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Angiotensin II induces nephrin dephosphorylation and podocyte injury: Role of caveolin-1

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ABSTRACT

Nephrin, an important structural and signal molecule of podocyte slit-diaphragm (SD), has been suggested to contribute to the angiotensin II (Ang II)-induced podocyte injury. Caveolin-1 has been demonstrated to play a crucial role in signaling transduction. In the present study, we evaluated the role of caveolin-1 in Ang II-induced nephrin phosphorylation in podocytes. Wistar rats-receiving either Ang II (400 ng/kg/min) or normal saline (via subcutaneous osmotic mini-pumps, control) were administered either vehicle or telmisartan (3 mg/kg/min) for 14 or 28 days. Blood pressure, 24-hour urinary albumin and serum biochemical profile were measured at the end of the experimental period. Renal histomorphology was evaluated through light and electron microscopy. In vitro, cultured murine podocytes were exposed to Ang II (10^{-6} M) pretreated with or without losartan (10^{-5} M) for variable time periods. Nephrin and caveolin-1 expression and their phosphorylation were analyzed by Western-blotting and immunofluorescence. Caveolar membrane fractions were isolated by sucrose density gradient centrifugation, and then the distribution and interactions between Ang II type 1 receptor (AT1), nephrin, C-terminal Src kinase (Csk) and caveolin-1 were evaluated using Western-blotting and co-immunoprecipitation. Podocyte apoptosis was evaluated by cell nucleus staining with Hoechst-33342.

Ang II-receiving rats displayed diminished phosphorylation of nephrin but enhanced glomerular/podocyte injury and proteinuria when compared to control rats. Under control conditions, podocyte displayed expression of caveolin-1 in abundance but only a low level of phospho moiety. Nonetheless, Ang II stimulated caveolin-1 phosphorylation without any change in total protein expression. Nephrin and caveolin-1 were co-localized in caveolae fractions. AT1 receptors and Csk were moved to caveolae fractions and had an interaction with caveolin-1 after the stimulation with Ang II. Transfection of caveolin-1 plasmid (pEGFPC3-cav-1) significantly increased Ang II-induced nephrin dephosphorylation and podocyte apoptosis. Furthermore, knockdown of caveolin-1 expression (using siRNA) inhibited nephrin dephosphorylation and prevented Ang II-induced podocyte apoptosis. These findings indicate that Ang II induces nephrin dephosphorylation and podocyte injury through a caveolin-1-dependent mechanism.

1. Introduction

Podocytes and slit diaphragm (SD) play a critical role in the maintenance of glomerular filtration barrier (GFB) [1]. Proteinuric glomerulopathies, including focal segmental glomerulosclerosis (FSGS) [2], minimal change disease (MCD) [3] and diabetic nephropathy [4], display by podocyte injury in the form of foot process effacement. In addition to leaky GFB, glomerular hypertension as a consequence of the activated renin-angiotensin-aldosterone system (RAAS) was implicated for enhanced proteinuria [5–7]. During the last decade, the direct effect of Ang II in the induction of podocyte injury is being recognized increasingly [8]. The multiple effects of Ang II are mediated predominantly through AT1 receptor, which is coupled to heterotrimeric G proteins and involved in different second-messenger signal transduction pathways [9]. Our previous studies have demonstrated that Ang II induced both podocyte injury and proteinuria [10]. However, the involved signaling mechanisms in Ang II-induced podocyte injury are not fully delineated.

Nephrin, known as the predominant molecule of slit diaphragm (SD), plays a key role in the maintenance of the GFB [11]. Nephrin belongs to the immunoglobulin superfamily of cell adhesion molecules and forms a zipper-like lattice structure in the slit diaphragm [11]. Six conservative tyrosine phosphorylation sites have been found in the intracellular domain of nephrin [12], which are phosphorylated by the Src family kinase (SFK) Fyn [12,13]. The phosphorylation events of nephrin are thought to be important for the survival status of podocytes and rearrangement of cytoskeletal proteins [14]. Some recent reports have demonstrated that Ang II can influence the nephrin expression, but the effects on phosphorylation of nephrin remain debated.

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Table 1
Systolic blood pressure and urinary albumin excretion in different groups (n = 6).

Unit	Systolic blood pressure (mm Hg)					Urinary albumin (mg/24 h)				
	0 day	7 days	14 days	21 days	28 days	0 day	7 days	14 days	21 days	28 days
Control	99.7 ± 1.53	98.26 ± 1.67	99.1 ± 1.33	102.8 ± 4.25	103.47 ± 4.39	12.7 ± 4.3	15.7 ± 4.98	13.8 ± 5.6	16.8 ± 5.3	17.2 ± 7.9
Ang II	101.3 ± 2.9	128.3 ± 3.7*	137.4 ± 2.05*	147.9 ± 2.95*	168.11 ± 4.97*	13 ± 3	69.1 ± 7.33*	115.8 ± 12.5*	169.7 ± 11.7*	200.3 ± 12.8*
Telmisartan + Ang II	99.5 ± 1.4	119.3 ± 1.1*△	120.3 ± 2.6*△	127.6 ± 1.94*△	132.22 ± 3.52*△	11.5 ± 2	41.9 ± 5.42*△	53.2 ± 9.8*△	88.52 ± 8.6*△	96.57 ± 10.9*△

Wistar rats received either vehicle (control), Ang II (400 ng/kg/min), or telmisartan (3 mg/kg/day) + Ang II (400 ng/kg/min) via mini-osmotic pumps for 28 days. Systolic blood pressure and urinary albumin per 24-h were measured on days 0, 7, 14, 21, 28.
*$p<0.05$ compared with control group of respective duration; △$p<0.05$ compared with Ang II infused group of respective duration.

Caveolin-1 is a key component of caveolae, which are 50–100 nm membrane invaginations and thought to play important roles in cell signaling transduction. Multiple signaling molecules are located in caveolae and their functions are modified by caveolin-1 [15]. Phosphorylation of caveolin-1 on tyrosine 14 may function to facilitate caveolin-1 interaction with other proteins in a stimulus-specific fashion [16,17]. Fyn has been found residing in podocyte caveolae, in which nephrin, podocin, CD2AP, and Neph1 are also found [18,19]. Here, we study the role of caveolin-1 in podocytes and hypothesize that caveolin-1 is participating in Ang II-nephrin signaling in podocytes.

2. Materials and methods

2.1. Animals

Thirty-six male SPF Wistar rats weighing between 140 and 160 g were purchased from Hubei Research Center of Experimental Animals and were maintained at a controlled temperature (23 ± 2 °C) and humidity (55 ± 5%) under an artificial light cycle, with a free access to tap water and standard rat chow. Embedded with osmotic mini-pump (Alzet model 2002 or 2004, CA), rats were randomly subjected to normal saline infusion, or Ang II infusion at 400 ng/kg/min, or Ang II at 400 ng/kg/min + telmisartan at 3 mg/kg/day by means of intragastric administration for 14 or 28 days. Systolic blood pressure was measured by tail cuff plethysmography in conscious, trained, and preheated rats. 24-hour urine was collected in metabolic cages and urinary albumin excretion rate was measured at days 7, 14, 21, and 28. Animals were sacrificed at days 14 and 28, and the blood was collected for serum creatinine, Ang II concentration analysis. Kidneys were infused by vanadate (a phosphatase inhibitor) before isolated and stored at −80 °C for biochemical and renal pathological analysis.

2.2. Cell culture

A conditionally immortalized murine podocytes were kindly provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York). Podocytes were maintained in RPMI 1640 medium (HyClone, USA) containing 10% heat-inactivated fetal calf serum (Gibco, USA), 100 U/mL penicillin G, and 100 μg/mL streptomycin in an incubator with 5% CO_2. During podocyte proliferation, the medium was mixed with 10 U/mL recombinant mouse interferon-γ (Sigma, USA), and the cells were maintained at 33 °C. Then podocytes were cultured at 37 °C to induce differentiation without interferon-γ for 10–14 days. 15–25 passages of podocytes were used in the following experiments. All experiments were performed in differentiated podocytes.

Table 2
Endogenous creatinine clearance rate (Ccr) and concentration of Ang II in plasma or renal tissue in different groups (n = 6).

	Day	Ccr (mL/min/100 g)	Concentration of Ang II	
			Plasma (pg/mL)	Renal tissue (pg/mL/mg)
Control	14	1.16 ± 0.37	270.5 ± 92.8	861.2 ± 107.5
	28	1.13 ± 0.42	300.74 ± 88.7	798.2 ± 97.5
Ang II	14	0.97 ± 0.21	1026.25 ± 148.2*	2438.7 ± 179.8*
	28	0.86 ± 0.16	1022.3 ± 132.7*	2326.4 ± 136*
Telmisartan + Ang II	14	0.83 ± 0.11	1381.5 ± 151.7*	2656.6 ± 155.9*
	28	1.07 ± 0.15	1339.3 ± 160.3*	2518.8 ± 139.1*

Wistar rats received either vehicle (control), Ang II (400 ng/kg/min), or telmisartan (3 mg/kg/day) + Ang II (400 ng/kg/min) via mini-osmotic pumps for 14/28 days. Endogenous creatinine clearance rate (Ccr) and concentration of Ang II in plasma or renal tissue were measured on days 14 and 28.
*$p<0.05$ compared with control group of respective duration.

2.3. Isolation of caveolar membrane fractions

Cells were washed in PBS, lysed in MES-buffered saline (MBS, 25 mM Mes [pH 6.5] and 150 mM NaCl) with 1% Triton X-100 and protease/phosphatase inhibitors, then solubilized by 10 passes through a 25-G needle and sonicated for 15 s on ice. Samples were equalized for protein and mixed with equal volume of 90% sucrose in MBS, then placed above with 35, 30, 25, and 5% sucrose in MBS, and centrifuged at 100,000 × *g* for 16 h at 4 °C. The caveolae fraction appeared at the 5 to 25% interface. From the top of each gradient, 12 equal fractions were collected and recentrifuged at 34,000 × *g* for 45 min, and separated on a 15% gel. Fractions 2 to 5 correspond to caveolae, as confirmed by immunoblotting for caveolin-1.

2.4. Western immunoblotting

Cells were lysed in RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) with protease/phosphatase inhibitors, and centrifuged at 12,000 rpm for 20 min at 4 °C. Then the protein samples were mixed with loading buffer and boiled at 95–100 °C for 5 min. The proteins were separated on 8 to 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to nitrocellulose membrane (GE Healthcare) by semidry blotting. Nephrin Guinea pig monoclonal antibody (1:1000; Progen), nephrin rabbit Phospho (pY1217) monoclonal antibody (1:1000; Epitomics), AT1 (N-10) rabbit polyclonal antibody (1:100; Santa Cruz), β-actin mouse monoclonal antibody (1:1000; Santa Cruz), caveolin-1 rabbit monoclonal antibody (1:1000; Cell Signaling Technology), phospho-caveolin-1 (Tyr14) rabbit polyclonal antibody (1:1000; Cell Signaling Technology) and Csk rabbit monoclonal antibody were used as primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (1:1000; Cell Signaling Technology) were used at 1:5000. Blots were visualized by the enhanced chemiluminescence reaction (Santa Cruz) and developed on the film.

2.5. Immunofluorescence assay

The frozen kidney sections were prepared using a cryostat and covered by bovine serum for 30 min at room temperature. The cell climbing film was fixed in 4% paraformaldehyde with 0.1% Triton X-100 for 30 min at 4 °C. Nephrin/phospho-nephrin or caveolin-1/phosphor-caveolin-1 antibodies were used as primary antibodies for overnight