Silencing of Acid Sphingomyelinase Gene Prevented Glomerular Oxidative Stress and Sclerosis in Hyperhomocysteinemic Mice

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ABSTRACT

Hyperhomocysteinemia (HHCy) has been reported to enhance ceramic production, thereby leading to activation of NADPH oxidase, and consequent glomerular oxidative stress and sclerosis. The present study was performed to determine whether acid sphingomyelinas (ASM), a ceramic producing enzyme, is implicated in the development of HHCy-induced glomerular oxidative stress and injury. After uninephrectomy and intra-cortical transfusion of vectors expressing shRNA against ASM, mice were fed a folate free (FF) diet for 8 weeks, which significantly elevated the plasma Hcy levels compared to mice on normal show diet (ND). By in vivo molecular imaging, it was found that transfected shRNAs were expressed in the renal cortices starting on day 3 and continuing for 27 days. In isolated mouse glomeruli after 8 weeks, real-time PCR showed down-regulated ASM activity. Glomerular injury significantly decreased ASM mRNA level by 46% and by 75%, respectively, when mice were on the ND and FF diet. Correspondingly, increases in glomerular NOX mRNA level and superoxide production measured by ESR in mice on the FF diet were substantially inhibited when ASM gene was silenced. Glomerular damages in mice with HHCy as shown by elevated urinary protein excretion (0.02 (0.01-0.06) mg/kg BW vs. 0.01 (0.005-0.02) mg/kg BW of control and glomerular damage index (2.5±0.05 vs. 4.7±0.33 of control) were also significantly attenuated by ASM gene silencing. Confocal microscopy demonstrated that Hcy-induced podocyte injury as shown by reduction of podin expression and increase of desmin production was improved by ASM gene silencing. These observations reveal that ASM importantly mediates podocyte injury and glomerular injury associated with NADPHoxidation activation and local oxidative stress during HHCy (supported by NIH grant HL 091446, HL-73161 and DK49327).

METHODS

Animals. Eight weeks old uninephrectomized male C57BL/6J mice were used in the present study (Jackson Laboratories, Bar Harbor, Maine). Mice were fed either a normal diet (Normal Diet, Dyets Inc., Bethlehem, PA) or a folate free diet (FF diet, Dyets Inc., Bethlehem, PA) for 8 weeks.

Gene transfer into the kidney by ultrasound-microbubble technique. In anesthetized animals, the left kidney was exposed by a flank incision and then the left renal vein was exposed. A mixture of ASM shRNA plasmid (200 μg) and luciferase expression plasmid (25μg) in 1 ml saline containing 20% microbubble (Optison, GE HealthCare) was injected into the renal vein. An ultrasound transducer was applied directly onto the kidney with a continuous-wave output of 1-MHz at 5% power output for a total of 180 s with 30s intervals.

In vivo imaging. An aguane solution of leukin (150 μg/ml intraperitoneally) (Xenogen, Alameda, CA) was injected 5 minutes prior to imaging. The live anesthetized mouse was imaged using the IVIS200 in vivo imaging system (Xenogen Corp.). Raw values are reported as photons/sec/mm²/μr.

Bioclinical analysis and morphological examinations. Quantitative RT-PCR analysis, asa activity assay, ESR spectrometry for O₂ production, plasma Hcy measurement by HPLC and immunohistochemistry were performed as we described previously.

RESULTS

Figure 1. Representative images of in vivo and in vitro determination of gene transfection efficiency in the kidney. A, in vivo molecular imaging to confirm gene transfection in the kidney by detection of co-transfected reporter gene luciferase. The luciferase expression signal maintained observable for around one month. B, ex vivo imaging to show localization of transfected gene expression in the heemisected kidney on day 3 after gene delivery. C, in vivo imaging performed on day 3 after gene delivery. D, ex vivo imaging performed on day 3 after gene delivery. E, semi-quantitative RT-PCR analysis of ASM mRNA levels in glomeruli from scrambled RNA and ASM shRNA transfected mice after 8-week. ASM shRNA significantly inhibited enhanced ASM expression induced by FF diet. *P<0.05 vs. Normal Diet transfected scrambled RNA transfected; **P<0.01 vs. Normal Diet transfected scrambled RNA transfected.

Figure 2. Plasma Hcy concentrations were measured in scramble RNA or ASM shRNA transfected mice fed with or without folate free diet using HPLC. Plasma Hcy concentration of scramble RNA or ASM shRNA transfected mice fed a normal diet. FF diet treatment significantly increased the plasma Hcy levels in both scramble RNA or ASM shRNA transfected mice compared to the normal diet fed mice. *P<0.05 vs. Normal Diet treated scramble RNA transfected mice; **P<0.05 vs. FF Diet treated scramble RNA transfected mice.

Figure 3. Effects of normal diet and FF diet on ASM activity and ceramic expression in the glomeruli. FF Diet treatment significantly increased the ASM activity (A) compared to scramble RNA transfected mice fed on the normal diet. However, ASM shRNA obviously attenuated such increases. In addition, IHC results showed that the ceramic expression was enhanced in scramble RNA or FF diet in scramble RNA transfected glomeruli, but not in ASM shRNA transfected glomeruli. *P<0.05 vs. Normal Diet treated scramble RNA transfected mice; **P<0.05 vs. FF Diet treated scramble RNA transfected mice.

Figure 4. Effects of the normal and FF Diet on glomerular O₂ production in scramble RNA and ASM shRNA transfected mice. (A) Quantitative RT-PCR analysis of Nox4 mRNA levels in glomeruli from scramble and ASM shRNA transfected mice after 8-week gene transfection. The results showed that the FF diet increased Nox4 mRNA expression in scramble RNA transfected glomeruli and it was attenuated in ASM shRNA transfected mice. NADPH-oxidase-dependent O₂ production was measured by ESR spectrometry. Representative ESR spectra (B) showed SOD-inhibitable O₂ signals. The summarized data (C) demonstrated that ASM shRNA transfection significantly attenuated the O₂ production than in mice fed with a FF diet suggesting that ASM gene silencing suppresses local oxidative stress in the kidney. *P<0.05 vs. Normal Diet treated scramble RNA transfected mice; **P<0.05 vs. FF Diet treated scramble RNA transfected mice.

Figure 5. ASM gene silencing attenuated the FF diet-induced urinary total protein and albumin excretion and glomerular injury. The 24-hour urinary total protein and albumin excretion were significantly higher in both scramble RNA and ASM shRNA transfected mice fed FF diet than animals on the normal diet. However, the urinary total protein (A) and albumin excretion (B) increased significantly less in ASM shRNA transfected mice. Photomicrograph (C) showed typical glomerular structure in normal diet or FF diet-fed mice. Panel C depicts semi-quantitative scores of glomerular damage index (0-6). ASM shRNA transfected mice had less glomerular injury compared to scramble RNA transfected mice under the FF diet. The glomerular damage index was similar in both scramble RNA and ASM shRNA transfected mice on the normal diet. *P<0.05 vs. Normal Diet treated scramble RNA transfected mice; **P<0.05 vs. FF Diet treated scramble RNA transfected mice.

CONCLUSION

HHCy plays a critical role in mediating glomerular injury associated with NADPH oxidase activation and local oxidative stress during lilies.

ASM gene targeting may be a novel therapeutic strategy for prevention and treatment of glomerulosclerosis associated with HHCy.