Podocyte Specific Deletion of Acid Ceramidase Predisposes Mice to Obesity-Induced Glomerular Injury

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ABSTRACT

Acid ceramidase (AC) as a key regulatory enzyme involved in ceramide metabolism plays a critical role in the intracellular lipid metabolism and molecular trafficking. Given recent reports that obesity enhances ceramide production, leading to the activation of NADPH oxidase and consequent development of glomerular sclerosis, the present study was designed to test whether AC contributes to the development of glomerular injury associated with obesity. First, we generated and characterized the podocyte-specific AC knockout mice by cross breeding loxP-floxed AC$^{+/-}$ and nephrin cre promoter mice. The podocyte specific AC heterozygous (Ac$^{+/-}$/Neph^{cre}) and wild type (*AC*$^{+/+}$) mice were fed a high fat diet (HFD) or normal chow (ND) for 12 weeks to produce obesity. Immunohistochemical analysis demonstrated that AC expression was reduced in glomeruli of Ac$^{+/-}$/Neph^{cre} mice compared to AC$^{+/+}$ mice. In contrast, the ceramide level, and desmin expression were higher in glomeruli of Ac$^{+/-}$/Neph^{cre} than AC$^{+/+}$ mice. Furthermore, Western blot, real time RT-PCR and Immunohistochemical analyses showed that HFD significantly decreased the AC expression in Ac$^{+/-}$/Neph^{cre} mice, but not in AC$^{+/+}$ mice. Correspondingly, the urinary protein excretion was significantly higher in HFD fed Ac$^{+/-}$/Neph^{cre} than Ac$^{+/+}$ mice. In *in vitro* studies of podocytes, AC inhibitor, D-NMAPPD decreased the podocin expression, VEGF level and increased the desmin expression compared to control cells. In conclusion, our observations reveal that normal expression of AC contributes to the function of podocytes and the defect of this gene expression is a critical mechanism triggering podocyte injury and ultimately resulting in obesity-associated end-stage renal disease (supported by NIH grants DK54927 and DK104031).

METHODS

Animals: Eight weeks old male Ac$^{+/+}$ and podocyte specific acid ceramidase heterozygous (AC$^{+/-}$/Neph^{Cre}) mice were used in the present study. Mice were treated with either a normal diet or high fat diet for 12 weeks

Morphological examinations. The fixed kidneys were paraffin-embedded, sections were prepared and stained with periodic acid–Schiff stain (1). Glomerular injury index was calculated from 0 to 4 on the basis of the degree of glomerulosclerosis and mesangial matrix expansion as described previously. In general, we counted a total of 80-100 glomeruli in each kidney slice under microscope, when each glomerulus was graded level 0-4 damages. 0 represents no lesion, 1+ represents sclerosis of <25% of the glomerulus, while 2+, 3+, and 4+ represent sclerosis of 25% to 50%, >50% to 75%, and >75% of the glomerulus. A whole kidney average sclerosis index was obtained by averaging scores from counted glomeruli. The immunohistochemical analysis of renal tissue was performed to detect acid ceramidase and ceramide staining using anti-acid ceramidase (Santa Cruz, 1: 50 dilution) and anti-Ceramide (Alexis, 1: 50 dilution) antibodies.

Urinary total protein and albumin excretion measurement: The 24-hour urine samples were collected using metabolic cages and subjected to total protein and albumin excretion measurements. Total protein content in the urine was detected by Bradford method using a UV spectrophotometer. Urine albumin was detected by using a commercially available mouse albumin enzyme-linked immunoassay assay kit (Bethyl Laboratories, Montgomery, TX, USA) as we described previously [1, 2].

Immunofluorescence staining of cells: Cells were fixed in 4% PFA, and then blocked with 1% BSA for 30 min. After incubated with primary antibodies overnight, the cells were stained with Alex-585-labeled secondary antibodies. After washing, the cells were mounted with DAPI-containing mounting solution. Then, the slides were observed under a fluorescent microscope and images were taken [2].

ESR detection of superoxide ($O_2^{\cdot-}$) production. NADPH dependent $O_2^{\cdot-}$ production were measured by ESR spectrometry [1].

ELISA for vascular endothelial growth factor A (VEGF-A): Podocytes were treated with AC inhibitor or vehicle for 12 h. After the treatment, the supernatant was collected for ELISA assay of VEGF-A using a commercially available kit (R&D system, Minneapolis, MN).

BACKGROUND

Acid ceramidase (N-acylsphingosine deacylase, AC) is the lipid hydrolase responsible for the degradation of ceramide into sphingosine and free fatty acids with in the lysosomes. AC not only hydrolyzes ceramide into sphingosine but also can synthesize ceramidase from sphingosine and free fatty acids in vitro and in situ.

Acid ceramidase (AC) is a key regulatory enzyme involved in sphingolipid metabolism, and plays a role in the pathology of several diseases such as Farber disease, diabetes, Alzheimer disease, obesity and cancer.

Recent reports shown that obesity enhances ceramide production, leading to the activation of NADPH oxidase and consequent development of glomerular sclerosis. However, the role of acid ceramidase in the renal glomerular function remains elusive.

Hence, the present study was designed to test whether acid ceramidase contributes to the development of glomerular injury associated with obesity.

RESULTS

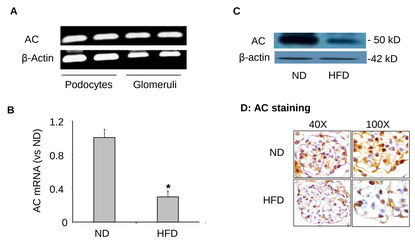

Fig. 1: Acid Ceramidase expression were determined by real time PCR (panel A and B), Western blot (panel C), and immunohistochemical (panel D) analysis. The acid ceramidase expression was significantly lower in glomeruli of high fat diet fed mice compared to normal diet fed mice. These results suggest that acid ceramidase is expressed in the glomeruli of C57BL/6J wild type mice. * significant difference (P<0.05) from normal diet fed mice. ND: Normal Diet; HFD: High fat diet, AC: Acid Ceramidase.

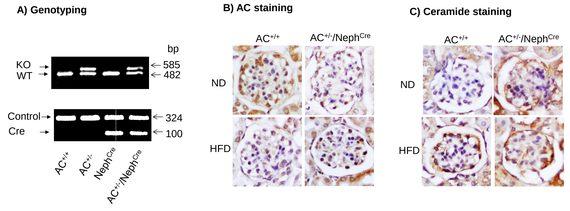

Fig. 2: A: Podocyte specific acid ceramidase heterozygous mice were genotyped using PCR. Detection of a PCR product at 585 bp indicates *Ac*$^{+/+}$, a PCR product of 482 bp indicates *Ac*$^{+/+}$ mice and while PCR product of 100 bp indicates Cre gene. B and C: The acid ceramidase expression was dramatically decreased in podocyte specific acid ceramidase heterozygous mice (*Ac*$^{+/-}$/*Neph*^{Cre}) mice compared to *Ac*$^{+/+}$ mice. After treatment with high fat diet further decreased the AC in both *AC*$^{+/+}$ and podocyte specific AC heterozygous mice. In contrast, ceramide expression was dramatically increased in Ac$^{+/-}$/Neph^{Cre} mice compared to Ac$^{+/+}$ mice.

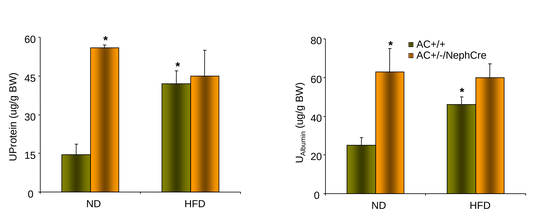

Fig. 3: Urinary total protein and albumin excretion in podocyte specific acid ceramidase heterozygous mice with or without high fat diet treatment. The 24-hour urinary total protein and albumin excretions were significantly higher in Ac$^{+/-}$/Neph^{Cre} mice compared to *Ac*$^{+/+}$ mice. However, the HFD treatment significantly enhanced the urinary total protein and albumin excretion in *AC*$^{+/+}$ mice, but not in Ac$^{+/-}$/Neph^{Cre} mice. * significant difference (P<0.05) from AC$^{+/+}$ mice fed a ND, # significant difference (P<0.05) from HFD treated *AC*$^{+/+}$ mice. These results suggest that inhibition of acid ceramidase gene pronounced the glomerular injury in mice.

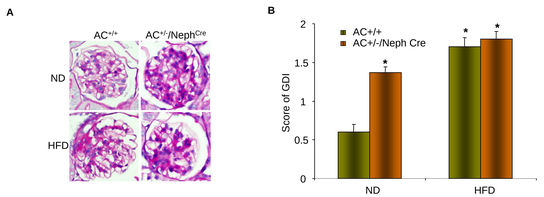

Fig. 4: Glomerular injury was more pronounced in Ac$^{+/-}$/Neph^{Cre} mice. Photomicrographs (panel A) show typical glomerular structure in normal diet or high fat diet treated Ac$^{+/-}$/Neph^{Cre} and *Ac*$^{+/+}$ mice. Panel B depicts semi- quantitative score of glomerular damage index (n=6). Morphological analysis showed that Ac$^{+/-}$/Neph^{Cre} mice had more glomerular damage compared to the *Ac*$^{+/+}$ mice with or without HFD treatment. The glomerular damage index was significantly higher in Ac$^{+/-}$/Neph^{Cre} mice compared to *AC*$^{+/+}$ mice. High fat diet treatment significantly increased the glomerular damage index in *Ac*$^{+/+}$ mice compared to the *Ac*$^{+/+}$ mice fed a normal diet. * significant difference (P<0.05) from Ac$^{+/+}$ mice fed ND. # significant difference (P<0.05) from HFD fed mice.

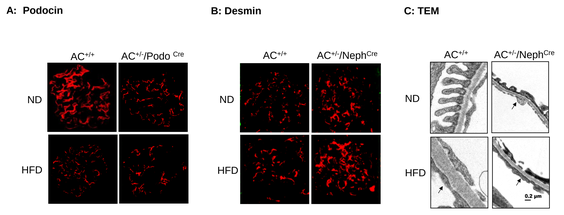

Fig. 5: Immunohistochemical analysis revealed that podocyte injury was more pronounced in podocyte specific AC heterozygous mice. Typical images of podocin (left panel) or desmin (middle panel) staining in glomeruli from Ac$^{+/-}$/Neph^{Cre} and *Ac*$^{+/+}$ mice with or without HFD treatment. High fat diet treatment decreased the podocin staining and increased the desmin staining in Ac$^{+/-}$/Neph^{Cre} than in *Ac*$^{+/+}$ mice (A and B). Under transmission electron microscopy, the intact structures of podocyte foot processes shown in glomeruli of mice on the ND were destroyed in Ac$^{+/-}$/Neph^{Cre} mice, as shown by evident foot process effacement in Ac$^{+/-}$/Neph^{Cre} mice with or without HFD (right panel). ND: Normal diet, HFD: High fat diet. These data reveal that lack of AC gene induces the podocyte damage in glomeruli.

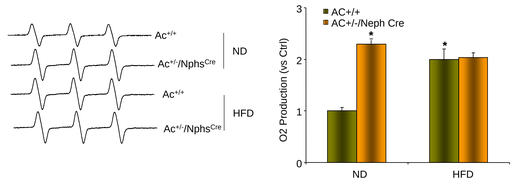

Fig. 6: Local oxidative stress was increased in the glomeruli of Ac$^{+/-}$/Neph^{Cre} mice. NADPH dependent $O_2^{\cdot-}$ production was measured by ESR spectrometry. Summarized data demonstrated that glomerular $O_2^{\cdot-}$ production was increased by 2.3 folds in glomeruli of Ac$^{+/-}$/Neph^{Cre} mice than in Ac$^{+/+}$ mice, suggesting that mice lacking acid ceramidase induce the local oxidative stress in the kidney. * significant difference (P<0.05) from Ac$^{+/+}$ mice fed a ND, # significant difference (P<0.05) from HFD treated *Ac*$^{+/+}$ mice.

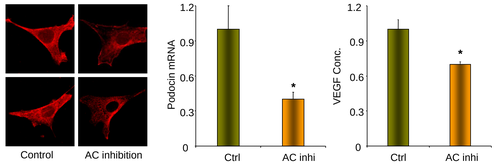

Fig. 7: Effect of acid ceramidase inhibitor (D-NMAPPD) on podocyte injury and VEGF production in cultured podocytes. Podocytes were treated with AC inhibitor for 12 h and measured the podocin expression by RT-PCR and indirect immunofluorescent method. The acid ceramidase inhibition decreased the podocin expression compared to control cells (Panel A and B). In addition, VEGF-A secretion in the culture medium was detected by ELISA. The AC inhibition decreased the VEGF production compared to control cells (Panel C). These results suggest that lack of AC has a functional damage in podocytes. * significant difference (P<0.05) from control group.

CONCLUSION

The observations reveal that normal expression of AC contributes to the function of podocytes and the defect of this gene expression is a critical mechanism triggering podocyte injury and ultimately resulting in obesity-associated end-stage renal disease

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