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ORIGINAL ARTICLE

Expression of cholecystokinin-1 receptor is correlated with proteinuria in human diabetic nephropathy

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Abstract This study aimed to examine the expression of cholecystokinin-1 receptor (CCK-1R) in the kidneys of type 2 diabetic nephropathy (DN) and the correlation of CCK-1R mRNA and proteinuria. Localization of CCK-1R in kidney of diabetic patient with nephropathy was examined by immunohistochemistry and in situ hybridization. The glomeruli did not express CCK-1R in either control or diabetic nephropathic kidneys. However, the expressions of CCK-1R protein and mRNA in tubules were significantly increased in DN, which had no relationship with the severity of DN. Furthermore, there was a positive correlation between the percentage of cells positive for CCK-1R mRNA and the degree of proteinuria. Increased CCK-1R expression could be demonstrated in the tubules and the CCK-1R might be implicated in the development of proteinuria in human DN.

Keywords Cholecystokinin-1 receptor · Proteinuria · Diabetic nephropathy

Introduction

The cholecystokinin-1 (CCK) receptors belong to the superfamily of G-protein-coupled receptors [1]. Two receptors for CCK have been identified: the CCK-1 and the CCK-2 receptor, which express [2–6] in the stomach, endocrine pancreas, adrenal gland, smooth muscles of gallbladder, brain nuclei and kidney. Not only do CCK receptors have a large number of inductive functions [7, 8], such as in the stimulation of secretion for somatostatin, leptin, insulin, and pancreatic polypeptide, but also they are involved in [9, 10] the regulation of gastrointestinal motility and contraction of gallbladder via mechanical actions. Previous studies demonstrated [11] the presence of CCK-2R by immunohistochemistry and Northern-blot RNA analysis in the rat kidney on tubules and collecting ducts, mediating changes in renal potassium and sodium absorption, while the localization and function of CCK-1R in the kidney remain poorly understood. Furthermore, identification of CCK-1R in human kidney tissues has not been reported in the previous literatures. DN is the major cause of chronic renal failure worldwide [12]. The pathophysiological processes that lead to DN include a classic view of metabolic and hemodynamic alterations [13–15]. Proteinuria, a biomarker of many serious renal diseases, is considered [13–15] as an important risk factor for the progression of DN.

Here, we investigated the CCK-1R expression in kidney biopsies from patients with type 2 DN. In addition, we assessed the relationship between clinical and immunohistochemical data. In this study, the CCK-1R expression at different stages of human DN was investigated using immunohistochemistry and in situ hybridization. We demonstrated that the induction of CCK-1R expression was associated with the degree of proteinuria in patients with type 2 DN.

Materials and methods

Human biopsies and patients

Normal human kidney tissues ($n = 6$) were acquired from uninvolved portions of surgically excised kidneys afflicted

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with the localized neoplasm. The other kidney tissues were obtained from 23 patients with DN. The patients underwent renal biopsy because of the presence of proteinuria, the absence of retinopathy, and a known history of diabetes. The morphological diagnosis of DN was made by two pathologists blinded to the source of tissues, with the observation of histologic changes of renal biopsy specimens. All formalin-fixed renal biopsy tissues were obtained with each patient's consent and in according with the Harbin Medical University Human Ethics Committee prior to the commencement of the study.

Biochemical analysis

The clinical data for each patient were retrieved from hospital files, including data on serum creatinine, creatinine clearance, 24-h urinary protein excretion, fasting plasma glucose, total protein at the time of renal biopsy, and duration of diabetes.

Morphology

Paraffin-embedded renal tissue for light microscopy was dewaxed using standard sequential techniques, and 3 μm-thick sections were stained with hematoxylin–eosin technique. The severity of DN was classified into three grades based on histopathological examination [16] of the renal biopsy specimens: DN stage I (six patients) showed mild mesangial expansion, DN stage 2 (eight patients) showed moderate mesangial expansion with less than 50% sclerosis and DN stage 3 (nine patients) showed extensive matrix expansion, Kimmelstiel–Wilson lesions global obliteration.

Immunohistochemistry (IMH)

Sections (4 μm-thick) obtained from paraffin-embedded tissue were dewaxed and washed with PBS. After incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity, sections were subjected to microwave irradiation in citrate buffer to enhance antigen retrieval, followed by blocking with normal goat serum (Santa Cruz) for 20 min. Then sections were incubated with mouse monoclonal antibody against CCK-1R (Dilution: 1:80, R&D Biotechnology Inc.). Primary antibody was incubated overnight at 4°C in a high-humidity chamber. Then each section was incubated with appropriate biotin-conjugated secondary IgG for 1 h at room temperature. After washed with PBS three times, the sections were stained with a 3,30-diaminobenzidine solution and then counterstained with hematoxylin. Negative controls included staining of issue sections with omission of the primary antibody.

In situ hybridization (ISH)

Biotin-labeled human CCK-1R probe was purchased from R&D Biotechnology Inc. The sections (4 μm-thick) were dewaxed with xylene and rehydrated through a series of decreasing ethanol solutions. The sections were treated with 2× SSC at 60°C for 10 min and washed with diethylpyrocarbonate (DEPC)-treated water. Sections were digested with proteinase K (Sigma Chemical, St. Louis, MO, USA) for 60 min at 37°C. After washed with DEPC-treated water, the sections were prehybridized in a hybridization buffer (20 μl each specimen) in a 38°C baking oven. Four hours later, the sections were drained, and then hybridized overnight with a digoxigenin (DIG)-labeled oligonucleotide probe in the hybridization buffer in a 38°C baking oven. The sections were washed with PBS four times after hybridization, then added to a mouse monoclonal anti-DIG antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody, and HRP-conjugated swine anti-rabbit IgG antibody. Color was developed with diaminobenzidine tetrahydrochloride in 0.05 M Tris–HCl, pH 7.6, and 0.03% H_2O_2. After being counterstained with hematoxylin, sections were rinsed, dehydrated, cleared in xylene and mounted. To evaluate the specificity of the reaction, some confirmations were taken as below: [1] control experiments were carried out without DIG-labeled oligonucleotide probe in the hybridization buffer or with a plasmid DNA; [2] the signal disappeared when RNase (100 μg/mL; Sigma) was added in 0.05 M tris after the digestion with proteinase K.

Statistical analysis

Data were expressed as mean ± SD. Statistical differences between two groups were analyzed by the unpaired student's t test and differences between multiple groups of data were assessed by ANOVA with Bonferroni test. Potential correlations between clinical parameters and CCK-1R mRNA were calculated using Spearman's algorithm. Statistical significance was defined as $P < 0.05$.

Results

Clinical parameters of DN patients and non-diabetic controls

There is no significant difference in mean age between DN patients and control patients (45.22 ± 5.38 vs. 46.67 ± 9.35 years, $P > 0.05$). The mean duration of diabetes for patients was 11.48 ± 4.05 years. Increased serum creatinine (2.29 ± 0.93 mg/dL), proteinuria (5.03 ± 3.40 g/day), and fasting plasma glucose (163.65 ± 40.99 mg/dL) were

Table 1 Clinical parameters in DN and non-diabetic patients

Parameter	DN ($n = 23$)	Non-diabetic ($n = 6$)
Age (years)	45.22 ± 5.38	46.67 ± 9.35
Duration of diabetes (years)	11.48 ± 4.05	0
Serum creatinine (mg/dL)	2.29 ± 0.93	0.92 ± 0.26*
Creatinine clearance (ml/min)	53.65 ± 17.40	77.50 ± 8.17*
Fasting plasma glucose (mg/dL)	163.65 ± 40.99	78.17 ± 10.23*
Proteinuria (g/day)	5.03 ± 3.40	NOT PRESENT
Total protein (g/dL)	6.49 ± 1.00	7.20 ± 0.53*

Data are expressed as means ± SD. * $P < 0.05$ versus control (no-diabetic)

observed in patients compared with the non-diabetic controls (serum creatinine: 0.92 ± 0.26 mg/dL; proteinuria: not present; fasting plasma glucose: 78.17 ± 10.23 mg/dL). Creatinine clearance and total protein among DN patients were decreased (53.65 ± 17.40 ml/min; 6.49 ± 1.00 g/dL) as compared with the control (77.50 ± 8.17 ml/min and 7.20 ± 0.53, respectively) ($P < 0.05$) (Table 1).

Immunohistochemistry for CCK-1R

In non-diabetic kidney tissues, there was slight staining of the CCK-1R expression in glomerular endothelial cells and mesangial cells as well as tubular cells. In DN, the expression of CCK-1R was significantly increased in tubular epithelial cells (12.40 ± 2.31 vs. 0.33 ± 0.10%, positive area, $P < 0.05$). However, the expression of CCK-1R was rare in glomerular cells as well.

Compared to control kidneys (0.33 ± 0.10%, positive area), the expression of CCK-1R was significantly upregulated during different stages of diabetic nephropathy (stage 1:11.76 ± 1.71%; stage 2:13.16 ± 2.43%; stage 3: 12.14 ± 2.59%, positive area; all of $P < 0.05$). In addition, there was no significant difference in each stage of diabetic nephropathy ($P > 0.05$, Fig. 1a–j).

Detection of CCK-1R mRNA by ISH

To further investigate whether the increased expression for CCK-1R is attributed to an increased mRNA expression, ISH was performed on paraffin-embedded renal tissue from

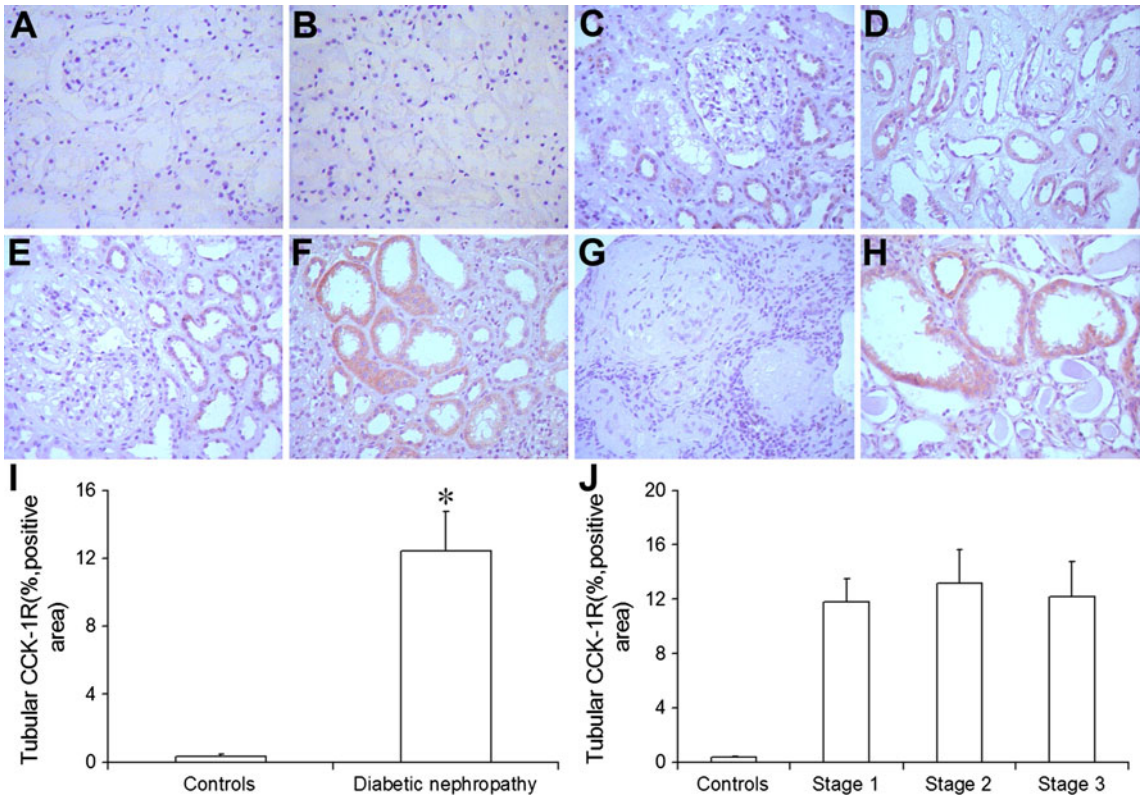

Fig. 1 Immunohistochemistry of CCK-1R in tubules from controls and DN kidney tissues. The expression of CCK-1R in control kidneys demonstrated the wide depletion of CCK-1R in glomeruli (**a**) as well as in tubules (**b**). Expression levels of glomerular (**c**, **e**, **g**) CCK-1R were absent with different stages of DN. Tubular (**d**, **f**, **h**) CCK-1R expression was increased in DN. The CCK-1R expression in tubules (**i**) was increased in DN compared to the control ($P < 0.05$), while the expressions in tubules (**j**) had no significant difference among biopsies in the different stages of DN ($P > 0.05$)

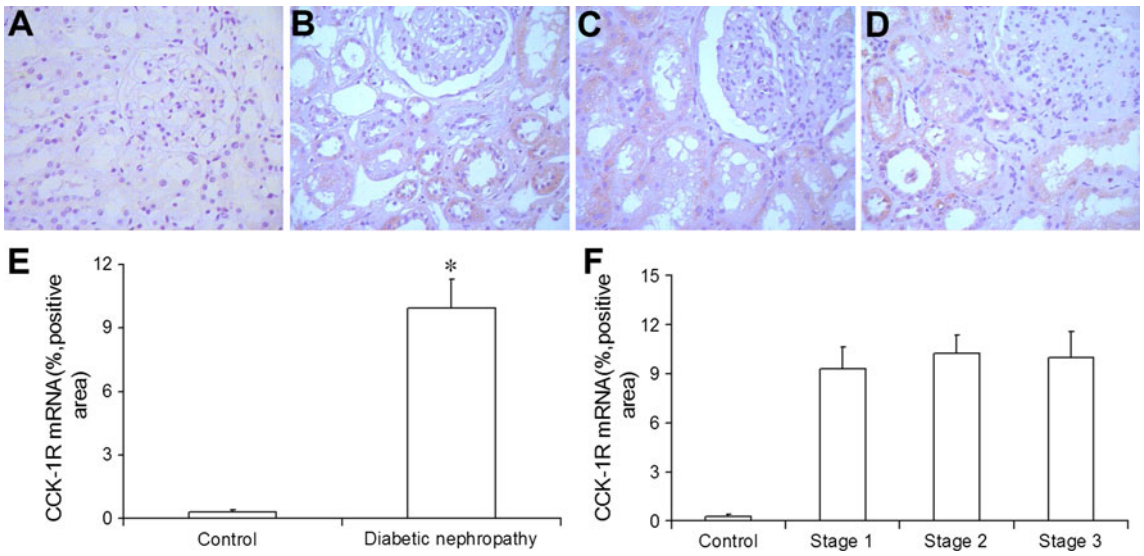

Fig. 2 In situ hybridization of CCK-1R mRNA in glomerular and tubules from the control and DN kidney tissues. Cells positive for CCK-1R mRNA were present in tubules of DN (**b–d**) whereas absent in the control tubules (**a**). Expression levels of glomerular CCK-1R mRNA were absent in both control and DN. CCK-1R mRNA expression was increased in the tubules (**e**) during DN compared to the control ($P < 0.05$), and had no significance in the different stages (**f**) of DN ($P > 0.05$)

patients with diabetic nephropathy and controls. We demonstrated cells positive for CCK-1R mRNA in renal tubular tissues of DN kidneys. However, there was little positive cells for CCK-1R mRNA in the glomerular area in DN. And the number of tubular cells positive for CCK-1R mRNA in DN was higher than the controls (9.91 ± 1.37 vs 0.28 ± 0.11%, positive area, $P < 0.05$). The percentage of cells positive for CCK-1R mRNA was not significantly different in each stage of DN (stage 1:9.33 ± 1.31%; stage 2:10.27 ± 1.10%; stage 3:9.98 ± 1.63%, positive area, all of $P > 0.05$). The specificity of CCK-1R mRNA signal detected by in situ hybridization was confirmed by the control studies (Fig. 2.)

Correlation between the expression of CCK-1R mRNA and clinical parameters

After demonstrating a co-localization of CCK-1R mRNA, we then analyzed the correlation between clinical parameters and the expression of CCK-1R mRNA. Interestingly, there was a close correlation between tubular CCK-1R mRNA and the degree of proteinuria in DN ($r = -0.613$, $P = 0.002$). However, the expression of CCK-1R mRNA was not correlated with other parameters such as age ($r = 0.253$, $P = 0.245$), duration of diabetes ($r = 0.270$, $P = 0.212$), serum creatinine ($r = -0.127$, $P = 0.564$), creatinine clearance ($r = 0.023$, $P = 0.915$), and fasting plasma glucose ($r = 0.135$, $P = 0.538$), etc. (Fig. 3).

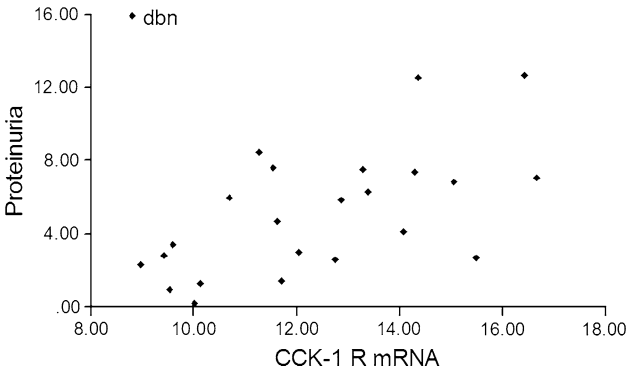

Fig. 3 There was a close correlation between CCK-1R mRNA in tubules and proteinuria in all DN patients. Correlations were calculated using Spearman's algorithm

Discussion

DN is one of the leading causes of end-stage renal failure and is associated with morbidity and mortality in the western world [17, 18]. Multivariate analysis of the RENAAL study illustrated that proteinuria was an independent risk factor that predicts renal outcomes in type 2 DN [19]. Previous studies on human biopsy samples emphasized [20, 21] the crucial role of tubules in the mechanism of proteinuria in DN patients, demonstrating the relationship between proteinuria, and tubules. However, reliable markers of tubules in DN patients are limited and difficult to interpret, especially in proteinuric states. The physiological and clinical function of the CCK-1R, which is involved in a variety of regulatory processes, has been

presented in previous literatures [22, 23]. CCK-1R can activate several signaling pathways [22, 23] such as the diacylglycerol (DAG), Ca^{2+} and protein kinase C (PKC) pathways. Previous studies showed [22, 23] supramaximal concentrations of CCK could induce pancreatitis in rats via CCK-1R by activating NF-κB. Till now, the study on the evaluation of CCK-1R expression in human renal tissues has not been reported yet. Therefore, we examined the expression of CCK-1R in this study. To our knowledge, this is the first time to examine the expression of CCK-1R in human kidney and whether there is a relevance between CCK-1R mRNA and proteinuria in human DN.

Although the CCK-1R protein was never observed in human kidneys, our data described the presence of CCK-1R established by immunohistochemistry. In this study, we investigated quantitative expressions of CCK-1R protein. As shown in Fig. 1, it was virtually absent in control glomeruli and tubules, as well as mesangial areas and capillaries. The expression levels were similar in the glomeruli of DN; however, those were positive in tubules of DN biopsies with a prominent staining pattern. In addition, the CCK-1R protein was present in biopsy tissues of DN throughout all disease stages. The progression of the disease did not relate to any significant differences in the tubules. Therefore, we can conclude that the precise localization of CCK-1R protein expression was in tubules of DN.

To further investigate the location of CCK-1R, we determined the expression of CCK-1R mRNA in controls and different stages of DN by non-radioactive in situ hybridization. As shown in Fig. 2, the in situ hybridization study using cRNA probes identified the distribution of CCK-1R mRNA, demonstrating the expression of CCK-1R mRNA localized exclusively in tubules of DN. Quantitative analysis of the in situ hybridization study clarified that the expression of CCK-1R was predominant in DN, whereas it was absent in control kidneys. Furthermore, the expression of CCK-1R mRNA had no significant difference compared between each stage of DN, showing that CCK-1R mRNA expression is independent of disease severity. Thus, our findings showed that CCK-1R in the human kidney tissues of DN was localized to tubules at both the protein and mRNA levels. Another interesting finding was the link between CCK-1R mRNA and clinical parameters assessed on the day of renal biopsy. As shown in Fig. 3, our results showed a significant correlation between the tubular CCK-1R mRNA expression and degree of proteinuria. However, no significant correlation was observed between the tubular CCK-1R mRNA and other clinical parameters. Our results could determine that the tubular CCK-1R may be a cause of proteinuria; however, the precise mechanisms of the upregulation of the tubular CCK-1R expression are not fully understood. We believe that the expression of CCK-1R in the progression of the disease is an important finding in terms of the various roles of diabetic nephropathy, since CCK-1R could activate several signaling pathways [24–29], which participates in the pathogenesis of proteinuria of diabetic nephropathy. In addition, CCK-1R plays a role in the induction and development of acute pancreatitis and irritable bowel syndrome [3, 4, 30]. CCK-1R might also activate inflammatory signaling pathways and then induce proteinuria. One particular finding to note is that there was a strongly significant correlation between the number of CCK-1R cells in diabetic tubuli and proteinuria in our study. Further studies are warranted to examine the regulatory mechanisms of the expression of CCK-1R and signaling pathways involved in proteinuria.

In summary, our study documents a robust increase in CCK-1R expression in tubules of human DN kidneys. This expression was specific to DN rather than the normal kidneys. Furthermore, the relationship between increased CCK-1R mRNA and the degree of proteinuria indicates that the expression of CCK-1R in tubules may be a marker of progressive DN. Further studies are necessary to elucidate the exact pathophysiological role of CCK-1R in DN.

Conflicts of interest The authors declare that they have no conflicts of interest.

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