Original Article

Novel Role of NOD2 in Mediating Ca\textsuperscript{2+} Signaling
Evidence From NOD2-Regulated Podocyte TRPC6 Channels in Hyperhomocysteinemia

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Abstract—Although hyperhomocysteinemia (hHcys) has been recognized as an important independent risk factor in the progression of end-stage renal disease and in the development of cardiovascular complications related to end-stage renal disease, the mechanisms triggering the pathogenic actions of hHcys are not yet fully understood. The present study was designed to investigate the contribution of nucleotide-binding oligomerization domain containing 2 (NOD2), an intracellular innate immunity mediator, to the development of glomerulosclerosis in hHcys. Our results showed that NOD2 deficiency ameliorated renal injury in mice with hHcys. We further discovered the novel role of NOD2 in mediating Ca\textsuperscript{2+} signaling and found that homocysteine-induced NOD2 expression enhanced transient receptor potential cation channel 6 (TRPC6) expression and TRPC6-mediated calcium influx and currents, leading to intracellular Ca\textsuperscript{2+} release, ultimately resulting in podocyte cytoskeleton rearrangement and apoptosis. Moreover, we found that nephrin expression was downregulated dependently by NOD2, and overexpression of nephrin attenuated homocysteine-induced TRPC6 expression in podocytes. The results add evidence to support the essential role of nephrin in mediating NOD2-induced TRPC6 expression in hHcys. In conclusion, our results for the first time establish a previously unknown function of NOD2 for the regulation of TRPC6 channels, suggesting that TRPC6-dependent Ca\textsuperscript{2+} signaling is one of the critical signal transduction pathways that links innate immunity mediator NOD2 to podocyte injury. Pharmacological targeting of NOD2 signaling pathways at multiple levels may help design a new approach to develop therapeutic strategies for treatment of hHcys-associated end-stage renal disease. (Hypertension. 2013;62:00-00.) • Online Data Supplement

Key Words: calcium signaling ■ homocysteine ■ NLR family ■ transient receptor potential channel

Hyperhomocysteinemia (hHcys) has been recognized as an important independent risk factor in the progression of end-stage renal disease and in the development of cardiovascular complications related to end-stage renal disease.\textsuperscript{1,2} Recent studies have demonstrated that homocysteine (Hcys) may directly act on glomerular cells to induce glomerular dysfunction and consequent glomerulosclerosis, leading to end-stage renal disease.\textsuperscript{3-6} However, so far there are no efficient Hcys-lowering and Hcys-detoxifying strategies being used in patients with cardiovascular and chronic kidney disease.\textsuperscript{7,8} Therefore, the key targets in the pathogenic pathways for Hcys-induced renal injury should be identified to direct toward prevention or treatment of end-stage renal disease associated with Hcys. Although several mechanisms are involved in the pathogenesis of high Hcys, such as oxidative stress, endoplasmic reticulum stress, and inflammation,\textsuperscript{1} the mechanisms triggering the pathogenic actions of Hcys are not yet fully understood.

Nucleotide-binding oligomerization domain containing 2 (NOD2), a member of the NOD-like receptor family, plays an important role in innate immune and adaptive response. Studies have revealed the association of NOD2 with several pathologies, including Crohn disease, atherosclerosis, and Alzheimer disease.\textsuperscript{9} In addition to being present in inflammatory cells, studies from our laboratory and by others have indicated that NOD2 is also highly expressed in the kidney,\textsuperscript{10} including renal proximal tubule epithelial cells,\textsuperscript{11} glomerular mesangial cells, endothelial cells, and podocytes.\textsuperscript{12} The expression within renal cells may position this innate intracellular protein as a sentinel participant in response to endogenous danger signals associated with renal disease. An elevated level of Hcys has been demonstrated to induce chronic inflammation in the vascular bed, including glomerulus.\textsuperscript{13,14} However, it remains unknown whether NOD2-mediated signaling in renal glomerular residential cells contributes to the pathogenesis of renal injury in hHcys.
Podocytes are unique terminally differentiated epithelial cells in the filtration barrier of the kidney; loss of podocyte functions is considered as an early and key event in the development of glomerulosclerosis. However, it remains unclear whether NOD2 is associated with changes in podocyte structure and functions in hHcys. Our study showed that NOD2 deficiency ameliorated renal injury in mice with hHcys. We further discovered the novel role of NOD2 in mediating Ca²⁺ signaling and demonstrated that NOD2-mediated transient receptor potential cation channel 6 (TRPC6) expression and activity via nephrin, which lead to intracellular Ca²⁺ release, ultimately result in podocyte dysfunction, suggesting that TRPC6-dependent Ca²⁺ signaling is one of the critical signal transduction pathways that links innate immunity mediator NOD2 to podocyte injury.

Materials and Methods
An extended Material and Methods section can be found in the online-only Data Supplement.

Animals
Twelve-week-old NOD2−/− mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Immunofluorescence
Immunofluorescent staining was performed as described using a LSM780 laser scanning confocal microscope (ZEISS, Oberkochen, Germany) equipped with a Plan-Apochromat 63×/1.4 objective.

Electrophysiological Analysis
The procedures for cell-attached patch-clamp recordings of TRPC6 channel currents from podocytes are presented in the online-only Data Supplement.

Statistics
Data are expressed as mean±SE. The significance of the differences in mean values between and within multiple groups was examined by 1-way ANOVA followed by Duncan multiple range test. P<0.05 was considered statistically significant.

Results

NOD2 Deficiency Ameliorated Renal Injury and Reduced TRPC6 Expression in Mice With hHcys
Our studies indicated that NOD2 expression was significantly increased in the kidney from hHcys-induced mice by folate-free diets (Figure 1A). Therefore, NOD2−/− mice were used to explore the role of NOD2 in the pathogenesis of hHcys. Our results showed that although plasma total Hcys levels were increased as the same levels of wild-type mice with folate-free diets (plasma total Hcys level >10 μmol/L is considered as hHcys; Figure 1B), albuminuria (Figure 1C) was significantly less in NOD2−/− mice with hHcys accompanied by decreased mesangial expansion (Figure 1D) and ameliorated podocyte injury (Figure 1E) compared with those of wild-type mice with hHcys. Meanwhile, NOD2 deficiency decreased the levels of proinflammatory mediators, including interleukin-1β, interleukin-6, tumor necrosis factor-α, monocyte chemoattractant protein-1, and intracellular adhesion molecule-1 in hHcys (Figure 1F). Moreover, Western blot (Figure 1G) and immunofluorescence (Figure 1H) analyses indicated that Hcys-induced TRPC6 expression was attenuated by NOD2 deficiency.

Hcys-Induced TRPC6 Expression Was Mediated by NOD2 in Podocytes
As shown in Figure 2A, Hcys enhanced NOD2 and TRPC6 expression in podocytes. Activation of NOD2 by muramyl dipeptide (MDP) also induced TRPC6 expression (Figure 2B). We further found that gene silencing of NOD2 by shRNA-NOD2 transfection (Figure 2C) blocked Hcys-induced TRPC6 expression (Figure 2D).

NOD2 Was Required for Hcys-Mediated Ca²⁺ Influx and Currents via TRPC6 in Podocytes
To confirm the contribution of NOD2 to Hcys-mediated Ca²⁺ influx, we evaluated Ca²⁺ influx into fluo-2–loaded podocytes stimulated by 1-oleoyl-2-acetyl-sn-glycerol, a TRPC6 channel agonist. It was found that Hcys enhanced 1-oleoyl-2-acetyl-sn-glycerol–stimulated Ca²⁺ influx, which was attenuated by NOD2 knockdown (Figure 3A). Consistently, application of 1-oleoyl-2-acetyl-sn-glycerol caused increased cation currents in podocytes. Moreover, the currents were substantially larger in podocytes with MDP or Hcys treatment, which were attenuated by NOD2 knockdown (Figure 3B).

NOD2-Mediated TRPC6 Channels Was Associated With Cytoskeleton Rearrangement and Apoptosis in Podocytes
As shown in Figure S1A in the online-only Data Supplement, MDP or Hcys altered cytoskeleton distribution as evidenced by the loss of actin filaments and a granular cytoplasmic pattern of actin distribution. Consistent with the effects of TRPC6 knockdown, the disruption of actin was dramatically ameliorated by NOD2 knockdown in Hcys-treated podocytes. Furthermore, flow cytometry analysis indicated that both Hcys and MDP induced podocyte apoptosis, which was inhibited by gene silencing of NOD2 or TRPC6 (Figure S1B).

TRPC6 Expression Was Associated With NOD2-Reduced Nephrin Expression in hHcys
We found that both Hcys (Figure 4A) and MDP (Figure 4B) decreased nephrin expression in podocytes. Further studies showed that overexpression of nephrin by pcDNA3.1-NPHS1 transfection (Figure 4C) attenuated Hcys-induced TRPC6 expression (Figure 4D). These results were consistent with in vivo study showing that decreased nephrin expression was recovered in NOD2−/− mice with hHcys (Figure S2).

Discussion
Among pattern-recognition receptors, the intracellular NOD-like receptors have recently been identified as key mediators of inflammatory and immune responses. The crucial role of NOD2, a member of the NOD-like receptor family, in inflammatory homeostasis is underscored by the observation that mutations in the NOD2 gene are associated with susceptibility to Crohn disease and Blau syndrome. This study was to investigate whether NOD2 contributes to renal injury in hHcys. We produced experimental hHcys by feeding mice with folate-free diets. Our results showed folate-free diets induced NOD2 expression. Furthermore, we found that NOD2 deficiency ameliorated hHcys-induced albuminuria, podocyte...
Han et al. NOD2 Mediates TRPC6 Expression and Activity

Injury, and glomerulosclerosis, indicating that NOD2 is an important element triggering renal injury in hHcys.

In this study, one of the most important findings is that we discovered a novel function of NOD2 in mediating TRPC6-dependent Ca\(^{2+}\) signaling in podocytes. Changes in podocyte function and structure are of pathogenetic relevance to human proteinuric kidney diseases. Several podocyte-associated proteins, including nephrin, podocin, and TRPC6, have emerged to provide critical insight into the pathogenesis of nephrotic syndromes. The discovery that gain-of-function mutations in the TRPC6 channel form a subset of familial forms of focal segmental glomerulosclerosis has focused attention on the basic cellular physiology of podocytes. In addition, elevated expression levels of TRPC6 in the podocyte slit diaphragm have been identified to cause podocyte injury and human renal diseases, such as minimal-change disease and membranous glomerulonephritis, suggesting that TRPC6 also plays a pivotal role in nongenetic forms of glomerular disease. Here, we found that TRPC6 expression was enhanced in the kidney from mice with hHcys, which was attenuated by NOD2 deficiency, indicating the role of NOD2 on the regulation of TRPC6 in hHcys.

As a Ca\(^{2+}\)-permeable channel, excess activation of TRPC6 results in a massive increase in intracellular Ca\(^{2+}\) release. Recent studies have examined the role of Ca\(^{2+}\) dynamics in podocyte function and their possible contributions to glomerular disease. A central working hypothesis has been that podocyte foot process effacement is mediated by rearrangement of the actin cytoskeleton and cell apoptosis via Ca\(^{2+}\) signaling. In this study, full characterization of TRPC6 channel gating and intracellular [Ca\(^{2+}\)] measurement provided convincing evidence, indicating that Hcys induced intracellular Ca\(^{2+}\)...
overload through, at least in part, NOD2-mediated TRPC6 expression and activity. We further demonstrated that NOD2 was associated with changes in cytoskeleton distribution and podocyte apoptosis via TRPC6.

Nephrin is essential for the maintenance of podocyte integrity; mutations in this gene cause the most severe form of congenital nephrotic syndrome. Although acting as an adhesion molecule, nephrin is a pivotal regulator of podocyte intracellular signaling, which is important in downstream phosphatidylinositol 3-kinase signaling and a negative regulator of nuclear factor-κB activity. TRPC6 interactions with nephrin near the slit diaphragm may underlie regulation of gating and trafficking of these channels and studies have reported that nephrin plays a role in modulating TRPC6 channel opening. Moreover, the absence of nephrin as shown in neonatal nephrin−/− mice led to increased expression of TRPC6 in podocytes compared with podocytes of wild-type littermates. Considering our previous study showing that activation of NOD2 reduced nephrin expression, it would be of great interest to elucidate whether NOD2-nephrin signaling contributes to the regulation of TRPC6 channel.

In this study, although we focus on the role of NOD2 in mediating Ca²⁺ signaling in podocytes, we cannot exclude that NOD2 in infiltration of immune cells or other renal resident cells also plays an important role in hHcys-induced renal injury. NOD2 in inflammatory cells may induce the activation of nuclear factor-κB and trigger a cell signaling cascade mediated by the stress-activated protein kinases, leading to the increase in the production of proinflammatory mediators. The renal resident cells then respond by these signaling molecules, ultimately resulting in renal injury. In addition, TRPC channels have been reported to be associated with the production of proinflammatory cytokines and inflammation. A very recent study has demonstrated that TLR4 activation of TRPC6-dependent Ca²⁺ signaling mediates endotoxin-induced lung vascular permeability and inflammation. Strikingly, except NOD2-mediated Ca²⁺ signaling directly contributing to cytoskeleton rearrangement and podocyte apoptosis, our preliminary results indicate that knockdown of TRPC6 can also attenuate NOD2-induced production of proinflammatory mediators in podocytes (data not shown). Therefore, it is very possible that NOD2-mediated Ca²⁺ signaling and its
proinflammatory action may build a signaling network to modulate related signaling in producing renal injury in hHcys. Further detailed studies are required to pinpoint the precise mechanisms both in vitro and in vivo.

It should be noted that the current studies suggest a direct causal link between NOD2 and TRPC6 in podocytes. However, we cannot rule out the involvement of other glomerular components and cannot avoid that all cells in the body are affected by systemic NOD2 deletion, which can lead to complex phenotypes that confound interpretation. Therefore, a podocyte-specific knockout of NOD2 mice may be needed to further confirm the current findings.

Figure 3. Nucleotide-binding oligomerization domain containing 2 (NOD2)–mediated transient receptor potential cation channel 6 (TRPC6) was required for homocysteine (Hcys)–induced Ca²⁺ influx and currents in podocytes. A, The 1-oleoyl-2-acetyl-sn-glycerol (OAG)–induced Ca²⁺ response was determined by Fura-2 ratiometry in podocytes transfected with scramble, shRNA-NOD2 under L-homocysteine (L-Hcys) treatment, as well as summarized data of peak calcium influx. B, Representative OAG–induced TRPC6–mediated whole-cell currents measured from these treated podocytes and average normalized current amplitude measured at −80 mV (white bars) and 80 mV (black bars) from these cells. *P<0.05 vs scramble, #P<0.05 vs vehicle of Hcys or MDP treatment (n=8).

Figure 4. Transient receptor potential cation channel 6 (TRPC6) expression was associated with nucleotide-binding oligomerization domain containing 2 (NOD2)–reduced nephrin expression in L–homocysteine (L-Hcys)–treated podocytes. A, Representative Western blot gel documents and summarized data showing that Hcys reduced nephrin expression in podocytes. B, Representative Western blot gel documents and summarized data showing that MDP reduced nephrin expression in podocytes. C, Representative Western blot gel documents and summarized data showing that overexpression efficiency of nephrin by pcDNA3.1-NPHS1 transfection. D, Representative Western blot gel documents and summarized data showing that overexpression of nephrin attenuated Hcys–induced TRPC6 expression. *P<0.05 vs control, #P<0.05 vs vehicle of Hcys or MDP treatment (n=8).
Perspectives
The current findings add an additional downstream pathway to NOD2 signaling and establish a previously unknown function of NOD2 in mediating Ca2+ signaling, suggesting that TRPC6-dependent Ca2+ signaling is one of the critical signal transduction pathways that links innate immunity mediator NOD2 to podocyte injury in hHcy. In addition, TRPC6 has been linked to hypertension, cardiac hypertrophy, cardiac fibrosis, and neuronal degeneration. Therefore, pharmacological targeting of NOD2-mediated Ca2+ signaling pathways via TRPC6 at multiple levels may help design a new approach to develop therapeutic strategies for prevention of deterioration of kidney function and for the treatment of hHcy-associated end-organ damage.

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Disclosures
None.

References

Novelty and Significance

What Is New?
• The present study discovered a previously unknown function of nucleotide-binding oligomerization domain containing 2 (NOD2) in mediating Ca2+ signaling, suggesting that transient receptor potential cation channel 6-dependent Ca2+ signaling is one of critical signal transduction pathways that links innate immunity mediator NOD2 to podocyte injury.

What Is Relevant?
• Except for the role of NOD2 in innate immune and adaptive response, NOD2-mediated transient receptor potential cation channel 6 expression and activity importantly contribute to the initiation and development of glomerulosclerosis. This finding underlines the crucial role of NOD2 in the pathogenesis of podocyte injury and proteinuria.

Summary
NOD2-triggered transient receptor potential cation channel 6-mediated Ca2+ signaling is an early pathogenic event of podocyte injury, and the development of strategies that target the activation of podocyte NOD2 may be a promising therapeutic intervention to prevent hyperhomocysteinemia-associated end-stage renal disease.
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A Novel Role of NOD2 in Mediating Ca²⁺ Signaling:
Evidence from NOD2-regulated Podocyte TRPC6 Channels in
Hyperhomocysteinemia

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Running title: NOD2 mediates TRPC6 expression and activity

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Materials and Methods

Animal studies: Twelve-week–old NOD2−/− and wild-type C57BL/6 (WT) mice were purchased from the Jackson laboratory (Bar Harbor, ME). To speed up the damaging effect of Hcys on the glomeruli, the mice were uninephrectomized. After a 1-week recovery period from uninephrectomy, the mice were maintained on a regular chow or a folate-free (FF) diet (Dyets Inc, Bethlehem, PA) to induce hHcys for 10 weeks. All protocols were approved by Institutional Animal Care and Use Committee of Shandong University and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. During recording days, blood and 24-h urine samples were collected. Plasma total Hcys was measured by fluorescence HPLC analysis as described1. Urinary albumin excretion was measured using a mouse albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). After blood samples were collected, the mice were euthanized and renal tissue was harvested for biochemical and molecular analysis and morphological examinations as we described previously2. Electron microscopic analysis was performed by the electron microscopic core lab of Shandong University.

Cell culture and treatments: Murine podocytes were cultured as described previously3. L-Hcys (25 to 100 µmol/L) and endotoxin-free NOD2 ligands L18-MDP (active L-D isomer, 2 µg/mL) were used to treat podocytes for 24 hours in this study.

Gene transfection: Small interference RNA to NOD2 (siRNA-NOD2) or TRPC6 (siRNA-TRPC6) was synthesized and constructed into pRNAT-U6.1/Neo to get shRNA-NOD2 or shRNA-TRPC6 by Biomics Biotechnologies Co., Ltd. (Nantong, Jiangsu, China). Full-length nephrin plasmid (pcDNA3.1-NPHS1) was the generous gift from Dr. Guohua Ding, Division of Nephrology, Renmin Hospital of Wuhan University, China. In these experiments, plasmids were transfected into podocytes by Lipofectamine 2000 (Invitrogen, Gaithersburg, MD). The DNA target sequence for shRNA-NOD2 (5'-GCTCTGTATTTGCGAGATATT-3') was designed based on the core sequence of mouse NOD2 cDNA (Accession number NM_145857). The target sequence for shRNA-TRPC6 (5'-CAGAAUAGCUUACAUUUUA-3') was designed based on the core sequence of mouse TRPC6 cDNA (Accession number NM_013838.2).

RNA extraction and RT-PCR: Total RNA was isolated from the kidney or cells and mRNA levels were analyzed by real-time RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA)4.

Western blot analysis: Total cellular lysates preparation and Western blot analysis were performed as described previously5. Antibody to NOD2 was from ProteinTech Group (Chicago, IL); Antibodies to nephrin and TRPC6 were from Abcam (Cambridge, MA). To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein β-actin (ProteinTech Group).

Immunofluorescence: Immunofluorescent staining was performed using a modified protocol as...
described previously. Sections were incubated with different primary antibodies, and were subsequently incubated with secondary fluorescein isothiocyanate (FITC)–conjugated antibody (1:100, Invitrogen, Carlsbad, CA, USA) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibody (1:100, Invitrogen). Nuclei were counterstained with DAPI (Roche, Mannheim, Germany, 1:2000). Images were obtained by confocal laser-scanning microscopy using a LSM780 laser scanning confocal microscope (ZEISS, Germany) equipped with a Plan-Apochromat 63×/1.4 objective. Images were assembled in the Adobe Photoshop 7.0 software package.

**Detection of cytokines and chemokines:** Chemokines and cytokines in the kidney were measured with enzyme-linked immunoabsorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

**Flow cytometry:** Cell apoptosis was determined by propidium iodide (PI)-Annexin V staining as described.

**Ca^{2+} fluorescent imaging measurement:** Imaging of intracellular Ca^{2+} release with Fura-2 was performed as described. Briefly, podocytes were loaded with 5 µmol/L of the calcium-sensitive, cell-permeable, intracellular fluorescence dye Fura-2-AM (Sigma-Aldrich). Fluorescence was measured using fluorescence spectrophotometry. TRPC6 was activated by 100 µmol/L 1-oleoyl-2-acetyl-sn-glycerol (OAG) and calcium influx was measured. Fluorescence data are presented as a 340/380 nm ratio and the ratio of Ca^{2+}-dependent fluorescence intensity to that at the basal level was quantified as the intracellular Ca^{2+} response. Data from selected cell populations were averaged, and statistical analysis was performed on multiple experiments.

**Electrophysiology:** Whole cell recordings were made using a modification of methods as described previously by an EPC-10 patch-clamp amplifier controlled by the Pulse software 8.8 (HEKA Elektronic, Germany). Briefly, the bath solution consists of 135 mmol/L NaCl, 5 mmol/L CsCl, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, and 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4. Pipette solutions consists of 2 mmol/L MgCl₂, 0.3 mmol/L CaCl₂, 145 mmol/L caesium methanesulfonate, 10 mmol/L HEPES, and 10 mmol/L EGTA, pH 7.2. 100 µmol/L OAG was used as an agonist for TRPC6 activation. For all whole-cell recordings, borosilicate patch pipettes had a resistance of 3-5 MΩ. The currents were induced by a 200-ms voltage ramp protocol (1 mV/ms, from 100 mV to -100 mV) every 3 seconds from a holding potential of 0 mV; 100µM OAG was applied for about 300 seconds. Analysis and display of patch-clamp data was performed with Igor Pro software version 6.0 (WaveMetrics, Lake Oswego, OR). Average current amplitudes at ~80 mV and 80mV were compared using the Student’s t-test.
References


Figure S1. NOD2-mediated TRPC6 channels were associated with rearrangement of the actin cytoskeleton and apoptosis in podocytes: A. Representative confocal microscopic images of F-actin using TRITC staining in podocytes with different treatments. B. Summarized data showing podocyte apoptosis determined by flow cytometric analysis. * P<0.05 vs. control, # P<0.05 vs. vehicle of Hcys or MDP treatment (n=8).
Figure S2. Decreased nephrin expression was recovered in NOD2−/− mice with hHcys. A. Representative confocal microscopic images showing nephrin expression in glomeruli from different group of mice with hHcys, synaptopodin was used as podocyte marker. B. Representative Western blot gel documents and summarized data showing that decreased nephrin expression was recovered in NOD2−/− mice with hHcys. *P<0.05 vs. control, # P<0.05 vs. WT mice with hHcys (n=10).