Podocyte Injury: The Role of Proteinuria, Urinary Plasminogen and Oxidative Stress

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Abstract:

Podocytes are the key target for injury in proteinuric glomerular diseases that result in podocyte loss, progressive focal segmental glomerular sclerosis (FSGS) and renal failure. Current evidence suggests that the initiation of podocyte injury and associated proteinuria can be separated from factors that drive and maintain these pathogenic processes leading to FSGS. In nephrotic urine aberrant glomerular filtration of Plasminogen (Plg) is activated to the biologically active serine protease Plasmin by urokinase type plasminogen activator (uPA). In-vivo inhibition of uPA mitigates Plg activation and development of FSGS in several proteinuric models of renal disease including 5/6 nephrectomy. Here we show that Plasminogen (Plg) is markedly increased in the urine in two murine models of proteinuric kidney disease associated with podocyte injury: Tg26 HIV-associated nephropathy and the Cd2ap\(^{-/-}\) model of FSGS. We show that human podocytes express uPA and three Plg receptors: uPAR, tPA and Plg-RKT. We demonstrate that Plg treatment of podocytes specifically upregulates NADPH oxidase isoforms NOX2/NOX4 and increases production of mitochondrial-dependent superoxide anion (O\(_2^\cdot\) ) that promotes endothelin-1 synthesis. Plg via O\(_2^\cdot\) also promotes expression of the B scavenger receptor CD36 and subsequent increased intracellular cholesterol uptake resulting in podocyte apoptosis. Taken together our findings suggest that following disruption of the glomerular filtration barrier at the onset of proteinuric disease, podocytes are exposed to Plg resulting in further injury mediated by oxidative stress. We suggest that chronic exposure to Plg could serve as a “second hit” in glomerular disease and that Plg is potentially an attractive target for therapeutic intervention.
Keywords: Plasminogen; Podocytes; Oxidative stress
Introduction:

Normal urine is essentially protein free. Proteinuria of increased severity is associated with progression of chronic kidney disease (CKD) independent of the etiology and baseline glomerular filtration rate (45, 58). Specialized endothelial cells, the glomerular basement membrane (GBM) and podocytes work in partnership to preserve the permselectivity of the kidney filter (24, 67, 74, 79). Podocytes, the component of the glomerulus most susceptible to injury, (8, 20, 22, 24, 36, 74) are highly differentiated cells that adhere to the GBM. Podocytes are critical for maintaining the integrity of the kidney filter (13, 22, 24, 36, 54, 60, 67). Independent of the cause of the injury, podocyte response follows similar pathways including disorganization of the actin cytoskeleton, foot process effacement, loss of the slit diaphragm (SD), detachment and death (24, 36, 67, 74, 75, 93). Podocytes’ ability to undergo cell division and compensate for injury and loss is limited (20, 24, 36, 67, 74, 92, 93). When podocyte loss exceeds 30%, persistent non-selective proteinuria, progressive glomerulosclerosis and progressive CKD ensue (20, 36, 91-93).

Clinically, proteinuria accompanied by continued podocyte loss and CKD progression is a hallmark of many glomerular diseases of different etiology including diabetic nephropathy and FSGS (8, 13, 24, 36, 54, 60, 67). To date it remains unclear whether persistent injury is driven by persistence of the initial pathogenic process or by a superimposed pathogenic process, a so-called “second hit model of podocyte injury.” The latter raises the question of whether continuous trans-glomerular passage of large circulating macromolecules might contribute to podocyte damage as a “second hit” (20, 24, 36, 46, 47, 54, 68, 93). In this context studies have shown that albuminuria itself
may contribute to tubule-interstitial disease and to glomerulosclerosis (11, 53).

Clinically, most of the mechanisms linking proteinuria to CKD progression remain to be fully defined (9, 13, 22, 54, 68). Data from clinical and animal models have documented significant quantities of biologically active urinary plasminogen (Plg) and the protease plasmin in nephrotic range proteinuria of diverse etiologies (6, 81). Furthermore, in patients in remission from nephrotic syndrome the urinary Plg/plasmin content is minimal (1) thereby supporting the concept that under physiologic conditions podocytes are not exposed to large concentrations of Plg; a fact further supported by its molecular size (81 KD), which is larger than albumin (3).

It is well established that after binding onto cell surface receptors (42, 51, 52, 59) Plg is protected from inhibitors, undergoes proteolytic activation and converts to the serine protease plasmin. In many cell types plasmin initiates responses linked to both its proteolytic as well as its non-proteolytic actions including cleavage of matrix proteins (62, 87), inactivation of VEGF (69), induction of reactive oxygen species (ROS) (85) as well as activation of TGF β (23, 34, 43).

Here we demonstrate that Plg is increased in the urine in two murine models of proteinuric kidney disease associated with podocyte injury: the Tg26 HIV-associated nephropathy mouse (37) and the Cd2ap−/− model of focal segmental glomerulosclerosis (76). We show that immortalized human podocytes constitutively express and synthesize uPA as well as the Plg receptors uPAR, tPA, and the novel trans-membrane receptor Plg R-KT.

Our studies show, to our knowledge for the first time, that podocyte binding of Plg upregulates NADPH oxidase isoforms NOX2 and NOX4 and elicits mitochondrial
production of superoxide anion (O$_2^-$) accompanied by: a) upregulation of the multifunctional scavenger receptor CD36 (77, 96) resulting in increased podocyte uptake of oxidized LDL (oxLDL) with subsequent apoptosis and b) induction of podocyte synthesis of endothelin-1 a molecule that acts in a paracrine/autocrine fashion, and has been implicated in podocyte-endothelial cell crosstalk and glomerular disease progression (14, 35, 41, 61). Epsilon Aminocaproic Acid (EACA), an inhibitor of cellular Plg binding and activation (51, 52), prevented all the injurious actions elicited by exposure of podocytes to Plg. In addition we demonstrated that Apocynin, AMPkinase and Mito Tempo, molecules that mitigate production of reactive oxygen species (ROS) (18, 19, 40), provided similar podocyte protection.

These data strongly support the conclusion that Plg–plasmin is a molecule that via previously unrecognized mechanisms that include oxidative stress can play a role as a promoter/contributor of a “second hit” type “of podocyte injury in proteinuric nephropathies. Our studies may contribute to development of therapeutic interventions in proteinuric kidney disease.
**Methods**

**Mouse studies**

Urine was collected from two proteinuric kidney disease models that have been well characterized: 5-week-old Cd2ap⁻/⁻ mice (25, 72, 76, 90, 94) and 6-week-old Tg26 mice (2, 4, 21, 32, 37, 38, 65). Urinary Plg was measured using 2μl urine with Elisa Kit (ICL E-90PMG) read at absorbance at 450 nm. Mouse creatinine was measured with an enzymatic assay kit (Crystal Chem: #80350).

**Human Podocyte Culture**

Human podocytes initially generated by MA Saleem Children’s Renal Unit and Academic Renal Unit, University of Bristol, UK (71) were generously provided by S. Merscher-Gomez and A. Fornoni, University of Miami, who also provided initial technical supervision of podocyte culture as reported (50, 64, 71). Briefly, human podocytes were cultured and differentiated in RPMI 1640 culture medium containing 10% FBS, 1% penicillin/streptomycin with or without 1% ITS, 50U/ml human interferon as described (50, 71). The immortalized normal human podocytes were propagated at 33°C and then thermoshifted for differentiation for 14 days at 37°C. Terminally differentiated podocytes were starved in serum free RPMI 1640 medium for 24 hours before the experiments were performed (24, 50, 64, 71).
Determination of $O_2^-$ production:

$O_2^-$ production was determined by using lucigenin enhanced chemiluminescence as described in our previous publications (96). Human podocytes were treated with Plg (1 μmol/L), the concentration of Plg found in human serum, (51, 52) for 6 hours as determined in experiments of time responses to Plg up to 24 hrs (not shown).

To determine the source of ROS, the cells were pre-incubated with the following compounds for 30 minutes before incubation with Plg: 1) NADPH oxidase inhibitors diphenyleneiodonium (DPI, 1 μmol/L, Sigma-Aldrich) and APOCY (100 μmol/L, Sigma-Aldrich) (12, 39, 40, 96), 2) AICAR, an agonist to AMP-activated protein kinase (100 μmol/L, Sigma-Aldrich) (19, 52, 86), 3) EACA (0.2 mol/L, Sigma-Aldrich) (42, 52), and 4) Amiloride (75 μM, Sigma-Aldrich)

Immunoblot analyses:

The cells were harvested with lysis buffer containing a cocktail of protein inhibitor (Complete Mini, Roche Diagnostics GmbH, cat: 11836153001). Protein was quantified by Bio-Rad assay, and 30 mg of total protein was first subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated overnight in a cold room with primary rabbit anti-CD36, anti-tPA, anti-WT1 and anti-uPAR (Santa Cruz Biotechnology), anti-Phospho-AMPK, anti-AMPK, rabbit anti-NOX 4 (Santa Cruz Biotechnology) and anti-NOX2 (AbCam) followed by incubation with a peroxidase-conjugated secondary antibody for 1 hour. All the Ab used equivalence-of-protein loading and transfer was confirmed by re-blotting the samples with anti-β-actin antibody. Immune reactive bands were detected by chemiluminescence and quantified by
densitometry. Relative quantities of each protein were normalized by β-actin (Santa Cruz Biotechnology) and expressed as fold increase vs. control (96).

**Determination of total cellular cholesterol content:**

Human podocytes in six-well dishes were incubated with or without Plg (1 μmol/L) for 24 hours followed by incubation with oxLDL (50 μg/ml) for another 24 hours (96). Total cellular cholesterol was extracted by adding 1 ml hexane/isopropanol (3:2, v:v) to the wells. The samples were evaporated by using a SpeedVac to remove the solvent and then re-dissolved in 50 ml ethanol. Cholesterol was determined with an enzyChrom AF cholesterol assay kit according to the manufacturer’s instructions (Bioassay Systems, Hayward, CA). The assay results were normalized according to the sample protein content.

**Apoptosis assay:**

A total of $2 \times 10^6$ cells were seeded in 75cm$^2$ flasks and treated for 24 hrs with one of following conditions: vehicle, Plg (1 μmol/L), oxLDL (50 μg/ml), or incubation with plasminogen followed by oxLDL (50 μg/ml). The cells were trypsinized and washed with PBS twice. Apoptosis was measured using the annexin V-fluorescein isothiocyanate apoptosis detection kit (Roche Diagnostics, Indianapolis, IN, USA) followed by flow cytometry.
Statistics:

The results were expressed as mean ± SD. Statistical analyses were performed by ANOVA with Bonferonni’s correction for multiple comparisons, followed by Scheffe’s test. Significance was assumed at P< 0.05.
Results

Urinary Plasminogen is increased in proteinuric mouse models

We measured urinary Plg normalized to urinary creatinine in two independent models of proteinuric kidney disease. Cd2ap−/− mice provide a well-characterized model of focal segmental glomerulosclerosis with proteinuria developing between 2 and 3 weeks of age. Urinary Plg from 5-week old Cd2ap−/− mice (mean urine protein/cr ratio = 125.4 mg/mg) was markedly elevated when compared to wild type littermate controls (30.96 versus 0.09 ng/mg; p: 0.007, Figure 1). We also detected elevated urine Plg in 6-week old mice from the Tg26 model (mean urine protein/creatinine ratio 83.1 mg/mg) of HIV nephropathy (2.56 versus 0.49 ng/mg; p: 0.008).

Plasminogen receptor expression and superoxide upregulation in podocytes

In cultured human podocytes, we established the expression of uPA as well as three well-characterized Plg receptors that are known to be blocked by the lysine analogue EACA: uPAR, tPA and the novel receptor R-KT (Figure 2A) (42, 51, 52, 59). Previous studies in vivo and in vitro have demonstrated that several isoforms of NADPH oxidase including NOX2, and NOX4 are present in podocytes and that ROS originating from NADPH oxidase play an important role in ROS mediated podocyte injury. NOX4 is the most abundant isoform in podocytes (10, 19, 31, 80). RTPCR (Figure 2B and 2C) and western blotting (Figure 2D and 2E) showed that Plg significantly up-regulated podocyte NOX4 and NOX2 expression. This was prevented by prior incubation with EACA thereby demonstrating that Plg binding and activation was required (51, 85).
Exposure of podocytes to Plg increased NADPH oxidase–mediated $O_2^-$ production (Figure 3A) that was prevented by the inhibitors of NADPH oxidase diphenyleneiodonium (DPI), and by apocynin (APOCY) (39, 40).

Plg concomitantly reduced the pAMPK/AMPK ratio (Figure 3B). AICAR, 5-aminomidazole-4-carboxamide-1-riboside, is known to phosphorylate and activate AMPK (18, 86). Pre-treatment of podocytes with AICAR prior to exposure to Plg significantly prevented the reduction in pAMPK/AMPK ratio and concurrently inhibited $O_2^-$ production (Figure 3A). This suggests an important role of this stress-sensing kinase in the biology of podocyte injury associated with oxidative stress (18, 86).

Expression of the scavenger receptor CD36 is upregulated by Plasminogen

The B scavenger receptor CD36, which is present in many cells including podocytes, (49) recognizes, binds and internalizes oxLDL (77, 96). Plg significantly upregulated podocyte expression of CD36 (Figure 4A). This paralleled the increased production of $O_2^-$ induced by Plg (Figure 3A). The up-regulation of CD36 was prevented by EACA as well as by the NADPH inhibitor APOCY and AICAR, the agonist of AMPK (18, 19, 86). Importantly, the significant inhibition of Plg up-regulation of CD36 by EACA, APOCY and AICAR indicates the interdependence between Plg activation, increased oxidative stress and CD36 up-regulation in podocytes.

Our group and others have shown that up-regulation of CD36 in human macrophages increases uptake of oxLDL accompanied by increased synthesis of ROS (77, 96). It is presently unknown whether upregulation of CD36 plays a similar role in podocytes. Similar to macrophages, podocytes treated with oxLDL for 24 hrs showed a
significant increase in cholesterol accumulation (Figure 4B) (96), which was further increased in podocytes treated with Plg plus oxLDL thereby suggesting that the increased oxLDL uptake was linked to Plg upregulation of CD36 (77, 96). The off-target effects of the diuretic amiloride as an inhibitor of uPA is well documented (30, 48, 88). We found that amiloride inhibited Plg induced upregulation of CD36 (Figure 5A) and ROS superoxide generation (Figure 5B).

Plasminogen enhances oxLDL-mediated podocyte apoptosis

Based on our novel findings that Plg up regulates CD36 (Figure 4A) and increases oxLDL uptake (Figure 4B), we investigated by flow cytometry the effects of oxLDL and Plg individually as well as together with oxLDL on podocyte apoptosis. As shown in Figure 6, both Plg as well as oxLDL alone slightly increased podocyte apoptosis. Importantly the combination of Plg and oxLDL had a clear additive effect that further significantly augmented podocyte apoptosis.

Plasminogen promotes Endothelin 1 synthesis

Studies in vivo and in vitro have shown that Endothelin-1 (ET1), acting in paracrine/autocrine fashion, induces oxidative stress and injury of glomerular endothelial cells and podocytes (14, 35, 41). It is currently unknown whether Plg promotes ET1 synthesis by podocytes and whether it is linked to oxidative stress by acting as a second messenger. As shown in Figure 7B we demonstrated that Plg promotes synthesis-release of ET1 that was prevented by EACA, APOCY and by the selective inhibitor of mitochondria ROS Mito Tempo (Figures 7A and 7B) (14). This
demonstrated for the first time the important role of Plg induced ROS, particularly mitochondrial ROS in promoting podocyte ET1 synthesis.

Discussion

Independent of the etiology, chronic severe non-selective proteinuria is a harbinger of progression of chronic kidney disease (CKD) (24, 27, 67, 74, 79). It has long been suspected that the relentless trans-glomerular passage of selected plasma macromolecules may contribute to podocyte injury as a “second hit”. Studies have shown that albuminuria itself may contribute to glomerular injury directly and indirectly via podocytes uptake of fatty acids (FFA) bound to albumin (11) as well as by sequestration of retinoic acid a molecule shown to be critical in protecting podocytes from injury (44, 57). Data from both patients and experimental animals with nephrotic syndrome have consistently shown their urine to contain large quantities of biologically active Plg and the protease plasmin (1, 5, 6, 81-84, 95).

It has been demonstrated that plasmin in nephrotic urine proteolytically activates ENaC, an effect that disappears during remission of the nephrotic syndrome (1, 5, 6, 81-84, 95). In vitro and in vivo studies have demonstrated the ability of plasmin to contribute to cellular injury and to inflammation via its proteolytic as well as its non-proteolytic actions (85). Interestingly previous studies in animals and in patients with proteinuric nephropathies treated with EACA, the inhibitor of Plg activation to plasmin (51, 52, 85), reported a marked reduction of proteinuria and glomerular injury (28).
We utilized two independent well-characterized models of progressive proteinuric kidney disease, the Cd2ap⁻/⁻ mouse model of FSGS and the Tg26 model of HIVAN. In both, urinary Plg was significantly elevated (Figure 1). We have also used cultured human podocytes (71) to further assist us in determining whether the Plg/plasmin system could potentially participate as a "second hit" type of podocyte injury linked to proteinuria.

In human podocytes we confirmed the expression of urokinase plasminogen activator uPA that is synthesized by podocytes and of Plg receptors uPAR, tPA and Plg R-KT (51, 52) (Figure 2A). uPAR has high-affinity for uPA, but has no intracellular domain, which limits its signaling capacity. Plg R-KT co-localizes with uPAR and is a unique trans-membrane protein that activates Plg because it exposes a C-terminal lysine on the cell’s surface (51, 52). In addition, Plg R-KT specifically interacts with tPA, which secures Plg on the cell surface and promotes its activation to plasmin that once bound to the cell surface is protected from its physiological inhibitors (51, 52, 85).

Here we demonstrated that Plg up-regulated mRNA and protein expression of the podocyte NOX2 and NOX4 (Figure 2) (10, 31), accompanied by significant production of the ROS superoxide anion $O_2^-$ as determined by lucigenin chemiluminescence. EACA prevented NOX2 and NOX4 up-regulation as well as $O_2^-$ production thereby demonstrating that these effects are associated with Plg binding and activation (Figure 2B-E). Next we determined that Apocynin, an inhibitor of NADPH oxidases, which are considered to be the major source of ROS mediated podocyte injury (10, 31), prevented the increase in $O_2^-$ production induced by Plg (Figure 3A).

AMPK is a serine/threonine kinase that is present in podocytes and acts as an
energy sensor (7, 47). Once AMPK is phosphorylated (pAMPK) it becomes activated and enables cells to reduce NADPH oxidase–dependent ROS formation (18, 19, 70, 86). We determined that exposure of podocytes to Plg reduced pAMPK (Figure 3B) and that AICAR, an activator of AMPK (18, 19, 70) normalized pAMPK and concomitantly inhibited Plg driven $O_2^-$ production (Figure 3A, 3B). Of note AICAR has been shown to have beneficial effects in diabetic nephropathy via similar mechanisms to those reported here (18, 19, 86). Hence AMPK may have a universal role in mitigating podocyte pathobiology linked to oxidative stress (10, 18, 19, 70, 86).

Podocyte ROS originate in the cytosol and particularly in mitochondria a major source of intracellular ROS production under physiologic and pathologic conditions (10, 14, 18, 31). Many studies have shown that sustained mitochondria ROS accumulation results in mitochondrial dysfunction (10, 14, 18). Here we showed that Mito Tempo, a selective inhibitor of mitochondrial ROS, reduced by 40% podocyte $O_2^-$ induced by Plg (Figure 6A). Based on our novel findings with AICAR and Mito Tempo we propose that both agents may have synergistic therapeutic benefits in proteinuric podocytopathies.

CD36 is an innate multifunctional membrane scavenger receptor that plays an important mechanistic role in atherosclerosis and in inflammation (77, 96). The B scavenger receptor CD36 is constitutive in many cells including podocytes, and recognizes, binds, and internalizes oxLDL and FFA (49, 56, 77, 96). We have shown in human macrophages that ROS up-regulate CD36 (96), and fosters increased uptake of OxLDL and foam cell formation. Mayrhofer et al (49) showed in preparations of glomeruli isolated from normal rats that CD36 is constitutively expressed in podocytes; in the same studies they demonstrated that in rats with PAN nephrosis podocyte CD36 was
up-regulated and promoted uptake of FFA and oxLDL resulting in podocyte injury and
development of FSGS. Furthermore, a recent study Souza et al (78) demonstrated that
the apolipoprotein A-I-mimetic peptide 5A a CD36 antagonist reduced glomerular and
tubular injury as well as CKD progression independent of blood pressure in 5/6
nephrectomized mice subject to AngII infusion (78). Similar reno-protection was
observed in 5/6 nephrectomized CD36KO mice (78). In the aggregate these in vivo
studies support the notion that CD36 plays an important role in the pathogenesis of
progressive CKD.

Here we determined that Plg significantly up-regulated CD36 expression (Figure
4A). This was prevented by inhibition with EACA of Plg binding as well as by inhibition
of O$_2^-$ synthesis by either APOCY or AICAR (Figure 4A). Importantly in subsequent
experiments we determined that podocytes take up OxLDL and that incubation with Plg
had an additive effect that resulted in increased podocyte oxLDL uptake (Figure 4B)
accompanied by podocyte apoptosis (Figure 6).

The off target effect of Amiloride as an inhibitor of uPA was documented in early
studies reported in the cancer literature (66, 89). In vivo experimental studies in Stroke
Prone SHR, Dahl salt sensitive and in 5/6 nephrectomy rats have shown that Amiloride
significantly reduces glomerular injury and proteinuria independent of blood pressure
(26, 33, 73). In patients with type 2 diabetic nephropathy Amiloride lowered urine
plasminogen excretion and activation, and albumin/creatinine ratio (55). In the current
studies we determined that Amiloride inhibits Plg induced O$_2^-$ production and CD36
upregulation in human podocytes (Figure 5). This might explain, at least in part, the
mechanisms involved in the salutary effects of Amiloride in clinical and experimental
proteinuric renal disease. These studies also provide strong support to our hypotheses regarding the potential role of Plg-plasmin in proteinuric renal diseases.

Elegant studies of biopsies from patients with FSGS presented evidence of endothelial–mitochondrial oxidative stress associated with podocyte-endothelial cell cross talk mediated by ET1 synthesized by podocytes (14, 15, 35, 41). Here we demonstrated that Plg induced synthesis and release of ET1 was inhibited by pre-treatment of podocytes with either EACA or the NADPH inhibitor APOCY (Figure 7B) (39, 40). Importantly, we made the new observation that selective inhibition of mitochondrial ROS with Mito-Tempo was sufficient to completely prevent Plg induced ET1 synthesis (Figure 7B) (14).

Crosstalk between mitochondria and NADPH oxidases is well documented and likely represent a feed-forward vicious cycle of ROS production in which activation of NADPH oxidases increase production of mitochondrial ROS and vice versa (16, 17). In podocytes Nox 4 is predominately localized in mitochondria (19, 31) and has been shown to produce hydrogen peroxide (H$_2$O$_2$). Our results with apocyanin that inhibits NOX2 and Mito Tempo would suggest (Fig 8) that Plg activation of NOX2 generates O$_2^-$, which activates mitochondrial Nox4 that reciprocally activates NOX2 via H$_2$O$_2$ (16, 17).

Overall our studies elucidated important new mechanisms that identified apoptosis, linked to the interaction of Plg-CD36 - ox-LDL as a potential “second hit” in podocyte injury and death associated with proteinuria (Figure 8) (20, 36, 47, 54, 68, 91, 93). Based on our studies we conclude that: 1) Plg activation on podocytes membranes can play a significant role in a “second hit” type of podocyte injury associated with chronic proteinuria independent of the initial etiology and 2) that oxidative stress
generated by Plg via NADPH oxidases plays a central role in these processes.

At the present time specific therapies for podocyte injury are not available. Our novel studies suggest that to slow CKD progression of proteinuric glomerular diseases would require multipronged strategies combining pharmacologically new molecules added to other agents such as biologicals that specifically target podocyte antigens, mitochondrial targeted antioxidants, activators of AMPKinase, ET1 receptor blockers/synthesis inhibitors, (14, 15, 18, 19, 35, 70) certainly RAAS inhibitors/ blockers, (8, 29, 63) and Amiloride, cautiously monitoring serum potassium. Further studies will be required to further define the pathogenic role of circulating Plg and potential benefits of its removal or inhibition. In summary we suggest that our studies provide a new biologic framework for the development of strategies for "comprehensive treatments" to arrest progression of proteinuric podocytopathies.

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Disclosures: None


Figure 1. Urine plasminogen levels are increased in mouse models of proteinuric kidney disease. Urine Plg/Cr is significantly increased in 6 week old Tg26 and 5 week old Cd2ap<sup>−/−</sup> mice. **P < 0.005

Figure 2. Plasminogen up-regulates NADPH oxidase isoforms NOX2 and NOX4:
Plg up-regulated mRNA and protein expression of NOX2 and NOX4 isoforms of NADPH oxidase that was significantly prevented by inhibition of Plg binding/activation by EACA. M = marker

Figure 3. Plasminogen induces production of Superoxide anion in human podocytes
A. Plg binds to human podocytes, is activated and induces production of superoxide anion. Plg (1 μmol/L, the Plg concentration in serum) significantly increased podocyte O$_2^-$ production; increased O$_2^-$ production was prevented by the NADPH oxidase and flavin containing oxidases diphenyleneiodonium (DPI) 1 μmol/L, the NADPH oxidase inhibitor APOCY (100 μmol/L), the agonist of AMP-activated protein kinase AICAR (100 μmol/L), as well as the inhibitor of Plg/plasmin activation EACA (0.2 mol/L). Data was expressed as mean ± SEM. *p < 0.05 vs. control, #p < 0.05 vs. plasminogen. n=6

B. pAMPK is active AMPK. Exposure of podocytes to Plg reduced pAMPK/AMPK ratio, and increased NADPH oxidase –mediated O$_2^-$ production. Activation of AMPK by AICAR, 5-aminoimidazole-4-carboxamide-1-riboside, significantly prevented
these effects by increasing the pAMPK/AMPK ratio and concomitantly reducing $O_2^-$ production. AMPK is a ubiquitous serine/threonine kinase that acts as an energy sensor and is expressed in the plasma membrane of podocytes; $^{62}$ pAMPK suppresses NADPH oxidase.$^{52-54, 62}$ Data are expressed as mean ± SEM. *$p < 0.05$ vs. control, #$p < 0.05$ vs. plasminogen. $n = 5$.

Figure 4. Expression of the scavenger receptor CD36 is upregulated by Plasminogen

A. Effects of Plg on scavenger receptor CD36 expression in human podocytes. Plg significantly increased CD36 expression, which was prevented by the NADPH oxidase inhibitor APOCY (10 μmol/L), Aicar (10 μmol/L) an agonist of AMP-activated protein kinase, as well as the inhibitor of Plg (plasmin) activation EACA (0.2mol/L). Data is expressed as mean ± SEM. *$p < 0.05$ vs. control, #$p < 0.05$ vs. plasminogen. $n = 5$.

B. Effect of Plg and oxLDL on total intracellular cholesterol content in human podocytes. Incubation of human PODC with oxLDL (50 mg/ml) resulted in a small but significant increase in total intracellular cholesterol content. Addition of Plg in the presence of oxLDL further increased intracellular cholesterol content, Data is expressed as mean ± SEM.*$p < 0.05$ vs. control and Control + oxLDL #$p < 0.05$ vs. Control group; $n = 6$ in each group.

Figure 5. Amiloride regulation of Plg-induced CD36 and ROS generation in podocytes. Amiloride abrogates Plg induction of CD36 (A) and ROS (B) in podocytes.

** $P < 0.005$, ****$P < 0.0001$
Figure 6. Exposure of human podocytes to Plg plus oxLDL has additive effects in promoting podocyte apoptosis:

A. Both Plg and oxLDL induced slight but significant podocyte apoptosis after 24hrs. The combination of Plg and oxLDL had a clear additive effect upon podocyte apoptosis driven by increased oxLDL uptake associated with Plg up-regulation of scavenger receptor CD36 (Figures 4A & B)

B. Podocyte apoptosis measured by flow cytometry.

Figure 7. Plasminogen promoted synthesis of Endothelin 1 was specifically prevented by Mito-Tempo an inhibitor of mitochondrial ROS

A. Plg induced O$_2^-$ production in podocytes that was partially prevented (40%) by Mito Tempo a selective inhibitor of mitochondrial oxidative stress.

B. Endothelin 1 synthesis/release was measured by ELISA in the supernatants of the various conditions. Plg increased podocyte synthesis of ET1 that was inhibited by APOCY and by Mito- Tempo. Important to note is that while Mito- Tempo only reduced ROS by 40% (A) it completely prevented ET1 synthesis in response to Plg suggesting that O$_2^-$ from mitochondria plays a critical role in modulating podocyte ET1 synthesis.

Figure 8. Working model of the participation of Plasminogen-Plasmin as a “second hit” type of podocyte injury associated with proteinuria.
Figure 1
Figure 2
Figure 3
**Figure 4**

**Panel A**

Protein Expression (CD36/β-actin)

- Ctr
- Plg
- Plg + EACA
- Plg + AICAR
- Plg + Apo

**Panel B**

Total intracellular cholesterol (mg/mg protein)

- Ctr
- Ctr + oxLDL
- Plg + oxLDL
Figure 5
Figure 6
Figure 7
**Plasminogen-Plasmin as a “second hit” of podocyte injury associated with proteinuria**

**Increased Proteinuria**

- **Plasminogen (Plg)**
  - **Plg Receptors**
    - **Plasmin**
      - **Podocyte**
        - **Increased ROS**
          - **Podocyte ET1 synthesis**
    - **Upregulation CD36 + oxLDL / FFA**
      - **Podocyte Apoptosis**

**Crosstalk Among Membrane - Cytosol-Mitochondria ROS production (Ongoing Feed-Forward Cycle)**

- **Nox2**
  - **Cytoplasm O$_2^-$**
    - **Mitochondria O$_2^-$**
      - **Mitochondria H$_2$O$_2$ (Nox4)**
        - **ROS overproduction**

**Figure 8**