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Research Article

Puromycin aminonucleoside increases podocyte permeability by modulating ZO-1 in an oxidative stress-dependent manner

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ABSTRACT

Puromycin aminonucleoside (PAN)-induced nephrosis is a widely studied animal model of human idiopathic nephrotic syndrome because PAN injection into rats results in increased glomerular permeability with the characteristic ultrastructural changes in podocytes similar to human nephrosis. To investigate the role of zonula occludens (ZO)-1 and oxidative stress on PAN-induced podocyte phenotypical changes and hyperpermeability *in vitro*, we cultured rat and mouse podocytes and treated with various concentrations of PAN. PAN treatment increased oxidative stress level of podocytes significantly with the induction of Nox4. In addition, PAN changed the ultrastructure of podocytes, such as shortening and fusion of microvilli, and the separation of intercellular gaps, which were improved by anti-oxidative vitamin C and Nox4 siRNA. PAN also disrupted the intercellular linear ZO-1 staining and induced inner cytoplasmic re-localization of ZO-1 protein, resulting in increased podocyte intercellular permeability. PAN reduced ZO-1 protein amount and mRNA expression in a dose-dependent manner, which means that PAN could also modulate ZO-1 protein transcriptionally. However, the decreased ZO-1 protein of podocytes by PAN was improved by Nox4 siRNA transfection. Furthermore, vitamin C mitigated the quantitative and distributional disturbances of ZO-1 protein caused by PAN. Our results demonstrate that the phenotypical changes of intercellular ZO-1 by oxidative stress via Nox4 likely contribute to the glomerular hyperpermeability caused by PAN.

1. Introduction

Proteinuria, a main clinical manifestation of podocytopathies, demonstrates increase in glomerular permeability caused by the ultrastructural and componental changes in podocytes and slit diaphragm (SD) with the retraction and effacement of the interdigitating foot processes [1–5]. The specialized, highly differentiated podocytes have a cell body and long, extending foot processes, which are separated by a filtration slit that is 25–60 nm wide and covered by a SD [4–6]. The molecular components of the SD are linked to the cytoskeletal structures by adapter proteins and vital for the maintenance of normal glomerular permselectivity [5–8]. One such molecule is zonula occludens (ZO)-1, expressed on the cytoplasmic surface of podocyte foot processes at the point of insertion of the SD and linked to the actin cytoskeleton on the other side [5,7–11]. Therefore, ZO-1 protein as a component of the SD plays a pivotal role in maintaining the glomerular permeability by connecting and maintaining both SD structure and actin cytoskeleton.

Puromycin aminonucleoside (PAN)-induced nephropathy is one of the well-described animal models of proteinuria. PAN specifically injured podocytes, leading to a flattening of podocyte foot processes, focal detachment from the glomerular basement membrane (GBM), actin cytoskeleton disorganization, decreased expression and abnormal distribution of SD proteins, coincided with the onset of proteinuria [12–14]. ZO-1 has been reported as one of the responsible SD proteins in PAN-induced nephropathy that may be associated with the development of proteinuria [14,15]. *In vivo* and *in vitro* studies have supported that PAN-induced glomerular injury is mediated via overproduction of reactive oxidative species (ROS) especially by podocytes acutely [16–18], which then may interact with biomolecules, leading to modification and potentially deleterious cellular consequences, including apoptosis [19,20]. Indirect support for the importance of

Abbreviations: BSA, bovine serum albumin; CM-H_2DCFDA, 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester; GBM, glomerular basement membrane; GEpC, glomerular epithelial cells; NADPH, nicotinamide-adenine dinucleotide phosphate; PAN, puromycin aminonucleoside; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SD, slit diaphragm; SEM, scanning electron microscopy; ZO-1, zonula occludens-1

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ROS in the pathogenesis of PAN comes from *in vivo* and *in vitro* studies utilizing therapy with various antioxidants. In this study, we applied an *in vitro* proteinuria model induced by PAN and found that PAN disrupted ZO-1 protein of podocytes quantitatively and spatially, therefore, subsequently increased their permeability via oxidative stress.

2. Materials and methods

2.1. Cell culture of rat glomerular epithelial cells (GEpCs) and mouse podocytes

We used two kinds of podocytes, rat glomerular epithelial cells (GEpCs) and mouse podocytes. We applied rat GEpCs to ROS analysis, morphology, and permeability assay, as rat GEpCs were grown compactly. We also applied differentiated mouse podocytes to most quantitative assays. Rat GEpCs, cloned from primary rat glomerular cultures, were characterized and provided by Kreisberg [21]. They were characterized by sensitivity to PAN, positive staining for Heymann antigen (gp330) and podocalyxin, whereas negative staining for factor VIII [21–23]. GEpCs were maintained as previously described [24]. Experiments were performed with cells between passages 15 and 18. Conditionally immortalized mouse podocytes were kindly provided by Dr. Peter Mundel (University of Harvard, Boston, MA, USA) and were cultured and differentiated for at least two weeks as described previously [25]. Both cell lines have been previously extensively characterized and used by many researchers including us. Cells were treated with 1–50 μg/mL of PAN (Sigma Chemical Co., St. Louis, MO, USA) dissolved in ethanol for 24 and/or 48 h. For the protective effect of antioxidant and nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase inhibitor, podocytes were treated with PAN and 30 μM vitamin C (Amresco Inc., Solon, OH, USA) or 100 μM apocynin (Sigma Chemical Co., St. Louis, MO, USA), an inhibitor of NADPH oxidase activity, before 2 hours for PAN treatment. Controls were treated with vehicle only.

2.2. Expression of Nox4, a NADPH oxidase subunit

Expression of NADPH oxidase subunits in mouse podocyte cellular protein was analyzed by Western blotting. At the end of incubation with additives, the confluently grown cell layers were washed twice with PBS and subsequently extracted in 4 M guanidinium-HCl, 2% CHAPS, and protease inhibitors containing 100 mM 6-aminohexanoic acid, 10 mM benzamidine HCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4 °C overnight and stored at −20 °C till further analysis. Protein concentrations determined with a Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, USA) as previously described [24]. Protein extracts was loaded to SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories). Transferred membranes were incubated with primary antibodies and subsequent horseradish peroxidase–conjugated anti-goat/rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a secondary antibody, bands were visualized the enhanced chemiluminescence system (Amersham Biotech Ltd., Bucks, UK). Primary antibodies were all purchased from Santa Cruz Biotechnology and as followings; rabbit anti-MOX1(Nox1; H-15) antibody, goat anti-gp91^{phox} (C-15) antibody, rabbit anti-p47-phox(H-195) antibody, goat anti-p67^{phox} (N-19) antibody, goat anti-Nox4 (N-15) antibody, rabbit anti-p22^{phox} (FL-195) antibody, and goat anti-β-tubulin antibody.

2.3. Small interference RNA (siRNA) for Nox4 transfection

One day before transfection, the culture medium was removed and differentiated podocytes were cultivated in antibiotics-free RPMI 1640 medium supplemented with 10% FBS. Cells were transfected with siRNA using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's introductions. Briefly, Nox4 siRNA (sc-41587, Santa Cruz Biotechnology) or control scrambled siRNA (Santa Cruz Biotechnology) were diluted into each 6-well plate with Transfection Medium (Opti-MEM, Invitrogen) and incubated for 5 min. In parallel, Lipofectamine was diluted with Transfection Medium (Opti-MEM). Diluted Lipofectamine reagent and siRNA were mixed and incubated at room temperature for 20 min. The medium was replaced with Opti-MEM and the siRNA/Lipofectamine mixture was added to the cells. After replacing the transfection mixture with RPMI 1640 medium supplemented with 10% FBS after 5 h, the inhibitory effect of siRNA for NOX4 was confirmed by Western blotting.

2.4. ROS assay

The intracellular production of ROS was assayed using the fluoroprobe 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA). To examine the effect of PAN on ROS generation, confluently grown rat GEpCs were stimulated with 1–50 μg/mL PAN for 2 and 24 h. To determine the effect of vitamin C on PAN-stimulated ROS generation, GEpCs were co-treated with PAN and 30 μM vitamin C. After treatment, cells were loaded with 10 μM CM-H2DCFDA. Cells were examined at 30 min by immunofluorescence microscopy and also immediately analyzed by ELISA reader at 480 nm excitation and 530 nm emission wavelengths.

2.5. Monolayer permeability assay

As rat GEpCs were grown compactly to be applicable to permeability assay, rat GEpCs were seeded and grown confluently in monolayer pattern on the surface of cellulose semi-permeable membranes (Millicell-HA, Millipore Corp., Billerica, MA, USA) having 0.45-μm of pore size. Hydrostatic pressure was applied continuously from basolateral to apical aspect by the equalization of each media volumes. After each incubation times (0, 2, 4, 6, 8 h), experimental media were washed completely with PBS and fresh maintenance media without serum were replaced into each aspect. Then, 1 μg/mL of bovine serum albumin (BSA) was put into the basolateral media and the amount of BSA filtered into apical chamber for 2 h at 37 °C was determined with sandwich ELISA method [24].

2.6. Scanning electron microscopy (SEM)

SEM was performed on rat GEpCs and mouse podocytes grown confluently on the surface of cellulose semi-permeable membranes (Millicell-HA, Millipore Corp., MA, USA). After being washed twice with PBS, the monolayered cells were fixed with 5% glutaraldehyde and post-fixed with 2% osmium tetroxide, followed by dehydration with progressive concentrations of ethanol. These samples were then passed through critical point drying and gold-coated before examination on a SEM (Hitachi S-570, Hitachi Science Systems Ltd., Hitachinaka, Japan).

2.7. Immunofluorescence staining

Rat GEpCs and mouse podocytes that were grown on type I collagen-coated glass coverslips incubated for 24 hours were fixed in 4% paraformaldehyde, permeabilized in PBS, blocked with 10% normal goat serum, and stained with polyclonal rabbit anti-rat ZO-