates mTOR signaling which leads to podocyte mass increase, hypertrophy and apoptosis as well as proteinuria.