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Low molecular weight fucoidan protects renal tubular cells from injury induced by albumin overload

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Albuminuria is a causative and aggravating factor for progressive renal damage in chronic kidney disease (CKD). The aim of this study was to determine if low molecular weight fucoidan (LMWF) could protect renal function and tubular cells from albumin overload caused injury. Treatment with 10 mg/g bovine serum albumin caused renal dysfunction, morphological changes, and overexpression of inflammation and fibrosis associated proteins in 129S2/Sv mice. LMWF (100 mg/kg) protected against kidney injury and renal dysfunction with decreased blood creatinine by 34% and urea nitrogen by 25%, increased creatinine clearance by 48%, and decreased significantly urinary albumin concentration. *In vitro* proximal tubule epithelial cell (NRK-52E) model showed that LMWF dose-dependently inhibited overexpression of proinflammatory and profibrotic factors, oxidative stress and apoptosis caused by albumin overload. These experimental results indicate that LMWF protects against albumin overload caused renal injury by inhibiting inflammation, fibrosis, oxidative stress and apoptosis, which suggests that LMWF could be a promising candidate drug for preventing CKD.

Chronic kidney disease (CKD), which is defined by glomerular filtration rate less than 60 mL/min/1.73 m^2 or albuminuria more than 30 mg per day (or urinary albumin/creatinine ratio > 30 μg/mg), has become a public health issue. The global prevalence of CKD is 8~16%. CKD has been considered as an important independent risk factor for cardiovascular diseases and greatly increases risk and mortality of cardiovascular diseases[1]. Therefore, it is necessary to develop novel drugs to prevent and treat CKD.

Proteinuria or albuminuria is a common feature of CKD and often associates with glomerular dysfunction, tubular lesion and interstitial injury. Albuminuria is not only a marker of renal injury, but also a causative or aggravating factor for progressive renal damage[2]. Normally, most of filtered albumin (about 70%) is reabsorbed by the proximal tubules. Excessive reuptake of albumin can cause tubulointerstitial inflammation, oxidative stress, fibrosis, and tubular cell injury and death by activating a series of signaling pathways.

In animal models, albumin overload activates protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) pathways that lead to inflammation by increasing expression of fractalkine, monocyte chemotactic protein 1 (MCP-1) and RANTES (regulated upon activation, normal T-cell expressed and secreted)[3–6]. Inflammation and oxidative stress are key mediators in CKD[7]. Inflammation sets up the fibrotic stages through recruitment of inflammatory cells and activation of profibrotic factors. It has been found that albumin overload can induce overexpression of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF) and accumulation of extracellular matrix in proximal tubule cells[8,9]. Albumin can increase NADPH oxidase activity and generation of reactive oxygen species (ROS) in proximal tubule cells[10]. Moreover, tubular cell apoptosis, a common feature of end-stage renal disease, occurs in albumin overload models[11–14].

Fucoidan represents a family of L-fucose-enriched sulfated polysaccharides. Low molecular weight fucoidan (LMWF, <10 kD) is obtained by free radical, mineral acid, organic acid, and enzymatic hydrolysis of fucoidan[15]. LMWF has multiple biological activities including anti-coagulant, anti-cancer, anti-inflammation, and anti-oxidation[16–18]. Our previous studies found that LMWF protects kidney from renal ischemia-reperfusion

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Author Contributions

H.Z. and B.Y. are correspondence authors. Q.Z. provided the drug of LMWF. H.Z and B.Y. conceived the study. H.Z., B.Y. and Y.J. analyzed and interpreted the data. Y.J., Y.S., L.W. and Y.L. performed the experiments. Y.J., H.Z. and B.Y. wrote the manuscript. Final approval was given by all authors.

Additional Information

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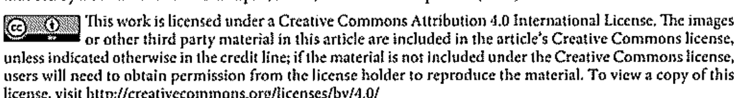

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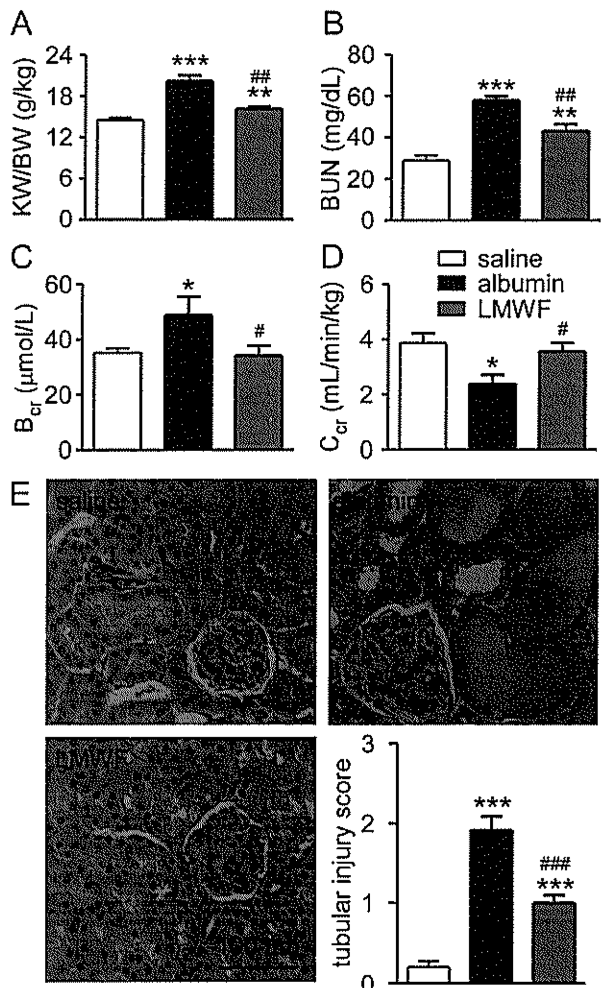

Figure 2. **Blood chemistry, kidney index and renal morphology of experimental mice.** Blood and kidney samples were collected and analyzed after albumin treatment for 3 weeks. (A) The ratio of kidney weight to body weight (KW/BW). (B) Blood urea nitrogen (BUN). (C) Blood creatinine (B_{cr}). (D) Creatinine clearance (C_{cr}). (E) Representative images stained with HE and tubular injury score. Values were means ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 *vs.* saline group. #P < 0.05, ##P < 0.01 and ###P < 0.001 *vs.* albumin group (ANOVA for A–E).

LMWF reduced proinflammatory factors and fibrosis related proteins in albumin treated mice. To test whether LMWF could influence inflammation and fibrosis in albumin overload mice, kidneys were collected after albumin treatment for 3 weeks and analyzed by Western blot. As shown in Fig. 3, expression levels of proinflammatory factors cyclooxygenase-2 (COX-2) and MCP-1 were significantly increased in albumin

cells and fibroblasts leading to tubulointerstitial inflammation and fibrosis. NF-κB is also an upstream regulator of inflammation and fibrosis[28]. As a result, LMWF could protect the kidney by reducing inflammation.

Our previous studies showed that LMWF can ameliorate diabetic nephropathy in GK rats and STZ-induced diabetic rats by inhibiting epithelial-mesenchymal transition and fibrotic process[29]. We found that LMWF significantly reduced fibrotic factors, such as CTGF, FN, and Col IV, which were increased by albumin overload.

Studies have demonstrated that LMWF has a great potential as an antioxidant *in vivo* and *in vitro*. LMWF could reduce MDA content and increase SOD activity in diabetic GK rats and acute renal ischemia-reperfusion injury[19,30]. Besides, it also directly inhibits ROS production induced by high glucose in cultured rat cardiomyocytes[30]. As far as we know, oxidative stress is a contributor to the progression and development of CKD. For containing large numbers of mitochondria, renal proximal tubular epithelial cells are susceptible to ROS[31]. In pathologic conditions, a surplus of ROS in tissue leads to oxidative stress with various injurious consequences such as inflammation and fibrosis[32]. Nox4 expressed abundantly in renal proximal tubule[33,34]. MDA is a prominent product of lipid peroxidation and also a biomarker for oxidative stress[35]. We found that LMWF might inhibit albumin-induced oxidative stress through suppressing generation of ROS, MDA, and expression of Nox4.

It is demonstrated that apoptosis is induced by albumin in glomeruli and tubulointerstitium in Wistar rats after uninephrectomy[36]. Albumin induces apoptosis mainly through the extrinsic pathway, the intrinsic pathway and the endoplasmic reticulum stress pathway[11,12,14,37]. It was reported that 30 TUNEL-positive glomerular parietal epithelial cells of 241 glomeruli counted are found in Balb/c mice treated with 10 mg/g BSA for 3 weeks[38]. It implies that the number of apoptotic cells constitute a little proportion in mice treated with 10 mg/g albumin for 3 weeks. In our study, apoptosis was not found in albumin overload mice by measuring expression of apoptosis related proteins. However, albumin treatment significantly increased apoptosis in NRK-52E cells exhibiting elevated apoptotic index and increased apoptosis-associated proteins expression, which were reduced by LMWF treatment. Besides, LMWF also decreased the phosphorylation of JNK and p53, which suggests that LMWF might protect renal tubule cells from albumin induced apoptosis at least partly through the extrinsic pathway.

Fucoidan is a group of large molecules and has low bioavailability. LMWF, small pieces of fucoidan, exhibits more preferable biological activity. We demonstrate that LMWF has excellent potential to prevent renal injury in albumin overload CKD model for the first time. However, we did not find the key modulatory factors to link all pathogenic processes in renal injury caused by albumin. LMWF may act in several ways to protect against renal damage. *In vivo* use of LMWF was safe for mice due to no influence on body weight and general condition. Fucoidan does not show toxicity *in vitro* and *in vivo*[39]. It was reported that the dose of LMWF up to 2000 mg/kg body weight in mice is safe and has no significant genotoxic concern[40].

In conclusion, our data indicate that LMWF may ameliorate albumin overload caused renal dysfunction and proximal cell damages mainly through inhibiting inflammation, fibrosis, oxidative stress and apoptosis. The experimental results suggest that LMWF may be a candidate drug for preventing and treating CKD.

Materials and Methods

Source of LMWF. LMWF was isolated from *L. japonica* commercially cultured in Qingdao, China, as described previously[41]. Its average molecular weight is about 7 kD determined by high performance steric exclusion chromatography analysis. LMWF (100 mg/mL) was dissolved in normal saline for animal treatment and in phosphate buffered saline (PBS) for cell incubation, and was filtered with 0.22 μm membrane filters.

Albumin solution. For animal treatment, BSA (A7906, Sigma, St. Louis, MO) was dissolved in normal saline and the final concentration was 330 mg/mL. For cell incubation, albumin (200 mg/mL) solution was prepared in PBS. Both solutions were filtered with 0.22 μm membrane filters. Albumin solution was detected by using Limulus reagent kit (Xmhsjc, China) and had little endotoxin.

Cell culture. Rat renal proximal tubule epithelial (NRK-52E) cells were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), 100 U/mL penicillin, 100 μg/mL streptomycin in an atmosphere of 5% CO_2/95% O_2 at 37 °C. At 70~80% confluence, the cells were changed to serum-free medium and incubated for additional 24 h. The cells were treated with different concentrations of albumin, LMWF or ERK inhibitor PD98059 (513000, Calbiochem) for various periods of time as scheduled.

TUNEL assay. NRK-52E cells were plated in 96-well culture plates. At 70~80% confluence, cells were incubated with 20 mg/mL albumin with or without LMWF at different concentrations (1, 5, 10, or 20 μg/mL) for 72 h. TUNEL assay was conducted using the *in situ* Cell Death Detection kit (Roche Applied Science) following the manufacturer's instruction. The images were captured by Leica fluorescence microscope (Germany). Positive staining in ten sections per well from three wells was quantified. The apoptotic index was defined as (number of apoptotic cells/total number of nucleated cells × 100).

Measurement of ROS. The formation of ROS was measured using DCFH-DA (2′, 7′-dichlorofluorescin diacetate) (D6883, Sigma, St. Louis, MO). NRK-52E cells were plated in 6-well dishes. At 70~80% confluence, cells were incubated with 20 mg/mL albumin with or without LMWF at different concentrations (1, 5, 10, or 20 μg/mL) for 24 h. Then the cells were treated with DMEM containing 20 μmol/L DCFH-DA. After incubation of 30 min at 37 °C, formation of ROS was detected. The images were captured by Leica fluorescence microscope (Germany).

Animal model. Male 129S2/Sv mice weighting 21~24 grams, at age of 9 weeks, were acquired from the Animal Center of Peking University Health Science Center. The mice were maintained on a standard diet and had free access to water. For experiment, mice were housed in cages in a light, temperature and humidity controlled

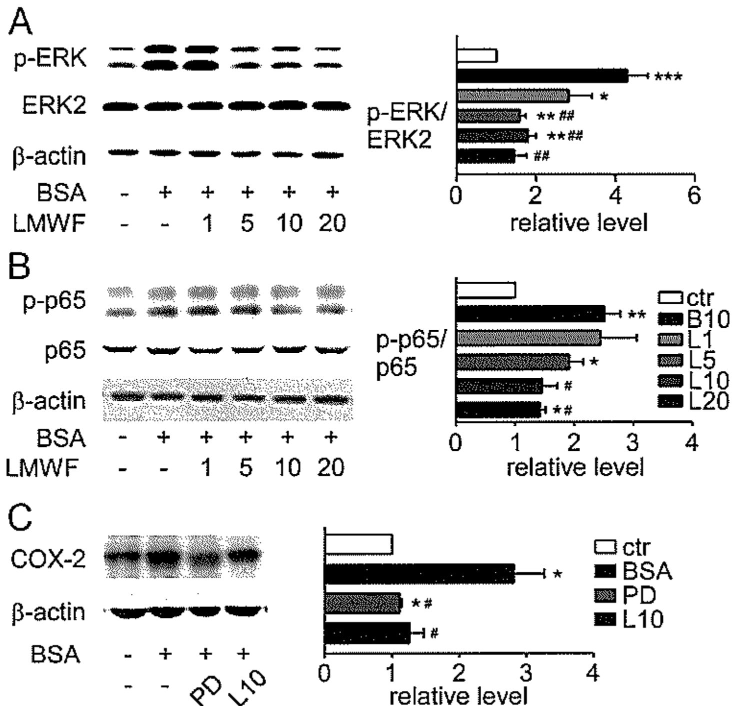

Figure 5. LMWF blocked NF-κB pathway activation induced by albumin in NRK-52E cells. (A) Expression levels of p-ERK, ERK2, and β-actin were detected by Western blot analysis. NRK-52E cells were pretreated with 1~20 μg/mL LMWF or PBS for 2 h and then were exposed to 10 mg/mL albumin or PBS for 15 min. Representative blots (left) and relative ratios of protein levels (right) are shown. (B) Expression levels of p-p65, p65, and β-actin in NRK-52E cells incubated with 1~20 μg /mL LMWF or PBS under 10 mg/mL albumin or PBS treatment for 48 h were determined by Western blot analysis. Left graph shows the representative blots and the right graph shows the density ratios. (C) Effect of ERK inhibitor PD98059 on albumin induced COX-2 expression. Cells were incubated with 10 mg/mL albumin or PBS in the presence or absence of LMWF or 20 μmol/L ERK inhibitor PD98059 (PD) for 48 h and were collected for Western analysis. Left graph shows the representative blots and the right graph shows the density ratios. Means ± SEM, n = 3~4. *P < 0.05, **P < 0.01 and ***P < 0.001 *vs.* PBS control group. #P < 0.05 and ##P < 0.01 *vs.* albumin group (ANOVA).

COX-2 and MCP-1 expression. LMWF also significantly reduced the overexpression of fibrosis-associated factors, CTGF and FN, in albumin treated cells in a dose-dependent manner.

LMWF suppressed NF-κB pathway activation induced by albumin in NRK-52E cells. Activated ERK could lead to NF-κB pathway activation that plays a central role in inflammation[3]. NRK-52E cells were pretreated with LMWF for 2 h and then incubated with albumin for 15 min. Western blot analysis showed that the expression levels of ERK2 did not change in each group. However, albumin increased significantly the phosphorylation of ERK that was significantly reduced dose-dependently by LMWF (Fig. 5A). As shown in Fig. 5B, LMWF inhibited significantly phosphorylation of p65 induced by albumin in a dose-dependent manner. To explore whether the activation of ERK signaling was involved in the increase of COX-2 expression, we detected the effect of ERK inhibitor PD98059 on albumin induced COX-2 expression. As shown in Fig. 5C, PD98059 at 20 μmol/L, as well as LMWF at 10 μg/mL, reversed the increased expression of COX-2 induced by albumin. The results suggest that COX-2 expression increased by albumin is partly dependent on the activation of ERK signaling pathway.