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Lipid peroxidation regulates podocyte migration and cytoskeletal structure through redox sensitive RhoA signaling

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ABSTRACT

Early podocyte loss is characteristic of chronic kidney diseases (CKD) in obesity and diabetes. Since treatments for hyperglycemia and hypertension do not prevent podocyte loss, there must be additional factors causing podocyte depletion. The role of oxidative stress has been implicated in CKD but it is not known how exactly free radicals affect podocyte physiology. To assess this relationship, we investigated the effects of lipid radicals on podocytes, as lipid peroxidation is a major form of oxidative stress in diabetes. We found that lipid radicals govern changes in podocyte homeostasis through redox sensitive RhoA signaling: lipid radicals inhibit migration and cause loss of F-actin fibers. These effects were prevented by mutating the redox sensitive cysteines of RhoA. We therefore suggest that in diseases associated with increased lipid peroxidation, lipid radicals can determine podocyte function with potentially pathogenic consequences for kidney physiology.

1. Introduction

Podocytes are highly specialized cells in the glomeruli, and an integral part of a healthy glomerular filtration barrier. Any impairment in their adhesion to the glomerular basement membrane [1,2] or in their foot processes [3,4] can lead to cell deformity, foot process effacement and ultimately to the loss of the cell, thus compromising barrier function. Loss of more than ~30% of podocytes is detrimental to a glomerulus [5–7] leading to glomerulosclerosis and chronic kidney disease (CKD), which is a major complication in diabetes. Therefore, podocyte preservation is critical. However, treatments aimed at two major factors in diabetes, hyperglycemia or hypertension, do not prevent CKD. Podocyte loss is also typical in obesity-related glomerulopathy (ORG) in normoglycemic individuals [8]. Thus, besides high glucose and hypertension, there must be additional pathogenic factors that can cause podocyte depletion. Numerous studies suggested “oxidative stress” as a common denominator in the pathogenesis of kidney disease [9–15] and, at least in part, in podocyte loss [7,16–19]. NADPH oxidase or mitochondrial pathways have been proposed to be main sources of ROS generation in the kidney, resulting in podocyte injury [7,20,21]. ROS has also been shown to alter gene expression in podocytes and induce inflammation and injury through the granulocyte macrophage-colony-stimulating factor [22]. Mechanisms leading to podocyte dysfunction and specifications as to which “ROS” are able to elicit such a response remain elusive. To address this issue, we propose that a lipotoxic environment accompanying diabetes is conducive to the formation of lipid peroxyl radicals ($LOO^{\bullet}$) and their end-products, lipid hydroperoxides.

Lipid radicals are formed during lipid peroxidation [23], which is heightened in diabetes [24]. Their stable end-products, lipid hydroperoxides (LOOH), will cross biological membranes [25,26]. Many of these end-products, termed as reactive lipid species (RLS) [27] are electrophilic (4-hydroxynonenal, F-isoprostanes) and readily react with nucleophile amino acid residues, particularly cysteine residues of proteins [27]. The key aspect of this notion is that even very low levels of oxidized lipids elicit biological response, through covalently modifying cysteine residues of proteins [27,28]. Cysteine residues of protein thiols are particularly sensitive to oxidation, thus lipid radical metabolites modulate cellular processes through protein thiol signaling [29,30]. Therefore, it is reasonable to suggest that lipid radicals/RLS initiating protein thiol signaling through cysteines may be a driving redox mechanism under conditions of oxidative stress leading to podocyte injury. Here, we address this hypothesis *in vitro* by exposing podocytes to lipid peroxyl radicals and propose a mechanism through investigating the role of RhoA, which is a redox sensitive [31] master regulator protein.

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2. Methods

2.1. Materials

All chemicals were from Sigma (St. Louis, MO), purest grade available unless otherwise stated. Antibodies from various sources are described in each section specifically.

2.2. Cell culture

Conditionally immortalized SV-40T podocytes were a generous gift from Dr. Katalin Susztak's (University of Pennsylvania, Philadelphia, PA) and Dr. Farhad Danesh's (Baylor, Houston, TX) laboratories. They were cultured and differentiated as described by Shankland et al. [32]. Briefly, podocytes were maintained under growth permissive conditions at 33 °C with mouse IFNγ supplementation (20 U/ml) in RPMI 1640 media (Thermo Scientific, Waltham, MA). For differentiation, cells were switched to 37 °C without IFNγ for at least 11 days. Since these cells carry the temperature-sensitive variant of the SV40T antigen, it allows podocytes to proliferate at 33 °C. Inactivation of the large T antigen at 37 °C results in cell cycle exit and differentiation into mature podocytes with interdigitating processes. Differentiation was verified by synaptopodin expression. Experiments were performed with differentiated podocytes plated on 6 well plates or glass bottom dishes coated with collagen I.

2.3. RhoA mutation

Cys16, Cys20 or both cysteines (Cys16/20) on RhoA were mutated to alanine (C16A and C20A) using site-directed mutagenesis. These cDNAs were then subcloned into adenoviral shuttle vectors for the construction of recombinant adenoviral vectors, as described. pEGFP-RhoA was purchased from Addgene (Plasmid # 23224). RhoA cDNA was subcloned into the *pAC.CMV* shuttle vector using EcoRI and BamHI sites in both vectors. *pAC.CMV* RhoA was used as a template to generate the C16A and C20A mutations in RhoA. *pAC.CMV* RhoA C16A was used as a template to generate the RhoA C16A/C20A double mutant. All mutations were generated using the QuikChange Site-Directed Mutagenesis kit, according to the manufacturer's protocol, and verified by dideoxy sequencing (PBRC Genomics Core) prior to construction of recombinant adenoviral vectors. Primers used for site-directed mutagenesis are as follows:

C16A: (F) 5′ GTTGGTGATGGAGCC**gc**TGGAAAGACATGCTTG 3′ and (R) 5′ CAAGCATGTCTTTCCA**gc**GGCTCCATCACCAAC 3′
C20A: (F) 5′ GAGCCTGTGGAAAGACA**gc**CTTGCTCATAGTCTTCAG 3′ and (R) 5′ CTGAAGACTATGAGCAAG**gc**TGTCTTTCCACAGGCTC 3′
C16A/ C20A: (F) 5′' GAGCC**gc**TGGAAAGACA**gc**CTTGCTCATAGTC TTC 3′ and (R) 5′ GAAGACTATGAGCAAG**gc**TGTCTTTCCA**gc**GGCTC 3′

Recombinant adenoviruses were generated by cotransfection of the pAC.CMV RhoA shuttle vectors and pJM17 viral genome into HEK293 cells. All four adenoviruses were confirmed to be insert positive and E1A negative. To deliver the constructs into podocytes, cells were transduced with the adenoviral constructs at day 11, and incubated with the virus for 48 h. (Delivery and transduction efficiency were titrated and verified using an adenovirus-GFP construct and Western blots see Suppl. Fig. 2). Experiments were performed with cells containing mutated RhoA.

2.4. Podocyte migration and time lapse imaging of live cells

Podocytes were differentiated on collagen coated 6 well plates for 11 days, washed and switched to serum-free RPMI 1640 media before experiments. To study the effects of lipid radicals, cells were incubated with the alkyl radical donor 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) at different concentrations (10 and 25 mM, for 4 h at 37 °C to produce 0.8 and 2 μmol/min radical generation, respectively). All incubations were in serum free RPMI 1640 media. After treatments, control or treated cells were washed twice with RPMI 1640 and placed into fresh media with 10% serum supplementation. A "wound" was scratched with a 200 μl sterile pipette tip onto the cell monolayer across each well. Pictures of the wounded area were taken on a Zeiss Axiovert 200 microscope at 72 h to count the number of cells migrating into the wound (4 different viewing areas per well, duplicate wells for each group, two independent experiments). In the live cell time lapse monitoring experiments, a 6-well plate of "wounded" podocyte cells was placed into a humidifying chamber with CO_2 thermostat and temperature control (37 °C) of a Leica DM6000 microscope. Coordinates of four different view area positions per well were programmed into the microscope software and cell migration was followed real time for 72 h. A picture of each position was taken in every 30 min (total of 144 frames per position) and compiled into video files at the end of experiments (see online Supplement for videos). Time-lapse videos were then analyzed using an Image J Manual Tracking plug-in. At least two independent experiments were performed for each group.

2.5. F-actin fiber analysis

To assess podocyte cytoskeletal F-actin fiber changes, an F-actin specific green fluorescence conjugated (488 nm) antibody was used (Cytoskeleton Inc, Denver, CO). After exposing podocytes to AAPH for 4 h, cells were washed with sterile PBS, fixed in 10% buffered formaldehyde for 30 min and incubated with the anti-F-actin antibody for 1 h. Cells were washed with PBS three times and were observed in 6 well plates using a Leica DM6000 fluorescent microscope (10 pictures per well, random viewing areas, duplicate wells per group, three independent experiments). F-actin anisotropy (how parallel the fibers are) and orientation were evaluated using the Image J plug-in "FibrilTool": confocal pictures of the actin fibers (green channel) were converted to greyscale images and at least 3–4 cells from each picture were analyzed with the plug-in.

2.6. RhoA activation

Podocytes were serum starved for 24 h before experiments to minimize endogenous RhoA amount and activity. Since activation of RhoA is a fast process, a time-course of RhoA activation was determined in preliminary experiments, and 5 min of activation was used with the radical donor AAPH. Cells were washed once with ice cold PBS and lysed rapidly on ice. The amount of active GTP-bound RhoA was measured from cell lysates equalized for protein content using a commercially available "G-LISA" assay (Cytoskeleton Inc., Denver, CO) that is specific for the active form of RhoA. A primary anti-RhoA (mouse) antibody provided in the assay kit was used (1:250). RhoA activity was expressed as fold-change, using untreated (normal or wild type) podocyte cell basal RhoA activity for normalization.

2.7. Statistical analysis

Experiments were done with podocytes that were closely similar in passage numbers (13 − 20) before differentiation. Data were expressed as mean ± SD. Statistical significance between groups was determined by one-way ANOVA followed by Bonferroni post-hoc test, or Student's *t*-test as appropriate. $p < 0.05$ was considered as statistically significant difference.

3. Results and discussion

To investigate the effects of lipid radicals on podocytes we have