Proteomics analysis reveals diabetic kidney as a ketogenic organ in type 2 diabetes

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Submitted 24 May 2010; accepted in final form 12 October 2010

Diabetic nephropathy (DN) has become the leading cause of end-stage renal disease among diabetic patients around the world (34, 49). Nephropathy develops in 20–40% of patients with diabetes, although the underlying mechanism remains incompletely characterized. During the development and progression of DN, a series of pathophysiological changes occur in the glomeruli and tubulointerstitium, thus leading to glomerular hyperfiltration, microalbuminuria, macroalbuminuria, and eventually glomerulosclerosis and tubulointerstitial fibrosis (18, 19, 35, 40). All of these pathophysiological changes are believed to be the result of an interaction between hyperglycemia-induced metabolic and hemodynamic changes and genetic predisposition (7). They are associated with alterations in a set of genes, including profibrotic cytokine transforming growth factor-β (TGF-β) and hemodynamic factor angiotensin II (27, 31). Blockade of the renin-angiotensin system and blood pressure control are effective in slowing the progress of DN, at least in a subpopulation of diabetic patients. Neutralizing antibodies against TGF-β also have certain beneficial effects on diabetic kidneys (36, 50). However, novel pathophysiological pathways identified and targeted are vital to reduce the increasing burden of this disease (39).

In the past decade, many high-throughput screening technologies, including gene microarray and proteomic techniques, have allowed researchers to define the pathophysiological mechanisms of many complicated diseases and to identify novel therapeutic targets for treatment (12, 21, 24). The rapidly developed proteomic technique, which can identify changes in protein expression, posttranslational modifications, protein-protein interactions, cellular and subcellular distribution, and temporal patterns of expression (48), offers an ideal approach to study the complicated functional network of proteins in diabetes and DN (26, 33). Proteome analysis of renal protein profiles in the diabetic condition may reveal additional novel mechanisms contributing to the progression of DN.

In the present study, we used two-dimensional electrophoresis (2DE) combined with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to quantitatively profile the expression of proteins in the kidneys of type 2 diabetic db/db mice. We found that the expression of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase 2 (HMGC2S), the key enzyme in ketogenesis, was increased fourfold in the kidneys of type 2 diabetic db/db mice. Consistently, the activity of HMGC2 in kidneys and 24-h urinary excretion of the ketone body β-hydroxybutyrate (β-HB) were significantly increased in db/db mice. Immunohistochemistry, immunofluorescence, and real-time PCR studies further demonstrated that HMGC2S was highly expressed in renal glomeruli of db/db mice, with weak expression in the kidneys of control mice. Because filtered ketone bodies are mainly reabsorbed in the proximal tubules, we used RPTC cells, a rat proximal tubule cell line, to examine the effect of the increased level of ketone bodies. Treating cultured RPTC cells with 1 mM β-HB significantly induced transforming growth factor-β1 expression, with a marked increase in collagen I expression. β-HB treatment also resulted in a marked increase in vimentin protein expression and a significant reduction in transforming growth factor-β expression, with a marked increase in collagen I expression. These findings demonstrate that diabetic kidneys exhibit excess ketogenic activity resulting from increased HMGC2S expression. Enhanced ketone body production in the diabetic kidney may represent a novel mechanism involved in the pathogenesis of DN.

diabetic nephropathy; ketone body; gene expression; epithelial-to-mesenchymal transition

DIABETIC NEPHROPATHY (DN) has become the leading cause of end-stage renal disease among diabetic patients around the world (34, 49). Nephropathy develops in 20–40% of patients with diabetes, although the underlying mechanism remains incompletely characterized. During the development and progression of DN, a series of pathophysiological changes occur in the glomeruli and tubulointerstitium, thus leading to glomerular hyperfiltration, microalbuminuria, macroalbuminuria, and eventually glomerulosclerosis and tubulointerstitial fibrosis (18, 19, 35, 40). All of these pathophysiological changes are

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MATERIALS AND METHODS

Chemical reagents. Immobilized pH gradient (IPG) strips were purchased from Bio-Rad Laboratories (Hercules, CA). All chemical reagents for electrophoresis were from Amersham Biosciences (Uppsala, Sweden). All cell culture medium and supplements were from Sigma (St. Louis, MO). Modified trypsin (sequence grade) was from Promega (Madison, WI).
Animal study. Sixteen-week-old male C57BKS/J db/db mice and age- and sex-matched nondiabetic littermate db/+mice as controls purchased from Jackson Laboratory were used. All animal experimental protocols were approved by the Peking University Institutional Animal Care and Use Committee. Fasting blood glucose, plasma insulin, serum triglyceride, and cholesterol levels were analyzed as previously described (5). Urinary albumin was measured by enzyme-linked immunosorbent assay (ELISA) (Albuwell kit, Exocell). Content of β-HB in plasma, urine, and kidney tissues was measured at the National Center of Biomedical Analysis with NMR spectroscopy as previously described (6).

Proteomic analysis. Kidneys were perfused with ice-cold PBS buffer to remove blood contamination. To prepare the renal protein samples, the whole kidney from each mouse was ground to powder in liquid nitrogen. The powder was suspended in ice-cold 10% TCA-acetone and incubated at −20°C overnight for protein precipitation. The precipitated proteins were centrifuged at 40,000 g and 4°C for 15 min. The pellets were collected, washed with ice-cold acetone twice, and then vacuum freeze-dried and stored as protein powder at −80°C. For 2DE, protein powders were dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 40 mM Tris, 2% CHAPS, 50 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.2% IPG buffer (pH 3–10), and sonicated for 3 min, and then centrifuged at 40,000 g and 4°C for 30 min.

Isoelectric focusing electrophoresis (IEF) was carried out with IPG strips (18 cm, pH 3–10) according to the manufacturer’s instructions. After quantification of protein content by the Bradford method, 200 μg of total mixed proteins from six control and six diabetic mice was loaded onto the gels in triplicate. Briefly, the strips were rehydrated without voltage for 4 h and then with 50 V for 8 h. IEF was programmed at 20°C with the following parameters: 500 V for 1 h, 1,000 V gradient for 1 h, 4,000 V gradient for 1 h, 8,000 V gradient for 1 h, and then 8,000 V until a total of 64,000 Vh was achieved. After reduction of 1% DTT for 15 min and alkylation of 2.5% iodoacetamide for another 15 min, the IPG strips were loaded onto 12% polyacrylamide gels in Tris-glycine buffer (25 mM Tris·HCl, 192 mM glycine, 0.1% SDS). Electrophoresis involved use of the Ettan DALT II system for 1 h at 2.5 W per gel and then at 15 W per gel until the dye front reached the bottom of the gel. Gels were stained by an optimized classic silver staining method appropriate for mass spectrometry identification.

The 2D gels were scanned with the Amersham Imagescanner, and the images were analyzed by ImageMaster 2D Platinum (Amersham Biosciences). Significant change in level was defined as protein spots with equal or greater than twofold expression change between the diabetic and control groups. For protein identification, gel pieces containing the target protein spots were excised, washed, and subjected to in-gel trypsin digestion as previously described (16). The tryptic digestes were desalted by POROS R2 (Applied Biosystems, Foster City, CA) and then cocystallized with a matrix of α-cyano-4-hydroxy-cinnamic acid spotted on the target wells. The dried matrixes underwent then Brüker autoflex MALDI-TOF MS (Bremen, Germany). Protein identity based on the peptide mass fingerprint was confirmed by Mascot search software (Matrix Science, Boston, MA) against the NCBIinr database; one incomplete cleavage was allowed, and alkylation of cysteine by carbamidomethylation, oxidation of methionine, and pyro-Glu formation of NH2-terminal Gln were considered possible modifications.

Western blot. Equal amounts of protein from each sample (20–100 μg) were separated by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (4°C, 200 mA for 2 h) by the Bio-Rad Mini PROTEAN 3. The membranes were blocked at room temperature for 1 h in PBS with Tween 20 (PBST) containing 5% skim milk and then incubated with polyclonal rabbit anti-HMGCS2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-E-cadherin (1:500; Cell Signaling), or monoclonal mouse anti-vimentin (1:1,000; Sigma) at 4°C overnight. The membranes were washed five times for 5 min each with PBST and then incubated in 1:5,000 horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The membranes were washed as above and developed with an enhanced chemiluminescence kit. After assay of target proteins, the membranes were stripped with 0.2 N NaOH (47) and reprobed with 1:1,000 diluted monoclonal tubulin antibody.

Isolation of renal glomeruli. Glomeruli for RNA extraction were isolated from control and db/db mice by the conventional sieving method. Briefly, the cortex of excised kidneys was dissected into pieces and underwent digestion with collagenase II (1 mg/ml) for 1 h at 37°C. The suspension was gently pressed though 100-, 70-, and 40-μm cell strainers sequentially and then the 40-μm cell strainer was inverted and washed twice with PBS. The flow-through was collected. Glomeruli were collected by spinning at 1,000 g for 10 min. The purity of isolated glomeruli was examined by visualization under a microscope.

RNA extraction and quantitative real-time RT-PCR. Total RNA from kidney tissue, RPTC cells, or mouse glomeruli was isolated with TRIzol reagent (Promega) and reverse transcribed by the reverse transcription system (Promega). Quantification of gene expression of HMGCS2, TGF-β1, and procollagen α1 chain of type I collagen (colIα1) was by quantitative real-time PCR. Complementary DNA was synthesized with the use of SuperScript III reverse transcriptase (Invitrogen, Carlsberg, CA) and oligo(dT) primer (Promega). Quantitative PCR involved the use of SYBR Green I (Molecular Probes, Eugene, OR) as a fluorescent probe. The primers were designed as follows: HMGCS2, forward: 5′-GGC TGT CAA AAC AGT GCT CA-3′ and reverse: 5′-GCA ATG TCA TCA CAG CAC AC-3′; TGF-β1, forward: 5′-TGA GTG GCT GTC TTC TGA CG-3′ and reverse: 5′-TGG ACT GAT CCC ATT ATT GAT T-3′; colIα1, forward: 5′-AGG CAT AAA GGG TCA TCG TG-3′ and reverse: 5′-ACC GGT GAC TTC ATC TTG GC-3′; and β-actin, forward: 5′-GGA TTC CTA TGT GGG TGA CG-3′ and reverse: 5′-CTT CTC CAT GTC GTC CCA GT-3′. Quantitative values were obtained as threshold-old PCR cycle number (Ct) when the increase in fluorescent signal of PCR product became an exponential growth. Target gene mRNA level was normalized to that of β-actin in the same sample as detailed previously (46). In brief, the relative expression of the target gene compared with that of β-actin was calculated as 2−ΔΔCt, where ΔΔCt = Ct target gene − Ct β-actin. Each sample was measured in duplicate or triplicate in each experiment. Moreover, melting curves for each PCR product were analyzed to ensure the specificity of the amplification product.

Immunohistochemical analysis and immunofluorescence staining. For immunohistochemical analysis, tissues were fixed in 4% paraformaldehyde overnight at 4°C and then dehydrated in an ascending ethanol series and paraffin embedded. After conventional deparaffinization and hydration, sections (3 μm) were treated with 3% H2O2 for 10 min to eliminate endogenous activity of peroxidase and then heated in a microwave oven in 1 mM EDTA (pH 8.0) for antigen retrieval. After being cooled at room temperature and washed with PBS, sections were blocked for 20 min in blocking solution (5% goat serum in PBS) and then incubated with polyclonal rabbit anti-HMGCS2 antibody (1:1,000, Santa Cruz Biotechnology) diluted in blocking solution overnight at 4°C. Sections were then washed in PBS and incubated with poly-peroxidase-conjugated goat-anti-rabbit IgG (Zhongshan Golden Bridge, Beijing, China) for 30 min at room temperature. After several washings with PBS, samples underwent successive diaminobenzidine (DAB) staining, hematoxylin staining, and dehydration and were mounted on cover glass. Serial sections were incubated with the same quantity of nonimmune rabbit IgG instead of primary antibody as negative controls.

Immunofluorescence staining was performed as previously reported (22). Briefly, 5-μm sections were fixed in ice-cold acetone and then permeabilized and blocked with 0.3% Triton X-100 and 100% goat serum. Primary antibodies were rabbit anti-HMGCS2 (1:1,000, Santa Cruz Biotechnology) and mouse anti-synaptotogpin (ready to
use. After three washes, the slides were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG. Stained images of glomeruli stained with each antibody were selected at random and analyzed by a person who was blinded to the study groups.

Determination of HMGCS2 activity in the kidney. HMGCS2 activity was determined by monitoring the disposal rate of absorbance at 30°C; 5 mM acetyl phosphate, 10 mM acetoacetate, and 5 l M acetoacety-CoA, and 5 l M acetyl-CoA from 10 μM acetyl-CoA, and 5-μl samples of extracts (25 μg protein) were added and then acetyl-CoA (100 μM) and 10 U of phosphotransacetylase (PTA) were added. HMGCS2 activity was measured as the difference in the rate before and after acetyl-CoA addition. By definition, 1 unit of enzyme activity causes 1 μmol of acetoacetyl-CoA to be transformed in 1 min.

RPTC cell culture. RPTC cells, a rat renal proximal tubule cell line, were generously provided by Dr. Zheng Dong (Medical College of Georgia, Augusta, GA). Cells were cultured in DMEM-F-12 medium supplemented with 1% fetal bovine serum, 5 mg/l insulin, 5 mg/l transferrin, 4 mg/l dexamethasone, 1% antibiotics, 1% 200 mM L-glutamine, and 5% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO2. The cells were split weekly, and the confluence reached 60%.

Measurement of TGF-β1 production and collagen secretion in RPTC cells. The TGF-β1 level in the supernatant of RPTC cell cultures was determined by a Quantikine ELISA Kit (R & D Systems) according to the manufacturer’s instructions. The soluble collagen content in the medium of cultured cells was determined with the Sircol solubilized collagen assay kit (Biocolor, Belfast, UK) as previously reported (5).

Statistical analysis. All data are given as means ± SE. Differences were analyzed by ANOVA and Student’s t-test. Statistical significance was defined as P < 0.05.

RESULTS

Animal data. The C57BKS/J db/db mouse, lacking the leptin receptor, is a well-documented model of type 2 diabetes exhibiting hyperglycemia, hyperinsulinemia, insulin resistance associated with hyperphagia, and obesity (1, 4, 37). The body weight of db/db mice at 16 wk of age was significantly increased by about twofold over that of db/m mice (Table 1). The blood glucose levels of db/m and db/db mice were 8.9 ± 0.5 and 17.9 ± 0.9 mmol/l, respectively (P < 0.001). Compared with db/m mice, db/db mice showed significantly higher plasma insulin and cholesterol levels (Table 1). To further evaluate the renal function of db/db mice, 24-h urinary albumin excretion was measured. At 16 wk of age, 24-h urinary albumin excretion was significantly higher in db/db mice than in db/m mice (588.7 ± 112.7 μg vs. 27.6 ± 8.4 μg), which is consistent with our previous findings in db/db mice (5).

Upregulation of HMGCS2 in diabetic kidneys. Protein spots with differential expression in three sets of gels were analyzed (Supplemental Fig. S1).1 Approximately 700 protein spots were visualized on the 2D gels. In total, 48 protein spots were significantly downregulated and 14 upregulated (P < 0.05). Five downregulated and three upregulated protein spots with the most significant expression changes underwent MALDI-TOF MS assay. All identified proteins are in Table 2. By using real-time PCR, we confirmed the changes of Acy3, Ldh, Nudt19, KCP1, and VH26 at the mRNA level (Supplemental Fig. S2). Among eight proteins, HMGCS2, the rate-limiting enzyme controlling the HMG-CoA pathway in ketogenesis, was significantly increased by more than fourfold (Fig. 1A). Increased HMGCS2 expression in diabetic kidneys was further confirmed by Western blot assay (Fig. 1B). To clarify whether the increase in HMGCS2 protein levels was due to increased gene transcription, quantitative real-time PCR

Table 1. Phenotypic characteristics of control and db/db mice

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ratio (db/db vs. control)</th>
</tr>
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<tbody>
<tr>
<td>Aspartoacylase-3</td>
<td>0.3</td>
</tr>
<tr>
<td>Epoxide hydrolase 2</td>
<td>0.4</td>
</tr>
<tr>
<td>G-lactate dehydrogenase</td>
<td>0.3</td>
</tr>
<tr>
<td>Nucleoside diphosphate-linked moiety X motif 19</td>
<td>0.3</td>
</tr>
<tr>
<td>Protein kinase C inhibitor protein 1</td>
<td>0.5</td>
</tr>
<tr>
<td>Mitochondrial 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (HMGCS2)</td>
<td>4.2</td>
</tr>
<tr>
<td>Aldolase 2, B isozyme</td>
<td>2.2</td>
</tr>
<tr>
<td>Similar to Ig heavy chain V–III region VH26 precursor</td>
<td>2.4</td>
</tr>
</tbody>
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Spot numbers correspond to those labeled in Supplemental Fig. S1.
that HMGCS2 was weakly expressed in kidneys of control mice (Fig. 4A). Real-time PCR analysis showed HMGCS2 mRNA expression higher, by 2.8-fold, in db/db kidneys than in db/m kidneys (Fig. 1C).

Increased HMGCS2 activity and ketone body production in diabetic kidneys. To determine whether upregulated HMGCS2 expression results in enhanced ketogenesis and increased ketone body production, HMGCS2 activity was measured in kidney homogenates from db/db and db/m mice. Very low HMGCS2 activity was detected in db/m kidneys (Fig. 2). In contrast, HMGCS2 activity was increased fivefold in db/db kidneys compared with db/m kidneys. Similarly, HMGCS2 mRNA and protein levels were significantly increased in the livers of db/db mice compared with those of db/m mice (Supplemental Fig. S3).

Because HMGCS2 is the key enzyme controlling ketone body formation in the liver (10) and β-HB accounts for ~70% of the ketone bodies (14), we measured β-HB levels in plasma, urine, and kidney tissues of db/m and db/db mice. Urinary β-HB excretion at 24 h was about fourfold higher in db/db mice than in control mice (Fig. 3A). Moreover, renal β-HB levels were significantly higher in db/db than control kidneys (Fig. 3B). In contrast, serum β-HB concentrations did not differ between db/m and db/db mice (Fig. 3C).

Localization of HMGCS2 in the kidneys of db/db mice. To further address the potential role of increased HMGCS2 expression in the kidneys of db/db mice, immunohistochemistry and immunofluorescence analyses were performed to determine intrarenal localization of HMGCS2 in kidneys. The specificity of HMGCS2 antibody was confirmed by the lack of specific staining with nonimmune IgG used as a negative control (Fig. 4A, inset). Immunohistochemical staining showed that HMGCS2 was weakly expressed in kidneys of control mice but highly expressed in renal glomeruli of db/db mice (Fig. 4A). This expression pattern was further confirmed by using another commercially available goat polyclonal antibody (Santa Cruz Biotechnology) (data not shown). Immunofluorescence study of HMGCS2 and synaptopodin, a glomerular podocyte-specific marker, further showed HMGCS2 highly expressed in glomerular podocytes and in other glomerular cells (Fig. 4B). Consistently, quantitative real-time PCR analysis revealed increased glomerular HMGCS2 mRNA expression in diabetic kidneys. HMGCS2 mRNA levels were 5.3-fold higher in freshly isolated glomeruli of db/db mice than in those of db/m mice (Fig. 4C). HMGCS2 mRNA levels were also significantly increased in renal cortical tubules (Fig. 4C).

Induction of TGF-β1 and collagen I expression and EMT by β-HB in cultured renal proximal tubule cells. Because filtrated ketone bodies are mainly reabsorbed and metabolized by the proximal tubules (8, 14), we analyzed the impact of increased ketone body exposure on RPTCs, a rat proximal tubule cell line. Treatment with 1 mM β-HB for 24 h significantly increased expression and secretion of TGF-β1 (Fig. 5A) and collagen I (Fig. 5B) in RPTCs, which is consistent with a previous report that β-HB induced TGF-β and collagen production in HK-2 cells (9). β-HB treatment also induced collagen IV and fibronectin expression in RPTCs (Fig. 5C). Furthermore, we evaluated histological changes, collagen I and IV expression, and EMT in kidneys of db/db mice. Histological examination showed that diabetic glomeruli exhibited increased mesangial expansion and fibrosis (Supplemental Fig. S4A). Immunohistochemistry study demonstrated that diabetic kidney exhibited increased expression of collagen I and IV (Supplemental Fig. S4, B and C) and loss of E-cadherin and

Fig. 1. Proteomic identification of increased 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) expression in diabetic kidneys and validation by Western blot and real-time PCR analysis. A: left: representative high-magnification images from 2-dimensional gels. Arrows indicate protein spot corresponding to HMGCS2. Right: quantitative measurements showed significant increase in HMGCS2 protein expression in db/db vs. db/m mouse kidneys. B: immunoblot assay verified that HMGCS2 protein was increased in diabetic kidneys (n = 6 for each group). C: real-time PCR analysis demonstrated that HMGCS2 mRNA levels of diabetic kidneys were significantly increased (n = 5 for each group). mRNA levels of HMGCS2 were normalized to those of β-actin. **P < 0.01, ***P < 0.001 vs. db/m.
gain in vimentin expression (Fig. 6A). We also examined the effect of β-HB treatment on EMT, which plays an important role in renal fibrosis and DN (32, 38). Western blot analysis showed that the cellular adhesion molecule E-cadherin, a specific marker of epithelial cell phenotype, was significantly inhibited with β-HB treatment, whereas the expression of vimentin, a myofibroblast marker, was significantly increased (Fig. 6B). At a dose as high as 1 mM, β-HB did not affect cell viability of RPTC cells (data not shown).

DISCUSSION

With the development of high-throughput technologies, proteomics is becoming a powerful tool for identifying potential mechanisms underlying many complicated diseases, including cancer, cardiovascular diseases, and diabetes mellitus (21, 33). One of the advantages of the proteomic approach is that it allows for comparison of protein profiles in normal and diseased tissues or cells, which may lead to the identification of new proteins or novel signaling pathways involved in the pathogenesis of disease (21). In the past decade, the proteomic technique has been used to define the pathophysiology of DN in various animal models (2, 40, 41, 43). Proteomic analysis of renal cortex and glomerular protein profiles from diabetic db/db mice were first reported by Tilton et al. (43) and Barati et al. (2). A number of dysregulated proteins were identified in these studies, which greatly advances our understanding of the mechanisms of DN. Our study is a step toward validation of the identified proteins in diabetic kidneys and elucidation of the role of novel signaling networks in the pathogenesis of DN.

In the present study, we used 2DE combined with MALDI-TOF MS to analyze protein profiles in the whole kidneys of db/db mice and lean control mice and identified a set of differentially regulated proteins. Among eight proteins identified, the changes of five proteins, Hmgcs2, Acy3, Ephx2, KCIP-1, and Nudt19, were in line with a previous study using renal cortex of db/db mice (39). Furthermore, we confirmed the changes of Acy3, Ldh, Nudt19, KCIP-1, and VH26 at the mRNA level. Among the proteins with altered expression, HMGCS2, the rate-limiting enzyme in ketogenesis and highly expressed in the kidneys of db/db mice. Because a previous report demonstrated that β-HB, the major ketone body, induces fibrotic processes in a human proximal tubule cell line (9), this finding suggests that ketone bodies may play an important role in the pathogenesis of DN. The increase of HMGCS2 expression in the kidneys of db/db mice was further supported by immunoblot and real-time PCR assays. As ex-
The activity of HMGCS2 was also significantly increased in the kidneys of \textit{db/db} mice. Because overexpression of HMGCS2 is associated with hepatic hyperketogenesis (44), these results suggest that the diabetic kidney may exhibit increased ketogenesis. In support of this, the level of the major ketone body, \(\beta\)-HB, was significantly higher in urine and kidney tissues from \textit{db/db} mice than in those from \textit{db/m} mice. Because the two groups did not differ in serum \(\beta\)-HB levels.

Fig. 4. Localization of HMGCS2 expression in diabetic kidneys. \(A\): immunohistochemical analysis of HMGCS2 in kidneys of \textit{db/m} and \textit{db/db} mice. Arrows indicate the glomeruli. NC, negative control (sections incubated with the same quantity of nonimmune rabbit IgG instead of primary antibody; shown for the specificity of the HMGCS2 antibody used). \(B\): immunofluorescence results showing HMGCS2 mainly expressed in podocytes but also in other cell types of glomeruli. Podocytes are labeled in green by a primary antibody against synaptopodin and a FITC-labeled secondary antibody. Colocalization of synaptopodin and HMGCS2 is displayed by yellow fluorescence in merged image. \(C\): measurement of HMGCS2 mRNA levels in freshly isolated glomeruli and cortical tubules of \textit{db/m} and \textit{db/db} mice; Top: morphological appearance and purity of isolated glomeruli; arrow indicates purified glomeruli. Bottom: HMGCS2 mRNA levels were examined by quantitative real-time PCR in glomeruli and cortical tubules compared with \textit{db/m} mice. The mRNA levels of HMGCS2 were normalized to those of \(\beta\)-actin. *\(P < 0.05\) vs. control (\(n = 4\)).

Fig. 5. Ketone body-induced transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) and extracellular matrix protein expression in cultured RPTC cells. \(A\): TGF-\(\beta\)1 mRNA expression (left) and production (right) after 24-h \(\beta\)-HB treatment (1 mM). \(B\): collagen I (coll1) mRNA expression (left) and supernatant collagen content (right) after exposure to 1 mM \(\beta\)-HB treatment for 24 h. \(C\): collagen IV (coll4; left) and fibronectin (right) mRNA expression were increased after \(\beta\)-HB treatment for 24 h. *\(P < 0.05\), **\(P < 0.01\) vs. control (\(n = 4\)).
increased renal and urinary ketone body levels may reflect enhanced ketogenesis in diabetic kidneys. It is also possible that increased glomerular filtration of \(\beta\)-HB and subsequent uptake by kidney may account for the elevated urinary and kidney \(\beta\)-HB levels seen in \(db/db\) mice. Since hepatic expression of HMGCS2 is increased in \(db/db\) mice, these mechanisms may help maintain normal plasma \(\beta\)-HB levels in the type 2 diabetes condition. Collectively, these results demonstrate that renal HMGC2 upregulation is, at least in part, responsible for increased ketone body production in kidneys of \(db/db\) mice and suggest that the diabetic kidney becomes a ketogenic organ.

It has been reported that HMGC2 mRNA and protein are present in suckling rat kidney, and renal cortex is able to produce low amounts of ketone bodies with oleate treatment (42). HMGC2 mRNA was also reported to be expressed in human kidney (17). However, little is known regarding the intrarenal localization of this enzyme. To further determine intrarenal localization of HMGC2 expression in diabetic kidneys, immunohistochemistry and immunofluorescence studies were performed. HMGC2 was found to be weakly expressed in nondiabetic kidneys but abundant in renal glomeruli of diabetic mice. High expression of HMGC2 in renal glomeruli was further supported by freshly isolated glomeruli from \(db/db\) mice exhibiting significantly higher mRNA levels of HMGC2 than those from \(db/m\) mice. In diabetic glomeruli, HMGC2 expression appeared to be mainly localized in podocytes, as assessed by immunofluorescence analysis. Thus podocytes may be one of the major sources of ketone bodies in diabetic kidneys. Although HMGC2 was predominantly expressed in the glomeruli of \(db/db\) mouse kidneys, low but detectable expression of HMGC2 was also found in renal cortical tubules. Therefore, renal cortical tubules, especially the proximal tubules, may also be responsible for increased ketone bodies in the kidney and urine.

Ketone bodies in Bowman’s capsule, either filtered or produced by glomeruli, are mainly reabsorbed in the proximal tubules; \(\sim 80\%\) of ketone bodies are absorbed by this nephron segment (8). In the physiological state, ketone bodies, including \(\beta\)-HB and acetoacetate, are mainly reabsorbed by tubular epithelial cells through sodium-coupled monocarboxylate transporters (SMCTs), as the major energy sources with lactic acids promoting substance transportation in the cells or by being transported back to circulation through proton-coupled monocarboxylate transporters (MCTs). Although little is known about the expression of SMCT and their regulation, peroxisome proliferator-activated receptor (PPAR)\(\alpha\) can up-regulate the MCT-1 expression in renal proximal tubular cells (13). In the present study, HMGC2 activity and ketogenesis were significantly elevated in the kidneys of \(db/db\) mice, which suggests that renal proximal tubule cells are exposed to high levels of ketone bodies in the diabetic condition. In line with a previous report (9), ketone body \(\beta\)-HB increased TGF-\(\beta\) expression and collagen production in cultured RPTCs. These effects appeared to be mediated by oxidative stress and are Smad3 dependent (9). Because EMT also plays an important role in the progression of DN (11, 32), the effect of \(\beta\)-HB on epithelial-to-mesenchymal transition (EMT) in renal proximal tubule cells (Fig. 6). Ketone body-enhanced epithelial-to-mesenchymal transition (EMT). A: representative photomicrographs of E-cadherin (top) and vimentin (bottom) immunohistochemistry in \(db/m\) (left) and \(db/db\) (right) mice. Magnification \(\times 400\). B, top: Western blot analysis of E-cadherin and vimentin expression in cultured RPTCs after 24-h \(\beta\)-HB treatment. Bottom: quantitation by computer-assisted densitometry. *\(P < 0.05\), **\(P < 0.01\) vs. control (\(n = 4\)).
the expression of E-cadherin and vimentin, specific molecular markers for epithelial and mesenchymal cells, respectively, was evaluated in RPTC cells. β-HB treatment resulted in significantly decreased E-cadherin expression and increased vimentin expression. These findings suggest that increased levels of β-HB may be involved in the EMT process in proximal tubule cells in the diabetic condition.

Lipotoxicity plays an important role in the pathogenesis of DN. In studies reported by Levi’s group (28, 45), expression levels of HMG-CoA reductase, a critical enzyme involved in cholesterol biosynthesis, were significantly increased in the kidneys of both type 1 and type 2 diabetes. These studies suggest that increased HMG-CoA reductase may account for cholesterol accumulation in diabetic kidneys. HMG-CoA, a key intermediate in the biosynthesis of cholesterol, is formed from acetyl-CoA and acetoacetyl-CoA by HMGC. Our finding that HMGC is significantly upregulated in diabetic kidney implies that increased renal HMGC may be an important factor contributing to cholesterol accumulation and lipotoxicity in diabetic kidneys, since HMGC can also produce cholesterol-convertible HMG-CoA inside the mitochondria (25).

In summary, we provide novel evidence that the expression and enzymatic activity of HMGC is significantly increased in the kidneys of db/db mice. Renal glomeruli may represent a major source of ketone bodies in the diabetic kidney. Exposure of proximal tubule cells to β-HB was associated with a significant induction of TGF-β1 and collagen I expression and the EMT process. Since ketoacidosis is predominantly a type 1 diabetic feature that may occur in type 2 diabetes, our results suggest that increased renal ketogenesis resulting from upregulation of HMGC may play an important role in the pathogenesis of DN in type 2 diabetes (Fig. 7). Renal HMGC may therefore represent a potential therapeutic target for the treatment of diabetic renal complications.

ACKNOWLEDGMENTS

We thank Dr. Zheng Dong (Dept. of Cellular Biology and Anatomy, Medical College of Georgia) for providing the RPTC cell line. We thank Dr. Jing Miao and Qian Wang for their help in tissue sectioning.

GRANTS

This study was supported by the Natural Science Foundation of China (30725033/81030003/30890041). Support for this project was also provided by the Ministry of Science and Technology of China (2010CB912503) (to Y. Guan).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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