Diabetes Induces Aberrant DNA Methylation in the Proximal Tubules of the Kidney

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ABSTRACT

Epigenetic mechanisms may underlie the progression of diabetic kidney disease. Because the kidney is a heterogeneous organ with different cell types, we investigated DNA methylation status of the kidney in a cell type–specific manner. We first identified genes specifically demethylated in the normal proximal tubules obtained from control db/m mice, and next delineated the candidate disease-modifying genes bearing aberrant DNA methylation induced by diabetes using db/db mice. Genes involved in glucose metabolism, including Slgt2, Pck1, and G6pc, were selectively hypomethylated in the proximal tubules in control mice. Hnf4a, a transcription factor regulating transporters for reabsorption, was also selectively demethylated. In diabetic mice, aberrant hypomethylation of Agt, Abcc4, Cyp4a10, Glut5, and Met and hypermethylation of Kif20b, Cldn18, and Slco1a1 were observed. Time-dependent demethylation of Agt, a marker of diabetic kidney disease, was accompanied by histone modification changes. Furthermore, inhibition of DNA methyltransferase or histone deacetylase increased Agt mRNA in cultured human proximal tubular cells. Aberrant DNA methylation and concomitant changes in histone modifications and mRNA expression in the diabetic kidney were resistant to antidiabetic treatment with pioglitazone. These results suggest that an epigenetic switch involving aberrant DNA methylation causes persistent mRNA expression of select genes that may lead to phenotype changes of the proximal tubules in diabetic kidney disease.


Diabetic kidney disease is the most common cause of CKD, and the number of patients with diabetic kidney disease continues to increase despite improved management of diabetes. This may stem, in part, from the irreversible nature of diabetic kidney disease. Epigenetic mechanisms have been suggested to play critical roles in the persistent phenotype changes of the blood vessels and organs and are likely to determine the incidence of diabetes-related complications. Aberrant increase in DNA methylation of the PGC-1a gene promoter is observed in the skeletal muscle of diabetic patients. DNA methylation changes of the kidney have also been observed in the parietal epithelial cells of diabetic animals and in the tubular compartment of patients with CKD. Proximal tubule (PT) cells of the kidney actively contribute to glucose homeostasis by reabsorbing glucose through the transporter Slgt2. PT cells, as the only part of the kidney expressing the appropriate enzymes for gluconeogenesis, such as Pck1

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and G6pc, also generate glucose by gluconeogenesis. Functional changes in the metabolism and transport are observed in the PT cells from an early stage in diabetes and are considered to be critically involved in the development of diabetic kidney disease. Epigenetic mechanisms may underlie PT-specific gene expressions and phenotype changes in diabetes, but the DNA methylation profile of PT cells has not been evaluated to date.

Genome-wide analyses revealed that the methylation state of only a fraction of the CpGs in the genome changes although in theory, that of every CpG can change. Comparison of the DNA methylation status among organs has led to the identification of differentially methylated regions (DMRs) in each organ. Such DMRs underlie tissue-dependent gene expressions and are considered to contain information about the fundamental functions of each organ. In previous studies, we identified DMRs by comparing the DNA methylation status in different organs, including the liver, cerebrum, and kidney. The DMRs observed in the kidney include those localized in kidney-specific transporters, which are hypomethylated in the kidney compared with other organs.

Because the kidney is a highly heterogeneous organ with more than a dozen different cell types, including each tubular component cell and interstitial and vascular cells, the DNA methylation status of the whole kidney is a summation of the methylation status of various cell types. In the present study, to evaluate the PT-specific DNA methylation status, we purified PT cells by sorting. We first identified PT-specific DMRs by comparing the DNA methylation status of the PT cells with that of the whole kidney. Alterations in the DNA methylation induced by diabetes were next delineated by comparing the DNA methylation status of the PT cells purified from diabetic and normal mice. Although DNA methylation is usually deemed to be relatively stable, hypermethylation of PGC-1α observed in obese patients can be reversed to the level seen in nonobese individuals after weight reduction induced by bariatric surgery. To evaluate the reversibility of the DNA methylation changes in the diabetic kidney, we determined the effects of antidiabetic therapy.

RESULTS

Purification of PT Cells and Identification of PT-Specific DMRs

PT cells were purified by sorting from the kidneys of db/m mice. Analysis of mRNA expressions revealed that markers of nephron segments other than PT, such as Na-K-2Cl cotransporter (NKCC2), Na+/Cl− cotransporter (NCC), and β-epithelial sodium channel (ENaC), expressed in the thick ascending limb, distal tubule, connecting tubule, and collecting duct, respectively, were excluded from the PT fraction, while mRNA of Sglt2 and Pck1, which are known to be expressed in PT, was enriched in the PT fraction (Figure 1, A–C). We screened for DMRs by comparing the DNA methylation profile of the PT cells with those of the whole kidney, liver, and cerebrum in a genome-wide manner (Supplemental Figure 1). Ontology analysis of genes neighboring the DMRs revealed that the genes expressed in the kidney were significantly enriched in the genes associated with the hypomethylated DMRs in the PT cells compared with other tissues (Supplemental Tables 1 and 2). We found the enrichment of genes related to mitochondrial function and the brush border, such as Sglt2 (Slc5a2) and Glut5 (Slc2a5), in the genes with PT-DMRs (Supplemental Tables 3 and 4). These data suggested strict regulation of the genes involved in metabolism and glucose uptake by DNA methylation.

We therefore analyzed the DNA methylation status of 55 representative DMRs associated with 42 genes, selected from the genes related to the kidney and metabolism, by combined
bisulfite restriction analysis (COBRA). Hierarchical clustering of the DNA methylation levels at these loci classified the PT cells and whole kidney into the same branch (Figure 2), suggesting similarity in the DNA methylation profiles between the PT cells and whole kidney. However, several CpGs exhibited significant differences in the DNA methylation status between the PT cells and whole kidney (Figure 2). Such PT-specific DMRs are considered to represent characteristic feature of the PT in the kidney. Among the PT-specific DMRs, those showing decreased methylation in the PT cells contained genes that are known to be the marker genes of PT cells, such as Sglt2 (Slc5a2), Pck1, Gcnt1 (glucosaminyl [N-acetyl] transferase 1, core 2); an enzyme involved in the formation of glycolipid, G6pc; and Hnf4a, a nuclear receptor. These genes were highly demethylated in the PT cells, while markedly methylated in the non-PT fraction (Figures 1D and 2B). As expected, mRNA expressions of these genes were mainly observed in the PT cells (Figure 1C). We also found that Cyp4a10 and Cyp4a14; both P450 enzymes; and Mgen5, a glycosidase that removes O-GlcNAc modifications, were demethylated and their expressions were elevated in the PT fraction. These results indicate that differential DNA methylation underlies cell-type–specific gene expressions within the kidney, just like differences among tissues, and that the genes described above may be expressed under epigenetic control.

Diabetes-Induced Changes in DNA Methylation and mRNA Expression

In the United Kingdom Prospective Diabetes Study, good glycemic control in the early stage of type 2 diabetes mellitus was associated with long-lasting beneficial effects on the risk of microvascular disease. This study also suggested that rearrangements induced in the early stage of diabetes may be important for the development of diabetic kidney disease. In the present study, therefore, we attempted to identify genes showing aberrant DNA methylation in the early stage of diabetic nephropathy, before the development of any apparent histologic changes. We obtained PT cells from 10-week-old db/db mice (Supplemental Figure 2) and compared the methylation status of PT cells purified from diabetic mice with that of PT cells purified from control animals by cluster analysis (Figure 2). PT cells obtained from the diabetic and control mice showed a rather similar DNA methylation patterns, but the methylation pattern significantly differed among the eight genes with potentially functional roles (Table 1, Supplemental Figure 3). Quantitative RT-PCR analysis demonstrated that the mRNA expressions of these genes in the PT cells were significantly different between the diabetic and control mice (Table 1, Supplemental Figure 3). An inverse correlation between expression and methylation was seen at these loci except for Met. This observation is concordant with previous reports indicating a generally, but not exclusively, negative correlation between DNA methylation and expression. Because we investigated DMRs in the promoter regions, the correlation of the expression and methylation levels of the DMRs located outside the promoter under diabetic conditions remains to be clarified.

Demethylation and Increased mRNA Expression of Agt in the PT

Because the expression of Agt in the PT is correlated with the progression of diabetic kidney disease, we further extended the analysis to the promoter region of this gene by bisulfite sequencing. In addition to the CpG at 470 bp downstream of the transcription start site (Table 1), CpGs 183 bp downstream and 592 bp upstream from the transcription start site were also significantly demethylated (Figure 3A). In situ hybridization revealed that Agt mRNA was mainly expressed in the PT cells of the medulla in the kidneys of control mice (Figure 3B). In diabetic mice, expression of Agt mRNA was markedly increased in the PT cells in the medulla. In addition, some glomerular and PT cells in the cortex also stained positive for Agt mRNA, although to a minor extent. These results indicate that Agt is induced mainly in the PT cells with concomitant DNA demethylation in diabetes.

Time-Dependent Epigenetic Changes of Agt

To delineate the time-dependent epigenetic changes, 5- and 8-week-old mice were analyzed. Demethylation of Agt was not apparent in the PT cells at week 5 (Figure 4A), when the blood glucose levels and body weight begin to increase (Supplemental Figure 4), while at week 8, significant DNA demethylation was observed in the diabetic kidney. These results indicate that the demethylation process takes place after the metabolic rearrangements begin to occur. In contrast, mRNA levels of Agt in the kidney were already elevated at week 5 in the diabetic kidney (Figure 4B). We next analyzed the histone modifications of H3K9 acetylation and H3K4 tri-methylation, both of which are associated with active transcription. H3K4 trimethylation was recently shown to be associated with persistent induction of PAI-1 in the endothelial cells in a mouse model of streptozotocin-induced diabetes. Analysis of histone modification by chromatin immunoprecipitation (ChIP) quantitative PCR revealed that H3K9 acetylation of the Agt promoter was increased at both weeks 5 and 8 in the diabetic kidney, while H3K4 tri-methylation was not significantly enriched in the promoter at week 5, but increased by week 8 (Figure 4, C and D). These results suggest that epigenetic changes start from H3K9 acetylation and gradually extend to H3K4 tri-methylation and DNA demethylation in the Agt promoter in the diabetic kidney.

Role of Epigenetic Changes in AGT Expression in Human Renal Proximal Tubular Epithelial Cells

The role of epigenetic modification in the expression of AGT was next investigated using human renal proximal tubular endothelial cells (HRPTECs). Incubation of HRPTECs with 5-Aza-2'-deoxycytidine, a DNA methyltransferase inhibitor, caused promoter demethylation at a CpG 367 bp
upstream of the transcription start site and mRNA induction of AGT (Figure 5, A and B). In addition, trichostatin A, a histone deacetylase inhibitor, increased histone acetylation of the promoter region and stimulated mRNA expression (Figure 5, C and D). Pretreatment of HRPTECs with 5-Aza-2′-deoxycytidine enhanced induction of Agt by trichostatin A. These results suggest that epigenetic changes are likely to play a causative role in the increased expression of Agt in the diabetic kidney. Demethylation of Agt seems to increase the basal levels of mRNA and to augment mRNA elevation in response to stimuli that induce histone acetylation in the promoter.

Resistance of DNA Methylation Changes to Antidiabetic Therapy

We next investigated whether the changes in DNA methylation in the diabetic kidney were responsive to antidiabetic therapy. Pioglitazone reduces blood glucose, albuminuria, and alterations in the glomerular gene expressions in db/db mice.28 Accordingly, pioglitazone significantly reduced the blood glucose levels and attenuated the increase in kidney weight and albumin excretion in the diabetic mice (Figure 6, A and B, Supplemental Figure 5A). In addition, pioglitazone prevented increases in the plasma levels of triglyceride and free fatty acid (Supplemental Figure 5B). Because changes in the mRNA levels of genes involved in lipid metabolism in the glomeruli are reported to be sensitive to pioglitazone,28 we next investigated the effect of pioglitazone on the proximal tubular cell expression of Acaca, an enzyme known to be involved in fatty acid synthesis. We found that the decrease in the mRNA expression levels of Acaca in the PT of diabetic mice was restored by pioglitazone (Supplemental Figure 5C). However, induction of Agt mRNA and demethylation of promoter DNA in the PT cells were not prevented by pioglitazone treatment. Aberrant DNA methylation, including demethylation of Abcc4 as well as increase in the methylations of Slco1a1 (Figure 6, C and D) and Clcn18 (Supplemental Figure 5D), and the corresponding mRNA changes of Abcc4 and Slco1a1 were also not inhibited by pioglitazone treatment. Changes in histone H3K9 acetylation and H3K4 tri-methylation were also resistant to pioglitazone treatment (Supplemental Figure 6).

DISCUSSION

DNA methylation studies in the diabetic kidney are rather limited compared with those of other tissues affected by
diabetes, such as the skeletal muscle, liver, and pancreatic islets, even though kidney disease is a serious complication of diabetes. This is partly because changes in the DNA methylation status of the whole kidney are a summation of the changes in the component cell populations and the actual methylation changes in individual cell types. To circumvent this problem, we collected PTs and first characterized the DNA methylation status in the PTs of normal mice. We revealed many functionally important genes that are demethylated in the PT. Hypomethylation of Slc2a5, Pck1, and G6pc and predominant expressions of their mRNAs in the PT indicates that DNA methylation underlies selective glucose handling by the PT in the kidney. In addition to glucose, PT cells also reabsorb amino acids, phosphate, and bicarbonate. Specific expression of Hnf4a in the PT seems to play critical roles in the maintenance of various transporters because Fanconi syndrome, resulting from a malfunction of the PT, is known to accompany maturity-onset diabetes of the young type 1 that is caused by inactivating mutations in human HNF4a. It would be reasonable to consider that the segmental expression of reabsorption genes in the PT is controlled by DNA methylation of key nuclear receptors such as HNF4a. Many nuclear and cytoplasmic proteins, including transcription factors and histone, are modified by the O-GlcNAc moiety. Mgea5 regulates cellular processes by eliminating the O-GlcNAc modification. The present study revealed relative mRNA enrichment and decreased DNA demethylation of Mgea5 in the PT, although the difference between PT and non-PT fractions was only moderate compared with that of the other genes described above. The preferential distribution of Mgea5 may have some relation to the active handling of glucose in the PT, but the functional role remains to be precisely determined.

By comparing the DNA methylation status of the sorted PT, we identified genes bearing aberrant DNA methylation that can potentially modify the progression of diabetic kidney disease. Met, which encodes the hepatocyte growth factor receptor, is downregulated in the diabetic kidney. Decreased Met levels with altered DNA methylation may contribute to kidney injury because stimulation of HGF signaling reduces interstitial fibrotic changes in the diabetic kidney. Persistent changes in mRNA levels with altered DNA methylation of Abcc4, which is involved in the urinary excretion of drugs such as furosemide and thiazides, and of Abcc4 in the PT may exert its effect on BP in a paracrine manner. In addition, Cyp4a14, another Cyp4a enzyme involved in BP regulation, was also significantly demethylated in the PT, where its predominant expression has been observed. Predominant expression of these Cyp4a enzymes in the PT seems to be regulated at the level of DNA methylation. Increased expression and further DNA demethylation of Cyp4a10 was observed in the PT of the diabetic kidney. This potentially leads to sodium wasting, but the contribution of Cyp4a10 in the PT to BP still needs to be determined.
With all components of the renin-angiotensin system present in the kidney, including Agt in the PT, locally generated angiotensin II has been suggested to play a significant role in sodium transport, maintenance of BP, and development of kidney injury. Although basal levels of Agt protein in the kidney are derived from the liver as a result of reabsorption by the PT after filtration, overexpression of Agt in the PT induces salt-sensitive hypertension. A pathophysiologic role for induced Agt expression in the PT and the resultant local activation of the renin-angiotensin system has also been suggested, although direct proof must await further studies. We demonstrated that the elevated expression of Agt mRNA in the PT was under the control of epigenetic mechanisms. In the PT of the db/db mice, elevation of the Agt mRNA expression preceded demethylation of DNA of the promoter region of Agt. Similarly, H3K9 acetylation of the Agt promoter was increased at both weeks 5 and 8 in the diabetic kidney, while H3K4 tri-methylation was increased only at week 8. These observations indicate that aberrant epigenetic changes gradually accumulate in the Agt promoter in the diabetic kidney. Among these epigenetic changes, H3K9 acetylation is the most sensitive marker of elevated Agt mRNA expression. Because an inhibitor of HDAC induced a marked increase of the Agt mRNA level in HRPECs, histone acetylation seems to act as a driving mechanism. The observation that DNA demethylation and increased H3K4 tri-methylation developed in the same time course is concordant with the previously reported colocalization of hypermethylated DNA and increased H3K4 tri-methylation in cancer cells. H3K4 tri-methylation and DNA demethylation could be induced by active transcription, and DNA demethylation may also act as a stabilizing mechanism in mRNA maintenance because an inhibitor of DNMT elicited a moderate but significant increase of the Agt mRNA level.

In contrast, methylation of Cldn18 markedly increased in mice with diabetes. Expression of Cldn18 mRNA was not observed, even with extended PCR cycles (data not shown), in the PT of control or diabetic mice. The closed chromatin status of Cldn18, already present in normal mice, seems to...
be further stabilized by the increase in DNA methylation in the diabetic kidney. Such stabilization has been frequently observed in tumor cells. Although its physiologic significance seems to be limited, hypermethylation of genes such as Cldn18, which show large DNA methylation changes, may serve as a good candidate marker for staging of diabetic kidneys. The finding that both increase and decrease in methylation were observed in diabetic kidneys suggests that changes in DNA methylation may somehow depend on the transcription status of each gene, possibly involving multiple chromatin-modifying factors. In the present study, we focused on the selected genes for analysis of aberrant DNA methylation induced by diabetes; therefore, further genome-wide analysis between control and diabetic animals would reveal the other genes with pathophysiologic importance.

Pioglitazone has been reported to attenuate glomerular injury in db/db mice, although the changes in glomerular gene expressions were, in some part, resistant to antidiabetic therapy. These results suggest a wide range of responsiveness to diabetic control. In the present study, pioglitazone inhibited blood glucose elevation, dyslipidemia, albuminuria, renal hypertrophy and the decrease of Acaca mRNA expression in the PT. In contrast, the altered DNA methylation and histone modifications were refractory to pioglitazone treatment. These observations suggest that epigenetic changes at the level of DNA methylation represent and underlie the persistent mRNA changes that potentially lead to phenotypic changes in the diabetic kidney. However, because we observed refractoriness of the response in only three selected genes, further analysis is needed to draw the general conclusion that aberrant DNA methylation defines the phenotypic changes in diabetes. Epigenetic changes could be triggered by metabolic abnormalities before the start of pioglitazone administration and be maintained by some stimuli, such as albuminuria and blood glucose levels, which pioglitazone did not prevent completely. Although stricter control of the blood glucose levels may attenuate the DNA methylation changes, the results that pioglitazone did not prevent aberrant DNA methylation may have some clinical relevance, given that reduction of the blood glucose levels to within the normoglycemic range is rarely achieved in many diabetic patients.

We used db/db mice with leptin receptor mutation as the model for diabetic kidney disease. Although leptin receptor mutation is a very rare cause of human type 2 diabetes, the kidney conditions in the db/db mice mimic many aspects of human kidney disease, particularly the changes in the early stage. Important factors and pathways for the development of diabetic kidney disease have been clarified using
whether aberrant DNA methylation in candidate disease-modifying genes, identified in the present study, is also induced in human diabetic kidney disease remains to be validated in future studies. In this regard, aberrant methylation of ABCC4 has been reported in the tubular compartment obtained from diabetic patients, while no changes in the DNA methylation status in other genes, which were observed in the present study, such as AGT, were detected in the study by Ko et al.6 Since the study by Ko et al. included patients with CKD without diabetes in the diseased cases, and diabetic patients without renal dysfunction in the controls for the first screening, some genes bearing aberrant DNA methylation specific for diabetes may not have been detected. Alternatively, purification of the PT, which can eliminate the influence of other cell populations, such as distal tubules, interstitial cells, and vascular compartment, may have led to the identification of the genes specifically altered in the PT in the present study.

In conclusion, the present study revealed genes showing differential DNA methylation within the kidney and indicated the importance of cell-type–specific analysis in organs with multiple components. The finding that ontology and pathway analysis of hypomethylated PT-DMRs illuminated terms of mitochondrial function and biogenesis coincides with the enrichment of mitochondria in the PT and suggests the strict regulation of mitochondrial function, such as metabolism of glucose, lipid, and energy, in the PT by DNA methylation. Indeed, many functionally important molecules in metabolism and transport are under the control of DNA methylation in the PT. Aberrant DNA methylation induced by diabetes was resistant to oral treatment with pioglitazone, a commonly used anti-diabetic agent, and underlies persistent mRNA alterations, which likely lead to phenotype changes in the diabetic kidney. Exploration of the mechanisms underlying aberrant methylation of the target genes revealed in the present study could pave the way to the development of novel therapeutic means to prevent and/or reverse progression of the diabetic kidney disease.

CONCISE METHODS

Detailed methods are available in the Supplemental Material.

Animals

Animal care and treatment complied with the standards described in the Guidelines for the Care and Use of Laboratory Animals of the University of Tokyo. Male C57BLKS/J db/db and db/m mice, 5–10 weeks old, were purchased from Japan CLEA (Tokyo, Japan).

Sorting of the PT Cells

PT cells were stained with Lotus tetragonolobus lectin (Vector Laboratories, Burlingame, CA) as the marker and were sorted using FACSAria III (BD Biosciences, San Jose, CA).

Cell Culture

HRPTECs (Lonza, Walkersville, MD) were cultured in growth medium (REGM, Lonza) at 37°C in an atmosphere containing 5% CO2, and used at passages between 3–5.

DNA Methylation Analysis

DNA methylation profile of PT cells (db/m) was analyzed genome-wide by a microarray-based analysis, D-REAM, as described previously.10,42
Representative DMKs were analyzed by COBRA using a microchip electrophoresis system, MutiNA (Shimadzu, Kyoto, Japan) for quantification. Agt promoter was analyzed by bisulfite sequencing.

**Analysis of mRNA Levels**

Quantitative RT-PCR analysis was performed with ABI StepOne Plus detection system. The signals of in situ hybridization were visualized using NBT-BCIP solution (Sigma-Aldrich), an alkaline phosphate color substrate.

**ChIP Assay**

ChIP assay was performed using antiacetylated histone H3K9 (#9671; Cell Signaling Technology, Beverly, MA) or anti–tri-methylated histone H3K4 (07–473; EMD Millipore, Bedford, MA) antibodies.

**Statistical Analyses**

Multiple parametric comparisons were performed by ANOVA, followed by Fisher protected least-significant-difference test. Comparisons between two groups were performed by the t test. P values <0.05 were considered to represent statistically significant differences.

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