Original

Expression and pathophysiological significance of carbohydrate response element binding protein (ChREBP) in the renal tubules of diabetic kidney

Susumu Suzuki¹, Atsushi Yokoyama¹, Erika Noro¹, Satoshi Aoki², Kyoko Shimizu¹, Hiroki Shimada¹ and Akira Sugawara¹

¹Department of Molecular Endocrinology, Tohoku University Graduate School of Medicine, Sendai 980-8575, Japan
²Division of Nephrology, Endocrinology and Vascular Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

Abstract. Carbohydrate response element binding protein (ChREBP), a glucose responsive transcription factor, mainly regulates expression of genes involved in glucose metabolism and lipogenesis. Recently, ChREBP is speculated to be involved in the onset and progression of diabetic nephropathy (DN). However, there exists no report regarding the localization and function of ChREBP in the kidney. Therefore, we analyzed the localization of Chrebp mRNA expression in the wild type (WT) mice kidney using laser microdissection method, and observed its dominant expression in the proximal tubules. In diabetic mice, mRNA expression of Chrebp target genes in the proximal tubules, including Chrebpβ and thioredoxin-interacting protein (Txnip), significantly increased comparing with that of WT mice. Co-overexpression of ChREBP and its partner Mlx, in the absence of glucose, also increased TXNIP mRNA expression as well as high glucose in human proximal tubular epithelial cell line HK-2. Since TXNIP is well known to be involved in the production of reactive oxygen species (ROS), we next examined the effect of ChREBP/Mlx co-overexpression, in the absence of glucose, on ROS production in HK-2 cells. Interestingly, ChREBP/Mlx co-overexpression also induced ROS production significantly as well as high glucose. Moreover, both high glucose-induced increase of TXNIP mRNA expression and ROS production were abrogated by ChREBP small interfering RNA transfection. Taken together, high glucose-activated ChREBP in the renal proximal tubules induce the expression of TXNIP mRNA, resulting in the production of ROS which may cause renal tubular damage. It is therefore speculated that ChREBP is involved in the onset and progression of DN.

Key words: Carbohydrate response element binding protein (ChREBP), Diabetic nephropathy, Renal tubular damage, Reactive oxygen species

CARBOHYDRATE RESPONSE ELEMENT BINDING PROTEIN (ChREBP) is a glucose responsive transcription factor that heterodimerizes with Mlx and binds to the carbohydrate response element (ChoRE) [1], and is composed of two isoforms α and β [1]. In human, ChREBP is expressed in the liver, adipose tissue, and kidney, and mainly regulates expression of genes involved in glucose and lipid metabolism [2-6]. ChREBP has also been suggested to be involved in the etiology and its complications of diabetes mellitus [7, 8]. In particular, there exist several reports indicating that ChREBP plays important roles in the onset and progression of diabetic nephropathy (DN) [9-12], which is well known to be developed by long-term duration suffering from diabetes mellitus. However, the detailed localization as well as function of ChREBP in the kidney has not been well understood so far. Therefore, in order to elucidate the relationship between ChREBP and the onset/exacerbation of DN, precise investigation to obtain this kind of information is necessary.

Each kidney is composed of a million of nephrons which consist of the glomeruli, renal tubules, and collecting ducts. Each segment contains its respective original function and is known to interact functionally each other. Recently, it has been reported that high glucose-activated Chrebp in glomerular mesangial cells induce glomerular disorders in diabetic mice [11]. However, there has been no report regarding the function of ChREBP in the renal tubules and collecting ducts. Since renal tubular damages, as well as glomerular damages, have recently been
recognized as an important etiology of DN [13], it is plausible that ChREBP in the renal tubules may also be involved in its onset/exacerbation. In order to elucidate the relationship between ChREBP and DN, we here examined the detailed localization and expression of Chrebps in the kidney using wild type (WT) and diabetic mice, and studied its function in the renal tubules in the presence of high glucose using human proximal tubular epithelial cell line.

**Materials and Methods**

**Animals and experimental design**

We used inducible nitric oxide (NO) synthase (iNOS) transgenic mice (iNOS-TG) [14] which carries NO synthase type 2 cDNA under the control of the insulin promoter. The mice overexpress iNOS specifically in the pancreatic β-cells, resulting in the hyperglycemic state due to the destruction of β-cells. Their water and diet were available randomly. The mice were weaned at 4 weeks of age and were thereafter fed with high protein/high calorie diet for 10 weeks in order to apply a load to the kidney [15]. WT (n = 7) and iNOS-TG (n = 4) mice were sacrificed at their 14 weeks of age. All experimental procedures conformed to “Regulations for Animal Experiments and Related Activities at Tohoku University,” and were reviewed by the Institutional Laboratory Animal Care and Use Committee of Tohoku University, and finally approved by the President of University.

**Blood and urine examination**

Blood samples were collected from mice tails. Blood glucose was measured by gluestest mint (Sanwa Kagaku Kenkyusho, Aichi, Japan). Urine samples were spot urine. Urinary albumin was measured by Bio-Rad CFX Connect™ Real-Time System (Bio-Rad, California, USA). Ribosomal protein lateral stalk subunit P0 (RPLP0) was used as a housekeeping gene for data normalization.

**Real-time quantitative PCR analyses**

At the sacrifice of mice, after PBS perfusion, their kidneys were immediately embedded in OCT and frozen in dry ice to prepare blocks. The blocks were cut into sections of 10 μm thick by cryostat (Leica CM1950; Leica, Tokyo, Japan) and were subjected to toluidine blue staining. These sections were then separated into the glomeruli, proximal tubules, and renal medulla using laser microdissection microscope (Leica LMD7000). Total RNA of separated tissue was isolated with ISOGEN (Nippon gene, Tokyo, Japan) according to the manufacturer’s instructions. The extracted total RNA was there- after reverse-transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara-bio, Shiga, Japan). Real-time PCR was carried out using KAPA SYBR Fast qPCR kit (Kapa Biosystems, Massachusetts, USA). PCR application was Bio-Rad CFX Connect™ Real-Time System (Bio-Rad, California, USA). Ribosomal protein lateral stalk subunit P0 (RPLP0) was used as a housekeeping gene for data normalization.

**Primer sets**

Primer sets for real-time PCR were showed in Table 1 (m: mouse, h: human). Locations of primer sets of ChREBP (Chrebp) which were designed to recognize both ChREBPα (Chrebpα) and ChREBPβ (Chrebpβ) or ChREBPβ (Chrebpβ) alone are shown in Fig. 1.

**Cell culture**

Human renal proximal tubular HK-2 cells were purchased from ATCC (ATCC CRL-2190) and were cultured in Dulbecco’s modified Eagle medium (DMEM) (low glucose) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. After transfection either with expression vector or small interfering RNA (siRNA), the cells were incubated either with high glucose (25 mM D-glucose), low glucose (5.5 mM D-glucose), or without D-glucose (0 mM D-glucose plus 25 mM L-glucose) for 24 hours. Thereafter, the cells were either subjected to measure ROS or total RNA isolation using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) to perform real-time quantitative PCR analyses.

**Transient transfection of expression vectors**

cDNA clones of ChREBPβ and Mlxβ were isolated from cDNA library derived from human hepatocellular carcinoma-derived HepG2 cells purchased from ATCC (ATCC HB-8065). Thereafter, ChREBPβ cDNA was subcloned into pQCIH vector (Takara-bio) with FLAG-tag at its N-terminal (FLAG-ChREBPβ), and Mlxβ cDNA was subcloned into pcDNA3.1 (+) vector (Invitrogen, California, USA) with Myc-tag at its N-terminal (Myc-Mlxβ). pQCIH vector containing FLAG-tag alone was also generated (FLAG). The vectors were transiently transfected to HK-2 cells cultured in 24 well plates (0.8 μg/well) using lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Transfection of siRNA and shRNA**

HK-2 cells were transfected with two different sets of ChREBP specific siRNA (Invitrogen) (Table 1) or negative control scramble oligonucleotides (Qiagen, Hilden, Germany) using lipofectamine 2000 according to the manufacturer’s instructions. Each ChREBP specific
siRNA can knockdown both ChREBPα and ChREBPβ mRNA. Efficiency of the knockdown was validated by quantitative PCR as described above. Two sets of TXNIP specific shRNA (shTXNIP1: GTGGAGGTGTG TGAAGTTA, shTXNIP2: CTCAGAGCAGCCCTATCTTTA) were generated using pSUPER RNAi System™ (OligoEngine, Washington, USA) according to the manufacturer’s instructions. Either control shRNA [16], shTXNIP1, and shTXNIP2 was transfected into HK-2 cells using lipofectamine 2000.

**ROS measurement**

The measurement of ROS was carried out using DCFDA/H2DCFDA—Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Cambridge, UK) according to the manufacturer’s instructions. Measurement application was performed in multi-plate-reader FlexStation3 (Molecular Devices Japan, Tokyo, Japan).

**Immunohistochemistry**

Kidney tissues from 14-week-old mice were fixed in 4% paraformaldehyde, embedded in paraffin, and thereafter sectioned. Sections were cut into 3 μm slices and were stained with anti ChREBP antibody (1:400, ab92809; Abcam). The stained images were obtained using Nikon Eclipse 50i (Nikon, Tokyo, Japan).

**Statistical analysis**

Date were given as mean ± SEM and were analyzed by Student’s t test (Figs. 2–4) and ANOVA followed by Tukey post-hoc tests (Figs. 5–7). Each analysis values were considered statistically significant when p values were less than 0.05 (*: p < 0.05, **: p < 0.01, ***: p < 0.001).

**Results**

**Chrebp expression mainly localizes in the proximal tubules of WT mouse kidney**

WT mice renal sections were separated into the glomeruli, proximal tubules, and renal medulla by laser microdissection method, and their total RNA was thereafter isolated. Collecting accuracy of each section was evaluated by the quantitation of the mRNA expression level of each tissue marker gene (Fig. 2A). The quantitation of Chrebp mRNA expression level revealed that Chrebp was predominantly expressed in the proximal tubules (Fig. 2B). Furthermore, immunohistochemical analyses of Chrebp protein also revealed its predominant expression in the renal tubules (Fig. 2C).

**Chrebp mRNA expression in the proximal tubules of WT and diabetic mice kidney**

We next compared the expression level of Chrebp mRNA between WT and diabetic (iNOS-TG) mice in the proximal tubules. Throughout the observation period (from 8 to 14 weeks), blood glucose levels of iNOS-TG mice were significantly higher than those of WT mice.
Since \(iNOS\)-TG mice are a model of mild type 1 diabetes \([14]\), their body weights were significantly lower than those of WT mice consistent to their progression of diabetes (Fig. 3B). In addition, UACR of \(iNOS\)-TG mice was significantly higher than that of WT mice (Fig. 3C). Moreover, since UACR of \(iNOS\)-TG mice was at the level of microalbuminuria (\(\geq 30 \text{ mg/g}\)), in contrast to that of WT mice, during the observation period (Fig. 3C), the \(iNOS\)-TG mice may possibly be at the stage of early diabetic nephropathy (DN) \([17]\). Regarding the expression level of \(Chrebp\) (the sum of \(Chrebp\alpha\) and \(Chrebp\beta\)) mRNA in the proximal tubules, there was no significant difference between WT and \(iNOS\)-TG mice (Fig. 3D).

**Increased mRNA expression of \(Chrebp\) target genes in the proximal tubules of diabetic mouse kidney**

We next compared the mRNA expression levels of \(Chrebp\) target genes in the proximal tubules between WT and \(iNOS\)-TG mice. We first examined the mRNA expression of \(Chrebp\beta\), which is one of \(Chrebp\) isoforms and is the primary target gene of glucose-activated \(Chrebp\alpha\) \([1, 6]\), and observed its significant increase in \(iNOS\)-TG mice than in WT mice (Fig. 4A). Moreover, the mRNA expression levels of other \(Chrebp\) target genes including lipid synthesis regulator gene acetyl-CoA carboxylase (\(Acc\)) (Fig. 4B), gluconeogenesis regulator gene glucose-6-phosphatase catalytic subunit (\(G6pc\)) (Fig. 4C), and oxidative stress regulator gene thioredoxin-interacting protein (\(Txnip\)) (Fig. 4D) were significantly higher in \(iNOS\)-TG mice than in WT mice. These results suggest that the activated \(Chrebp\) in the proximal tubules of diabetic kidney actually function to induce the expression of its target genes including \(Chrebp\alpha\) and \(Txnip\).

**ChREBP/Mlx co-overexpression induces \(TXNIP\) mRNA expression and ROS production in human renal proximal tubular cells in the absence of glucose**

In order to examine if ChREBP is involved in the production of ROS in the proximal tubules, we co-overexpressed ChREBP (FLAG-ChREBP\(\beta\)) and its partner Mlx \([4]\) (Myc-Mlx\(\beta\)) in human renal proximal tubular HK-2 cells by transient transfection. As shown in Fig. 5A, when ChREBP and Mlx were co-transfected into HK-2 cell in the absence of D-glucose (L-glucose 25 mM), both \(TXNIP\) mRNA expression (Fig. 5A) and ROS production (Fig. 5B) were significantly induced as well as in high glucose (D-glucose 25 mM). These results indicate that ChREBP/Mlx heterodimer-induced \(TXNIP\)
mRNA expression may contribute to the production of ROS in the renal tubular cells.

**High glucose-activated endogenous ChREBP is involved in the induction of TXNIP mRNA expression and ROS production in human renal proximal tubular cells**

In order to confirm the involvement of high glucose-activated endogenous ChREBP in the induction of TXNIP mRNA expression and ROS production, we transfected two different sets of ChREBP specific siRNA into HK-2 cells. In contrast to ChREBPβ mRNA expression which is significantly induced by high glucose (Fig. 6B), high glucose did not induce ChREBP (the sum of ChREBPα and ChREBPβ) mRNA expression (Fig. 6A) as previously described [18]. Transfection of each ChREBP specific siRNA, which can knockdown both ChREBPα and ChREBPβ mRNA, significantly abolished mRNA expression of both ChREBP (Fig. 6A) and ChREBPβ (Fig. 6B), indicating its specificity. Transfection of each ChREBP specific siRNA into HK-2 cells also significantly abolished both high glucose-induced TXNIP mRNA expression (Fig. 6C) and ROS production (Fig. 6D). Moreover, transfection of each TXNIP specific shRNA significantly decreased high-glucose induced TXNIP mRNA expression (Fig. 7A) and ROS production (Fig. 7B). These data indicate that high glucose-activated endogenous ChREBP is involved in the induction of TXNIP mRNA expression and ROS production in the renal tubular cells.
Fig. 3  *Chrebp* mRNA expression in the proximal tubules of WT and diabetic mice kidney.

(A) Body weight of WT and *iNOS*-TG mice. (B) Blood glucose level of WT and *iNOS*-TG mice. (C) UACR of WT and *iNOS*-TG mice. (D) *Chrebp* mRNA expression in the proximal tubules of WT and *iNOS*-TG mice. Values represent the mean ± SEM (WT mice; *n* = 6, *iNOS*-TG mice; *n* = 4). *: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001 vs. WT mice. N.S., not significant; m, mouse.

Fig. 4  Induction of the mRNA expression of *Chrebp* target genes in the proximal tubules of diabetic mice.

mRNA expression of *Chrebpβ* (A), *Acc* (B), *G6pc* (C), and *Txnip* (D) in the proximal tubule of WT and *iNOS*-TG mice. Values represent the mean ± SEM (WT mice; *n* = 6, *iNOS*-TG mice; *n* = 4). *: *p* < 0.05, **: *p* < 0.01, ***: *p* < 0.001. m, mouse.
Discussion

In the present study, we have first examined the detailed localization of Chrebp mRNA and protein in the WT mice kidney, and have observed their dominant expression in the proximal tubules (Fig. 2). Among ChREBP isoforms, ChREBPα is a dominant isoform which is activated by high glucose via several mechanisms including dephosphorylation, and is thereafter translocated into the nucleus to bind to and activate ChREBPα of its target genes including ChREBPβ, ACC, G6PC, and TXNIP [1]. Interestingly, high glucose-induced ChREBPβ is known to downregulate ChREBPα mRNA expression via negative feedback loop in pancre-
atic β cells [1, 19]. Since mRNA expression of Chrebp target genes in the proximal tubules including Chrebpβ, Acc, G6pc, and Tnxip was significantly increased in diabetic mice kidney than in WT mice kidney (Fig. 4), it is suggested that endogenous Chrebp in the proximal tubules is activated by high glucose. In contrast, there was no difference between diabetic mice kidney and WT mice kidney regarding Chrebp (the sum of Chrebpα and Chrebpβ) mRNA expression in the proximal tubules (Fig. 3D), most likely due to the negative feedback loop described above [1, 18, 19].

Recently, several reports have described about the involvement of ChREBP in the onset/progression of DN [9-12]. Regarding DN, the proximal tubules have also been recognized to be involved in its etiology in addition to the glomeruli [13]. Moreover, megalin-mediated protein metabolic overload in the proximal tubules has been hypothesized as an etiology of DN [20]. We therefore examined the possible involvement of ChREBP in the etiology of DN in the proximal tubules using human proximal tubular HK-2 cells. Our study demonstrated that overexpression of ChREBP and its partner Mlx [4] induced TXNIP mRNA expression and ROS production as well as high glucose (Fig. 5). Additionally, knockdown of ChREBP using its specific siRNA abrogated high glucose-induced TXNIP mRNA expression and ROS production (Fig. 6). Moreover, knockdown of TXNIP also abrogated high glucose-induced ROS production (Fig. 7). ROS is well known as the inducer of apoptosis, and it causes cell damage [21]. In pancreatic β-cells, the induction of apoptosis via ChREBP and TXNIP has been reported to cause cell injury [22, 23]. Moreover, it has recently been reported that TXNIP-induced ROS promotes renal tubular injury in DN [24]. In the kidney, excess amounts of ROS are recognized to activate protein kinase C, mitogen-activated protein kinases, and various cytokines that eventually induce fibrosis via the increased expression of extracellular matrix genes, resulting in the end stage renal disease [25].

Taken together, our study suggests that the high glucose-activated ChREBP in the renal proximal tubules of diabetic kidney increases TXNIP mRNA expression to induce ROS production, which may result in the etiology of DN due to ROS-mediated renal tubular damage (Fig. 8).

Fig. 6 Involvement of high glucose-activated endogenous ChREBP in TXNIP mRNA induction and ROS production in human proximal tubular cells.

Transfection of two sets of ChREBP specific siRNA (siChREBP1 and siChREBP2) significantly abolished mRNA expression of ChREBP (A), high glucose-induced Chrebpβ (B) and TXNIP (C) as well as ROS production (D) in contrast to control siRNA (siControl). Values represented the mean ± SEM (n = 3 in A, B, C, n = 6 in D). *, p < 0.05, **, p < 0.01, ***, p < 0.001. h, human.
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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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