Progranulin protects against renal ischemia/reperfusion injury in mice

Meng Zhou[1,9], Wei Tang[2,9], Yi Fu[1], Xiaoying Xu[2], Ziying Wang[1], Yi Lu[3], Feng Liu[3], Xinying Yang[4], Xinbing Wei[1], Yan Zhang[1], Juan Liu[2], Xue Geng[1], Chun Zhang[5], Qiang Wan[6], Ningjun Li[7] and Fan Yi[1,8]

[1]Department of Pharmacology, Shandong University School of Medicine, Jinan, China; [2]Department of Pathogenic Biology, Shandong University School of Medicine, Jinan, China; [3]Department of Biochemistry and Molecular Biology, Shandong University School of Medicine, Jinan, China; [4]Institute of Pharmaceutical Analysis, School of Pharmacy, Shandong University, Jinan, China; [5]Department of Nephrology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; [6]Department of Nephrology, Provincial Hospital Affiliated to Shandong University, Jinan, China; [7]Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA, USA and [8]Institute of Nephrology, Shandong University, Jinan, China

Progranulin (PGRN), an autocrine growth factor, has multiple physiological functions and is widely involved in the pathogenesis of many types of diseases. The pivotal anti-inflammatory function of PGRN in rheumatoid arthritis encouraged us to examine the role of PGRN in acute kidney injury (AKI). We found that levels of PGRN were significantly reduced in the kidney in a mouse model of renal ischemia/reperfusion injury. We also observed that PGRN deficiency ($Grn^{-/-}$ mice) significantly aggravated renal injury as evidenced by higher serum creatinine, more severe morphological injury, increased tubular epithelial cell death, and tubulointerstitial neutrophil and macrophage infiltration versus wild-type mice. *In vitro*, we found that recombinant human PGRN attenuated hypoxia-induced inflammatory actions and apoptosis in proximal tubule epithelial cells, at least in part associated with a nucleotide-binding oligomerization domain containing 2 (NOD2)-mediated immune response. Importantly, pretreatment with or delayed administration of recombinant human PGRN protected against or promoted recovery from renal ischemia/reperfusion injury in wild-type and $Grn^{-/-}$ mice. Similar protective effects were also found in cisplatin-induced AKI. Thus, our findings provide a better understanding of the biological activities of PGRN in the kidney and suggest that PGRN may be an innovative therapeutic strategy for treating patients with AKI.

Kidney International advance online publication, 21 January 2015; doi:10.1038/ki.2014.403

KEYWORDS: acute kidney injury; hypoxia; inflammation; pattern-recognition receptors; progranulin

Correspondence: *Fan Yi, Department of Pharmacology, Shandong University School of Medicine, 44#, Wenhua Xi Road, Jinan, Shandong, 250012, China. E-mail: fanyi@sdu.edu.cn*

[9]Co-first authors, equally contributed to this work.

Received 29 June 2014; revised 3 October 2014; accepted 16 October 2014

Acute kidney injury (AKI) is a serious clinical complication with high morbidity and mortality, and renal ischemia/reperfusion injury (IRI) is a major cause of AKI for patients subjected to kidney, liver, or aortic surgery. Although the pathogenesis of AKI is multifactorial, an increasing number of studies have revealed that a robust inflammatory process engaging both innate and adaptive immune responses is believed to cause the initial renal injury and mediate long-term structural changes including interstitial fibrosis or repair.[1,2] The innate immune system is responsible for initiating immune responses to resolve infections and repair damaged tissues, and is activated through pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).[3,4] Abnormalities in PRR activation lead to excessive inflammation.[5] However, there is no effective therapy available to treat or prevent ischemic AKI.[6,7] Therefore, it is essential to develop a new strategy to ameliorate renal injury after ischemia.

Progranulin (PGRN), also known as GRN-epithelin precursor, is a 593-amino-acid autocrine growth factor containing seven-and-a-half repeats of a cysteine-rich motif. PGRN is abundantly expressed in epithelial cells, macrophages, and is also expressed in a broad range of other tissues and cell types, such as skeletal muscle, adipose tissue, hematopoietic cells, T cells, and dendritic cells (DCs).[8] PGRN has a vital role in the maintenance and regulation of the homeostatic dynamics of the normal tissue development, regeneration, proliferation, and the host–defense response, and therefore it is widely involved in the pathogenesis of many kinds of diseases, including autoimmune disorders, atherosclerosis, and cancer.[9–11] Recent studies have highlighted the importance of PGRN in inflammatory responses, although the exact function of PGRN may vary depending on the stage and components involved in inflammation. In acute skin injury, PGRN increases the accumulation of neutrophils, macrophages, and blood vessels, and it acts directly on the

isolated dermal fibroblasts and endothelial cells to promote division, migration, and the formation of capillary-like tubule structures in the wound.[12] However, in acute infection, PGRN inhibits lipopolysaccharide-mediated cytokine release from macrophages.[13] PGRN has also exhibited a protective role in chronic inflammation.[10] Administration of recombinant human PGRN (rPGRN) significantly alleviated inflammatory responses in rheumatoid arthritis animal models.[10,14] In addition, a recent study has also shown that PGRN binds with Toll-like receptor 9 (TLR-9) and assists in the recruitment of CpG oligodeoxynucleotides in macrophages, a process that is essential for the elimination of infection, indicating that PGRN is critical in innate immunity against microorganisms.[15] Although the role of PGRN in the kidney is unclear, it is well known that acute and chronic immune responses elicited by ischemia are of importance in the functional deterioration of the kidney.[16] Therefore, the present study was designed to elucidate the function of PGRN in ischemic AKI. Our results showed that PGRN protected against renal IRI, and we identified for the first time that PGRN serves as a negative regulator of immunity, at least in part, by the regulation of nucleotide-binding oligomerization domain containing 2 (NOD2) mediated immune response. A better understanding of the function of PGRN will provide unexpected opportunities for developing new therapies for various inflammation-related diseases.

RESULTS

PGRN was significantly reduced in the kidney from a mouse model of renal IRI

Real-time polymerase chain reaction (Figure 1a) and western blot (Figure 1b) analyses showed that PGRN levels were time dependently reduced in the kidney after 30 min of renal ischemia followed by different time points of reperfusion, which was further confirmed in paraffin-embedded sections of kidney tissues by immunohistochemical staining (Figure 1c). Meanwhile, no staining with anti-PGRN in the kidney from PGRN-deficient mice ($Grn^{-/-}$ mice) was observed, indicating the specificity of the PGRN immunostaining. To further define the tubular segment specificity of PGRN expression in the kidney, we used double immunostaining for PGRN (green) and various tubular markers (red) in the kidney. The following segment-specific tubular markers were used: proximal tubule, aquaporin-1 (AQP1); distal tubule, calbindin D28k; and collecting duct, aquaporin-3 (AQP3). As shown in Figure 1d, PGRN was mainly expressed in proximal tubules and distal tubules. Interestingly, on the contrary, the plasma levels of PGRN were significantly increased compared with those of controls (Supplementary Figure S1), indicating the tissue or cell-specific expression patterns under stress conditions.

PGRN deficiency exacerbated renal injury after ischemia/reperfusion (I/R)

The $Grn^{-/-}$ mice were phenotypically normal and had no appreciable defect in kidney morphology and function. However, PGRN deficiency significantly aggravated renal injuries in AKI induced by I/R. Compared with controls, $Grn^{-/-}$ mice after I/R displayed higher elevation of serum creatinine (SCr, Figure 2a) and blood urea nitrogen (BUN, Figure 2b); more severe morphological injury, as evidenced by loss of the brush border, tubule dilatation, and cast formation (Figures 2c-d); and increased cell death, as demonstrated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (Figures 2e-f).

PGRN deficiency increased inflammatory responses in the kidney after I/R

PGRN deficiency enhanced the levels of proinflammatory mediators by enzyme-linked immunoabsorbent assay (ELISA, Figure 3a) and real-time polymerase chain reaction (Supplementary Figure S2) analyses. Consistently, neutrophil (Figures 3b and d) and macrophage (Figures 3c and e) accumulation was further increased in the kidney from ischemic $Grn^{-/-}$ mice, indicating that loss of PGRN signaling exacerbates renal inflammation.

PGRN decreased hypoxia-induced inflammatory responses and apoptosis in proximal tubule epithelial cells

In vitro, we used different approaches to mimic hypoxia condition, including chemical anoxia/recovery induced by incubating proximal tubule epithelial cells (HK-2) in glucose-free medium with antimycin A (AA)/2-deoxyglucose (2-DG) for ATP depletion (90 min, anoxia) and then in glucose-replete complete growth medium (recovery) (Figure 4a), oxygen–glucose deprivation (Figure 4b), and $CoCl_2$ treatment (Figure 4c); all of them reduced PGRN levels. To further investigate the function of PGRN, recombinant human PGRN (rPGRN) was prepared (Supplementary Figure S3) and used in this study. We found that rPGRN reduced the levels of proinflammatory mediators (Figure 4d), cell apoptosis (Figure 4e), caspase-3 activity (Figure 4f) and Bax/Bcl-2 ratio (Figure 4g) in HK-2 cells with AA/2-DG treatment.

PGRN negatively regulated NOD2-mediated signaling

Compared with wild-type (WT) mice, ischemic $Grn^{-/-}$ mice displayed higher levels of NOD2 in the kidney (Figure 5a). *In vitro*, we found that NOD2 was upregulated in HK-2 cells (Figure 5b) under hypoxia stress. Moreover, we treated HK-2 cells with MDP (a NOD2 agonist) for the activation of NOD2. It was found that MDP induced the activation of NF-κB signaling pathway, as documented by increased levels of NF-κB subunit phospho-p65, phospho-IκBα, IκBα degradation (Figure 5c), and the nuclear translocation of p65 (Figure 5d), which were attenuated by rPGRN treatment. We further observed that rPGRN inhibited the MDP-enhanced production of proinflammatory mediators (Figure 5e) and cell apoptosis (Figure 5f) in HK-2 cells.

Both pretreatment with and delayed administration of rPGRN protected against renal IRI in mice

The procedure of rPGRN treatment was summarized in Figure 6a. We found that both WT and $Grn^{-/-}$ mice