Original Paper

Loss of the Protein Cystathionine β -Synthase During Kidney Injury Promotes Renal Tubulointerstitial Fibrosis

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Key Words

Renal tubulointerstitial fibrosis • Cystathionine β -synthase • Renal inflammation

Abstract

Background/Aims: Renal tubulointerstitial fibrosis (TIF) is the common pathway of progressive chronic kidney disease. Inflammation has been widely accepted as the major driving force of TIF. Cystathionine β -synthase (CBS) is the first and rate-limiting enzyme in the transsulfuration pathway. CBS is considered to play protective role in liver and pulmonary fibrosis, but its role in TIF remains unknown. The purpose of this study was to investigate the potential role and mechanism of CBS in renal inflammation and TIF. **Methods:** Renal function, tubulointerstitium damage index score, extracellular matrix (ECM) deposition, and the expressions of collagen I, collagen III, fibronectin, CD3, CD68, IL-1 β , TNF- α were measured in sham operation and unilateral ureteral obstruction (UUO) rats. Proteomics and gene array analysis were performed to screen differentially expressed molecules in the development of renal inflammation and TIF in UUO rats. The expression of CBS was detected in patients with obstructive nephropathy and UUO rats. We confirmed the expression of CBS using western blot and real-time PCR in HK-2 cells. Overexpression plasmid and siRNA were transfected specifically to study the possible function of CBS in HK-2 cells. **Results:** Abundant expression of CBS, localized in renal tubular epithelial cells, was revealed in human and rat renal tissue, which correlated negatively with the progression of fibrotic disease. Expression of CBS was dramatically decreased in the obstructed kidney from UUO rats as compared with the sham group (SHM). In addition, knocking down CBS exacerbated extracellular matrix (ECM) deposition, whereas CBS overexpression attenuated TGF- β 1-induced ECM deposition in vitro. Inflammatory and chemotactic factors were also increased in CBS knockdown HK-2 cells stimulated by IL-1 β .

Conclusions: These findings establish CBS as a novel inhibitor in renal fibrosis and as a new therapeutic target in patients with chronic kidney disease.

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Introduction

Chronic kidney disease (CKD) is a global public health issue with increasing attributable mortality and incidence and prevalence of end-stage kidney disease (ESRD), which leads to a rising burden worldwide [1]. Renal tubulointerstitial fibrosis (TIF) is the common pathway of progressive CKD with different etiologies. The characteristic pathological manifestations of TIF are the continuous deposition of extracellular matrix (ECM), such as fibronectin (FN) and collagens, accompanied by renal tubular atrophy and renal vascular remodeling, which leads to irreversible loss of nephron construction and function in the form of ESRD [2]. Thus, preventing or postponing renal tubulointerstitial fibrosis in the early stages of CKD is a critical strategy to prevent and treat ESRD [3].

Renal tubulointerstitial inflammatory responses have multiple consequences—some harmful while others healing, is one of the four pivotal cellular responses of renal fibrosis [4]. Renal fibrosis is almost always preceded by the infiltration of inflammatory cells, including lymphocytes, monocytes/macrophages, dendritic cells, and mast cells [5]. Although inflammation is an integral part of the host defense mechanisms in response to injury, non-resolving inflammation is a major driving force in the development of fibrotic disease [5-9]. The presence of factors, such as interleukin-1 β (IL-1 β), transforming growth factor- β_1 (TGF- β_1) et al. [10,11], while the renal inflammatory process persists, leads to development of interstitial fibrosis [12].

Renal fibrotic disorders are generally refractive to current therapies [3]. However, the concrete and fundamental mechanisms of renal inflammation remain elusive and effective drugs are scarce. Despite the importance of inflammation in renal tubulointerstitial fibrosis, the pathogenesis of renal inflammation has not been fully explained until now, it is believed to involve some novel proteins, mechanisms, or drug targets associated with the inflammation in the pathogenesis of TIF.

In the present study, we used isobaric tags for relative and absolute quantitation (ITRAQ) and gene array to analyze the differential expression of proteins and genes in the kidney of sham and unilateral ureteral obstruction (UUO) rats. Cystathionine β -synthase (CBS) was identified as one of the most significantly down-regulated proteins in the renal cortex of UUO rats. CBS, enriched in the outer cortex, especially in cells of the proximal convoluted tubule, is the first and rate-limiting enzyme in the transsulfuration pathway [13]. Homocysteine (Hcy) is detoxified through the transsulfuration pathway. It has been reported that the progression of renal inflammation and fibrosis is associated with abnormal metabolism of Hcy, a precursor of H₂S [14-16]. Recent human and animal studies have demonstrated the involvement of H₂S in various diseases, including renal inflammatory damage, TIF, and ESRD [17-19]. CBS deficiency may lead to pro-inflammatory states [20] such as vascular remodeling [21], impaired angiogenesis [22] and endothelial dysfunction [23]. However, whether CBS has certain roles in TIF is still unknown. In view of the importance of CBS in the transsulfuration pathway, the relationship among homocysteine, H₂S, renal inflammation, and TIF, we hypothesize that CBS may play important role in renal inflammation and TIF.

In this study, we found that CBS is down-regulated in rat and human TIF using a systemic biology method. Moreover, the decreased CBS expression was accompanied by infiltration of inflammatory cells, expression of IL-1 β , and deposition of ECM. In vitro cell studies in which CBS was overexpressed or knocked down highlighted the fundamental role of CBS in regulating the amount of fibronectin and collagen I associated with TGF- β_1 stimulation. In addition, we found that silencing CBS in vitro increased the expression of inflammatory factors (TNF- α , IL-6) and chemokines (MCP-1) in HK-2 cells stimulated by IL-1 β . Taken together, to our knowledge, this presents the first investigation of the mechanistic involvement of CBS in the progression of renal inflammation and interstitial fibrosis.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of the Xiangya Hospital (Central South University, Hunan, China, Permit number: 201312477). Human kidney sections were obtained from surgical specimens of obstructive nephropathy (without other disease) and non-tumor normal tissue adjacent to renal carcinoma. All patients signed official informed consent forms before operation, allowing retention of their information in the hospital database for research. All animals were subject to the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China guidelines. Moreover, all methods involving humans were tested in accordance with relevant guidelines and regulations.

Reagents and Antibodies

Recombinant Human Transforming Growth Factor β_1 (TGF- β_1) was purchased from Peprotech (USA). Recombinant Human Interleukin-1 β (IL-1 β) was purchased from Cell Signaling Technology (USA). The primary antibodies to CBS, fibronectin, collagen I and collagen III were purchased from Abcam (UK). CD68 antibody was purchased from AbD Serotec (UK), and CD3 antibody was purchased from BD Pharmingen (USA). The primary antibody to β -actin and secondary antibodies were purchased from Sigma-Aldrich (USA). The BCA Protein Assay Kit was purchased from Thermo (USA). IL-1 β and TNF- α enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D (USA). CBS, TNF- α , MCP-1, and IL-6 quantitative polymerase chain reaction (qPCR) primer was purchased from Generay Biotech Co., Ltd (Shanghai, CHINA). CBS siRNA and control siRNA were purchased from GenePharma Co., Ltd (Suzhou, CHINA). Expression plasmids encoding human PcDNA3.1 and PcDNA3.1-CBS were obtained from Open BioSystems (Changsha, CHINA). Lipofectamine™ 2000 Transfection Reagent was purchased from Invitrogen (USA).

Animals

Male Sprague-Dawley rats (180 to 200g) were purchased from Slac Laboratory Animal Center (Shanghai, CHINA). Animals were housed in a pathogen-free environment with a 12-hour light-dark cycle and unrestricted access to a standard rat chow diet and water. Rats were randomly assigned into the sham operation group and the UUO group, with 5 rats in each group. UUO was induced by ligation of the left ureter according to the procedure previously described [24,25]. All the rats were sacrificed at 14 days after the sham or UUO operation. Half of the left kidney was harvested and fixed in 10% neutral buffered formalin for pathologic examination and immunohistochemistry assay. The remaining half of the left kidney was preserved in liquid nitrogen for other analyses.

Quantitative proteomics using iTRAQ labeling and mass spectrometry

For iTRAQ screening, the renal cortexes isolated from SHM rats and UUO rats were lysed several times by freeze-thawing, followed by sonication. The extracted proteins were reduced and alkylated as described in the iTRAQ protocol (Applied Biosystems, Foster City, CA, USA). Isobaric tagging iTRAQ reagent (1 unit in ethanol) was added directly to the protein digest (70% ethanol final), and the mixture was incubated. Proteins were subjected to the conventional procedure with off-line 2D LC-MS/MS. Peptides were eluted with a linear gradient. Then, the LC-MS/MS analysis was conducted with samples injected into the trap column. To identify the peptides, database searches were performed with ProteinPilot 2.0.1 software (Applied Biosystems) using Paragon and ProGroup algorithms against the Swiss-Prot rat database [26]. We selected differentially expressed proteins using peptide confidence >95% criteria, and fold-changes <0.7 were set as cut-off values. The differentially expressed proteins were matched with the results of gene array through the code of SwissProt. If the protein levels reached the requirements of expression fold-changes <0.7 compared to SHM, we regarded it as a meaningful differential protein.

Gene Array

Total RNA was isolated from the renal cortex with Trizol Reagent. RNA was then purified with QIAGEN RNeasy mini columns (QIAGEN, USA) and re-suspended in 50 μ l diethylpyrocarbonate-treated water, according to the manufacturer's protocol. Microarray analysis was conducted on the two groups by a GeneChip® Rat Genome 230 2.0 Array (Rat 230 2.0) (Affymetrix, USA), which includes more than 31,000 probe sets representing about 28,700 well-substantiated rat genes. Average linkage clustering analysis,

based on a centered Pearson correlation, was implemented in the Cluster program by Java Tree view 1.0.12® software [27]. If the RNA levels reached the requirements of mRNA expression ratio-changes <0.4 compared to SHM, we regarded it as a meaningful differential gene.

Histopathological assessment and immunohistochemistry

Formalin-fixed kidneys were embedded in paraffin and prepared in 4 µm sections for hematoxylin-eosin (HE) and Masson trichrome staining. The tubulointerstitial damage index and tubulointerstitial fibrosis score were graded as previously described [28].

Immunohistochemistry (IHC) staining was performed in paraffin sections as previously reported [29]. Slides were incubated with a primary antibody against collagen I (1:500), collagen III (1:800), CD3 (1:200), CD68 (1:200), or CBS (1:500). The positive staining cell numbers of CD3 and CD68 in 20 randomly selected fields at 400x magnifications in the cortex and outer medulla were analyzed. The staining of collagen I, collagen III, and CBS was measured in 10 randomly selected fields at 200x magnifications in the cortex using computerized morphometry (ImagePro Plus 6.0 software, Media Cybernetics, Bethesda, MD). All of the analyses were conducted by two individual pathologists in a double-blind manner as previously described [30].

qPCR

Total RNA was extracted from kidney tissue using Trizol (Life Technologies, USA) reagent according to the manufacturer's instructions. The cDNAs were synthesized from 1 µg of the total RNA in a 20 µl reaction system using a RevertAid First Strand cDNA Synthesis Kit (Life Technologies, USA). The specific primers used for CBS and β-actin were designed based on the GenBank sequences and synthesized by Generay Biotech Co., Ltd (Shanghai, CHINA). The CBS primer pair consisted of forward primer 5'-GACCAAGTTCCTGAGCGACA-3' and reverse primer 5'-CGGAGGATCTCGATGGTGTG-3', the collagen α (1)I primer pair consisted of forward primer 5'-CAACAATTCCTGGCGTTACCTT-3' and reverse primer 5'-AAGCCCTGTATTCCGCTCCTT-3', the MCP-1 primer pair consisted of forward primer 5'-CTCAGCCAGATGCAATCAAT-3' and reverse primer 5'-GCTTCTTTGGGACACTTGCT-3', the TNF-α primer pair consisted of forward primer 5'-CCTGTGAGGAGGACGAACAT-3' and reverse primer 5'-TTTGAGCCAGAAGAGGTTGAG-3', the IL-6 primer pair consisted of forward primer 5'-CTTCGGTCCAGTTGCCTTCT-3' and reverse primer 5'-GTGAGTGGTGTCTGTGTGG-3'. β-actin was utilized as a loading control with forward primer 5'-TCAGGTCATCACTATCGGCAAT-3' and reverse primer 5'-AAAGAAAGGGTGTAACGCA-3'. The qPCR quantitation for individual target mRNA expression was performed with a CFX96 Real-time Detection System (Bio-Rad, USA) using a Thermo SYBR Green qPCR kit (Thermo, USA). The amount of specific mRNA in each sample was calculated based on the cycle threshold (CT) values, which were standardized with the quantity of the housekeeping gene β-actin. Further calculation and statistical analysis was based on the comparative 2^{-ΔΔCT} method [31].

Cell culture and transfection

Human proximal tubular epithelial cells (HK-2) were purchased from American Type Culture Collection (ATCC, Rockville, MD, U.S.). HK-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, USA) in a 5% CO₂ incubator at 37°C.

Cells were seeded into 12-well plates in complete culture medium and then simulated with 10 ng/mL of recombinant human TGF-β₁ for 12 h, 24 h, and 48 h respectively. Whole cell protein was collected for Western blot analyses.

For transfection experiments, cells were seeded into 12-well culture plates in complete medium containing 10% FBS without penicillin or streptomycin for 24 h. Transfections were performed with siRNA or overexpression plasmid using Lipofetamine 2000 according to the manufacturer's instructions. The humo CBS siRNA oligos were as follows: 5'-GGCGGCGAAGAACGAAAUUUAUUCGUUCUUCAGCCGCCTT-3'. Six hours after transfection, cell culture mediums were exchanged for fresh medium with or without 10 ng/mL of recombinant human TGF-β₁, and whole cell protein or RNA was collected at 48 h after transfection. To test the influence of CBS on inflammation, cells were incubated with 5 ng/mL of recombinant human IL-1β at 24 h after transfection with CBS siRNA, cell RNA was collected at 48 h after transfection.

Western blot analysis

Kidney tissue and cells were lysed with a buffer containing 20 mM Tris-HCl [pH 7.4], 4% sodium dodecyl sulfate, and 10% glycerol. Lysates were boiled at 100°C for 10 minutes. Protein concentration was determined using the BCA Protein Assay Kit according to the manufacturer's protocol. Proteins were separated by 8% or 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride (PVDF) membranes (Millipore, USA). The membranes were incubated in 1X TBS-T [0.05% Tween 20, 20 mmol/l Tris-HCl, and 150 mmol/l NaCl (pH 7.6)] containing 5% skim milk for 1 hour at room temperature before incubation overnight at 4°C with the primary antibodies against CBS (1:2000), FN (1:800), or β -actin (1:5000). Membranes were washed three times with TBS-T and then incubated with HRP-conjugated secondary antibodies at 1:5000 dilution for 1 hour at room temperature. Signals were developed by enhanced chemiluminescence (ECL) reagents (GE Healthcare, England) and X-ray film (Kodak, USA) as previously described [32]. Bands were quantified using ImageJ as previously described [33].

ELISA

Renal cortex tissue homogenate was prepared using mechanical attrition treatment [34]. IL-1 β and TNF- α protein concentrations were detected by ELISA kits according to manufacturer's instructions as previously described [35].

Statistical Analysis

Data are expressed as mean $\pm$ SD. Statistical analysis of data was performed with SPSS 22.0 software (SPSS Inc., IL, USA). Comparison among groups was made with one-way ANOVA. Multiple-comparison tests were applied only when a significant difference was determined by the ANOVA; p-values < 0.05 were considered to be statistically significant. Each experiment was repeated at least three times with similar results.

Results

Kidneys from UUO rats developed TIF and displayed significant inflammation

UUO is a well-established experimental model of obstructive nephropathy characterized by increased ECM deposition, which leads to tubulointerstitial fibrosis. We observed increased blood urea nitrogen (Bun), serum creatinine (Scr), and serum uric acid (UA) in UUO rats compared with those in SHM (Fig. 1A-C), which was consistent with previous study [25]. The renal tubulointerstitial injury score (Fig. 1D-E) and ECM deposition (Fig. 1F-G) were significantly increased in the obstructed kidney, confirmed by HE and Masson's trichrome staining, respectively. More specifically, immunohistochemistry staining confirmed that collagen I and collagen III deposition in the renal interstitium was markedly elevated (Fig. 1H-K), and western blot indicated enhanced fibronectin accumulation in the UUO kidney (Fig. 1L-M). In addition to ECM deposition, we analyzed the inflammation state in UUO rats. Immunohistochemistry staining showed increased infiltration of CD3-positive lymphocytes and CD68-positive macrophages in the renal interstitium after UUO operation (Fig. 2A-D). ELISA analysis detected that the protein concentration of inflammation cytokines in the renal cortex tissue homogenate, IL-1 β and TNF- α for example, was elevated in the UUO (Fig. 2E-F). These results certified that we successfully conducted the tubulointerstitial fibrosis model, and the inflammation state involved TIF.

Fig. 1. Renal tubulointerstitial fibrosis in UUO rats. Blood urea nitrogen (Bun), B. serum creatinine (Scr), C. serum uric acid (UA) in rats, D. HE stain (x200) and corresponding E. Tubulointerstitial injury score, F. Masson's trichrome staining (x200) and corresponding G. Tubulointerstitial fibrosis score. Collagen I (H) and Collagen III (I) stained by immunohistochemistry (x200) and corresponding semi-quantitative analysis results (J) and (K). Representative Western blot of FN in kidney tissue (L) and quantitative analysis of FN protein expression (M). Data expressed as means $\pm$ SD, n=5. *P < 0.05 vs. sham group.

Fig. 2. Renal tubulointerstitial inflammation in UUO rats. CD68 (A) and CD3 (B) stained by immunohistochemistry (x200) and corresponding semi-quantitative analysis results (C) and (D). IL-1 β (E) and TNF- α (F) protein concentrations in UUO renal tissue homogenate detected by ELISA. Data expressed as means $\pm$ SD, n=5. [#]P < 0.05 vs. sham group.

CBS expression was significantly decreased in the renal cortex of UUO rats

To assess the potential target proteins involved in TIF, we extracted renal cortex protein of UUO rats for proteomic and gene array analysis. Proteomic analysis yielded a total of 1105 proteins, which were identified by Protein Pilot at a confidence level of 95%. Through the primary proteomic analysis in the protein data bank of Swiss-Prot, we detected 294 proteins with fold-changes <0.7. After matching these proteins to the differentially expressed gene detected by the United Affymetrix GeneChip profiling with ratio-changes <0.4, 18 proteins were confirmed to be down-regulated at the transcriptional level as well as translational level in the renal cortex of UUO rats compared with those in SHM. Among them, we successfully found the CBS protein (Table 1).

To further examine CBS expression in UUO rats, we analyzed the protein and mRNA level expressions of CBS in rat kidney tissue. Firstly, we conducted IHC staining of CBS in rat kidney sections. As expected, CBS was mainly expressed in the cytoplasm of proximal renal tubular epithelial cells in normal kidneys, and was significantly decreased in obstructed kidneys (Fig. 3A-B). Secondly, the down-regulation of CBS protein expression in UUO rat kidney tissue was con-

firmed by Western blot analysis (Fig. 3C-D). Thirdly, qPCR tests were conducted to detect the mRNA expression change of CBS in the UUO model, which was proven to be reduced as well (Fig. 3E). Collectively, the above observations showed that the expression of CBS was decreased in TIF.

Table 1. Differentially expressed proteins in proteomics analysis

No.	Gene Symbol	Protein Name	Swiss-prot accession number	UUO/S HM
1	CALB1	Calbindin	P07171	0.0373
2	RGN	Regucalcin	Q03336	0.1556
3	ST1C2	Sulfotransferase 1C2	Q9WUW8	0.1754
4	CGL	Cystathionine gamma-lyase	P18757	0.2089
5	S22A2	Solute carrier family 22 member 2	Q9R0W2	0.3221
6	DPYS	Dihydropyrimidinase	Q63150	0.3837
7	ACSF2	Acyl-CoA synthetase family member 2, mitochondrial	Q499N5	0.4529
8	APOC3	Apolipoprotein C-III	P06759	0.4529
9	GABT	4-aminobutyrate aminotransferase, mitochondrial	P50554	0.4699
10	PYC	Pyruvate carboxylase, mitochondrial	P52873	0.4966
11	S1C2A	Sulfotransferase 1C2A	Q9WUW9	0.4966
12	SCLY	Selenocysteine lyase	Q68FT9	0.52
13	ARLY	Argininosuccinate lyase	P20673	0.5297
14	KHK	Ketohexokinase	Q02974	0.5598
15	GSHB	Glutathione synthetase	P46413	0.6194
16	CBS	Cystathionine beta-synthase	P32232	0.6194
17	M2GD	Dimethylglycine dehydrogenase, mitochondrial	Q63342	0.6855
18	PPBT	Alkaline phosphatase, tissue-nonspecific isozyme	P08289	0.6918

CBS decreased in human obstructive nephropathy

Recent research has shown that CBS is closely related to fibrotic diseases [36]. However, the role of CBS in human TIF remains unclear. Our preceding results showed that CBS is down-regulated in the kidney of UUO rats. Thus, we analyzed CBS expression in human obstructive nephropathy, which is one of the most common forms of TIF without other confounding etiologies. CBS expression in 10 cases of non-tumor normal tissue adjacent to renal carcinoma and 9 patients with obstructive nephropathy (Table 2) was examined by IHC. In accordance with the literature, CBS was mainly detected in the cytoplasm of proximal renal tubular epithelial cells (Fig. 4A). A significant decrease of CBS was observed in patients with obstructive nephropathy (Fig. 4B).

CBS was down-regulated in TGF- β_1 -stimulated HK-2 cells

To better explore the relationship between CBS and renal fibrosis, we established an in vitro model imitating fibrosis. Since CBS mostly expresses in the proximal convoluted tubule, HK-2, a human proximal tubule epithelial cell line from a normal adult human [37], was employed, and TGF- β_1 was used to induce TIF in vitro [32]. HK-2 cells were stimulated with 10 ng/mL TGF- β_1 for 12 h, 24 h, or 48 h respectively. Western blot assay showed that CBS protein significantly decreased along with FN accumulation after treatment with TGF- β_1 for 48 h (Fig. 5A-C). Thus, it is intriguing to explore whether the overexpression of CBS would attenuate TGF- β_1 -induced FN and collagen I accumulation in HK-2 cells.

Overexpression of CBS attenuated TGF- β_1 -induced fibronectin and collagen I accumulation in HK-2 cells

We overexpressed CBS in HK-2 cells using plasmid transfection with PcDNA3.1 and PcDNA3.1-CBS with or without 10 ng/mL TGF- β_1 treatment for 48 h. Notably, overexpression of CBS attenuated fibronectin and collagen I accumulation in HK-2 cells after TGF- β_1 treatment (Fig. 5D-H). These results suggested that CBS could contribute to alleviate TIF. To consolidate this hypothesis, we detected the influence of CBS knockdown in renal tubulointerstitial fibrosis in vitro.

Knockdown CBS enhanced TGF- β_1 -induced fibronectin and collagen I accumulation in HK-2 cells

We knocked down CBS in HK-2 cells using siRNA oligo strand against CBS. Surprisingly,

Fig. 3. Expression of CBS in UUO rats. Representative immunohistochemistry (IHC) results (200x) and corresponding B. Histogram of CBS expression in rat kidney sections. C. Representative Western blot of CBS in rat kidney tissue and D. Quantitative analysis of CBS protein expression. E. CBS mRNA expression in rat kidney tissue detected by qPCR. Data expressed as means $\pm$ SD, n=5. #P < 0.05 vs. sham group.

fibronectin accumulated in HK-2 cells after CBS knock-down even without the stimulation of TGF- β_1 . Moreover, treatment with 10 ng/mL TGF- β_1 for 48 h induced further accumulation of fibronectin and collagen I in CBS-deficient HK-2 cells (Fig. 5I-M).

Knock down CBS enhanced IL-1 β -induced expression of MCP-1, TNF- α , and IL-6 in HK-2 cells

Since inflammation plays critical roles in renal tubulointerstitial fibrosis, and inflam-

Table 2. Nephropathy patients information

No.	Gender	Age (years)	Diagnose
1	Female	66	Renal carcinoma (left side)
2	Male	52	Renal carcinoma (right side)
3	Female	77	Renal carcinoma (left side)
4	Male	53	Renal carcinoma (right side)
5	Male	59	Renal carcinoma (right side)
6	Male	68	Renal carcinoma (right side)
7	Female	67	Renal carcinoma (left side)
8	Male	64	Renal carcinoma (right side)
9	Male	54	Renal carcinoma (right side)
10	Male	68	Renal carcinoma (left side)
11	Female	67	Ureter and kidney stone, hydronephrosis (right side)
12	Female	51	Kidney stone and hydronephrosis (right side)
13	Female	53	Ureter and kidney stone, hydronephrosis (right side)
14	Female	53	Kidney stone and hydronephrosis (right side)
15	Male	65	Ureter stone and hydronephrosis (left side)
16	Female	62	Ureter and kidney stone, hydronephrosis (left side)
17	Female	66	Ureter and kidney stone, hydronephrosis (right side)
18	Female	67	Ureter stone and hydronephrosis (right side)
19	Male	46	Ureter and kidney stone, hydronephrosis (left side)

Control: 1-10; Obstructive Nephropathy: 11-19

Fig. 4. Expression of CBS in human renal tissue. Representative immunohistochemistry (IHC) results (x200) and B. Histogram of control (n=10) and patients with obstructive nephropathy (n=9). Data expressed as means $\pm$ SD. [#]P < 0.05 vs. Control group.

mation state is reported to be able to regulate CBS expression [38], we further explored the relationship between CBS and inflammatory reactions in TIF. We used recombinant human IL-1 β stimulation to induce inflammation in HK-2 cells. CBS mRNA was significantly decreased after treatment with 5 ng/ml IL-1 β for 24 h (Fig. 6A). Meanwhile, marked elevation of the transcriptional expressions of MCP-1, TNF- α , and IL-6 were detected by qPCR. After transfection of siRNA against CBS, IL-1 β induced sharply increasing mRNA expressions of MCP-1, IL-6, and TNF- α in the CBS deficient HK-2 cells (Fig. 6B-D).

Discussion

Understanding the fundamental pathways that lead to renal fibrosis is essential for developing better therapies for human CKD [4]. The amount of ECM deposition correlates with the severity of renal tubulointerstitial fibrosis [2]. However, our knowledge in terms of the identities of ECM accumulation-inducing factors during the pathogenesis of renal tubulointerstitial fibrosis remains limited. Although TGF- β_1 appears to be the primary driver of kidney fibrosis, a vast array of additional molecules may have modulating roles [4]. Additionally, TGF- β_1 knock-out (KO) mice develop severe multi-focal autoimmune inflammatory lesions and die within 3 weeks after birth [39], which indicates that TGF- β_1 may not be a good drug target, making it necessary to find additional modulating molecules. Through the proteomic and genomic analysis, we found that the introduction of inflammation and TIF was accompanied by significant reduction in CBS in the UUO model, a rodent model of fibrotic nephropathy resembling human CKD.

CBS, abundant in kidneys, livers, lungs, and other organs, is the first and rate-limiting enzyme in the transsulfuration pathway, which plays critical roles in detoxification of Hcy and production of endogenous H₂S [40, 41]. Hyperhomocysteinemia (HHcy) has a close relationship with several disorders in CKD [42]. Researches provide evidence to support the causal role of HHcy in the development of CKD and outlines several cellular and molecular mechanisms by which Hcy induces renal injury. These mechanisms include inflammation [43, 44], oxidative stress [45], endoplasmic reticulum stress [46], DNA hypomethylation [47, 48], et al. Recent studies have revealed protective effects of H₂S in renal disease progression and failure [16, 49]. Abnormal metabolism of H₂S is associated with fibrosis pathogenesis, causing damage to the structure and function of different organs [50]. H₂S supplementation prevents HHcy-associated renal damage, in part, through its antioxidant properties [16, 38].

Recently, an increasing amount of researches have indicated that CBS is closely connected with fibrotic diseases. Mani et al found that CBS-deficient mice develop inflammation, fibrosis, and hepatic steatosis [51]. Hamelet et al also observed enhanced pulmonary fibrosis

Fig. 5. CBS regulated TGF- β_1 -induced ECM accumulation in HK-2 cells. Representative Western blot of CBS and FN in HK-2 cells treated with TGF- β_1 for different time, quantitative analysis of B. CBS and C. FN protein expression. D. Representative Western blot of overexpression of CBS in HK-2 cells with or without TGF- β_1 treatment, quantitative analysis of E. CBS and F. FN protein expression. G. CBS and H. collagen I mRNA fold

expression after overexpression of CBS in HK-2 cells with or without TGF- β_1 treatment, detected by qPCR. I. Representative Western blot of knockdown of CBS by siRNA in HK-2 cells with or without TGF- β_1 treatment, quantitative analysis of J. CBS and K. FN protein expression. L. CBS and M. collagen I mRNA fold expression after knockdown of CBS by siRNA in HK-2 cells with or without TGF- β_1 treatment, detected by qPCR. Data expressed as means $\pm$ SD, n=3. #, *, Δ , $\star$ P < 0.05 vs. different groups as indicated in each graph respectively.

Fig. 6. CBS regulated IL-1 β -induced inflammation in HK-2 cells. A. CBS mRNA fold expression after knockdown of CBS by siRNA in HK-2 cells with or without IL-1 β treatment. TNF- α (B), IL-6 (C), and MCP-1 (D) mRNA fold changes after knockdown of CBS by siRNA in HK-2 cells with or without IL-1 β treatment. Data expressed as means $\pm$ SD, n=3. #, *, Δ , $\star$ P < 0.05 vs. different groups as indicated in each graph respectively.

in CBS-deficient mice [35]. Tan et al reported that in a carotid arterial vein patch mice model, compared with WT mice, CBS^{-/-} mice exhibited thicker neointima with increased elastin and collagen deposition [52]. Muthuram et al conducted transverse aortic constriction (TAC) to induce pressure overload, and found that overexpression of CBS attenuated cardiac hypertrophy and fibrosis as well as reduced mortality after TAC [53]. These results suggest that CBS may be critical in protecting mammals from organ fibrosis.

Although some researches have reported the alteration of CBS expression in UUO models, almost all the studies focused on the role of H₂S instead of CBS [48,49]. The exogenous H₂S, released via sodium hydrosulfide (NaHS), can ameliorate damage associated with short-term ureteral obstruction, but the reno-protective effect was depending on the direct increasing of H₂S or enhancement of CBS or both of them remained to be elucidated. Moreover, NaHS generates supra-physiological quantities of H₂S spontaneously in solution and has a half-life of only 15 minutes, which make it difficult to be a potential drug. Researcher claims that

future studies might focus on the potential to intervene fibrosis by targeting the pathway of endogenous H₂S-producing enzymes [44]. However, the relationship between CBS and TIF remained to be determined. This study intended to elucidate the role and mechanism of CBS in renal interstitial inflammation and TIF.

First of all, we confirmed that the mRNA and protein expression of CBS was dramatically down-regulated in obstructive kidneys accompanied by CD3- and CD68-positive inflammation cells infiltration, IL-1 β production, and ECM deposition. Therefore, based on the literature and our results, we postulated that CBS might be a critical regulator in inflammation and ECM deposition in kidney diseases.

Furthermore, we detected CBS expression change in obstructive nephropathy in human. Our results showed that CBS was abundantly present in normal human kidney tissue, in which the majority of renal tubules stained positive for CBS, which aligns with a previous study indicating that CBS activity was found in the renal proximal tubule [54]. As expected, to our knowledge, it is the first report that the expression of CBS was sharply down-regulated in interstitial fibrosis accompanied with ECM deposition in the kidney tissue from obstructive nephropathy patients.

Next, to further confirm the role of CBS in fibrosis, we knocked down and overexpressed CBS in TGF- β_1 , an essential fibrogenic factor, stimulated HK-2 cells. Results showed that knocking down CBS exacerbated ECM deposition, whereas CBS overexpression attenuated TGF- β_1 induced fibronectin and collagen I production. These in vitro findings are consistent with the induction of TIF accompanied by decreased CBS expression in vivo by the UUO operation. Thus, we speculated that CBS might prevent the progress of TIF.

Unresolved inflammation is one of the most important initiators that promote progressive TIF, which usually culminate in ESRD [6]. Kidney inflammation involves cells of the immune system as well as activation of intrinsic renal cells, with the consequent production and release of pro-fibrotic cytokines and growth factors that drive the fibrotic process [55]. In view of the fact that CBS deficiency may cause a pro-inflammation state [56], we speculated that the anti-fibrotic effect of CBS may partially depend on its anti-inflammatory effect. As one of the crucial inflammatory cytokines, IL-1 β initiates the production of cytokines and chemokines, exacerbates intrinsic renal cell damage [57], and mediates the disruption of interstitial ECM balance [58]. Furthermore, our results indicated that the IL-1 β production was significantly up-regulated in UUO rats. Therefore, we chose IL-1 β as the stimulator to induce inflammation in HK-2 cells. We found that IL-1 β decreased CBS mRNA expression in HK-2 cells, as well as inducing a sharp increase of MCP-1, IL-6, and TNF- α mRNA expression in the CBS-deficient HK-2 cells. Taken together, these findings suggested significant interrelationships between CBS expression and inflammation in renal disease.

Conclusion

In summary, this study indicated that CBS is one of the ECM accumulation-decreasing factors during the pathogenesis of renal tubulointerstitial fibrosis. The regulating effect of CBS may be mediated by its anti-inflammatory function, and overexpression of CBS may have renal protective effects. However, the concrete mechanism through which CBS exerts its anti-fibrotic and anti-inflammatory effects requires further study. What is more, our proteomics and genomic analysis showed that the hypoexpression is much more pronounced for Cystathionine gamma-lyase (CSE), another important enzyme in the transsulfuration pathway of Hcy metabolism [38], than for CBS, suggesting that the pathologic change detected in UUO rats may be also dependent on this selective impairment of CSE and that the inflammatory upraise may be relevant to a uneven handling of homocysteine and related sulfur metabolites [44]. Also other down-regulated proteins involved in calcium-modulation, such as Calbindin or Regucalcin, could be interesting. Further researches are needed to explore the other down-regulated proteins roles in TIF.

Disclosure Statement

The authors have no conflict of interest to disclose.

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