Less expression of prohibitin is associated with increased Caspase-3 expression and cell apoptosis in renal interstitial fibrosis rats

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SUMMARY AT A GLANCE

The authors reported a reduced expression of prohibitin and that this is associated with the increased Caspase-3 expression and cell apoptosis in renal interstitial fibrosis rats in a rat UUO model. These findings suggest that prohibitin plays a protective role against renal interstitial fibrosis and regulates the expression of Caspase-3, which induces cell apoptosis in UUO rats. The authors acknowledged the limitation in that they have not performed in vitro experiments to show that the causal effects of prohibitin on cell apoptosis using cultured renal cells cannot be established.
Renal interstitial fibrosis (RIF) is a common feature of chronic kidney disease, regardless of the aetiology of the primary renal syndrome. Tubule-interstitial changes, including tubular degeneration and interstitial cell infiltration, are a hallmark of common progressive chronic diseases that lead to renal failure. Elevation of transforming growth factor-β1 (TGF-β1) and accumulation of extracellular matrix (ECM) in renal interstitium are the most important features of RIF.

Unilateral ureteral obstruction (UUO), used extensively as a model of progressive RIF, results in rapid parenchymal deterioration. These alterations are also a common feature associated with a variety of kidney disorders, such as chronic kidney disease and end-stage renal disease, and the increase of renal tubular epithelial cell (RTEC) apoptosis is a critical detrimental event that leads to chronic kidney injury in association with renal fibrosis.

Prohibitin (PHB), a ubiquitous protein, plays a number of different molecular functions and is mainly located on the inner mitochondrial membrane and nuclei. PHB could play a pivotal role in the processes of cell apoptosis. The overexpression of PHB could protect the mitochondria from oxidative-stress-induced injury. When the function of mitochondria is confused, the expression of TGF-β1 will be upgraded and Caspase-3 expression will be increased. TGF-β1 is an important cytokine to induce the accumulation of ECM. The increased PHB could suppress renal interstitial fibroblasts proliferation and halt the progression of RIF. So, PHB might take part in the development and progression of RIF.

As mentioned above, we drew a hypothesis that there was an association between PHB and Caspase-3/cell apoptosis. This investigation was conducted to explore whether PHB was associated with the Caspase-3 expression/cell apoptosis in RIF rats induced by UUO.

**METHODS**

**Animal model**

The Animal Care and Use Committee of Guangxi Medical University approved all protocols. Twenty-four male Wistar rats (6 weeks old) were purchased from the Experimental Animal Center of Guangxi Medical University, Nanning, China. The rats were randomly divided into two groups: the sham operation group (SHO); n = 12, respectively. The ureter was ligated at approximately 1 cm below the renal hilum with 3-0 silk suture. The abdominal wound was closed, and rats were returned to the cages. Control rats underwent abdominal incision and approximation with no ligation of the ureter. Six rats of the two groups were killed at 14 days and 28 days after surgery, respectively, and their renal tissues were collected for histological and molecular biology determination.

**Renal morphology**

After 10% neutral formaldehyde fixation, the renal tissues were dehydrated through a graded ethanol series and embedded in paraffin. Sections were prepared on a microtome and stained with masson’s trichrome staining. Renal pathology was observed by light microscope, the severity of the renal lesion was presented by the RIF index. Blue granular or linear deposits were interpreted as positive areas for collagen staining. Semi-quantitative evaluation was performed by computer-assisted image analysis (DMR + Q550, Leica Co., Germany). The area of positive staining for fibrosis was measured at 400-fold original magnification in 50 fields (ignoring the fields containing glomerular parts) and expressed as a percentage of the total area. The extent of interstitial fibrosis was scored as absent (0), involving less than 25% of the area (1), involving 26–50% of the area (2), and involving greater than 50% of the area (3). RIF index was obtained by the formula as follow: GSI = (0 × n0 + 1 × n1 + 2 × n2 + 3 × n3)/(n0 + n1 + n2 + n3) = (0 × n0 + 1 × n1 + 2 × n2 + 3 × n3)/50. All the fields were selected from coded sections for each rat at random and the scores obtained by two investigators were averaged.

**Apoptosis assay**

Cell apoptosis was examined by the TdT mediated dUTP nick end labelling (TUNEL) assay (Roche Inc., Basel, Switzerland) as described previously. Six slides from each kidney were evaluated for percentage of apoptotic cells by using the TUNEL assay. Then 10 watch fields, which didn’t include the glomerular parts, were chosen at random under a microscope on each section. Brown staining of cell nuclei was considered as apoptotic cells. Positive brown cells and total cells were counted. The formula for apoptosis index as the indicator of apoptosis was as follows: cell apoptosis index = positive cells/total cells × 100%. The scores obtained by two investigators were averaged.

**Immunohistochemical analysis of the protein expressions of PHB, Caspase-3, TGF-β1, collagen-IV and fibronectin**

Renal tissue fixed with 4% buffered paraformaldehyde was embedded in paraffin, and 4 μm thick sections were stained. The positive area was measured quantitatively using a computer-aided manipulator (DMR + Q550, Leica Co., Germany). For immunohistochemical analysis of PHB, Caspase-3, TGF-β1, collagen-IV (Col-IV) and fibronectin (FN), the sections were deparaffinized, washed with phosphate-buffered saline (PBS), and treated with 3% H2O2 in methanol for 10 min. All sections were then incubated with anti-PHB antibody (1:300) (Neomarker Lab., Co., USA), anti-Caspase-3 antibody (1:200) (Thermo Fisher Scientific, Co., Runcorn, UK), anti-TGF-β1 antibody (1:100) (Zhongshan, Co., Beijing, China), anti-Col-IV antibody (ready-to-use kit) (Bo Shide, Co., Wuhan, China) and anti-FN antibody (1:50) (Zhongshan, Co., Beijing, China), respectively. After incubation with second antibody immunoglobulin (Shanghai Changdao, Co., Shanghai, China), the sections were stained with diaminobenzidine (Maixin Bio, Co., Fuzhou, China). The positive area of PHB, Caspase-3, TGF-β1, Col-IV or FN in renal tissue was measured. During evaluation of the interstitial areas, fields containing glomerular parts were ignored. All of the
PHB with Caspase-3/cell apoptosis

Fig. 1: Statistical parameters in two groups. *P < 0.01 compared with SHO. Col-IV, collagen-IV; FN, fibronectin; GU, model group subjected to unilateral ureteral obstruction; PHB, prohibitin; RF, renal interstitial fibrosis; SHO, sham operation group; TGF-β1, transforming growth factor-β1.
Renal morphology was normal in sham group (A1 and A2). Renal tubular structure was severely, collapsed lumen, diffusive infiltration of fibroblast in renal interstitium, and collagen formation in the majority of extracellular matrix in GU group (blue = collagen) (A3 and A4), especially in A4. Cell apoptosis (SHO: B1: 14 days, B2: 28 days; GU: B1: 14 days, B2: 28 days) and representative samples of immunohistochemical stainings for prohibitin (PHB) (SHO: C1: 14 days, C2: 28 days; GU: C3: 14 days, C4: 28 days), Caspase-3 (SHO: D1: 14 days, D2: 28 days; GU: D3: 14 days, D4: 28 days), transforming growth factor-β1 (TGF-β1) (SHO: E1: 14 days, E2: 28 days; GU: E1: 14 days, E2: 28 days), collagen-IV (Col-IV) (SHO: F1: 14 days, F2: 28 days; GU: F3: 14 days, F4: 28 days), and fibronectin (FN) (SHO: G1: 14 days, G2: 28 days; GU: G3: 14 days, G4: 28 days) were observed in two groups. PHB protein expression was found at normal renal tissues with a positive distribution in interstitial cells and tubular epithelial cells (C1 and C2). However, the staining for PHB was mainly located in renal tubular epithelial cell (RTEC). Furthermore, the expression of PHB was markedly downregulated in damaged interstitial and tubular epithelial cells in the GU group (C3 and C4), especially in C4. The staining for PHB was mainly located in RTEC (especially in distal tube cells). Positive stainings (in brown) for TGF-β1, Col-IV and FN were strong in the area of extracellular matrix in the GU group than those in the SHO group, especially at 28 days of the GU group. The staining for Caspase-3 in the GU group (D3 and D4) was much more marked when compared with that in the SHO group (D1 and D2), especially in D4. Caspase-3 was also mainly located in the RTEC and the apoptotic cell in our observation was mainly derived from RTEC. GU, model group subjected to unilateral ureteral obstruction. Magnification x400.

Real time reverse transcription polymerase chain reaction to detect PHB mRNA expression in renal tissue

Renal tissue was homogenized and total RNA was extracted with TRIzol (Beijing Tiangen, Co., China). Ultraviolet spectrophotometer measuring absorbance, agarose gel electrophoresis confirmed that there had been no degradation of RNA by visualizing the 18S and 28S RNA bands under ultraviolet light. Primers were designed according to primer design principles by Primer Premier 5.0. The primers for PHB and internal control β-actin were as follows: F 5′-TGGCGTTAGCGGTACAGGAG-3′ and R 5′-GAGGATCCGTAGTGTATGTGAC-3′ for PHB; F 5′-GCCCCCTGAGGACACCCTG-T3′ and R 5′-ACGCTCGTGGTACAGATCTCA-3′ for β-actin. One microgram total RNA from the renal tissue of each rat was reverse transcribed into cDNA with an ExScript RT reagent kit (Takara Biotechnology, Co., Dalian, China). PHB and β-actin were amplified with SYBR Premix Ex Taq (Beijing Tiangen, Co., China). Gene expression of β-actin was also measured in each sample and used as an internal control for loading and reverse transcription efficiency. The analysis for each sample was performed in triplicate. The average threshold cycle (Ct, the cycles of template amplification to the threshold) was worked out as the value of each sample. The data for fold change was analyzed using $2^{-\Delta \Delta Ct}$. For example, the ΔCt for PHB mRNA expression in GU group at 14 days was as follows: $\Delta C_{\text{GU}} = (C_{\text{TMB}} - C_{\text{Tbeta-actin}})_{14\text{day, GU group}}$ and the fold change for PHB mRNA expression in GU group in 14 day was $2^{-\Delta \Delta C_{\text{PHB}}}$.

Statistical analysis

The data were shown as mean ± standard deviation (SD). Independent-Samples T-test was performed to determine the differences between the SHO group and GU group, and the Pearson’s correlation coefficients were used to determine the relationships between the indicators for detection. A value of $P < 0.05$ was considered as significant. Statistical analysis was performed using the statistical package for social studies SPSS version 13.0 (SPSS, Chicago, IL, USA).

RESULTS

Renal morphology

More collagen deposition, fibroblast proliferation and diffuse lymphocyte filtration in the renal interstitium of GU group were observed when compared with those in the SHO group (Fig. 2). The index of RIF in GU was notably elevated when compared with that in SHO ($P < 0.01$; Fig. 1).

Cell apoptosis

The staining for cell apoptosis was significant in renal interstitium in the GU group than that in the SHO group (Fig. 2), especially at 28 days, and the cell apoptosis index was significantly increased in the GU group when compared with that in SHO ($P < 0.01$, Fig. 1). Interestingly, the apoptotic cell in our observation was mainly derived from RTEC (Fig. 2).

Protein expression of PHB, Caspase-3, TGF-β1, Col-IV or FN

When compared with those in the SHO group, in the GU group, the protein expression of PHB in renal interstitium was significantly weakened ($P < 0.01$, Figs 1,2) and protein expressions of Caspase-3, TGF-β1, Col-IV and FN in renal interstitium were significantly increased (all $P < 0.01$, Figs 1,2). PHB and Caspase-3 were mainly located in the RTEC in our observation (Fig. 2).

mRNA expression of PHB

Renal tissue of the GU group showed consistently lower PHB mRNA expression, when compared with that in SHO (9 weeks: SHO vs GU = 1.023-fold vs 0.372-fold, 13-week: SHO vs GU = 1.015-fold vs 0.280-fold; all $P < 0.01$; Fig. 1).

Correlation analysis

There was a negative correlation between PHB protein and index of RIF, cell apoptosis index, or protein expression of...
Caspase-3, TGF-β1, Col-IV or FN (r = -0.825, -0.886, -0.863, -0.817, -0.948, -0.953; each P < 0.01).

DISCUSSION

Renal interstitial fibrosis, associated with extensive accumulation of ECM constituents in the cortical interstitium, is directly correlated to progression of renal disease. Overexpression and deposit of ECM, such as Col-IV and FN, are the important characteristics of RIF. The impaired RTEC plays a crucial role in the progress of RIF. Of all the cytokines and growth factors, TGF-β1 plays the most important role when compared with others, and the increased expression of TGF-β1 is closely correlated with the development of RIF. TGF-β1 is known to be one of the major mediators, which leads to RIF by inducing the production of ECM (Col-IV and FN) in renal interstitium. So, TGF-β1, Col-IV and FN are the important indicators to evaluate the grade of RIF lesion and the progression of RIF. Caspase-3 is a pivotal effector of the apoptosis machinery and Caspase-3 activity is associated with cell apoptosis. The elevation of cell apoptosis is associated with the development of RIF. In this investigation, those indicators were evaluated.

Prohibitin is regarded as an apoptosis-regulating protein. The PHB might play a protective role against the injury in cells or tissue in some studies. Liu et al. conducted a study in cardiomyocytes and their data indicated that PHB could protect the cardiomyocytes from oxidative stress-induced damage, and that increasing PHB content in mitochondria constituted a new therapeutic target for myocardium injury. Muraguchi et al. performed an investigation in H9C2 cardiomyocytes and found that PHB might function as a survival factor against hypoxia-induced cell death. Ko et al. reported that hepatocyte-specific PHB deficiency resulted in marked liver injury, oxidative stress, and fibrosis with development of hepatocellular carcinoma, suggesting that PHB was a tumour suppressor in hepatocytes. The results from those studies mentioned above drew a consistent conclusion that PHB could protect the cells or tissue from reactive oxygen species (ROS) induced injury.

There were some observations reported that the PHB might be observed in renal tissue and these studies found that PHB might play a protective role in kidney against renal disease. Guo et al. observed that PHB protein was positively expressed at normal renal tissues, strongly downregulated in renal biopsy specimens from patients, and negatively correlated with the degrees of tubulointerstitial lesions, and they also conducted a study in rat kidney fibroblasts cell line and found that the overexpression of PHB suppressed the renal interstitial fibroblasts proliferation and cell phenotypic change induced by TGF-β1. Wu et al. performed a study in rats with renal tubular atrophy and interstitial fibrosis induced by aristolochic acid and found that the expression of PHB protein was downregulated in renal tissue of rats. Quan et al. observed that the expression of prohibitin-2 (homologue of PHB147) was downregulated in RTEC stimulated by elevated uric acid, which might promote trans-differentiation of RTEC, and they also noted that prohibitin-2 was associated with RTEC apoptosis due to uric acid. Those reports consistently agreed that PHB was a protective factor, and Quan et al. found that prohibitin-2 was associated with RTEC apoptosis in vitro. It was similar to our result in vivo. However, there was not any investigation performed in vivo to report that there was an association between PHB expression and the expression of Caspase-3 or the cell apoptosis in renal interstitium of RIF rats. This study was performed to explore this association in RIF rats induced by UUO.

Results from our study showed that protein expression of Caspase-3, TGF-β1, Col-IV or FN, indexes of RIF and cell apoptosis were more markedly increased in the GU group than those in SHO group, especially at 28 days. We also found that the impaired RTEC was the main contributor for RIF progression in theUUO model. It could draw a conclusion that the RIF model induced by UUO in our study was successful. However, the pathological mechanism of RIF was not elucidated. In this study, we found that PHB was mainly located in RTEC and PHB expression was negatively correlated with protein expression of Caspase-3, TGF-β1, Col-IV or FN, index of RIF or cell apoptosis index. The PHB expression in the normal control group was more marked when compared with that in the GU group. In conclusion, PHB suppressed the development of RIF and alleviated the protein expression of Caspase-3, TGF-β1, Col-IV or FN, and weakened the indexes of cell apoptosis and RIF. As those mentioned above, PHB was associated with the expression of Caspase-3/apoptotic cell in renal interstitium of UUO rats.

Prohibitin expression (mRNA or protein) in SHO group was much more marked than that in the GU group in our observation. We speculated that the mechanism was as follows: The PHB expression in the GU group was weakened, which induced the generation of ROS. The increased ROS might upregulate the expression of TGF-β. The disorder of TGF-β might induce the expressions of Col-IV and FN, and overexpression TGF-β could upregulate the expression of Caspase-3. The increased Caspase-3 was associated with cell apoptosis. So, the over-accumulation of ECM was observed and index of RIF and the number of apoptotic cells were increased.

Interestingly, in our investigation, we found that PHB and Caspase-3 mainly located in RTEC, and the apoptotic cell was mainly derived from RTEC. We speculated that the injury of RTEC was an early event and might play a pivotal role in the progression of RIF in UUO rats. So, how to protect the RTEC against injury was very important in the prevention of RIF. More attention should be paid to the event of impaired RTEC in future study. Furthermore, in our study, we also found that the PHB mainly located in RTEC, and there was only a minimal expression in mesangial cells of glomerulus. The PHB expression in glomerulus was markedly weak when compared that in renal interstitium in UUO rats (figure and
data not shown). The location of PHB was similar to that in Guo et al. It might give us some new insights to explore the association of PHB with renal disease.

However, there was a limitation in our study. In this observational study, we only found that the PHB was associated with caspase-3 expression/cell apoptosis. Cell culture using RTEC in vitro and transfection with small inhibitory RNA of PHB to decrease the PHB gene expression might be needed in future to investigate the effect of PHB on caspase-3/cell apoptosis in UUO rats.

In conclusion, less expression of PHB was associated with the increased expression of Caspase-3/cell apoptosis in RIF rats, although the detailed mechanisms were not fully elucidated. So, how to upregulate the expression of PHB is very important for prevention of RIF, and PHB might be a potential therapeutic target for prevention of the cell injury. However, cells culture in RTEC and so on, and inhibition of signalling pathway of PHB need to be conducted to explore its detailed mechanism in the further.

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