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Activation of Nod-Like Receptor Protein 3 Inflammasomes Turns on Podocyte Injury and Glomerular Sclerosis in Hyperhomocysteinemia

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Abstract—Inflammasome is a multiprotein complex consisting of Nod-like receptor protein 3 (NALP3), apoptosis-associated speck-like protein (ASC), and caspase 1 or 5, which functions to switch on the inflammatory process. The present study hypothesized that the formation and activation of NALP3 inflammasomes turn on podocyte injury leading to glomerulosclerosis during hyperhomocysteinemia (hHcys). RT-PCR and Western blot analysis demonstrated that murine podocytes expressed 3 essential components of the NALP3 inflammasome complex, namely, NALP3, ASC, and caspase 1. Treatment of podocytes with l-homocysteine induced the formation of NALP3 inflammasome complex, an increase in caspase 1 activity, podocyte cytoskeleton rearrangement, and decreased production of vascular endothelial growth factor from podocytes, which were all blocked by silencing the ASC gene or inhibiting caspase 1 activity. In mice with hHcys induced by feeding them a folate-free diet, NALP3 inflammasome formation and activation in glomerular podocytes were detected at an early stage, as shown by confocal microscopy, size exclusion chromatography of the assembled inflammasome complex, and increased interleukin-1 β production in glomeruli. Locally silencing the ASC gene in the kidney significantly reduced NALP3 inflammasome formation and interleukin 1 β production in glomeruli of mice with hHcys. Pathologically, hHcys-associated albuminuria, foot process effacement of podocytes, loss of podocyte slit diaphragm molecules, and glomerulosclerosis at the late stage were significantly improved by local ASC gene silencing or by caspase 1 inhibition. In conclusion, NALP3 inflammasome formation and activation on stimulation of homocysteine are important molecular mechanisms triggering podocyte injury and ultimately resulting in glomerulosclerosis in hHcys. (*Hypertension*. 2012;60:154-162.) • [Online Data Supplement](#)

Key Words: homocysteine ■ inflammatory machinery ■ podocytes ■ end-stage renal disease

The inflammasome was identified recently as the cellular machinery responsible for activation of inflammatory processes.¹ Among different types of inflammasomes, the Nod-like receptor protein 3 (NALP3) inflammasome is well characterized in a variety of mammalian cells, which is characteristic of a proteolytic complex mainly composed of the NALP3 (or nucleotide leikin-rich polypeptide 3), the adaptor protein apoptosis-associated speck-like protein (ASC), and caspase 1.² Caspase 1 is vital for the production of mature interleukin (IL) 1 β and IL-18 in response to a variety of agonists or stimuli. It has been reported that IL-1 β is an important cytokine with a broad range of biological activities³ involved in kidney injury and repair⁴ and that in glomeruli it is mainly produced by podocytes.⁵ The active mature IL-1 β is formed by cleavage of the inactive pro-IL-1 β precursor by caspase 1, which is activated in a large

multiprotein complex, namely, the inflammasome. The NALP3 inflammasome has been reported to be activated by bacterial toxins¹ or pathogen-associated molecular patterns, such as muramyl dipeptide and other stimuli. NALP3 can also detect endogenous stress-associated danger signals, such as ATP,¹ monosodium urate crystals,⁶ or β -amyloid, which may be a major mechanism producing local sterile inflammation. With respect to the functional relevance, the NALP3 inflammasome has been implicated in the pathogenesis of various metabolic diseases, including diabetes mellitus, gout, silicosis, acute myocardial infarction, and liver toxicity.^{2-3,6} Given the fact that hyperhomocysteinemia (hHcys) is also regarded as a metabolic disorder attributed to the failure of the clearance of homocysteine (Hcys), the inflammasome may be an attractive candidate as an initiating molecular switch to turn on the inflammatory response observed in hHcys.

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From the Departments of Pharmacology and Toxicology (C.Z., K.M.B., M.X., J.M.A., X.L., P.-L.L.) and Physiology and Biophysics (Q.L.), Medical College of Virginia, Virginia Commonwealth University, Richmond, VA; current address (C.Z.): Department of Nephrology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

C.Z. and K.M.B. contributed equally to this work.

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Correspondence to Pin-Lan Li, Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, 410 N 12th St, Richmond, VA 23298. E-mail pli@vcu.edu

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Hyperhomocysteinemia is an important pathogenic factor both in the progression of end-stage renal disease and in the development of cardiovascular complications related to end-stage renal disease.⁷ Previously, we have demonstrated that chronic elevations of plasma Hcys levels importantly contribute to the development of glomerular disease independent of hypertension in hHcys mice and rat models.⁸ Studies from our laboratory^{8,9} and by others¹⁰ have demonstrated that Hcys induces podocyte injury and extracellular matrix accumulation and inhibits their degradation in glomeruli, which ultimately leads to glomerulosclerosis and loss of renal function.⁸ Although these studies increased our understanding of hHcys-associated glomerular injury and sclerosis, the precise mechanism mediating podocyte injury and activating the local inflammatory response in glomeruli has not been fully clarified.

In this regard, earlier reports have shown that hHcys increased levels of inflammatory cytokines and plays a crucial role in hHcys-induced endothelial damage and atherosclerosis.^{11,12} It was also reported that hHcys enhanced the production of monocyte chemoattractant protein 1 in glomerular mesangial cells and tubular epithelial cells, and blockade of these inflammatory processes completely protected the kidney from hHcys-associated damage, supporting the view that hHcys-induced renal injury is associated with its ability to induce inflammation.¹³ These studies raised the possibility that Hcys may activate an inflammatory response resulting in glomerulosclerosis and end-stage organ damage during hHcys. However, it remains unknown how Hcys activates or initiates the local inflammatory response in renal glomerular residential cells and whether Hcys-induced activation of inflammasomes serves as an early mechanism mediating glomerular injury and sclerosis. The present study tested the hypothesis that hHcys may induce NALP3 inflammasome formation and activation in podocytes and thereby lead to hHcys-associated podocyte dysfunction or injury and consequent glomerular sclerosis.

Materials and Methods

Animals

Twelve-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used in the present study. All of the protocols were approved by the institutional animal care and use committee of the Virginia Commonwealth University. To speed up the damaging effects of hHcys on glomeruli, all of the mice were uninephrectomized, as described in previous studies.^{9,14} After a 1-week recovery period from the uninephrectomy, mice were fed a normal diet or a folate-free (FF) diet (Dyets Inc, Bethlehem, PA) for 1, 2, or 4 weeks to induce hHcys. In another series of experiments, ASC short hairpin RNA (shRNA) or a scrambled shRNA (Origene, Rockville, MD) plasmid with a luciferase expression vector was cotransfected into the kidneys of mice via intrarenal artery injection with help of the ultrasound microbubble gene delivery system, as we described previously.⁹ After delivery of plasmids into the kidney, these uninephrectomized mice were maintained on a normal or a FF diet for 4 weeks. In additional experimental groups, mice were injected with Z-WEHD-FMK (WEHD, R&D System, Minneapolis, MN), a caspase 1 inhibitor (1 mg/kg per day, IP) during the FF diet treatment. One day before euthanizing these mice, 24-hour urine samples were collected using mouse metabolic cages. After blood samples were collected, the mice were euthanized and renal tissues

were harvested for biochemical and molecular analysis, as well as morphological examinations, as we described previously.⁸

Size-Exclusion Chromatography

Size-exclusion chromatography (SEC) was performed in podocytes or isolated mouse glomeruli, as described previously.¹⁵ For a detailed method, please see the online-only Data Supplement. All other methods are described in the online-only Data Supplement.

Results

Activation of NALP3 Inflammasomes by Hcys in Cultured Podocytes

Using cultured murine podocytes, we first characterized the formation and activation of NALP3 inflammasomes. RT-PCR analysis demonstrated that NALP3, ASC, and caspase 1 mRNAs were detectable in these cultured podocytes, as well as in the normal mouse renal glomeruli (Figure 1A). By immunocytochemical analyses, the protein expression of these inflammasome components was further confirmed in podocytes (Figure 1B). On stimulation of L-Hcys (the active form of Hcys), mRNA levels of NALP3, ASC, and caspase 1 significantly increased in a concentration-dependent manner, as detected by real-time RT-PCR (Figure 1C).

We next analyzed the assembly of NALP3 inflammasome proteins as a complex in podocytes by SEC. As shown in Figure 1D, total proteins from podocytes were eluted through a Sepharose 6 SEC column, and all of the proteins were separated into different fractions according to their size and detected by Western blot analysis. It was observed that the specific bands for NALP3, ASC, and caspase 1 were located in the low-molecular-weight fractions under control conditions. However, on stimulation of L-Hcys for 24 hours, these bands specific to inflammasome components migrated into high-molecular weight fractions, which were termed "inflammasome fractions" (Figure 1E). Furthermore, confocal microscopic analysis demonstrated that colocalization of NALP3 with ASC or caspase 1 was increased in podocytes on L-Hcys stimulation compared with control podocytes (Figure 1F), indicating the aggregation or assembly of these inflammasome molecules, namely, the formation of NALP3 inflammasome complex in podocytes.

Effects of ASC Gene Silencing and Caspase 1 Inhibition on Podocyte Inflammasome Activation and Functional Changes Induced by L-Hcys

As shown in Figure 2A, ASC small interfering RNA transfection in podocytes markedly inhibited the L-Hcys-induced colocalization of NALP3 with ASC or caspase 1. Consistent with these findings, ASC gene silencing or blockade of caspase 1 activity dramatically blocked the Hcys-induced caspase 1 activity (Figure 2B) and IL-1 β production (Figure 2C) in podocytes. Vascular endothelial growth factor- α secretion was dramatically reduced in podocytes treated with L-Hcys, and this Hcys-induced decrease in vascular endothelial growth factor- α secretion was substantially blocked by ASC gene silencing or inhibition of caspase 1 activity (Figure 2D).

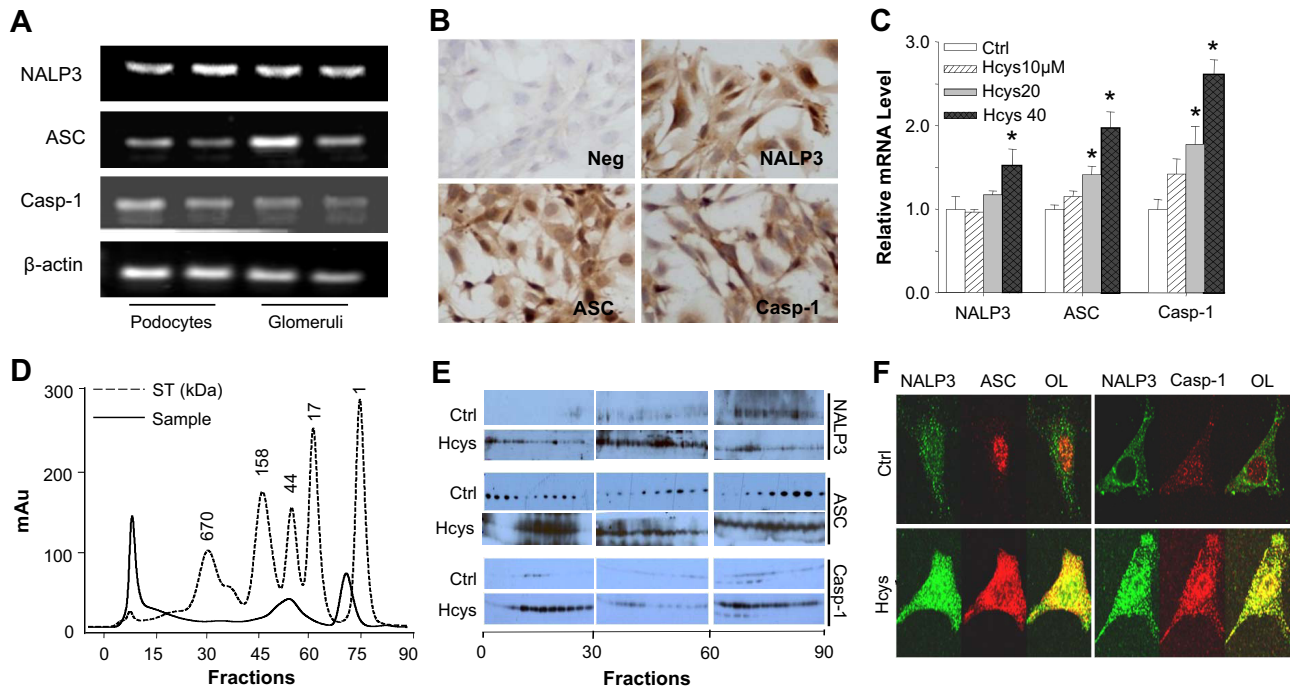


Figure 1. Hcys-induced activation of Nod-like receptor protein 3 (NALP3) inflammasomes in cultured podocytes. **A**, RT-PCR gel document showing the expression of NALP3, apoptosis-associated speck-like protein (ASC), and caspase 1 in cultured podocytes. **B**, Immunocytochemical staining of NALP3, ASC, and caspase 1 in podocytes (original magnification, $\times 100$). **C**, mRNA expression of NALP3, ASC, and caspase 1 in podocytes with or without stimulation of L-homocysteine (Hcys; $n=6$). \square , control; ▨ , Hcys 10 μM ; ▩ , Hcys 20; \blacksquare , Hcys 40. **D**, Elution profile of proteins (optical density at 280 nm) from podocytes by size-exclusion chromatography (SEC). The molecular mass scale was determined using a wide range of molecular mass standard. ST indicates standard. **Dotted line**, ST (kDa); **solid line**, sample. **E**, SDS-PAGE of proteins fractions via SEC from control and Hcys-treated podocytes that were probed with anti-NALP3, ASC, or caspase 1 antibodies, respectively. **F**, Representative confocal microscopic images showing colocalization of NALP3 (green) with ASC (red) or NALP3 (green) with caspase 1 (red, Casp-1) in podocytes (original magnification, $\times 400$). OL indicates overlay. $*P<0.05$ vs control.

Activation of Podocyte NALP3 Inflammasomes in Mice With hHcys

In mice on the FF diet, the plasma total Hcys levels were gradually increased, starting from the first week on the FF diet (control, 5.8 ± 1 ; first week, 9.9 ± 1 ; second week, 14.9 ± 2 ; fourth week, 18 ± 1 $\mu\text{mol/L}$). As shown in Figure 3A, under normal condition, NALP3, ASC, and caspase 1 were expressed at a low level within glomeruli, and very little colocalization of these molecules could be detected by confocal microscopy. The FF diet increased the colocalization of NALP3 with ASC or caspase 1 in a time-dependent manner, as shown by large yellow spots or patches in glomeruli of mice. The Pearson correlation coefficient (PCC) of NALP3 with ASC or caspase 1 was summarized in Figure 3B. Such colocalization of NALP3 molecules suggests the formation of inflammasomes in glomeruli. Furthermore, SEC demonstrated that inflammasome components (NALP3 and ASC) markedly shifted to large molecular fractions in glomeruli of hHcys mice (Figure S2A and S2B). Although podocin staining as a podocyte marker was found decreased in podocytes even in the first week of the FF diet feeding, we could still detect the colocalization of podocin with NALP3 or caspase 1, which was more remarkable in the second and fourth weeks (Figure 3C and 3D). Consistent with the aggregation of these inflammasome molecules in the glomeruli, the caspase 1 activity and IL-1 β production were increased even in the first week of FF diet treatment (Figures

3E, 3F, S2C, and S2D), suggesting the activation of NALP3 inflammasomes. In addition, the creatinine clearance was gradually decreased in mice fed the FF diet starting from the second week (control, 3.4 ± 0.6 ; first week, 3.3 ± 0.8 ; second week, 2.6 ± 0.6 ; fourth week, 1.7 ± 0.2 $\mu\text{L/min}$ per gram of body weight).

Blockade of Glomerular Inflammasome Formation and Activation by ASC shRNA and Caspase 1 Inhibition

To further determine the role of NALP3 inflammasome activation in glomerular injury, we transfected ASC shRNA into the kidney via the renal artery to silence the ASC gene locally. As illustrated in Figure 4A, the ASC shRNA transfection substantially suppressed the hHcys-induced colocalization of NALP3 with ASC or caspase 1 in the glomeruli. The summarized data were shown in Figure 4B. Using podocin as a podocyte marker, the inflammasome formation in glomeruli induced by the FF diet was found again to be mainly located in podocytes, as demonstrated by colocalization of podocin with NALP3 or caspase 1, which was substantially blocked by local ASC gene silencing (Figure S4A and S4B). Consistent with decreased aggregation of inflammasome components in the glomeruli, FF diet-enhanced caspase 1 activity and IL-1 β production were markedly attenuated in glomeruli of ASC shRNA transfected mice. Similarly, caspase 1 inhibitor WEHD also reduced the

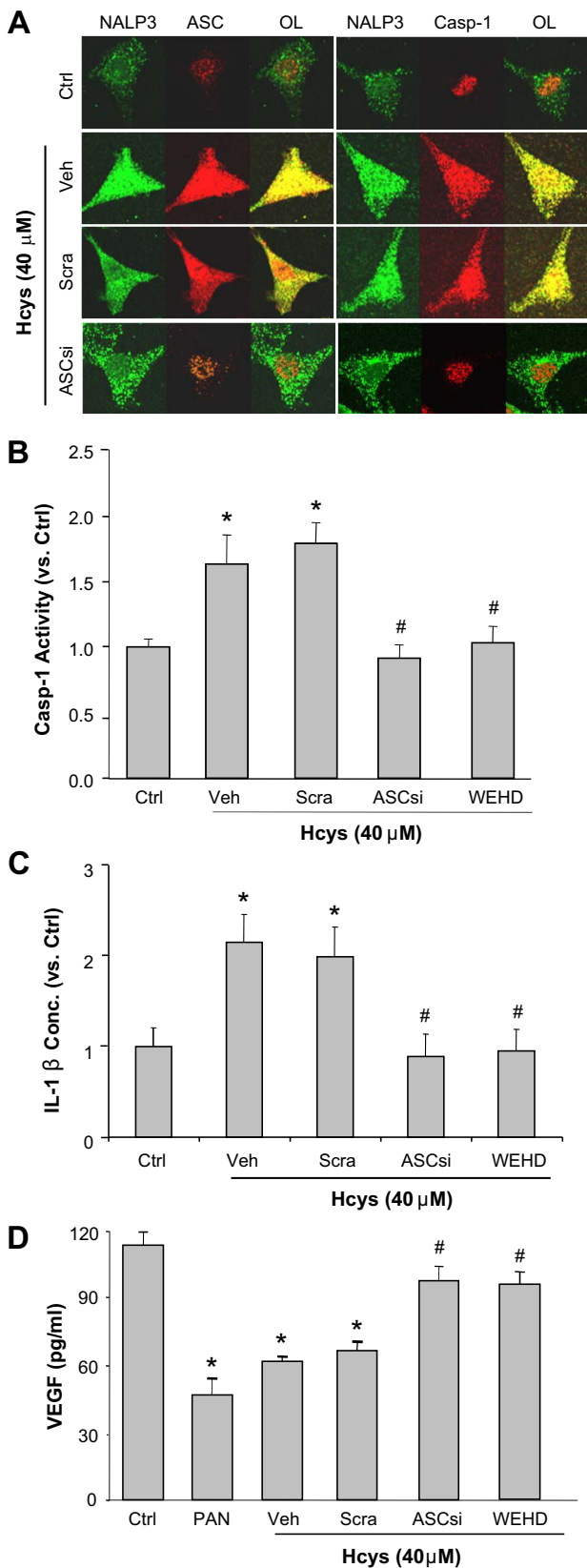


Figure 2. Effects of apoptosis-associated speck-like protein (ASC) gene silencing and caspase 1 inhibition on inflammasome activation and functional changes in podocytes. **A**, Representative confocal microscopic images showing colocalization of Nod-like receptor protein 3 (NALP3; green) with ASC (red) or caspase 1 (red; Casp-1) in podocytes (original magnification,

FF diet–induced increase in caspase 1 activity and IL-1 β production (Figure 4C and 4D).

Effects of Silencing ASC Gene or Inhibition of Caspase 1 Activity on hHcys-Induced Glomerular Damage

As shown in Figure 5A, the scrambled shRNA-transfected mice on the FF diet had severe albuminuria. When the ASC gene was silenced in the kidney and caspase 1 inhibited by WEHD, albuminuria in mice on the FF diet was significantly improved (Figure 5A). By periodic acid Schiff staining, morphological examinations showed typical sclerotic changes in glomeruli of scrambled shRNA-transfected mice on the FF diet, such as mesangial expansion, collapse of glomerular capillaries, and hypercellularity in these glomeruli (Figure 5B). Correspondingly, the glomerular damage index increased significantly in mice fed the FF diet (Figure 5C). Renal ASC gene silencing or WEHD treatment significantly blocked the glomerular damage induced by the FF diet (Figure 5B and 5C). Under transmission electron microscopy, the intact structures of podocyte foot processes shown in glomeruli from mice on the normal diet were destroyed by hHcys after 4 weeks, as shown by evident foot process effacement in scrambled shRNA-transfected mice on the FF diet. In contrast, podocytes of ASC shRNA-transfected or WEHD-treated mice on the FF diet had relatively normal ultrastructures (Figure 5D). In addition, real-time RT-PCR and immunofluorescence analyses demonstrated that the expression of nephrin, a podocyte functional marker, significantly decreased in scrambled shRNA-transfected mice on the FF diet, but this decrease in nephrin expression was not seen in ASC shRNA-transfected or WEHD-treated mice on the same diet. In contrast, the expression of desmin significantly increased in scrambled shRNA-transfected mice on the FF diet but not in ASC shRNA-transfected or WEHD-treated mice (Figure S4C and S4D). Furthermore, we determined the effect of ASC shRNA transfection on FF diet–induced mean arterial pressure and heart rate in mice. It was found that body weight, mean arterial pressure, and heart rate were similar in ASC shRNA-transfected mice fed a normal diet or FF diet (Table S1, available in the online-only Data Supplement).

Discussion

The major goal of the present study was to determine whether NALP3 inflammasomes are activated in glomerular podocytes and thereby lead to podocyte dysfunction and subsequent glomerular injury during hHcys. Our results demonstrated that hHcys induces NALP3 inflammasome formation and activation in podocytes even at a very early stage of Hcys stimulation (24 hours in vitro and 1 week in vivo). This inflammasome activation served as an intracellular molecular machinery to initiate the inflammatory response and to

$\times 400$); OL indicates overlay. **B**, Caspase 1 activity. **C**, Interleukin (IL) 1 β production. **D**, Vascular endothelial growth factor (VEGF)-A secretion in podocytes with or without treatment of L-homocysteine (Hcys) and/or ASC short hairpin (sh)RNA and caspase 1 inhibitor. Ctrl indicates control; Veh, vehicle; Scra, scrambled siRNA; Casp-1, caspase 1; WEHD: Z-WEHD-FMK; n=5. * $P < 0.05$ vs control; # $P < 0.05$ vs Hcys.

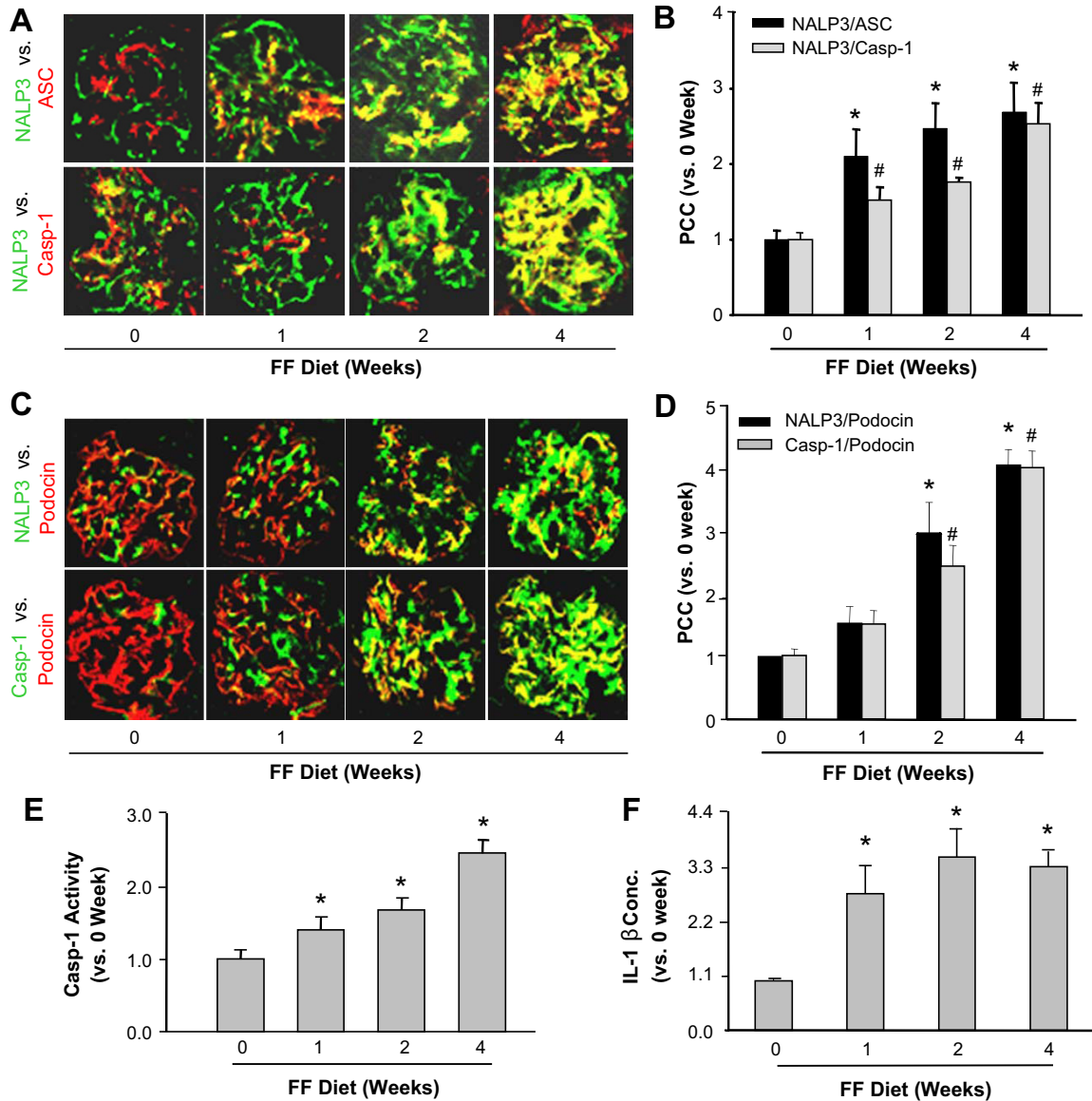


Figure 3. Formation and activation of podocyte Nod-like receptor protein 3 (NALP3) inflammasomes during hyperhomocysteinemia (hHcys) in mice. **A**, Colocalization of NALP3 (green) with apoptosis-associated speck-like protein (ASC; red) or caspase 1 (red) in mouse glomeruli on the normal diet or folate-free diet (FF diet). Casp-1 indicates caspase 1. **B**, Summarized data showing the fold changes in Pearson correlation coefficient (PCC) for the colocalization of NALP3 with ASC or caspase 1 (n=6). ■, NALP3/ASC; □, NALP3/Casp-1. **C**, Colocalization of podocin (red) with NALP3 (green) or caspase 1 (green) in mouse glomeruli. **D**, Summarized data showing the fold changes in PCC for the colocalization of podocin with NALP3 or caspase 1 (n=6). ■, NALP3/Podocin; □, Casp-1/Podocin. **E**, Caspase 1 activity. **F**, Interleukin (IL) 1β concentrations in mouse glomeruli (n=6). *P<0.05 vs 0 week.

directly damage podocytes in glomeruli, ultimately leading to glomerular sclerosis. Silencing of the ASC gene and inhibition of caspase 1 activity almost completely blocked podocyte injury and late glomerular dysfunction and sclerosis. These results indicate that activation of NALP3 inflammasomes in podocytes may be an early mechanism turning on podocyte injury and consequent glomerulosclerosis during hHcys.

It has been reported that nonmicrobial or sterile inflammation is an important pathological process in many kidney diseases, including hHcys-associated glomerulosclerosis.¹⁶ In particular, the residential renal cells have been found to play an important role in initiating renal inflammation and kidney damage. In this regard, a delicate study by Niemir et al⁵

revealed that podocytes are the major source of glomerular IL-1β, which participates in the progression of many nonproliferative forms of human glomerulonephritis by induction of a local inflammatory process. Many other studies from humans and animals, such as rats and mice, have also demonstrated that podocytes are one of the major sources of glomerular IL-1β under various pathological conditions and that activation of inflammasomes may contribute to IL-1β production.^{5,17-19} Because activation of the NALP3 inflammasomes has been known to cause caspase 1 activation and cleavage of pro-IL-1β and pro-IL-18 into their active and mature form,²⁰ namely, IL-1β or IL-18, we first characterized the expression and activity of this inflammasome complex in murine podocytes and determined whether it is indeed in-

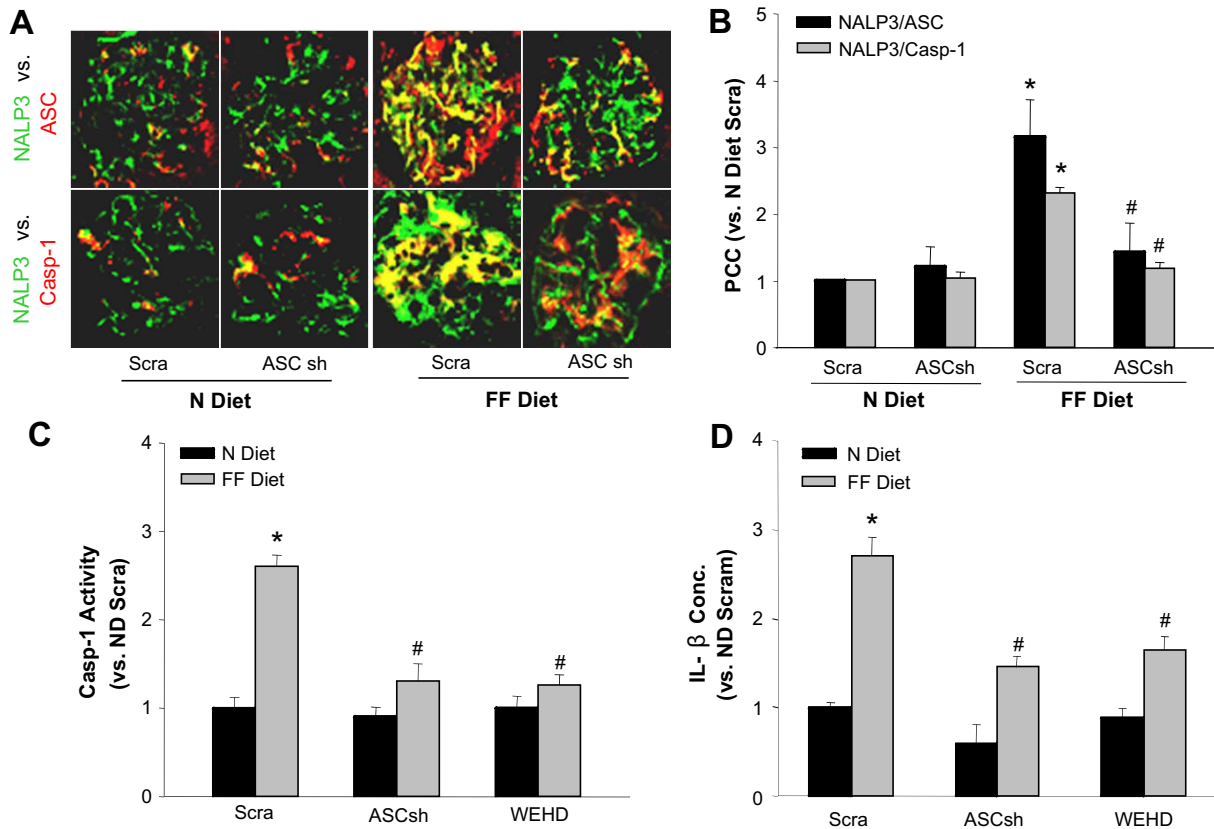


Figure 4. Effects of apoptosis-associated speck-like protein (ASC) short hairpin (sh)RNA or caspase 1 inhibition on podocyte inflammasome formation and activation during hyperhomocysteinemia (hHcys). **A**, Colocalization of Nod-like receptor protein 3 (NALP3; green) with ASC (red) or caspase 1 (red) in mouse glomeruli. **B**, Summarized data showing the fold changes in Pearson correlation coefficient (PCC) for the colocalization of NALP3 with ASC or caspase-1 (n=6). ■, NALP3/ASC; □, NALP3/Casp-1. **C**, Caspase 1 activity in different groups of mice (n=6). **D**, Interleukin (IL) 1 β production in mouse glomeruli (n=6 per group). **C** and **D**, ■, N diet; □, FF diet. N Diet indicates normal diet; FF diet, folate-free diet; Casp-1, caspase 1; Scra, scrambled shRNA-transfected; ASC sh, ASC shRNA-transfected. * P <0.05, vs scrambled shRNA-transfected mice on the N diet; # P <0.05, vs scrambled shRNA-transfected mice on the FF diet.

involved in Hcys-induced podocyte injury. By various approaches, such as RT-PCR, Western blot analysis, immunohistochemistry, and SEC, it was found that the main NALP3 inflammasome molecules, such as NALP3, ASC, and caspase 1, were expressed in murine podocytes. Importantly, L-Hcys stimulation induced the formation of the NALP3 inflammasome complex in podocytes, as shown by colocalization of NALP3 with ASC or caspase 1 using confocal microscopy, by an increase in NALP3-specific large molecular fractions detected by SEC, and by biochemical analysis of caspase 1 activity and production of IL-1 β . These results clearly suggest that the NALP3 inflammasomes are functioning in podocytes and that Hcys stimulation can lead to its activation. Although this type of inflammasome was firstly characterized in immune cells, recent studies have demonstrated that it can be detected in various nonimmune cells, including intrinsic glomerular cells^{17,21,22} and other residential cells in the brain, heart, and vessels.^{23,24} Although there are some reports that NALP3 and other inflammasomes can be activated in glomeruli during different pathological conditions,¹⁷ the results from the present study provide the first experimental evidence that L-Hcys activates NALP3 inflammasomes in podocytes, which may be an important patho-

genic mechanism responsible for glomerular injury during hHcys.

Another interesting finding of the present study is that Hcys directly induced podocyte dysfunction in vitro, as shown by a decrease in podocin expression, the disruption of actin cytoskeleton, and the decrease in vascular endothelial growth factor production in these cells. This Hcys-induced podocyte dysfunction was almost completely blocked by caspase 1 inhibition or ASC gene silencing. These results imply that podocytes are not only a glomerular cell type with intracellular inflammatory machinery featured by the formation and activation of NALP3 inflammasomes but also a cell type that is a target of inflammatory factors derived from activated NALP3 inflammasomes. The present study did not attempt to define the mechanism mediating the effect of NALP3 inflammasome activation to induce podocyte dysfunction or injury. However, 2 main mechanisms may contribute to such effect of NALP3 inflammasome activation on podocytes. First, the production of inflammatory factors, such as IL-1 β , attributed to activation of NALP3 inflammasomes may act in an autocrine fashion to change podocyte function. Indeed, there were some reports that various inflammatory factors, including IL-1 β , can induce podocyte injury by

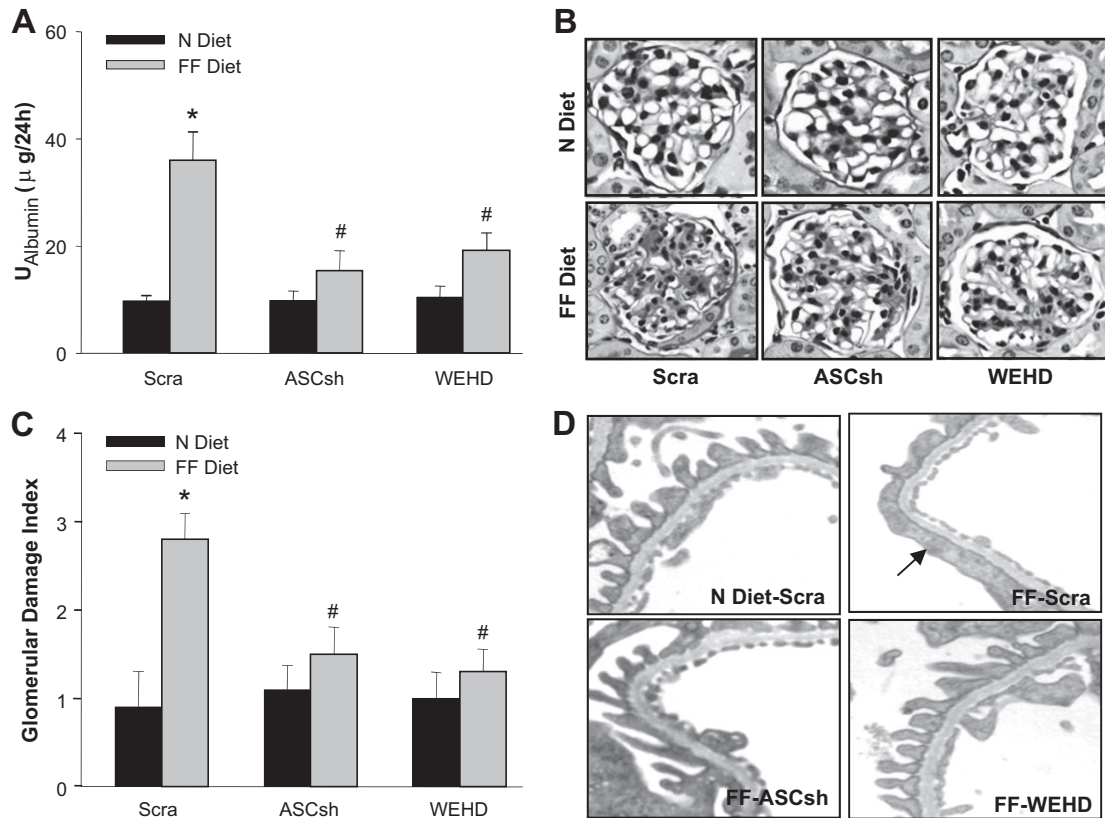


Figure 5. Effects of apoptosis-associated speck-like protein (ASC) gene silencing and caspase 1 inhibition on hyperhomocysteinemia (hHcys)-induced glomerular damage. **A**, Urinary albumin excretion in 6 groups of mice as indicated ($n=8$ per group). **B**, Periodic acid Schiff staining showing glomerular morphological changes (original magnification, $\times 400$). **C**, Summarized data of glomerular damage index in 6 different groups of mice ($n=6$ per group). **D**, ASC gene silencing and caspase 1 inhibition improved podocyte ultrastructure in FF diet-treated mice as shown by transmission electron microscopy examination. **Arrow** denotes the area of foot process effacement in WT mice on the FF diet ($n=3$). Original magnification: $\times 8000$. **A** and **C**, ■, N diet; □, FF diet. N Diet indicates normal diet; FF diet, folate-free diet.

reduction of nephrin production.^{21,25} Another mechanism may be related to the intrinsic functional changes in podocytes during the formation and activation of NALP3 inflammasomes, which may lead to intracellular signaling alterations and thereby result in podocyte dysfunction. Some ongoing studies in our laboratory will further clarify these mechanisms.

In animal experiments, we produced experimental hHcys by feeding mice with the FF diet. It was found that the FF diet gradually increased the plasma total Hcys levels starting from the first week. Our confocal microscopy and SEC experiments indeed detected the formation and activation of NALP3 inflammasomes in the glomeruli of mice on the FF diet, which mainly occurred in podocytes given the colocalization of increased NALP3 molecules with podocin. Correspondingly, glomerular caspase 1 activity and IL-1 β production were dramatically elevated during hHcys, suggesting the activation of NALP3 inflammasomes in glomeruli. In particular, most of these changes related to the formation and activation of NALP3 inflammasome could be seen at a very early stage of hHcys (first week on the FF diet), when functional or structural changes in glomeruli could not be seen. These data may afford a reasonable explanation for previous results reported by others that podocytes are a major source of IL-1 β seen in different kinds of glomerular injuries

or sclerosis.^{5,17,19} Consistent with our findings, some recent reports demonstrated that NALP3 mRNA expression increased, and this inflammasome can be activated in different glomerular diseases. It is assumed that NALP3 serves as a sensor of danger factors, and consequent activated inflammasomes may integrate several triggering signals leading to the secretion of IL-1 β , ultimately resulting in glomerular inflammatory injury.²⁶ However, a recent study has shown that NALP3 inflammasomes may not be activated in glomeruli or podocytes in anti-glomerular basement membrane glomerulonephritis or by lipopolysaccharide.²⁷ It is possible that, under some pathological conditions, such as anti-glomerular basement membrane glomerulonephritis or on stimulation of lipopolysaccharide, activation of NALP3 inflammasomes is dominant in immune cells, such as dendritic cells. However, in other pathological conditions that were caused by some autoinflammatory stimuli or factors such as hHcys, hyperglycemia, increased plasma uric acid, and cholesterol, activation of NALP3 inflammasomes may occur in glomerular residential cells at the early stage of such pathological processes.

To further confirm the role of podocyte NALP3 inflammasome activation in the development of glomerular injury during hHcys, a well-established in vivo gene silencing strategy was used for delivery of ASC shRNA into the

kidney.⁹ Using this method, ASC shRNA was introduced into renal cortical tissue including glomeruli, and renal local ASC expression could be dynamically monitored in vivo and thereby guided functional studies. At the end of experiments, real-time RT-PCR was conducted routinely to confirm the inhibitory efficiency of local gene silencing in the glomeruli. We demonstrated that ASC gene silencing substantially attenuated the NALP3 inflammasome formation and activation, caspase 1 activity, and IL-1 β production, as well as glomerular damage in hHcys mice. In contrast, ASC shRNA transfection did not alter the mean arterial pressure and heart rate in C57BL/6J WT mice fed a normal or FF diet. Similarly, inhibition of caspase 1 in the kidney also ameliorated production of IL-1 β and glomerular injury during hHcys. These data further support the view that podocyte and glomerular NALP3 inflammasome formation and activation occur during hHcys and that this NALP3 inflammasome activation importantly contributes to the initiation or development of glomerulosclerosis independent of hypertension. The present study did not aim to elucidate the exact mechanism of hHcys-induced NALP3 inflammasome activation in podocytes. However, NADPH oxidase-derived superoxide production may be an important mechanism mediating hHcys-induced inflammasome activation in podocytes (unpublished observations). Targeting this inflammasome at the stage of its assembling or activation may be a novel strategy to prevent the development of glomerular injury or sclerosis in hHcys. In this regard, although a recent study reported that ASC knockout mice did not protect the kidney from ischemia-reperfusion injury,²⁸ NALP3 knockout mice had less interstitial fibrosis as compared with their wide-type littermates.²⁶ These observations, together with our findings in the hHcys mouse model, point to the idea that renal NALP3 inflammasome formation and activation may be crucial mechanisms mediating a renal inflammatory response that may directly cause podocyte dysfunction or indirectly induce accumulation of inflammatory cells, such as T cells or macrophages, in glomeruli, ultimately leading to glomerular injury and sclerosis. It is plausible that NALP3 inflammasome activation is an important early mechanism triggering or promoting chronic renal tissue damage, leading to fibrosis and sclerosis.

Our further experiments indeed confirmed that podocytes can be damaged by activation of NALP3 inflammasomes in mice with hHcys. It was found that ASC gene silencing or caspase 1 inhibition by WEHD protected the podocytes from hHcys-induced injury. Transmission electron microscopy examinations showed that foot process effacement induced by hHcys was dramatically alleviated in mice transfected with ASC shRNA or injected with WEHD, suggesting that podocyte ultrastructure was improved in these mice. Moreover, the expression of the most important slit diaphragm molecule, nephrin, was almost completely recovered in mice treated with ASC shRNA or WEHD. In contrast, increase of a classic podocyte marker, desmin, was found to be attenuated in ASC-transfected hHcys mice, further indicating that hHcys-induced podocyte injury was significantly attenuated because of reduced NALP3 inflammasome activation in these cells. Such protection of podocyte structure and function from hHcys-induced injury further confirms the important role of

NALP3 inflammasome activation in the development of glomerular injury.

Perspectives

The present study revealed a new triggering mechanism of hHcys-induced glomerular injury that is characterized by the formation and activation of an NALP3 inflammasome complex in podocytes. This NALP3 inflammasome activation may represent a novel early event leading to podocyte dysfunction and injury, initiating glomerulosclerosis during hHcys. Based on these findings, the development of strategies that target the activation of podocyte NALP3 inflammasomes may be a promising therapeutic intervention to prevent hHcys-associated glomerular damage.

Sources of Funding

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Disclosures

None.

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Novelty and Significance

What Is New?

- The present study clarified a novel intracellular mechanism, namely, the formation and activation of inflammasomes, which turn on podocyte injury and glomerular inflammation, leading to glomerulosclerosis during hHcys.

What Is Relevant?

- Podocyte and glomerular NALP3 inflammasome formation and activation occur during hHcys, and NALP3 inflammasome activation importantly

contributes to the initiation and development of glomerulosclerosis independent of hypertension.

Summary

NALP3 inflammasome formation is an early pathogenic event of podocyte injury and glomerular sclerosis, and, therefore, it may be an important, novel therapeutic target for treatment of hHcys-associated glomerular disease or other chronic degenerative diseases.

ONLINE SUPPLEMENT

**ACTIVATION OF NALP3 INFLAMMASOMES TURNS ON PODOCYTE INJURY
AND GLOMERULAR SCLEROSIS IN HYPERHOMOCYSTEINEMIA**

Chun Zhang ^{1,3,*}, Krishna M. Boini ^{1,*}, Min Xia ¹, Justine M. Abais ¹, Xiang Li¹,
Qinglian Liu ², Pin-Lan Li ¹

¹ Department of Pharmacology & Toxicology and ² Physiology and Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, USA

³ Current Address: Department of Nephrology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

* Both authors contributed equally to this work.

Running title: NALP3 Inflammasomes in podocytes

Send Correspondence and Reprint Request to:

Pin-Lan Li, M.D, Ph.D.
Department of Pharmacology and Toxicology
Medical College of Virginia
Virginia Commonwealth University
410 N 12th Street, Richmond, VA 23298
Tel: (804) 828-4793
Fax: (804) 828-4794
E-mail: pli@vcu.edu

SUPPLEMENTARY MATERIALS AND METHODS

Cell culture: A conditionally immortalized mouse podocyte cell line, kindly provided by Dr. Klotman PE (Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA), was cultured on collagen I-coated flasks or plates in RPMI 1640 medium supplemented with recombinant mouse interferon- γ at 33°C. After differentiation at 37°C for 10-14 days without interferon- γ , podocytes were used for the proposed experiments. L-Hcys (a pathogenic form of Hcys) was used and its concentration and incubation time in cell culture dishes were chosen based on our previous studies¹ and some preliminary experiments.

ASC siRNA transfection: ASC siRNA was purchased from Qiagen (Valencia, CA, USA), which was confirmed to be effective in silencing the ASC gene in different cells by the company. The scrambled RNA (Qiagen, Valencia, CA, USA) was also confirmed as non-silencing double-strand RNA and was used as a control. Podocytes were serum-starved for 12 h and then transfected with ASC siRNA or scrambled siRNA using siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA). After 18 h of incubation at 37°C, the medium was changed and L-Hcys (40 μ mol/L) added into the medium for indicated time spans in different protocols.

Real-time reverse transcription polymerase chain reaction (RT-PCR): Total RNA from cultured podocytes or isolated mouse glomeruli was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol described by the manufacturer. The primers used in this study were synthesized by Operon (Huntsville, AL, USA) and the sequences were: for NALP3 sense TACGGCCGTCTACGTCTTCT, antisense CGCAGATCACACTCCTCAA; for ASC sense ACAGAAGTGGACGGAGTGCT, antisense CTCCAGGTCCATACCAAGT; for caspase-1 sense CACAGCTCTGGAGATGGTGA, antisense TCTTTCAAGCTTGGGCACTT; for nephrin sense CCCGGACACCTGTATGACGAG, antisense CCGCCACCTGGTCGTAGATT; for desmin sense CAGTCTACACCTGCGAGATT, antisense GGCCATCTTACATTGAGC; and for β -actin sense TCGCTGCGCTGGTCGTC, antisense GGCCTCGTCACCCACATAGGA.

Caspase-1 activation assay: Caspase-1 activity was detected using a commercially available kit (Biovision, Mountain View, CA, USA), which was used to represent activation of NALP3 inflammasomes. The data was expressed as the fold changes compared with control cells.

Indirect immuno-fluorescent staining and confocal microscopy: For colocalization of inflammasome molecules in podocytes, cultured cells were fixed in 4% PFA for 15 minutes. After being rinsed with phosphate-buffer saline (PBS), the cells were incubated overnight at 4°C with goat anti-NALP3 (1:200, Abcam, Cambridge, MA, USA) and rabbit anti-ASC (1:50, Enzo, Plymouth Meeting, PA), or goat anti-NALP3 (1:200) and anti-caspase-1 (1:100,

Abcam, Cambridge, MA, USA). To colocalize inflammasome molecules and podocyte markers in the mouse kidney, double-immunofluorescent staining was performed using frozen tissue slides. After fixation, the slides were incubated overnight at 4°C with goat anti-NALP3 (1:200) and rabbit anti-ASC (1:50), or goat anti-NALP3 (1:200) and anti-caspase-1 (1:100). In additional experiments, tissue slides were probed with anti-NALP3 or caspase-1 antibody together with anti-podocin antibody (1:400, Sigma, St. Louis, MO, USA) to show the localization of inflammasome molecules in podocytes. After washing, these slides probed with primary antibodies were incubated with Alexa-488- or Alexa-555-labeled secondary antibodies for 1 h at room temperature. After being mounted with DAPI-containing mounting solution, the slides were subjected to examinations using a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan), with photos being taken and the colocalization of NALP3 with ASC or caspase-1 analyzed by the Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). The data was expressed as Pearson correlation coefficient (PCC) as we described previously ².

Size-exclusion chromatography (SEC): Podocytes or isolated glomeruli were homogenized with the following protein extraction buffer: 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L Na EDTA, 1 mmol/L Na EGTA, and 1 × protease inhibitor cocktail set I (Calbiochem, Gibbstown, NJ, USA). Samples were then centrifuged at 18,000g for 10 min at 4°C and run on a Sepharose 6 size-exclusion chromatography column with the following buffer: 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% octylglucoside, and 1× protease inhibitor cocktail. Fractions (200 μl) were collected starting at the void volume time. 5× sample buffer was added directly to the fractions, which were then heated at 95°C for 5 min and resolved in SDS-polyacrylamide gel electrophoresis gels followed by Western blot analysis ³. Protein standards were run on a column under identical conditions and absorbance at 280 nm was used to analyze the fractions.

Western blot analysis: Protein from podocyte lysate or mouse kidney tissues was run on an SDS-PAGE gel, transferred on to PVDF membrane and blocked. Then, the membrane was probed with primary antibodies against NALP3, ASC, or caspase-1 (1:500 dilution) overnight at 4°C followed by incubation with horseradish peroxidase-labeled immunoglobulin G. The immunoreactive bands were detected by chemiluminescence methods and visualized on Kodak Omat film. β-actin was reprobed to serve as a loading control. The intensity of the bands was quantified by densitometry.

Direct fluorescent staining of F-actin: To determine the role of NALP3 inflammasome activation in Hcys-induced cytoskeleton changes, podocytes were cultured in 8-well chambers. After pretreatment with vehicle, caspase-1 inhibitor (Z-WEHD-FMK) or transfected with ASC siRNA or scrambled siRNA, the cells were treated with L-Hcys (40 μmol/L) or puromycin aminonucleoside (PAN, 100 μg/mL, Sigma, St. Louis, MO, USA) for 24 h. After washing with PBS, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100, and blocked with 3% bovine serum albumin. F-actin was stained with rhodamine-phalloidin (Invitrogen, Carlsbad, CA,

USA) for 15 min at room temperature. After mounting, the slides were examined by a confocal laser scanning microscope. Cells with distinct F-actin fibers were counted as we described previously¹. Scoring was obtained from 100 podocytes on each slide in different groups.

ELISA for vascular endothelial growth factor A (VEGF-A) and IL-1 β in podocytes and glomeruli: After transfection with ASC siRNA, scrambled siRNA, or pretreatment with Z-WEHD-FMK and its vehicle, podocytes were incubated with L-Hcys (40 μ mol/L) for 24 h. A specific podocyte injury compound, puromycin aminonucleoside (PAN, 100 μ g/ml) was used to treat cells for 24 h to serve as a positive control. The supernatant was collected for ELISA assay of VEGF-A using a commercially available kit (R&D system, Minneapolis, MN). In additional experiments, IL-1 β in the cell supernatant and mouse glomeruli were measured by a mouse IL-1 β ELISA kit from Bender Medsystems (San Diego, CA, USA) according to the protocol described by the manufacturer.

Transmission electron microscopy (TEM): For TEM observation of ultrastructural changes in podocytes, the kidneys were perfused with a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer. After fixation and dehydration with ethanol, the samples were embedded in Durcupan resin for ultra-thin sectioning and TEM examination by VCU electron microscopy core facility.

Isolation of mouse glomeruli, morphological examination, mean arterial pressure, plasma homocysteine, urinary albumin and creatinine measurements were performed as we described previously⁴⁻⁵.

Statistical analysis: All of the values are expressed as mean \pm SEM. Significant differences among multiple groups were examined using ANOVA followed by a Student-Newman-Keuls post hoc test. χ^2 test was used for testing the significance of ratio and percentage data. $P < 0.05$ was considered statistically significant.

SUPPLEMENTARY RESULTS

Effects of ASC gene silencing and caspase-1 inhibition on functional changes induced by L-Hcys. Using rhodamine-phalloidin to stain F-actin, podocytes treated with L-Hcys were found to have reduced and reorganized F-actin fibers, which was normally distributed along the longitudinal axis within the podocytes. In podocytes treated with L-Hcys, the remaining F-actin fibers were found mainly aggregated around the periphery of the cells, which was similar to the changes induced by a podocyte-selective toxic compound. After ASC gene silencing or inhibition of caspase-1 activity, the amount and distribution pattern of F-actin in podocytes were restored to normal (Figure S1). These changes in F-actin staining were summarized in Figure S1.

Efficiency of *in vivo* local transfection of ASC shRNA into the kidney. As shown in Figure S3A, the expression efficiency of the co-transfected luciferase gene was monitored daily using an *in vivo* molecular imaging system to insure an efficient introduction of target gene into the kidney cells. Even on the 4th day after the transfection, the gene expression could be detected. This expression continued for 4 weeks. As shown in a hemi-dissected kidney, almost all of the cortical regions exhibited efficient gene transfection, as shown in green fluorescence compared with the control kidney (Figure S3B). RT-PCR analysis demonstrated that FF diet treatment for 4 weeks significantly increased the ASC mRNA expression, which was substantially blocked in ASC shRNA-transfected glomeruli both on the normal diet or FF diet, indicating the successful silencing of ASC gene in mouse glomeruli (Figure S3C).

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Table S1: Body weight, plasma Hcys concentration, mean arterial pressure and heart rate in C57BL/6J WT mice transfected with or without ASC shRNA and fed a ND or FF diet (n= 4-5).

Parameters	Scra ND	ASCsh ND	Scra FF	ASCsh FF
Body Weight (gm)	27.7 ± 1.3	28.4 ± 1.5	27.8 ± 1.2	25.2 ± 0.6
Hcys Conc. (µM)	6.1 ± 1.2	6.8 ± 0.3	17.5 ± 1.1*	20.1 ± 2.1*
MAP (mmHg)	102.4 ± 3.4	107.5 ± 1.4	105.7 ± 2.0	105.4 ± 0.8
Heart Rate (beats/min)	563.2 ± 11.4	580.8 ± 8.0	581.4 ± 3.0	558.2 ± 13.0

ND: Normal diet, FF: Folate-free diet, MAP: Mean arterial pressure, Hcys: Homocysteine, ASCsh: ASC shRNA, Scra: Scrambled shRNA-transfected. The data was expressed as mean ± SEM. *p<0.05 as compared with Scra ND.

SUPPLEMENTARY FIGURES

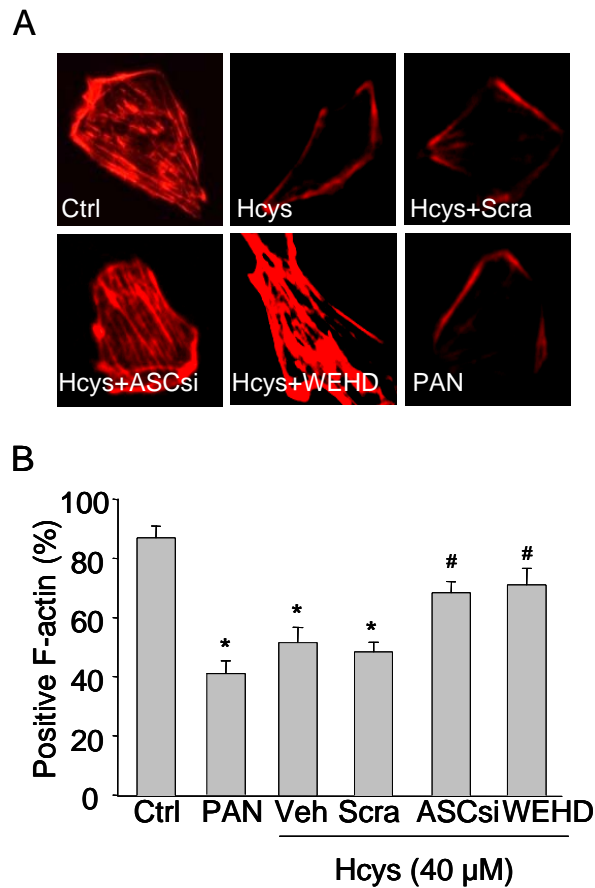


Figure S1. A. Representative microscopic images showing F-actin staining in podocytes using rhodamine-phalloidin staining (magnification, $\times 400$). B. Summarized data showing the rate of podocytes retaining distinct longitudinal stress fibers. Scoring was from 100 podocytes on each slide in different groups ($n=6$ batches of podocytes). Ctrl: control; Veh: vehicle; Scra: scrambled siRNA; Casp-1: Caspase-1; WEHD: Z-WEHD-FMK. * $P < 0.05$ vs. control; # $P < 0.05$ vs. Hcys.

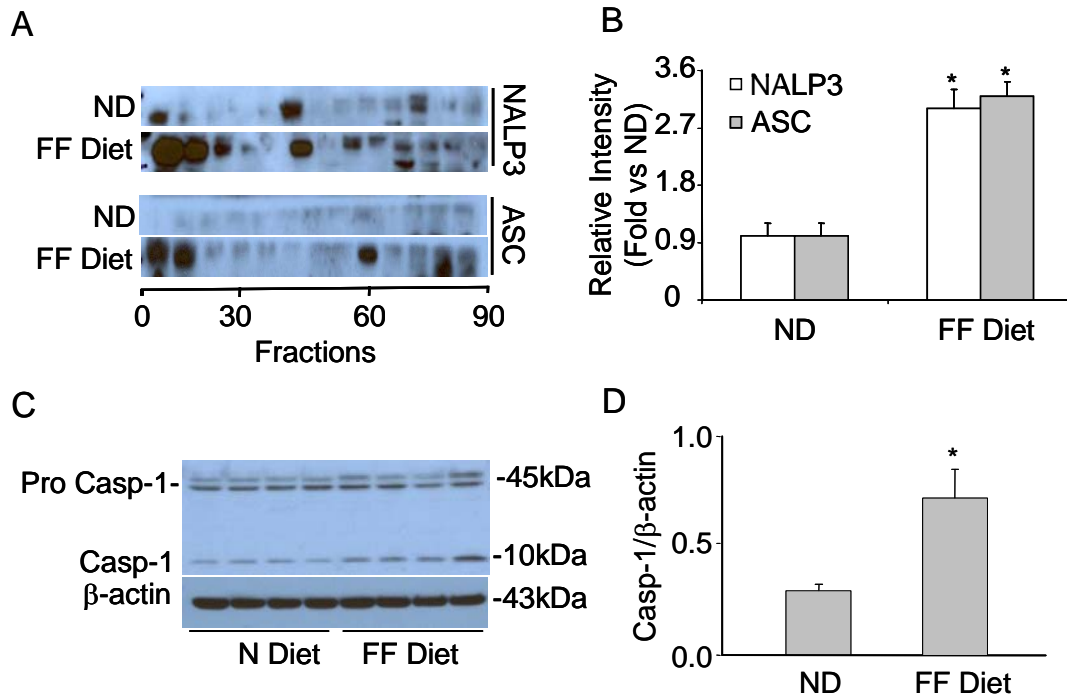


Figure S2. Formation and activation of NALP3 inflammasomes in glomeruli of hHcys mice. A. Western blot analysis of protein fractions obtained from glomeruli of normal diet and FF diet fed mice probed with anti-NALP3 and ASC antibodies. B. Summarized data showing the band intensities measured from the inflammasome complex fractions (fractions 7-21) of NALP3 and ASC (n=3). C. Total and cleaved caspase-1 expression in mouse glomeruli after 4 weeks of FF or normal diet treatment. D. Summarized data showing the quantification of cleaved caspase-1 expression, which was normalized to β -actin (n=7). Casp-1: Caspase-1, N diet: Normal diet, * $P < 0.05$ vs. ND.

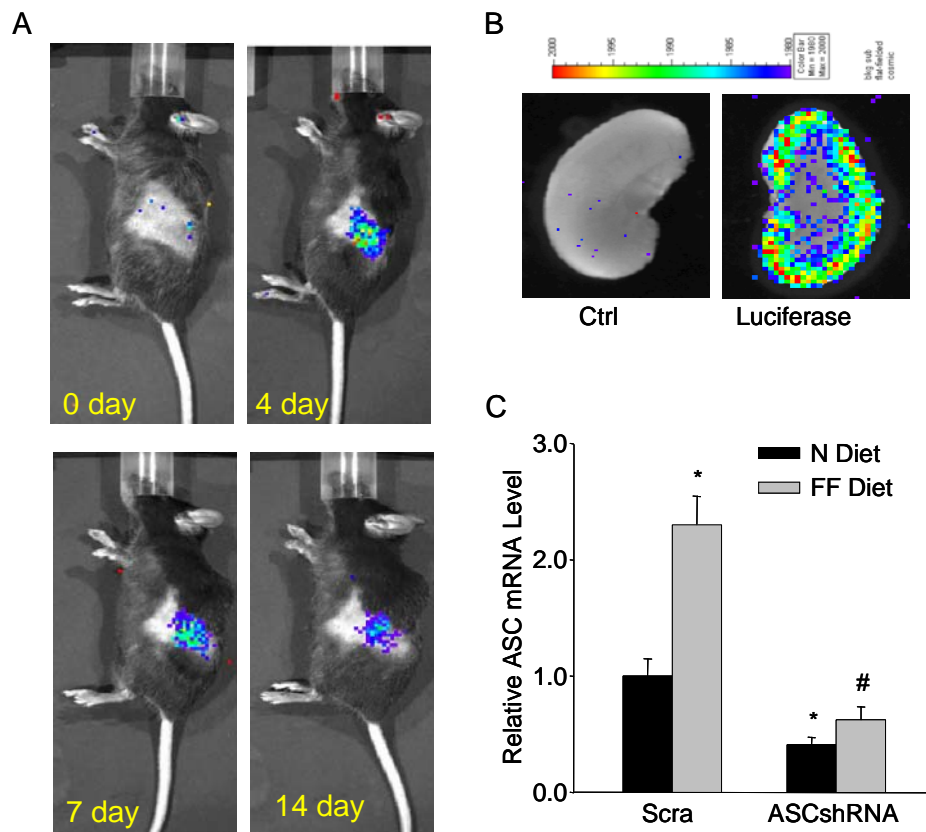


Figure S3. *In vivo* and *in vitro* determination of gene transfection efficiency in the kidney. A. Daily imaging confirmation of gene transfection in the kidney by an *in vivo* molecular imaging system. B. Localization of transfected gene expression in the hemi-dissected kidney on day 14 after gene delivery. C. Real-time RT-PCR detection of ASC mRNA after ASC shRNA delivery at 4 weeks after gene delivery (n=4). * $P < 0.05$, vs. scrambled shRNA-transfected mice on the N diet; # $P < 0.05$, vs. scrambled shRNA-transfected mice on the FF diet.

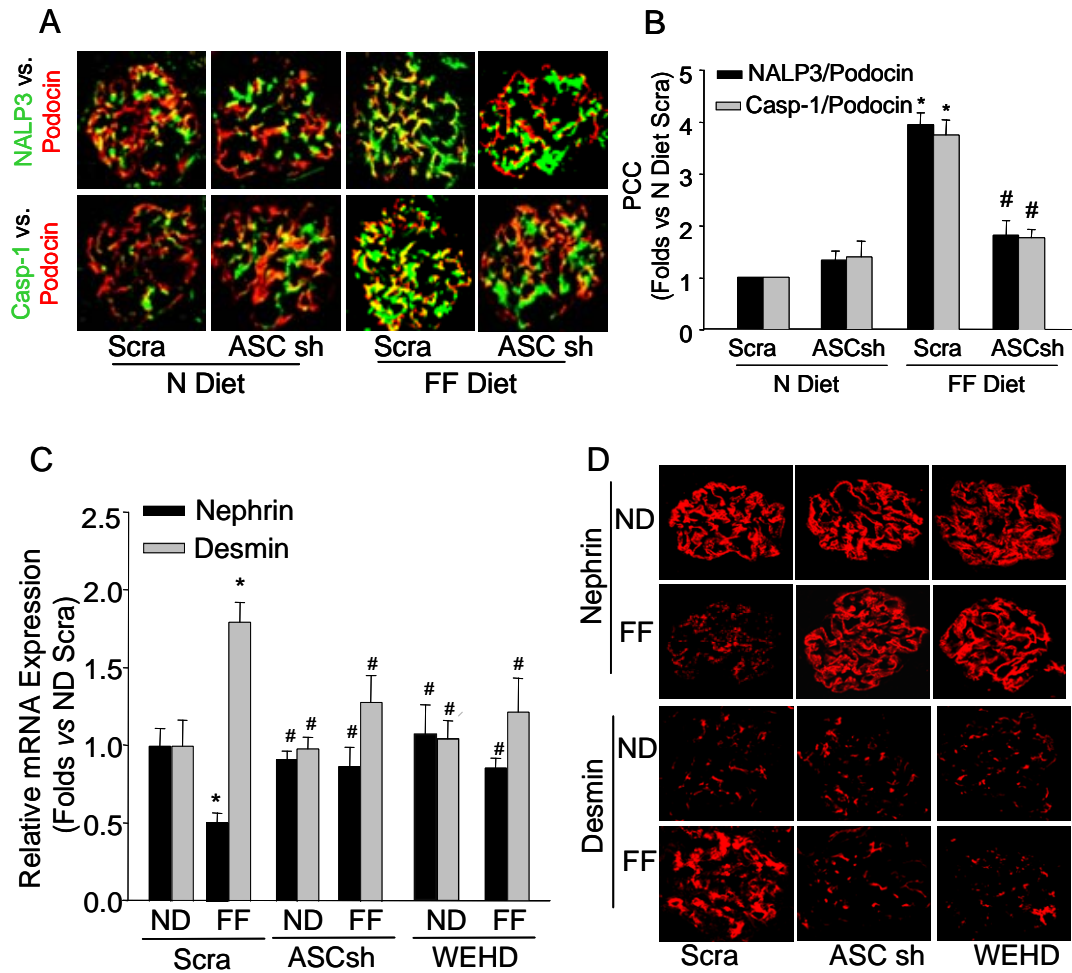


Figure S4. A. Colocalization of NALP3 (green) with podocin (red) and caspase-1 (green) with podocin (red) in mouse glomeruli. B. Summarized data showing the fold changes in PCC for the colocalization of podocin with NALP3 or caspase-1, C. Real-time RT-PCR analysis showing changes in the expression of nephrin and desmin in the glomeruli from different groups of mice (n=4 per group). D. Immunofluorescent staining of nephrin and desmin in the glomeruli from 6 groups of mice (n=6 per group). N Diet: Normal Diet; Casp-1: Caspase-1; Scra: Scrambled shRNA-transfected; ASC sh: ASC shRNA-transfected. * $P < 0.05$, vs. scrambled shRNA-transfected mice on the N diet (normal diet); # $P < 0.05$, vs. scrambled shRNA-transfected mice on the FF diet. (n=6).