Clinical and epidemiological studies have demonstrated that hyperhomocysteinemia (hHcys) is an important independent risk factor for the development of cardiovascular disease and end-stage renal disease. Although multiple approaches lowering the levels of homocysteine have been used in experimental studies and clinical trials, there is no effective therapy available to fully prevent homocysteine-induced injury. Therefore, identifying key molecules in the pathogenic pathways may provide clues to develop new therapeutic strategies for the treatment of hHcys-associated injury beyond lowering the plasma homocysteine levels. In this study, we found that the levels of progranulin (PGRN), an autocrine growth factor, were significantly reduced in the kidney and heart from a mouse model of hHcys. We further observed that in hHcys, PGRN-deficient mice significantly exacerbated cardiorenal injury as evidenced by higher levels of urinary albumin excretion, more severe renal morphological injuries, including pronounced glomerular basement membrane thickening and podocyte foot process effacement, and adverse myocardial remodeling versus wild-type mice. Mechanistically, we found that PGRN-mediated Wnt/β-catenin signaling was one of the critical signal transduction pathways that links homocysteine to cardiorenal injury. Importantly, we finally provided direct evidence for the therapeutic potential of PGRN in mice with hHcys by pretreatment with recombinant human PGRN. Collectively, our results suggest that PGRN may be an innovative therapeutic strategy for treating patients with hHcys.

Abstract—Hyperhomocysteinemia (hHcys) is an important independent risk factor for the development of cardiovascular disease and end-stage renal disease. Although multiple approaches lowering the levels of homocysteine have been used in experimental studies and clinical trials, there is no effective therapy available to fully prevent homocysteine-induced injury. Therefore, identifying key molecules in the pathogenic pathways may provide clues to develop new therapeutic strategies for the treatment of hHcys-associated injury beyond lowering the plasma homocysteine levels. In this study, we found that the levels of progranulin (PGRN), an autocrine growth factor, were significantly reduced in the kidney and heart from a mouse model of hHcys. We further observed that in hHcys, PGRN-deficient mice significantly exacerbated cardiorenal injury as evidenced by higher levels of urinary albumin excretion, more severe renal morphological injuries, including pronounced glomerular basement membrane thickening and podocyte foot process effacement, and adverse myocardial remodeling versus wild-type mice. Mechanistically, we found that PGRN-mediated Wnt/β-catenin signaling was one of the critical signal transduction pathways that links homocysteine to cardiorenal injury. Importantly, we finally provided direct evidence for the therapeutic potential of PGRN in mice with hHcys by pretreatment with recombinant human PGRN. Collectively, our results suggest that PGRN may be an innovative therapeutic strategy for treating patients with hHcys. (Hypertension. 2017;69:259-266. DOI: 10.1161/HYPERTENSIONAHA.116.08154.)

Key Words: cardiac hypertrophy ■ glomerular filtration barrier ■ homocysteine ■ PGRN ■ Wnt pathway

Clinical and epidemiological studies have demonstrated that hyperhomocysteinemia (hHcys) is an important independent risk factor for the development of cardiovascular disease and end-stage renal disease. Although multiple approaches lowering the levels of homocysteine (Hcys) have been used in experimental studies and clinical trials, there is no effective therapy available to fully prevent Hcys-induced cardiac and renal injury. Therefore, identifying key molecules involved in the pathogenesis of hHcys will provide new therapeutic strategy for treating patients with hHcys.

Progranulin (PGRN), an autocrine growth factor, has been identified in various tissues and is involved in a diversity of physiological and pathological processes, including tissue development, host-defense response, insulin resistance, and modulation of inflammation. Recent studies highlight the protective role of PGRN in chronic inflammation. Tang et al have reported that PGRN directly binds to tumor necrosis factor receptors and disturbs the tumor necrosis factor–α–tumor necrosis factor receptor interaction. Administration of recombinant human PGRN (rPGRN) significantly alleviates inflammatory responses in rheumatoid arthritis animal models. In cardiovascular system, PGRN deficiency exacerbates atherosclerosis in apolipoprotein E knockout mice by modulation of local and systemic inflammation. Recently, we also demonstrate that PGRN serves as a negative regulator of immunity by regulation of nucleotide-binding oligomerization domain containing 2–mediated immune responses in acute kidney injury. However, it keeps unknown the role of PGRN in the pathogenesis of hHcys. In this study, we found that PGRN levels were significantly reduced in the kidney and heart in mice with hHcys by pretreatment with recombinant human PGRN. Collectively, our results suggest that PGRN may be an innovative therapeutic strategy for treating patients with hHcys.

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

Animals
Twelve-week-old male PGRN-deficient (Grn−/−) mice and wild-type C57BL/6 mice were purchased from the Jackson laboratory (Bar Harbor, ME).
Isolation of Glomeruli
Isolation of glomeruli was performed and confirmed as described, and the purity of glomeruli was estimated to be >98%. (Representative isolated glomeruli are shown in Figure S1 in the online-only Data Supplement.)

Echocardiography
Echocardiography was performed with a Vevo770 imaging system (VisualSonics, Toronto, Canada) using a 30-MHz high-frequency transducer.

Immunofluorescence Staining and Confocal Microscopy
Immunofluorescent staining and images obtained by an LSM780 laser scanning confocal microscope (ZEISS, Oberkochen, Germany) system were performed as described.

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

Results
PGRN Was Reduced in the Kidney and Heart From a Mouse Model of hHcys
Compared with controls, the levels of PGRN were reduced in the renal cortex, isolated glomeruli, and renal tubules from mice with hHcys; a more significant PGRN decrease was observed in glomeruli than in tubules from mice with hHcys (Figure 1A). To define the expression patterns of PGRN, we used double immunofluorescent staining for PGRN (green) and various markers for major renal parenchymal cells (red). Although PGRN was expressed in all these cells, a significant decrease in the expression of PGRN was observed in podocytes, glomerular endothelial cells (GECs), and distal tubules, and there were no obvious changes in other tubular...
areas, including proximal tubules and collecting ducts in mice with hHcys (Figure 1B). We also found that PGRN was downregulated in the heart from mice with hHcys (Figure 1C). Interestingly, unlike previous studies showing an increase in the plasma levels of PGRN in mice with acute renal injury8 or diabetic nephropathy,10 there were no significant changes in the plasma in mice with hHcys (Figure 1D). In vitro, PGRN was significantly reduced in podocytes, GECs, cardiomyocytes, and human cardiac microvascular endothelial cells in response to Hcys rather than proximal tubule epithelial cells and mesangial cells (Figure 1E), indicating the tissue- or cell-specific expression patterns of PGRN under different stress conditions, and a local rather than systemic effect of PGRN contributes to the regulation of cardiorenal function.

**PGRN Deficiency Exacerbated Cardiorenal Dysfunction in Mice With hHcys**

PGRN-deficient (Grn−/−) mice were fed with folate-free diets for 10 weeks to induce hHcys. As shown in Table, although PGRN deficiency had no effects on the levels of Hcys, glucose, and blood pressure compared with wild-type controls, PGRN deficiency aggravated renal injuries in mice with hHcys as evidenced by higher levels of urine albumin-to-creatinine ratio (Table) and glomerulosclerosis (Figure 2A). Transmission electron microscopy analyses further revealed more severe glomerular basement membrane (GBM) injuries, including pronounced GBM thickening and podocyte foot process effacement (Figure 2B). At the molecular levels, the reduced expression of podocyte markers, including nephrin, podocin, and synaptopodin (Figure 2C; Figure S2), and the loss of tight-junction proteins, including ZO-1 and occludin, in the kidney were further exacerbated by PGRN deficiency (Figure 2D). In addition, PGRN deficiency enhanced the levels of proinflammatory mediators (Figure 2E) in renal cortex, and a more significant increase was observed in isolated glomeruli than in tubular areas from mice with hHcys (Figure S3). In the heart, mice with hHcys showed more spherical left ventricle (LV) chamber shape, LV dilatation, and myocyte hypertrophy with increased cardiomyocyte width. PGRN deficiency exacerbated the altered LV shape and the enlarged cardiomyocytes (Figure 2F). At the end of week 10 after the mice were fed with folate-free diets, we examined cardiac functions by echocardiography. As shown in Table, Hcys significantly decreased cardiac function in wild-type mice as evidenced by decreases in ejection fraction % and fractional shortening %, which were aggravated in Grn−/− mice. We also measured cardiac remodeling–related parameters and found that PGRN deficiency induced more LV enlargement and wall thinning.

**Administration of rPGRN Protected Against Cardiorenal Dysfunction in Mice With hHcys**

The level of endogenous plasma PRGN in hHcys-treated groups was ≈0.6 μg/mL, which had no obvious difference compared with control mice. According to pharmacokinetic profile, Hcys-treated mice were administrated intraperitoneally rPGRN (5 mg/kg body weight) twice per week. The plasma PGRN levels could reach to >5 μg/mL after exogenous administration at 6 hours and keep the relative high level for at least 72 hours (Figure 3A). Although we found that administration of rPGRN after 12 hours slightly increased

<table>
<thead>
<tr>
<th>Variables</th>
<th>WT Mice</th>
<th>Grn−/− Mice</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normal Diet</td>
<td>FF Diet</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>29.55±1.60</td>
<td>27.12±0.52</td>
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<tr>
<td>Kidney weight, g</td>
<td>0.27±0.02</td>
<td>0.28±0.01</td>
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<td>Plasma total Hcys, μmol/L</td>
<td>4.65±0.42</td>
<td>14.22±1.21*</td>
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<td>Glucose, mmol/L</td>
<td>5.96±0.68</td>
<td>5.71±0.45</td>
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<tr>
<td>UACR, mg/g</td>
<td>34.48±5.49</td>
<td>65.83±3.23*</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>468.54±9.03</td>
<td>476.25±14.7</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
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<tr>
<td>Systolic</td>
<td>122.64±5.48</td>
<td>112.26±5.31</td>
</tr>
<tr>
<td>Diastolic</td>
<td>76.03±4.96</td>
<td>67.60±4.27</td>
</tr>
</tbody>
</table>

EF indicates ejection fraction; FF, folate free; FS, fraction shortening; Hcys, homocysteine; hHcy, Hyperhomocysteinemia; LVIdD, left ventricular internal dimension at the end diastole; LVpWd, left ventricular posterior wall thickness at the end diastole; UACR, urine albumin-to-creatinine ratio; and WT, wild-type. Values are mean±SEM for 10 mice in each group.

*P<0.05 vs WT normal diet mice.
†P<0.05 vs WT mice with hHcys.
the plasma glucose levels and came back to the normal level after 72 hours (Figure S4), rPGRN significantly attenuated the increase in proteinuria (Figure S4) accompanied by reversed mesangial expansion and ameliorated podocyte injury in mice with hHcys (Figure 3B; Figure S5), as well as the decreased production of proinflammatory mediators in the kidney (Figure 3C). In addition, the protective role of rPGRN in dilated cardiomyopathy was also verified. Treatment with rPGRN prevented the loss of cardiac function as reflected by the recovery of attenuated LV ejection fraction and LV fractional shortening, as well as improved LV enlargement and posterior wall thinning (Figure 3D through 3F; Figure S6). Meanwhile, we also found that rPGRN ameliorated diastolic dysfunction as indicated by decreased A wave and Aa velocity and increased E/A and Ea/Aa ratio in mice with hHcys (Figure S6). Collectively, our study demonstrated that rescue therapy with rPGRN promoted kidney and cardiac repairs in mice with hHcys.

Figure 2. Progranulin (PGRN) deficiency exacerbated cardiorenal dysfunction in mice with hyperhomocysteinemia (hHcys). A, Photomicrographs showing typical glomerular structure changes in different groups of mice. B, Representative photomicrographs and quantifications of mean glomerular basement membrane (GBM) thickness, mean foot process width, and the number of foot processes in different groups of mice by transmission electron microscopy (TEM) analyses. C, Representative immunofluorescent staining for podocyte markers, including nephrin, podocin, and synaptopodin in the kidney from different groups of mice. D, Representative Western blot gel documents and summarized data showing tight-junction protein levels, including ZO-1 and occludin in the kidney from different groups of mice. E, Relative levels of proinflammatory mediators in renal cortex from different groups of mice. F, Heart size (1 mm/unit on the scale ruler), representative histological cross-sectional anatomy at the papillary muscle level, and hematoxylin and eosin (H&E) staining of left ventricular (LV) longitudinal and transverse sections showing altered LV chamber and the cardiomyocytes. *P<0.05 vs control, †P<0.05 vs WT mice with hHcys (n=10). FF indicates folate free.
PGRN Negatively Regulated Wnt/β-Catenin Signaling Pathways

Among Wnt genes that are closely associated with renal injury, including Wnt1, Wnt2b, Wnt3, Wnt3a and Wnt4, we found that Wnt1 was upregulated in the kidney from mice with hHcys by mRNA (Figure 4A) and Western blot analyses (Figure 4B), which was further enhanced by PGRN deficiency. We also found that the upregulation of Wnt1 was mainly in glomeruli as observed by Western blot and immunofluorescent analyses (Figure S7). To examine the biological consequence of Wnt1 induction, we next investigated the activation of β-catenin. The amount of Hcys-induced dephosphorylated β-catenin was dramatically enhanced, as well as the total β-catenin expression levels. Because the phosphorylation of β-catenin by GSK-3β leads to its degradation via the ubiquitin/proteasome pathway, we next examined the cellular activity of GSK-3β. Our results showed that the increase in β-catenin levels was associated with an increase in phospho-GSK-3β (Ser9) levels, which inactivated GSK-3β. PGRN deficiency enhanced the effect of Hcys on phospho-GSK-3β, β-catenin in the kidney (Figure 4C). Consistently, administration of rPGRN inhibited Hcys-induced Wnt/β-catenin signaling in the kidney from mice with hHcys (Figure 4D).

PGRN Ameliorated Hcys-Induced Podocyte Dysfunction Through Inhibition of Wnt/β-Catenin Signaling Pathways

In consistent with the results from animal studies, in vitro, we found that Hcys-enhanced levels of active β-catenin were blockaded in podocytes by rPGRN treatment (Figure S8). As well as rPGRN (Figure 5A), Dkk1, an unique inhibitor of the canonical Wnt signaling pathway, recovered the expressions of nephrin and podocin in podocytes under Hcys treatment (Figure 5B). In addition, we further observed that both Dkk1 and rPGRN improved podocyte cytoskeleton rearrangement (Figure 5C) and inhibited Hcys-induced apoptosis (Figure 5D). The protective effects of rPGRN were also observed in GECs (Figure S9) and cardiomyocytes (Figure S10).

Discussion

This study for the first time demonstrates that PGRN protects against cardiorenal injury in mice with hHcys (Figure 5E). Considering that hHcys alone does not have effects on blood pressure and no significant changes in blood pressure were observed in wild-type and Grn−/− mice with hHcys, as well as mice with rPGRN treatment in this study, we suggest that the beneficial effects of PGRN in hHcys may be because of its anti-inflammatory role independent of blood pressure.

In the kidney, the glomerular filtration barrier comprises a fenestrated capillary endothelium, GBM, and podocyte slit diaphragm. Among them, GECs have a unique feature of fenestrations responsible for handling a large amount of filtration. Podocytes play an important role in GBM turnover, the maintenance of glomerular filtration barrier, and the regulation of glomerular filtration. Injuries of any of these components result in glomerular capillary permeability, thereby, leading to proteinuria and glomerular disease. In this study, we found that PGRN deficiency exacerbated inflammatory responses and podocyte fusion and effacement, destroyed GBM, and increased proteinuria in hHcys,
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providing direct evidence for the essential role of PGRN in maintaining renal function. In addition, studies have demonstrated that cardiovascular-related mortality and morbidity are associated with Hcys. Ventricular enlargement, myocardial hypertrophy, and adverse myocardial remodeling are principal features in the development of heart failure. Experimental animal studies have also demonstrated that the increased levels of Hcys contribute to cardiac hypertrophy, which is further confirmed in human heart. In this study, we found that PGRN deficiency exacerbated Hcys-induced LV dilatation and hypertrophy in mice with hHcys, indicating that PGRN is a central target molecule for maintaining cardiorenal functions.

Mechanistically, we found that PGRN negatively regulated Wnt/β-catenin signaling, which is an evolutionarily conserved developmental signaling cascade that exhibits a pivotal function in the regulation of a variety of biological processes in the tissue development and in the pathogenesis of human diseases. Although in the kidney, Wnt/β-catenin signaling is indispensable for nephron formation and becomes functionally silent after differentiation in the adult kidney, emerging evidence has indicated that Wnt/β-catenin is reactivated after renal injury and plays a critical role in promoting renal injury through effects on regulatory molecules, such as Snail1, TRPC6 (transient receptor potential channel 6), and angiotensin II type I receptor. In particular, both Wnt and β-catenin are specifically activated in podocytes from patients with focal segmental glomerulosclerosis and diabetic nephropathy, suggesting the clinical relevance of Wnt pathway to human proteinuric kidney diseases.

In cardiovascular system, Wnt signaling is centrally involved in myocardial remodeling after pathological injuries. Studies from Nakagawa et al suggest that the sustained activation of Wnt/β-catenin signaling in endothelial cells might be a cause of heart failure. Although functional genomic analyses have shown the involvement of Wnt signaling pathways in PGRN deficiency in human fetal neural progenitors, the contributions of Wnt signaling and the association between PGRN and Wnt cascade in hHcys-induced cardiorenal dysfunction keep unknown. In this study, we found that PGRN negatively regulated Wnt/β-catenin signaling pathways in hHcys, which had multiple functions in different cell types by regulation of cell fate determination, the expression of podocyte differentiation markers, and endothelial cell permeability, and so on. Collectively, these data clearly indicate that PGRN serves as a protective factor in hHcys, at least in part, by negative regulation of Wnt/β-catenin signaling (Figure 5E).

Considering that administration of rPGRN significantly alleviated inflammatory responses in rheumatoid arthritis animal models and both pretreatment with and delayed administration of rPGRN protected against acute kidney injury in mice, we further detected the effect of rPGRN in mice with hHcys.
One of the most striking findings was the therapeutic efficacy of rPGRN for the treatment of mice with hHcys. Administration of rPGRN attenuated disease progression and ameliorated Hcys-induced cardiorenal injury, indicating that PGRN is essential for conferring cardiorenal protection and may be an innovative therapeutic strategy for treating patients with hHcys.

**Perspectives**

Although several multicenter, prospective, case–control studies have demonstrated that Hcys is an independent risk factor of cardiovascular disease and end-stage renal disease, and a decrease in the plasma level of Hcys reduces the risk of coronary heart disease,\(^2\) the beneficial effects of Hcys-lowering therapy by daily folic acid or some B vitamins are not conclusive.\(^2\) The current findings indicate that PGRN-mediated Wnt/β-catenin signaling is one of the critical signal transduction pathways that links Hcys to cardiorenal injury, suggesting that PGRN may be an innovative therapeutic strategy for the treatment of hHcys-associated end-organ damage. In addition, endothelial dysfunction tends to be the initial event in macrovascular complications, such as peripheral arterial disease, coronary artery disease, and stroke, and in microvascular complications, such as nephropathy and retinopathy. In this study, we found that PGRN protected against endothelial injury not only in GECs but also in human cardiac microvascular endothelial cells, suggesting that PGRN may even have therapeutic potential in treating these diseases in a vast range of medical specialties.

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**Disclosures**

None.
References


What Is New?

- The present study explored a novel molecular mechanism indicating that progranulin (PGRN)-medicated Wnt/β-catenin signaling is one of the critical signal transduction pathways that links homocysteine to cardiorenal injury and further provided direct evidence for therapeutic potential of PGRN in hyperhomocysteinemia.

What Is Relevant?

- A better understanding of the mechanisms responsible for homocysteine-induced cardiorenal injury and identifying the protective role of PGRN in the pathogenic pathways may provide clues to develop new therapeutic strategies for the treatment of hyperhomocysteinemia-associated end-organ damage beyond lowering the plasma hyperhomocysteinemia levels.