(Pro)renin receptor regulates autophagy and apoptosis in podocytes exposed to high glucose

Caixia Li and Helmy M. Siragy

Division of Endocrinology and Metabolism, University of Virginia Health System, Charlottesville, Virginia

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Li C, Siragy HM. (Pro)renin receptor regulates autophagy and apoptosis in podocytes exposed to high glucose. Am J Physiol Endocrinol Metab 309: E302–E310, 2015. First published June 16, 2015; doi:10.1152/ajpendo.00603.2014.—High glucose reduces autophagy and enhances apoptosis of podocytes. Previously, we reported that high glucose induced podocyte injury through upregulation of the (pro)renin receptor (PRR). We hypothesized that increasing PRR reduces autophagy and increases apoptosis of mouse podocytes exposed to high glucose via activation of the PI3K/Akt/mTOR signaling pathway. Mouse podocytes were cultured in normal (5 mmol/l) or high (25 mmol/l) d-glucose for 48 h. High glucose significantly increased mRNA and protein levels of PRR, phosphorylation of PI3K/Akt/mTOR, and p62. In contrast, high glucose decreased activation of UNC-51-like kinase-1 (ULK1) by phosphorylating Ser757 and protein levels of microtubule-associated protein-1 light chain 3B (LC3B)-II and Lamp-2. Bafilomycin A1 increased LC3BII and p62 accumulation in high-glucose-treated cells. High glucose reduced the autophagic flux. Confocal microscopy studies showed significant reduction in the protein level of LC3B in response to high glucose. Cyto-ID autophagy staining showed a significant decrease in autophagosome formation with high glucose. In the absence of PRR, activation of Akt with sc-79 or mTOR with MHY-1485 increased p62 accumulation. Caspase-3/7 activity and apoptosis monitored by TUNEL assay were significantly increased in podocytes treated with high glucose. PRR siRNA significantly reversed the effects of high glucose. Based on these data, we conclude that high glucose decreases autophagy and increases apoptosis in mouse podocytes through the PRR/PI3K/Akt/mTOR signaling pathway.

podocyte; high glucose; (pro)renin receptor; autophagy; apoptosis

HIGH GLUCOSE CAUSES GLOMERULAR CELL INJURY, including mesangial cells (23), podocytes (8), and endothelial cells (51), leading to loss of renal function and eventual development of end-stage renal disease (7, 17). Podocytes are a key component of glomerular basement membrane and maintain the glomerular filtration function (34, 35). Our recent studies demonstrated that high glucose decreased podocyte expression of the slit diaphragm-associated proteins nephrin and podocin and increased albumin filtration and podocyte damage. These effects were mediated through upregulation of (pro)renin receptor (PRR) expression (33). However, the mechanisms involving PRR in mediating podocyte injury are unknown. PRR are widely expressed in the kidney, mainly in the glomerular mesangial cells (22), podocytes (8), vascular smooth muscle cells (38), and renal tubule cells (1).

Autophagy is a major catabolic pathway for proteins and organelles delivered to lysosomes and vacuoles, where they get degraded and recycled (46). It also has key roles in cell survival and development through maintaining a balance between synthesis and degradation (55). Many pathological conditions, including diabetes mellitus, cancer, and neurodegenerative diseases are associated with abnormal autophagy (15, 26, 46, 54). PRR is a subunit of V-ATPase, which mediates acidification of autolysosome (12). Recent studies demonstrated a role for PRR in regulating autophagy through modulation of lysosome function (39, 43). Interestingly, other studies have demonstrated that V-ATPase is necessary for activation of mammalian target of rapamycin (mTOR), which reduces autophagosome formation and in turn inhibits autophagy (25, 59). These studies suggested both stimulatory and inhibitory roles for PRR on autophagy (4).

High glucose activates the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (50). Interestingly, PRR was shown to have similar effects in neuronal cells (41). Activation of the PI3K-Akt and MAPK/Erk1/2 signaling pathways induces phosphorylation of mTOR, resulting in UNC-51-like kinase-1 (ULK1) inactivation and eventual inhibition of autophagy (27, 37, 53, 57). These effects were supported by accumulation of endogenous p62, a marker for reduced autophagy (5, 29). Currently, the role of PRR in the activation of the PI3K/Akt/mTOR signal pathway and ULK1 under high-glucose conditions is not known.

In this study, we investigated the role of PRR in regulating autophagy and cell survival in podocytes exposed to high glucose. Our results demonstrated that high glucose reduced autophagosome formation and enhanced apoptosis in podocytes via upregulation of PRR. These effects are mediated by activation of the PI3K/Akt/mTOR signaling pathway.

MATERIALS AND METHODS

Cell culture, treatment, and reagents. Conditionally immortalized mouse podocytes (kindly provided by Dr. Mark Okusa, Division of Nephrology, University of Virginia Health System, Charlottesville, VA) were cultured as described previously (3, 35). Cells were cultured on collagen I-coated flasks or plates (Corning Incorporated) in RPMI 1640 medium (Life Technologies) supplemented with recombinant mouse interferon-γ (IFN-γ; eBioscience) at 33°C. After differentiation at 37°C for 10–14 days without IFN-γ, podocytes were used for the proposed experiments. Podocytes were cultured for 48 h in medium containing 25 mmol/l d-glucose (high glucose; Life Technologies) for the experimental groups and 5 mmol/l D-glucose plus 20 mmol/l l-glucose (normal glucose; Life Technologies) for the control groups. Bafilomycin A1 (10 nm), Akt activator sc-79 (4 µg/ml), and mTOR activator MHY-1485 (2 µM) were purchased from EMD Millipore as described (9, 24).

PRR siRNA transfection. Transfection with PRR siRNA or scrambled siRNA was performed in six-well plates using the siLentFect lipid reagent (Bio-Rad) according to the manufacturer’s instructions. One hundred micromoles of Accell mouse PRR siRNA-SMARTpool (target sequences: 5'-CGAUAAGAUUGAAUUUUCC-3', 5'-CGGUAUACCUAAGUUUAU-3', 5'-UGGUUUGAGAGAGAU-
UUA-3', and 5'-GGACCAUCUUAGGGCAAA; Thermo Scientific Dharmacon Research) was used for each well. After a 6-h incubation in transfection reagent, the cells were then switched to normal medium for overnight recovery. A scrambled siRNA (target sequences: 5'-AATTCTCCGAACGTGTCACGT-3'; Qiagen), which was confirmed as nonsilencing double-stranded RNA, was used as control for siRNA experiments.

Real-time reverse transcription polymerase chain reaction. Total RNA from cultured podocytes was extracted using TRIzol reagent (Invitrogen) according to the protocol as described by the manufacturer. Aliquots of total RNA (1 µg) from each sample were reverse-transcribed into cDNA according to the instructions of the first-strand cDNA synthesis kit manufacturer (Bio-Rad). Equal amounts of the reverse transcriptional products were subjected to PCR amplification, using SYBR Green as the fluorescence indicator on a Bio-Rad iCycler.

Western blot analysis. Western blot analysis was performed as described previously (33). In brief, homogenates from cultured podocytes were prepared using lysis buffer containing protease inhibitor. After boiling for 5 min at 95°C in a 5× loading buffer, 20 µg of total proteins were subjected to SDS-PAGE (Bio-Rad), transferred onto a PVDF membrane (Millipore), and blocked by solution with 5% dry milk (Bio-Rad). Then, the membrane was probed with primary antibodies of anti-PRR (1:1,000 dilution, ab40790; Abcam), anti-p-Akt, etc.

Fig. 1. Effect of (pro)renin receptor (PRR) siRNA on light chain 3B (LC3B) accumulation and autophagosome formation and protein levels of LC3BII, Lamp-2, and p62 in podocytes treated with high glucose. A: immunofluorescence staining of LC3B staining shown in green, 4,6-diamidino-2-phenylindole (DAPI) shown in blue (n = 4). B: summarized data show the fold change of relative LC3B fluorescence. C: Cyto-ID autophagosome staining shown in green, DAPI shown in blue (n = 4). D: summarized data show the fold change of relative autophagosome fluorescence. E: Western blot analysis of LC3B protein level in response to treatment with normal or high glucose for 48 h (n = 4 for each group). F: Western blot analysis of Lamp-2 protein level in response to treatment with normal or high glucose for 48 h (n = 4 each group). G: Western blot analysis of p62 protein level in response to treatment with normal or high glucose for 48 h (n = 4 for each group). NG, normal glucose, 5 mmol/l D-glucose; HG, high glucose, 25 mmol/l D-glucose; AP, autophagosome. Black bars, scrambled (Scr) siRNA; gray bars, PRR siRNA. Data are presented as means ± SE. *P < 0.05 vs. NG; #P < 0.05 vs. HG + Scr siRNA.
autophagosome formation ($P < 0.05$; Fig. 1, C and D). In scrambled siRNA-treated podocytes, high glucose reduced LC3B accumulation ($P < 0.01$; Fig. 1B), autophagosome formation ($P < 0.01$; Fig. 1D), and protein levels of LC3BII and Lamp-2 ($P < 0.01$; Fig. 1, E and F) while increasing p62 levels ($P < 0.05$; Fig. 1G). In high-glucose-treated podocytes, PRR siRNA significantly increased LC3B accumulation ($P < 0.01$; Fig. 1B), autophagosome formation ($P < 0.01$; Fig. 1D), and LC3BII levels ($P < 0.01$; Fig. 1E) while reducing p62 levels ($P < 0.05$; Fig. 1G). PRR siRNA did not influence Lamp-2 level (Fig. 1F).

**Influence of high glucose and PRR siRNA on PRR.** Compared with normal glucose, high glucose significantly increased expression of PRR mRNA by 172% ($P < 0.01$; Fig. 2A) (33). Under normal conditions, PRR siRNA treatment significantly decreased PRR protein levels by 63% ($P < 0.01$; Fig. 2B). In the presence of high glucose, PRR siRNA treatment significantly attenuated PRR protein levels by 75% ($P < 0.001$; Fig. 2B).

**Influence of high glucose and bafilomycin A1 on autophagic flux, p62 level, and colocalization of LC3 puncta and Lamp-2.** Compared with normal glucose, high glucose significantly reduced autophagic flux (Fig. 3A), and bafilomycin A1 alone significantly increased p62 protein level by 52% ($P < 0.05$; Fig. 3B). In high-glucose-treated cells, bafilomycin A1 further increased p62 by 76% ($P < 0.05$; Fig. 3B). Compared with normal glucose, high glucose significantly decreased colocalization of LC3B accumulation and Lamp-2. Bafilomycin A1 significantly increased LC3BII accumulation and Lamp-2 levels under normal- or high-glucose conditions. Bafilomycin A1 decreased colocalization of LC3B and Lamp-2 in cells treated with high glucose compared with normal glucose (Fig. 3C).

**RESULTS**

**Influence of high glucose and PRR siRNA on LC3B accumulation, autophagosome formation, and protein levels of LC3BII, lamp-2, and p62.** Compared with scrambled siRNA treatment and under normal glucose conditions, PRR siRNA increased LC3B accumulation ($P < 0.01$; Fig. 1, A and B), LC3BII, and p62 levels ($P < 0.05$; Fig. 1, E and G) and
Influence of high glucose and PRR siRNA on phosphorylation of PI3K p85α (Tyr508), Akt (Ser473), p-mTOR (Ser2448), and p-ULK1 (Ser757). High glucose significantly increased protein levels of p-PI3K p85α (Tyr508) by 68% ($P < 0.01$; Fig. 4A) and p-Akt (Ser473) by 40% ($P < 0.05$; Fig. 4B) and had no effect on protein levels of total PI3K p85 (Fig. 4A) or total Akt (Fig. 4B). In normal-glucose-treated cells, PRR siRNA did not cause significant changes in protein levels of p-PI3K p85α (Tyr508; Fig. 4A) or p-Akt (Ser473; Fig. 4B). In contrast, in high-glucose-treated podocytes, PRR siRNA treatment caused significant reduction in levels of p-PI3K p85α (Tyr508) by 36% ($P < 0.05$; Fig. 4A) and p-Akt (Ser473) by 29% ($P < 0.05$; Fig. 4B).

Compared with normal glucose, high glucose significantly increased p-mTOR (Ser2448) by 133% ($P < 0.05$; Fig. 4C). In normal-glucose-treated cells, PRR siRNA did not induce significant changes in protein levels of p-mTOR (Ser2448) or total mTOR (Fig. 4C). In high-glucose-treated cells, PRR siRNA treatment caused a significant reduction in the level of p-mTOR (Ser2448) by 52% ($P < 0.05$; Fig. 4C) and had no effect on total mTOR (Fig. 4C).

High glucose significantly increased p-ULK1 (Ser757) by 84% ($P < 0.05$; Fig. 4D) and did not affect total ULK1 protein (Fig. 4D). PRR siRNA did not cause significant change in levels of p-ULK1 (Ser757) or total ULK1 in normal-glucose-treated cells (Fig. 4D). In high-glucose-treated cells, PRR
siRNA treatment caused a significant reduction in the level of p-ULK1 (Ser757) by 42% (P < 0.05; Fig. 4D) and had no effect on total ULK1 (Fig. 4D).

Influence of Akt/mTOR activators in the absence of PRR on p62 levels in podocytes. Compared with the control group, sc-79, an Akt activator, significantly increased p-Akt (Ser473) by 77% (P < 0.05; Fig. 5A) in scrambled siRNA treatment cells and 73% (P < 0.05; Fig. 5A) in PRR siRNA treatment cells. MHY-1485, an mTOR activator, increased p-mTOR (Ser2448) by 63% (P < 0.05; Fig. 5C) in scrambled siRNA treatment cells and 105% (P < 0.05; Fig. 5C) in PRR siRNA treatment cells. sc-79 significantly increased p62 levels by 40% (P < 0.01; Fig. 5B) in scrambled siRNA treatment cells and 78% (P < 0.05; Fig. 5B) in PRR siRNA treatment cells. MHY-1485 significantly increased p62 levels by 46% (P < 0.05; Fig. 5D) in scrambled siRNA treatment cells and 110% (P < 0.01; Fig. 5D) in PRR siRNA treatment cells.

Influence of high glucose and PRR siRNA on caspase-3/7 activity and apoptosis. High glucose significantly increased caspase-3/7 activity by 139% (P < 0.01; Fig. 6A) and proportions of apoptotic cells in podocytes by 420% (P < 0.01; Fig. 6, B and C). Under normal glucose conditions, PRR siRNA did not cause change caspase-3/7 activity or the number of apoptotic cells (Fig. 6, A and B). In high-glucose-treated cells,
PRR siRNA treatment caused significant reduction in caspase-3/7 activity by 27% \((P < 0.001; \text{Fig. } 6\text{A})\) and cell death by 79% \((P < 0.01; \text{Fig. } 6, \text{B and } \text{C})\).

**DISCUSSION**

Autophagy contributes to the degradation of long-lived or damaged proteins and excessive or dysfunctional cell organelles and is a major homeostatic and quality control mechanism to maintain cellular integrity \((20)\). Decreased autophagy is associated with decreased podocin expression and increased albumin flux across podocytes that can be reversed by rapamycin, one of the autophagy activators \((15, 54, 56)\).

In the present study, we investigated the role of PRR in high-glucose-mediated reduction in autophagy and increased apoptosis in podocytes and explored the mechanisms contributing to these pathological processes. Our results showed that high glucose significantly increased PRR mRNA and protein level and activated the PI3K/Akt/mTOR signaling pathway. High glucose reduced activation of ULK1, levels of LC3B, and autophagic flux and enhanced p62 accumulation and apoptosis. In the absence of PRR, activation of Akt or mTOR increased p62 accumulation, confirming involvement of Akt and mTOR in inhibiting autophagy. Downregulation of PRR levels attenuated high-glucose-induced activation of the PI3K/Akt/mTOR signaling pathway and p62 accumulation and apoptosis and improved autophagosome formation. These results indicated direct involvement of PRR in high-glucose-induced autophagy dysfunction and apoptosis in podocytes.

Previous studies showed that podocyte-specific total PRR deletion in the nonhyperglycemic mouse during early development in utero led to massive albuminuria that contributed to increased mortality \((38, 42)\). These studies suggested an im-
important role in the development of the kidney (39, 43). In present study, we performed partial deletion of PRR in fully developed podocytes. Previous studies demonstrated that activation of mTOR by V-ATPase (including PRR) (4) led to autophagy inhibition (27). Interestingly, the podocyte-specific mTOR-knockout mouse had similar renal damages like the PRR-knockout model (11), suggesting that PRR is important for mTOR function. To our knowledge, the role of PRR in regulating autophagy under high-glucose conditions has not been elucidated.

In present study, we demonstrated that high glucose reduced autophagy activity mainly through reduction in autophagosome formation. These results are consistent with previous reports (15, 20, 54) suggesting involvement of hyperglycemia in inhibiting autophagy. We hypothesized that reduction in autophagy by high glucose is mediated by enhanced activity of PRR/PI3K/Akt/mTOR signaling pathway and inactivation of ULK1, leading to reduction in LC3BII and autophagosome formation. Previous studies showed that high glucose activated the PI3K/Akt signaling pathway inhibits autophagy through mTOR activation (32, 48). mTOR is activated at Ser2448 phosphorylation and Ser2481 autophosphorylation (14, 42, 47). Our data in podocytes treated with high glucose clearly demonstrated an increased protein level of PRR, activation of the PI3K/Akt signaling pathway, and mTOR phosphorylation at Ser2448. Downregulation of PRR levels inhibited high-glucose-induced PI3K/Akt/mTOR activation, suggesting that this receptor is upstream of this signaling pathway. These findings are consistent with reports showing decreased mTOR activity with inhibition of V-ATPase (4, 12, 28, 59). To our knowledge, this is the first report showing that PRR mediates high-glucose-induced activation of the PI3K/Akt/mTOR signaling pathway.

The serine/threonine kinase ULK1, also named Atg1, plays a key role in autophagy induction and progression (19, 21, 27, 45). ULK1 is inhibited by increased mTOR activity through Ser757 phosphorylation (27), preventing its association and activation by AMPK, thus inhibiting autophagy (13, 27). Atg8/LC3 members are essential components of autophagy, which binds p62/SQSTM1 to facilitate degradation of ubiquitinated proteins aggregated in autophagosome (40), such as p62 (5, 40). Reduction of autophagy results in p62 accumulation (29). LC3 directly binds Atg1/ULK1 through a conserved LC3 interaction region motif. This binding targets the Atg1-Atg13 complex to autophagosomes, where it promotes autophagosome maturation and/or fusion with vacuoles/lysosomes (2, 31). Lamp-2 is required for the proper fusion of autophagosomes with lysosomes. Lamp-2 depletion correlated with the accumulation of autophagosomes and absence of autolysosomes (16). In the present study, we showed that high glucose inhibited ULK1 activation by phosphorylating ULK1 at Ser757, decreased LC3B accumulation, autophagosome formation, and Lamp-2 protein level, and increased p62 accumulation, confirming reduction of autophagy. Downregulation of PRR re-
versed this process. Bafilomycin A1 increased LC3BII and p62 accumulation in high-glucose-treated cells and decreased autophagic flux and colocalization of LC3B and Lamp-2, which suggests that the reduction of autophagic proteins under high glucose was associated with reduced autophagy activity and not enhanced autophagic degradation. Activation of Akt in the absence of PRR increased p62 accumulation, confirming that activation of the PRR/P13K/Akt/mTOR signaling pathway led to a reduction in autophagy. To our knowledge, this is the first report showing that increased PRR level is involved in high-glucose-induced ULK1 inactivation, leading to decreased autophagosome formation. These results suggested that PRR plays an important role in high-glucose-induced reduction of autophagy in mouse podocytes.

Finally, we also tested the effect of increased PRR level on apoptosis by inhibiting autophagy. Previous reports showed that autophagy inhibits cell apoptosis by reducing caspase activation in the cytosol (6, 18, 30). Thus, suppression of autophagy may enhance cell death. Our results demonstrated that high glucose enhanced caspase-3/7 activation and the number of apoptotic cells. Downregulation of PRR reversed this effect. These findings may help develop a new therapeutic strategy for the management of podocyte injury in diabetes.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.L. and H.M.S. conception and design of research; C.L. performed experiments; C.L. and H.M.S. analyzed data; C.L. and H.M.S. interpreted results of experiments; C.L. prepared figures; C.L. and H.M.S. drafted manuscript; H.M.S. edited and revised manuscript; H.M.S. approved final version of manuscript.

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