Associations between local acidosis induced by renal LDHA and renal fibrosis and mitochondrial abnormalities in patients with diabetic kidney disease

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During the progression of diabetic kidney disease (DKD), renal lactate metabolism is rewired. The relationship between alterations in renal lactate metabolism and renal fibrosis in patients with diabetes has only been partially established due to a lack of biopsy tissues from patients with DKD and the intricate mechanism of lactate homeostasis. The role of lactate dehydrogenase A (LDHA)-mediated lactate generation in renal fibrosis and dysfunction in human and animal models of DKD was explored in this study. Measures of lactate metabolism (urinary lactate levels and LDHA expression) and measures of DKD progression (estimated glomerular filtration rate and Wilms’ tumor-1 expression) were strongly negatively correlated in patients with DKD. Experiments with streptozotocin-induced DKD rat models and the rat renal mesangial cell model confirmed our findings. We found that the pathogenesis of DKD is linked to hypoxia-mediated lactic acidosis, which leads to fibrosis and mitochondrial abnormalities. The pathogenic characteristics of DKD were significantly reduced when aerobic glycolysis or LDHA expression was inhibited. Further studies will aim to investigate whether local acidosis caused by renal LDHA might be exploited as a therapeutic target in patients with DKD. (Translational Research 2022; 249:88–109)

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Submitted for Publication January 25, 2022; revision submitted May 23, 2022; Accepted for Publication June 22, 2022.
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https://doi.org/10.1016/j.trsl.2022.06.015
The role of lactate dehydrogenase A (LDHA) mediated lactate generation in renal fibrosis and dysfunction in human and animal models of diabetic kidney disease (DKD) was explored in this study. Measures of lactate metabolism and measures of diabetic kidney disease progression were found to be strongly negatively correlated in DKD patients.

**Translational Significance**

Our study provides insights into pathogenesis of DKD and hypoxia-mediated lactic acidosis, which leads to fibrosis and mitochondrial abnormalities. Local acidosis by renal LDHA might be identified as a treatment target for DKD.

**METHODS**

**Human subjects with chronic DKD.** Patients with DKD \((n = 53)\) and healthy controls \((n = 16)\) were recruited from Korea University Ansan Hospital, Seoul, South Korea. The included patients were diagnosed with type 2 diabetes and had proteinuria with a urine albumin-to-creatinine ratio greater than 30 mg/g. The healthy controls were recruited from the same hospital and had normal renal function and blood pressure. The study was approved by the institutional review board, and all participants provided written informed consent.
2 diabetes at least 6 months prior to inclusion and had different urinary albumin and creatinine levels. Urine and blood samples were collected in the early morning from all study participants after fasting for 8 hours. Human kidney tissues were collected from kidney transplant recipients and subjects who died from brain conditions or from ordinary deaths who agreed to organ donation. The inclusion criteria were as follows: (1) age ≥ 19 years old; (2) healthy normal subjects and patients with diabetes presenting with mild Chronic Kidney Disease (CKD) and severe CKD who were diagnosed by nephrologists based on concentrations of urinary albumin or creatinine; and (3) an evaluation of renal dysfunction by histological changes. Subjects with the following characteristics were excluded: (1) age < 19 years old, (2) patients with CKD receiving chronic dialysis or organ transplantation, (3) patients with a recent infection, and (4) patients with any other severe chronic or acute disease requiring treatment. All clinical and demographic parameters were recorded for each participant. The recruited patients were also classified into 3 groups according to the estimated glomerular filtration rate (eGFR) using the Modification of Diet in Renal Disease (MDRD) \(^{17}\) or the Chronic Kidney Disease Epidemiology Collaboration \(^{18}\) equation: group 1, ≥ 60 mL/min/1.73 m\(^2\); group 2, 30–59 mL/min/1.73 m\(^2\); and group 3, < 30 mL/min/1.73 m\(^2\). Patients with diabetes mellitus were defined as those who were previously diagnosed with diabetes mellitus, regardless of their current plasma glucose level. Informed consent was obtained from all participants before study entry under a protocol approved by the Ethics Committee of the Review Board of Korea University Ansan Hospital (KAH-IRB-AS14137) and the Korea National Institute of Health (IRB approval Nos. 2016-02-24-R-A and 2018-02-02-2C-A). Our study was conducted in accordance with the Declaration of Helsinki of Human Rights.

Animal studies. Eight-week-old male Sprague-Dawley rats (SLC, Inc., Shizuoka, Japan) weighing 240–290 g were used in the study (n = 32). All rats were housed under controlled temperature (22°C) and lighting (12:12-hour light-dark cycle) conditions and had free access to water and standard chow throughout the study. Diabetes was induced by administering an intraperitoneal injection of freshly prepared STZ (60 mg/kg body weight; Sigma–Aldrich, St. Louis, MO) dissolved in 10 mmol/L cold citrate buffer (pH 4.5) (Fig S1, Table S1). Blood glucose levels in tail capillary blood samples were measured using a portable glucose meter (Accucheck, Roche Diagnostics, Rotkreuz ZG, Switzerland). Rats were considered diabetic when the fasting blood glucose levels reached 300 mg/dL (16.7 mmol/L) 7 days after the STZ injection. After 2 and 8 weeks, the animals were placed individually in a metabolic cage and housed there for 24 hours for urine collection. After an additional 2 days, the animals were euthanized, and then blood and tissue samples were obtained. Two weeks after the STZ injection, the rats had the characteristics of prediabetes or early diabetes with hyperglycemia and hyperinsulinemia, but they did not exhibit renal fibrosis and dysfunction. In contrast, 8 weeks after the STZ injection, rats were diagnosed with DKD, which was characterized by high expression of renal fibrosis-related genes, a low creatinine clearance ability, and increased urinary protein excretion. All animals received care according to the Korea National Institutes of Health guidelines, and all animal experiments were approved by the Korean National Institutes of Health Animal Care and Use Committee ( Permit Number: KCDC-109-18-2A).

NMR analysis of patients with diabetic CKD. One-dimensional (1D) \(^1\)H NMR spectra were acquired using an Ascend 800 MHz AVANCE III HD Bruker spectrometer (Bruker BioSpin AG, Switzerland) with a triple-resonance 5-mm CPTIC cryogenic probe. The Bruker standard 1D \(^1\)H T2 filter (Car-Purcell-Meiboom-Gill) parameter was used to acquire 1D \(^1\)H spectra of the serum and urine samples. The NMR data were processed using TopSpin (ver. 3.1, Bruker BioSpin, Rheinstetten, Germany) and the 800 MHz Chenomx library (ver. 7.1, Chenomx, Edmonton, AB, Canada).

Metabolomic capillary electrophoresis time-of-flight mass spectrometry analysis of the kidney tissues from STZ-treated rats. The rat kidney tissue samples were analyzed for metabolic changes by Human Metabolome Technology Inc. (HMT, Yamagata, Japan). \(^{19}\) The kidney metabolic profile was analyzed using a capillary electrophoresis time-of-flight mass spectrometry system (Agilent 6210 TOF, Agilent Technologies Inc., Santa Clara, CA) in cationic and anionic modes. The relative area of each metabolite was computed using the \(^{13}\)C isotope ion peak. Among 280 putative peaks that were detected and annotated based on the HMT standard compound library, 110 target metabolites were quantified by comparing their concentrations, and thus 73 metabolites, which were present in at least 94% of all subjects, were detected (Table S2).
Standard Peak Area. Putative metabolites were then assigned from the HMT standard library and known-unknown peak library based on m/z values and MT. In addition, putative metabolites were assigned from the KEGG database20 and Human Metabolome Database database.19 Absolute quantification was performed for 110 metabolites, including glycolytic and TCA cycle intermediates, amino acids, and nucleic acids. All metabolite concentrations were calculated by normalizing the peak area of each metabolite to the area of the internal standard and using standard curves, which were obtained by single-point (100 μM) calibrations.

Assessment of renal function by measuring the urine protein-to-creatinine ratio and eGFR. The creatinine clearance rate (Ccr) and urine protein-to-creatinine ratio (UPCR) were calculated according to the following formula: Ccr = urinary creatinine level (mg/mL) × urine volume (mL/kg)/creatinine level in plasma (mg/mL); UPCR = spot urine (SU) protein level (mg/mL)/ SU creatinine level (mg/mL).21 The eGFR was calculated using the following methods: (1) IDMS-traceable MDRD equation,17 for women with a serum creatinine level >0.7, 144 × (serum creatinine level/0.7)^−1.209 × (0.993)^age; for women with a serum creatinine level ≤0.7, 144 × (serum creatinine level/0.7)^−0.329 × (0.993)^age; for men with a serum creatinine level >0.9, 141 × (serum creatinine level/0.9)^−1.209 × (0.993)^age; and for men with a serum creatinine level ≤0.9, 141 × (serum creatinine level/0.9)^−0.411 × (0.993)^age; (2) Chronic Kidney Disease Epidemiology Collaboration formula,18 for women, 186.3 × (serum creatinine level)^−1.154 × (age)^−0.203 × 0.742; and for men, 186.3 × (serum creatinine level)^−1.154 × (age)^−0.203.

Measurement of body weight and food or water intake. Body weight and food intake were measured at least once a week, as well as on the day drug administration started, just before dosing. Food and water intake were measured once or twice a week.

Biochemical analysis. Blood samples collected from human subjects without or with diabetic CKD and from rats that had fasted overnight (by cardiac puncture) were used to assess biochemical parameters, including hemoglobin, blood urea nitrogen (BUN), creatinine, uric acid, glycosylated hemoglobin (HbA1c), high-sensitivity C-reactive protein, fasting blood glucose (FBG), protein, albumin, total cholesterol, triglyceride, low density lipid-cholesterol, and high density lipid-cholesterol levels. These hematologic and renal function parameters were analyzed using an automated hematology analyzer (Siemens Healthcare Diagnostics, Germany) and standard automated enzymatic methods (Hitachi 912 automated analyzer), respectively, as previously described.22-23 The presence of albuminuria was evaluated by measuring albumin and creatinine level in a morning spot urine sample. Albuminuria was calculated by determining the ratio of albumin to creatinine levels in the urine samples. The excretion of 8-oxo-2′-deoxyguanosine (8-OHdG), an oxidative stress marker, and TGF-β1 was also measured in the urine and plasma of rats using an ELISA kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan).24

Cell culture and transfection. The rat mesangial cell line (RMC) (Cat.# CRL-2573, ATCC) was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 25 mM glucose, 0.37% additional sodium bicarbonate, 4 mM L-glutamine, and penicillin–streptomycin (100 U/mL) (Invitrogen) at 37°C with 5% CO2. After reaching 60%–70% confluence, cells were cultured in DMEM containing 25 mM glucose, antibiotics, and 1% FBS overnight followed by the addition of TGF-β1 (10 ng/mL, Sigma-Aldrich) and culture for another 48 hours. For transfection, RMCs at 60%–70% confluence were transfected with the scrambled siRNA (Scr.) (sc-37007, Santa Cruz Biotechnology, Dallas, TX) and LDHA siRNA (LDHA si) (#200627, Thermo Fisher Scientific) using Lipofectamine RNAiMAX reagent (Gibco, Gaithersburg, MD) according to the manufacturer’s protocol.

Animal tissue extraction. Rat kidney tissues (50 mg) were placed in a glass homogenizer containing 0.6 mL of radioimmunoprecipitation assay (RIPA) lysis buffer (Gibco). Completely homogenized tissues were incubated on ice for 20 minutes and centrifuged at 13,000 rpm for 10 minutes at 4°C. The solubilized proteins were detected using immunoblotting.

Tissue collection and sample preparation. For the histological evaluation, the rats (n=8 per group) were anesthetized with zoletil (5 mg/kg, Virbac Laboratories, Carros, France) after an 8-hour fast and transcardially perfused with heparinized saline and 4% paraformaldehyde. The kidneys were immediately retrieved and fixed with 10% neutral buffered formalin for 24 hours or freshly frozen.

Renal pathology. After dehydration, the fixed kidney tissues were embedded in paraffin, sliced into 5-μm-thick sections, and then stained with histological dyes, such as hematoxylin and eosin and periodic acid-Schiff stains, to evaluate the extent of the mesangial matrix in the mesangial and glomerular volumes using previously described methods.25,26 Four mice from each group were randomly selected, and at least 5 different slices from each tissue were randomly selected for specific staining. Kidney mesangial expansion, glomerular size, and the glomerulosclerosis index were assessed or evaluated according to the periodic acid-Schiff-positive area in the glomerulus (15 randomly
selected glomeruli per animal). The evaluation of glomerulosclerosis was divided into 5 grades (Grade 0, little change; Grade 1, 1%–25% (the percentage of the sclerotic lesion occupying the glomerulus area); Grade 2, 26%–50%; Grade 3, 51-75%; and Grade 4, 76%–100%) based on previous studies.27

**Western blot assay.** Tissues and cells were homogenized and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche and Thermo Fisher Scientific, respectively) at 4°C and then vortexed and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was mixed with Laemmli loading buffer, boiled for 4 minutes, and then separated on sodium dodecyl-sulfate polyacrylamide gel electrophoresis gels. Antibodies against LDHA (sc-137243), α-smooth muscle actin (α-SMA) (sc-53142), Wilms’ tumor-1 (WT-1) (sc-393498), MCT4 (sc-376140), fibronectin (sc-18825), cytochrome c oxidase 2 (COX2) (sc-19999), 8-OHdG (sc-393871), HO-1 (sc-390991), carnitine palmitoyltransferase 1 (sc-393070), peroxisome proliferator-activated receptor-α (sc-398394), actin (sc-84322), and tubulin (sc-166729) were used (Santa Cruz Biotechnology). Anti-HSP70 (#4872) and anti-PDK1 (#3062) antibodies were obtained from Cell Signaling Technology. Anti-MCT1 (ab156807) and anti-NRF2 (ab92946) antibodies were purchased from Abcam. Anti-COX4 (MA5-15078), anti-Collagen IV (14-9871-82) and anti-NRF2 (ab92946) antibodies were purchased from Abcam. Anti-COX4 (MA5-15078), anti-Collagen IV (14-9871-82) were purchased from Thermo Fisher Scientific. Data are presented as the means ± SEM. Significant differences between groups were examined using Student’s t test. P < 0.05 was considered statistically significant.

**Immunohistochemistry and immunocytochemistry.** Immunohistochemistry and immunocytochemistry analyses were performed as described in our previous studies.28,29 After the kidney tissues were fixed with 10% neutral formalin, embedded in paraffin, deparaffinized, and washed, the sections were treated with diluted blocking serum. Staining was performed with a Histostatin-Plus Kit (Zymed Laboratories, San Francisco, CA) according to the manufacturer’s instructions. Antibody staining was assessed and scored using the “quick score method.”26,30 Briefly, the proportion of positive cells was estimated and scored on a scale ranging from 1 to 10: 0%–4% = 1; 5%–14% = 2; 15%–24% = 3; 25%–34% = 4; 35%–44% = 5; 45%–54% = 6; 55%–64% = 7; 65%–74% = 8; 75%–84% = 9; and 85%–100% = 10. The protein expression level in the positively stained cells based on the average score was also categorized into three groups: low (score 0–1.49), middle (1.5–2.99), and high (≥3).

**Transmission electron microscopy (TEM).** After treatment with TGF-β1 for 48 hours, the cells were washed, trypsinized and resuspended. Cells were fixed overnight with a mixture of cold 2.5% glutaraldehyde (EMS, 16020) in 0.1 M phosphate buffer (pH 7.2) and 2% paraformaldehyde (Merck) in 0.1 M phosphate buffer (pH 7.2) and embedded in epoxy resin (EMS, 14120). The epoxy resin-mixed samples were loaded into capsules and polymerized at 60°C for 48 hours. Thin sections were sliced using an ultramicrotome (Leica, UC7, Wetzlar, Germany) and collected on a copper grid. Appropriate areas for thin sectioning were cut at 70 nm and stained with saturated 2% uranyl acetate (EMS, 22400) and lead citrate (EMS, 17800) before examination with a transmission electron microscope (Carl Zeiss, LIBRA 120, Oberkochen, Germany) at 120 kV. Images were captured with an Ultrascan 4000 CCD camera and First Light Digital Camera Controller (Gatan Inc., Pleasanton, Calif).

**Mitochondrial oxygen consumption rate.** Cell respiration (oxygen consumption rate [OCR]) and extracellular acidification rate (ECAR) was measured using a Seahorse XF24 Analyzer ( Seahorse Bioscience, North Billerica, MA).31 Cells were seeded on collagen-coated XF-24 plates at a density of 2 × 10^4 cells per well and incubated in DMEM containing 15% FBS overnight at 37°C with 5% CO₂. The cells were treated with 10 ng/mL TGF-β1 after transfection with or without the LDHA siRNA. Each cycle included 3 minutes of mixing, 2 minutes of waiting and measurement over 2 minutes. Three OCR measurements were obtained under basal conditions and upon sequential injection of 2 μM oligomycin, 1 μM fluoro-carbonyl-cyanide phe- nylhydrazone, and 0.5 μM rotenone plus antimycin A (Sigma-Aldrich). The OCR measurements were adjusted for cell numbers. Glycolysis was assessed by analyzing the ECAR in RMCs cultured under the same conditions.

**RNA extraction and PCR arrays.** Total RNA was extracted from 1 × 10^6 cells/mL, or kidney tissues using an RNeasy Mini Kit (Qiagen, Valencia, Calif) according to the manufacturer’s protocol. The RNA concentration and quality were immediately determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific), and aliquots of the total RNA were stored at −80°C until further use. Reverse transcription was performed with 1 μg of RNA using an iScript cDNA Synthesis Kit (Bio–Rad Laboratories, Hercules, Calif). Then, quantitative polymerase chain reaction (qPCR) was performed using SYBR GreenER qPCR Supermix (Invitrogen) according to the manufacturer’s instructions. The expression levels of messenger RNAs (mRNAs) were normalized to mouse β-actin as the internal standard. The following primers were used:
amplified cDNAs were mixed with RT2 SYBR Green/ROX master mix containing AMV Reverse Transcriptase (Promega). The fold change (2^−ΔΔCt) method was calculated with a reference gene (500 β-actin) as the reference gene. The normalized expression (2^−ΔCt) was used to calculate the fold change in mRNA abundance using the 2^−ΔΔCt method. Data are presented as the fold change.

The fold change (2^−ΔΔCt) method is the normalized gene expression (2^−ΔCt) in the test sample divided by the normalized gene expression (2^−ΔCt) in the control sample. Differences among experimental groups were analyzed using Student’s t test and compared with RT2 profiler PCR Array data analysis software version 3.5 (SA Biosciences). P < 0.05 was considered statistically significant.

Determination of the kidney NADH/NAD⁺ ratio. The NAD⁺ level was determined with a colorimetric method using an NAD⁺/NADH assay kit according to the manufacturer’s instructions (Abcam, Cambridge, MA). Briefly, 20 mg of kidney tissue were washed with cold PBS and homogenized (30–50 passages) in 400 μL of NAD⁺/NADH extraction buffer. The supernatant was collected after centrifugation at 14,000 rpm for 5 minutes at 4°C. The collected supernatant was filtered through a 10-kDa spin column (ab993349) to remove the enzymes that rapidly consume NADH before performing the assay. For determination of the total NAD⁺ (total NAD⁺ and NADH) and NADH content, 50 μL of supernatant was transferred into 96-well plates in triplicate. Two hundred microliters of the supernatant were heated at 60°C for 30 minutes to decompose NAD⁺, and 50 μL of the NAD-decomposed samples were transferred into 96-well plates in triplicate to measure NADH levels. NADH cycling enzyme/buffer mix (100 μL) was added to each sample and standard, mixed, and incubated at room temperature for 5 minutes to convert NAD⁺ to NADH. NADH developer (10 μL) was added to each reaction and incubated at room temperature for 5 hours before reading the OD of the plate at 450 nm. The standard was prepared according to the manufacturer’s protocol. The NADH/NAD⁺ ratio was calculated using the formula NADH/NAD⁺ (m) - NADH formula and reported as millimoles per kilogram of kidney tissue.

Lipid peroxidation assays. As a reflection of kidney levels of lipid peroxidation, malondialdehyde levels were determined using a colorimetric assay (EMD Biosciences, La Jolla, Calif). Fifty milligrams of kidney tissue were homogenized in 20 mM Tris-HCl, pH 7.4, and 500 μM 3,5-di-tert-butyl-4-hydroxytoluene, and the colorimetric reaction was performed according to the manufacturer’s instructions.

Mitochondrial function assays. Mitochondria were isolated by gradient centrifugation as described previously. Briefly, fresh kidney tissues (1 g) were homogenized in 8 mL of isolation buffer containing 220 mM D-mannitol, 70 mM sucrose, 10 mM Tris-HCl, 1 mM Ethylene glycol-bis(2-aminoethyl)-N,N,N’,N’-tetraacetic acid (EGTA), and 0.4% bovine serum albumin (pH 7.4). The homogenates were centrifuged at 850 × g for 10 minutes. The mitochondrial pellet was resuspended in a final wash buffer containing 220 mM D-mannitol, 70 mM sucrose, and 10 mM Tris-HCl (pH 7.4). Mitochondrial protein concentrations were determined using a DC Protein Assay Kit (Bio-Rad). The rates of ATP formation were measured using an ATP Colorimetric/Fluorometric Assay Kit (BioVision, Mountain View, Calif). Mitochondrial ROS production was measured from fresh mitochondrial suspensions using dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) as described previously. Briefly, the DCFH-DA stock solution was dissolved in 1.2 mM methanol and incubated in a dark room at 0°C. One milligram of kidney mitochondria and 2 μL of 3 mM DCFH-DA were added to a quartz cuvette (total volume of 0.3 mL). The mixture was incubated at 37°C for 2 minutes to allow the DCFH-DA probe to penetrate into the mitochondria. A negative control consisting of the same materials without mitochondria was used to correct the autooxidation rate of DCFH-DA. The units were picomoles of DCF formed per minute per milligram of protein. Mitochondrial and nuclear cytosolic fractions were isolated by differential centrifugation, and mtDNA and nuclear DNA were quantified as previously described to measure the mitochondrial DNA content. Briefly, kidney tissues were gently homogenized in 1 mL of isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM 3-(N-morpholino)propanesulfonic acid, 1 mM ethylene glycol tetraacetic acid, and 0.5% bovine serum
the 8-OHdG levels with a competitive ELISA kit.

sides. The nucleoside samples were used to determine
at 37 °C for 1 hour to hydrolyze the nucleotides to nucleo-
tase (Yoyobo, Osaka, Japan), and then, after purging
with a nitrogen stream, the mixtures were incubated at
digested into nucleotides by adding alkaline phospha-
tase (Yoyobo, Osaka, Japan), and then, after purging
with a nitrogen stream, the mixtures were incubated at

Urine preparation and urinary exosome isolation. Urine samples were collected from each rat (CTL- and STZ-
injected rats) or individual subjects (healthy individu-
als and patients with diabetic CKD) in sterile contain-
ers. ExoQuick-TC was used according to the
manufacturer’s instructions (System Biosciences). By
inverting the tube, 10 mL of urine were mixed with
2 mL of ExoQuick-TC solution. After an overnight
incubation at 4°C, the sample was centrifuged twice at
1500 × g for 40 and 5 minutes to remove any particu-
late matter, including cells and cell debris. The pellet
was resuspended in 100 L of PBS and stored at −80°C
after the supernatants were discarded. A 5-μL sample
was mixed with 5 μL of Laemmli lysis buffer to mea-
sure the protein concentration of isolated exosomes.
The protein concentration was determined using the
Bio–Rad DC Protein Assay (Bio–Rad, Hercules,
Calif). For analysis, 10 μg of protein were suspended in
200 μL of RIPA buffer, vortexed for 15 seconds and
incubated at room temperature for 5 minutes to allow
complete lysis. Laemmli buffer with beta-mercapto-
ethanol was added to the samples, which were then
boiled at 95°C for 5 minutes. Proteins were separated
on sodium dodecyl-sulfate polyacrylamide gel electro-
phoresis gels and transferred to polyvinylidene fluoride
membranes that were blocked with 5% nonfat milk in
PBS supplemented with 0.5% Tween-20 and immuno-
sified.

ATP measurement. Renal ATP levels were estimated
using an ATP Colorimetric/Fluorometric Assay Kit
(Abcam, ab83355, Cambridge, United Kingdom)
according to the manufacturer’s instructions. Briefly,
kidney tissue (20–30 mg) was freeze clamped by an
aluminum block precooled in liquid nitrogen and
immediately immersed in liquid nitrogen. Kidney sam-
pies were stored at −80°C. On the day of the assay, the
kidney samples were pulverized in liquid nitrogen and
homogenized in 6% perchloric acid. Homogenates
were centrifuged at 13,000 × g for 5 minutes at 4°C,
and the supernatants were neutralized to pH 7.8 with
2 M KOH, incubated on ice for 1 hour, and centrifuged
at 13,000 × g for 5 minutes at 4°C. Absorbance was
measured at 570 nm after 30 minutes of incubation
using a microplate plate reader (Multiskan GO,
Thermo Fisher Scientific). The ATP concentration
was calculated using an ATP standard curve and reported
in nmol/mg protein.

Lactate measurement. Lactate levels were measured
enzymatically in 96-well plates according to the manu-
ufacturer’s specifications (Lactate-Glo Assay, Prom-
ega). RMCs were washed with ice-cold PBS three
times and lysed with 22.5 μL of HCl (0.2 N). Cell
lysates were neutralized with 7.5 μL of 1 M Tris base
and incubated with 30 μL of detection reagent. Lumi-
nescence was recorded after 1 hour, and intracellular
lactate concentrations were determined from a standard
curve. Extracellular lactate production was measured
in the medium after background subtraction from fresh
medium. Lactate levels were normalized to the protein
content (Pierce BCA, Thermo Fisher Scientific) from
cells in duplicate plates after lysing cells with NP40
(1% vol/vol). Extracellular lactate levels were also
measured directly in the culture medium. Intracellular
lactate levels were measured in the cell pellet. Cells
were centrifuged briefly and suspended in 20 μL of
dH2O. The pellet was lysed by performing three freeze-
thaw cycles in a dry ice/water bath, and the amount
of lactate released was measured. Extracellular and
intracellular lactate concentrations were measured in
homogenized kidney tissues by homogenizing
3–15 mg of kidney tissues in 50 mM Tris, pH 7.5
(homogenization buffer) that had been premixed with
inactivation solution at an 8:1 (vol/vol) ratio (eg, 1 mL
of buffer + 0.125 of mL of inactivation solution). After
homogenization for 20–30 seconds, the tissue homog-
enate was neutralized by adding 0.125 mL of neutral-
ization solution and, if necessary, diluted to ensure the
linearity of the assay.

Multivariate and statistical analyses. Pearson’s correla-
tion analysis, partial least squares-discriminant analysis
(PLS-DA), cross-validation, and PLS regression analy-
ses were performed using SPSS 21.0 software. PCA
and PLS-DA for VIP analysis were conducted using
SIMCA-P v. 12.0 (Umetrics, Umea, Sweden). Differen-
ces in protein levels between groups were determined
using 1-way or 2-way ANOVA, followed by a post hoc
analysis using Bonferroni’s multiple comparison test.
The results are presented as the means ± standard errors
of the means (SEM), and P < 0.05 was considered sig-
ificant. The SPSS statistical package (SPSS Inc., ver-
sion 18.0, Chicago, IL) was used for all analyses.
RESULTS

Clinical characteristics of patients with DKD. In the present study, we collected human kidney tissues from kidney transplant recipients and from subjects who died of brain conditions or ordinary deaths who agreed to organ donation to characterize the remodeling of renal lactate metabolism in patients with diabetes and the mechanism of action of lactate on renal fibrosis in patients with DKD. We compared the changes in clinical characteristics, including renal function and nephropathy indices, between normal healthy subjects (n = 16) and patients with DKD (n = 53) (Table S3). Patients with DKD had higher FBG, BUN, creatinine, uric acid, and HbA1c levels than normal healthy controls, and these indices correlated with the reduction in the eGFR, an indicator of deteriorating renal function, and the increase in the UPCR, another indicator of renal dysfunction (Fig 1, A, left panel). However, no significant changes in renal function were observed in patients with mild DKD and microalbuminuria (urinary albumin excretion rate of 30 to 300 mg/24 h) compared to their normal counterparts. In contrast, significant renal dysfunction was observed in patients with severe DKD and macroalbuminuria (urinary albumin excretion rate >300 mg/24 h) (Fig 1, A, right panel and Table S3).

Increased urinary lactate levels are related to renal dysfunction in patients with DKD. Next, we investigated whether lactate is a differentially abundant metabolite in patients with DKD using 1H-NMR spectroscopy coupled with multivariate analysis. In the PLS-DA score plot for urine samples from all subjects, patients with DKD were separated from the normal healthy groups (Fig 1, B). Thirty-three metabolites (VIP >1 and P values < 0.05) were identified as important metabolites in the urine of patients with DKD (Table S4). Urinary lactate metabolite levels were significantly increased in patients with DKD (Fig 1, C, Table S3). A significant negative correlation between the eGFR based on the MDRD Study equation and urinary lactate metabolite levels was observed (Pearson’s correlation coefficient (r) = -0.38, P < 0.001) (Fig 1, D and Fig S2, A), suggesting a role for secreted urinary lactate as a predictive marker of renal dysfunction. Additionally, the levels of systolic blood pressure (SBP), BUN, creatinine, HbA1c, protein, albumin, cholesterol, LDL, spot urine protein (SU-P), SU-creatinine (SU-Cr), and UPCR were positively or negatively correlated with urinary lactate levels (r = 0.33 for SBP, r = 0.27 for BUN, r = 0.38 for Cr, r = 0.26 for HbA1c, r = -0.55 for protein, r = -0.62 for albumin, r = 0.33 for cholesterol, r = 0.28 for LDL, r = 0.35 for SU-P, r = -0.38 for SU-Cr, and r = 0.49 for UPCR; all P < 0.05) (Fig S2, A). No significant correlations of the urinary lactate level with body mass index or the levels of uric acid, high-sensitivity C-reactive protein (hs-CRP), FBG, and triglycerides were observed. When the participants were categorized into 3 groups according to the eGFR, the groups with a reduced or moderately reduced eGFR (<30 or 60–30 mL/min/1.73 m², respectively) had higher FBG and HbA1c levels than those with a normal eGFR (≥60 mL/min/1.73 m²) (P < 0.05) (Fig 1, E). Furthermore, type IV collagen (Col-IV), the primary collagen present in the basement membrane, was expressed at high levels in the kidneys of subjects with a moderately reduced eGFR (60–30 mL/min/1.73 m²) (Fig 1, F). Nevertheless, the increase in Col-IV expression was abrogated in subjects with a reduced eGFR (<30 mL/min/1.73 m²) (Fig S2, B). On the other hand, the expression of a tumor suppressor gene, WT-1, was markedly decreased in subjects with an eGFR of 60–30 mL/min/1.73 m² and an eGFR of <30 mL/min/1.73 m² (Fig S2, B). Additionally, the eGFR correlated with urinary lactate levels (P < 0.005) (Fig 1, G) but not with serum lactate levels (P = 0.713) (Fig S2, C). The urinary lactate concentration in samples from females was significantly higher than that in samples from males, but we did not detect a difference in serum lactate levels (Table S5). In women, higher excretion of urine TCO₂ and an increase in the urine pH were associated with gastrointestinal anion excretion, including lactate.

Increased LDHA levels are related to renal dysfunction in patients with DKD. We examined LDHA expression in human kidney tissues to evaluate lactate-mediated diabetic renal dysfunction. The LDHA protein was expressed at high levels in both glomeruli and tubular epithelial cells in the kidney tissues of patients with DKD (Fig 2, A), which was confirmed by Western blotting (Fig 2, C). The groups with high LDHA levels in human kidney tissues exhibited significant reductions in the eGFR, which correlated with the increased expression of α-SMA, a fibrosis marker, and the low expression of WT-1 (Fig 2, B and C). Next, we confirmed the involvement of monocarboxylate transporters, which transport pyruvate, lactate and ketone across the plasma membrane, in mediating the increase in urinary lactate levels in patients with DKD by examining the expression of MCT1 and MCT4, which are considered the most important transporters of lactate with a proton. Significant changes in the expression of MCT1 and MCT4 were not observed in kidney tissues from patients with DKD compared to healthy subjects (Fig 2, C), which was confirmed by immunohistochemistry for MCT1 in kidney tissues (Fig 2, D). Moreover, LDHA, which was expressed at high levels in the glomeruli of patients with DKD, colocalized with the
Fig 1. Correlation of urinary lactate production with a reduced eGFR in patients with diabetic CKD.

(A) The eGFR and UPCR were significantly reduced and increased, respectively, in patients with DKD (n = 53) compared with normal healthy subjects (n = 16) and in normal healthy participants (n = 16) and those with mild DKD (n = 11) and severe DKD (n = 42). Mild and severe DKD were defined by urinary albumin excretion ranging from 30 to 300 mg and ≥300 mg in a 24-hour urine sample, respectively. (B) PLS-DA plot of urinary lactate levels measured using CE-TOF-MS. (C) Urinary lactate excretion (concentration and fold change) was significantly increased in patients with DKD. (D) Correlation analysis between the eGFR and urinary lactate excretion. Pearson’s correlation coefficients ($r$) and $P$ values are shown. (E) The changes in FBG and HbA1c levels in participants with high ($≥60$ mL/min/1.73 m$^2$), moderately reduced (60–30 mL/min/1.73 m$^2$), and low ($<30$ mL/min/1.73 m$^2$) eGFR, an indicator of deteriorating renal function. (F) ICC analysis of collagen IV and WT-1 expression in the glomeruli of participants with three different eGFR levels (scale bar = 50 μm). Lower
oxidative stress marker 8-OHdG, suggesting important roles for LDHA and lactate production in renal dysfunction mediated by oxidative stress (Fig 2, E). This result is consistent with previous research revealing that LDHA bound to NADH transforms NADH as an electron donor to produce ROS.36

Additionally, we examined whether renal function indicators and HbA1c values were differentiated according to LDHA expression levels to further clarify the relative effect of glomerular LDHA expression on HbA1c levels, which is a useful indicator for diagnosing and monitoring people with diabetes.37 HbA1c levels were significantly increased in the groups with high LDHA expression (Fig 2, F); conversely, LDHA expression was significantly increased in the kidney tissues of subjects with diabetes presenting HbA1c levels ≥6.5 (Fig S2, D). Consistently, a significant decrease in renal function was observed in subjects with HbA1c levels ≥6.5% compared to those with HbA1c levels <6.5% (eGFR 67.1 ± 32.5 mL/min/1.73 m² vs 41.2 ± 38.2 mL/min/1.73 m², P < 0.005) (Fig 2, G). We also observed a positive correlation of HbA1c levels with urinary lactate levels (Fig 2, H) and the UPCR (Fig S2, E), whereas a negative correlation was observed between the eGFR and HbA1c levels (Fig 2, I). Collectively, we revealed that LDHA expression levels in human kidney tissues are associated with the extent of diabetes and kidney dysfunction.

**STZ-induced diabetic renal dysfunction is related to TGF-β1-mediated fibrotic and oxidative damage.** The STZ-induced diabetic renal injury group had significantly higher levels of aerobic glycolytic metabolites produced by rate-limiting enzymes related to glycolysis and gluconeogenesis (Fig 3, A and B), and citric acid, alanine and sedoheptulose-7-phosphate levels were decreased (Fig 3, B and C). Consistent with the human data, renal and urinary lactate levels were substantially increased in the diabetic rat model (Fig 3, C and D). Furthermore, the urinary exosomal level of WT-1, which reflects the variability in glomerular injury, was detected early at 2 weeks after the STZ injection and in patients with early diabetes before the occurrence of albuminuria. WT-1 expression increased with the progression of glomerular injury after 8 weeks and in patients with DKD (Fig 3, E). Additionally, a negative correlation was observed between increased urinary lactate levels and WT-1 expression in podocytes of STZ-treated rats (Fig 3, F).

We first examined the functional effect of enhanced aerobic glycolysis on STZ-induced rat models to explore the mechanism underlying TGF-β1-mediated renal fibrosis. Intriguingly, plasma lactate concentrations and renal LDHA expression were slightly increased in rats with STZ-induced diabetes at 2 weeks and were potentiated at 8 weeks (Fig 4, A and B). In contrast, the changes in other metabolic features of rats with STZ-induced DKD at 2 and 8 weeks exhibited differences. The fasting insulin level, ATP level, mitochondrial DNA concentration, and the OCR, which is indicative of mitochondrial respiratory activity, were consistently significantly increased 2 weeks after STZ administration; however, all of these measurements were significantly decreased at 8 weeks (Fig 4, A–C). The disparities in these metabolic reactions may be due to variations in their maladaptation to metabolic stress. Compensatory defense mechanisms protected against mild stress at 2 weeks after the STZ injection, whereas the rats exhibited altered metabolic reactions after 8 weeks of chronic stress. Similar to patients with DKD, no significant changes in MCT1 and MCT4 expression were observed in kidney tissues from STZ-injected rats compared to control rats (Fig 4, D), which were not correlated with the increase in urinary lactate concentration in STZ-injected rats (Fig 4, E). Next, we further examined whether changes in intracellular pH and intracellular lactate levels were present in the kidney tissues of rats with STZ-induced DKD. As expected, intracellular pH (Fig 4, F) and lactate levels (Fig 4, G) were significantly decreased and increased in both 2- and 8-week-old rats after STZ injection, respectively, and correlated with increased urinary lactate excretion (Fig 4, E), suggesting that intracellular and extracellular lactic acidosis may be responsible for STZ-mediated metabolic disorders and ultimately lead to renal fibrosis and dysfunction.

Next, we employed rat RMCs as an in vitro model and treated them with TGF-β1 (10 ng/mL) (Fig 4, H). Direct measurement of extracellular lactate levels in the media of RMCs showed that TGF-β1 substantially increased lactate production in RMCs (Fig 4, H, lower panel). Concomitantly, the pH of the RMC culture medium was decreased by TGF-β1, which was further
Fig 2. Increased renal LDHA levels in patients with DKD. Immunohistochemistry (IHC) and quantification of LDHA expression in kidney tissues from subjects with or without DKD (upper panel; scale bar = 50 μm, 100×).
characterized by a color change in the culture medium (Fig 4, H, upper panel), despite the lack of difference in the growth rate (data not shown), suggesting that TGF-β1 mediated lactic acidosis. Acidification of the culture media was also observed in the rat renal tubular epithelial cell line NRK52E after treatment with TGF-β1 (data not shown). Next, we examined whether extracellular acidification and elevated urinary lactate levels were also related to intracellular acidification and an increase in intracellular lactate levels. Intracellular acidification was detected using pHrodo Green AM and increase in intracellular lactate levels. Intracellular cellular acidification and elevated urinary lactate levels were also observed in the rat renal tubular culture media was also observed in the rat renal tubular TGF-β1 mediated lactic acidosis. Acidification of the culture media was also observed in the rat renal tubular epithelial cell line NRK52E after treatment with TGF-β1 (data not shown). Next, we examined whether extracellular acidification and elevated urinary lactate levels were also related to intracellular acidification and an increase in intracellular lactate levels. Intracellular acidification was detected using pHrodo Green AM and was significantly increased in TGF-β1-treated cells (Fig 4, I), consistent with an increase in intracellular lactate levels (Fig 4, I, right graph). Additionally, TGF-β1 treatment significantly increased the levels of LDHA and PDK1, an inhibitor of pyruvate dehydrogenase, in RMCs (Fig 4, J). The expression of α-SMA and fibronectin, genes related to renal fibrosis, was significantly increased in TGF-β1-treated cells, whereas changes in MCT1 and MCT4 expression were not observed. We compared the effects of exposure to lactate and to acidic culture media (pH 6.4) on the changes in fibrosis-related markers in TGF-β1-treated cells to confirm the direct effect of TGF-β1-induced lactic acidosis on fibrosis in RMCs (Fig 4, K). We revealed that the exposure of RMCs to lactate significantly potentiated TGF-β1-induced fibronectin and α-SMA expression. Additionally, these lactate-mediated changes in fibrosis markers were attenuated by adjusting the pH of the acidified culture medium to 7.2 using NaOH before incubation. Similar to the effect of lactate, cells cultured in pH 6.4 acidic media exhibited increased expression of fibronectin and α-SMA, changes that were potentiated by the addition of TGF-β1. Therefore, TGF-β1-mediated lactic acidosis was sufficient to induce fibrosis in RMCs.

Inhibition of aerobic glycolysis attenuates TGF-β1-mediated renal fibrosis. RMCs were treated with the glycolytic pathway inhibitors 2-deoxyglucose (2-DG) and dichloroacetic acid and the LDHA inhibitor oxamate in the presence or absence of TGF-β1 to further confirm the role of enhanced aerobic glycolysis in TGF-β1-mediated renal fibrosis (Fig 5, A). As expected, 2-DG dose-dependently diminished the TGF-β1-induced increase in fibronectin and α-SMA expression, which correlated with a reduction in LDHA protein levels (Fig 5, B). Additionally, blocking aerobic glycolysis with 2-DG markedly reversed the TGF-β1-induced increase in lactic acidosis, as characterized by an increase in the pH and a decrease in extracellular lactate levels in the culture media (Fig 5, C and D). Similarly, the inhibition of PDK1 and LDHA activities by dichloroacetic acid and oxamate, respectively, also significantly abolished the TGF-β1-induced expression of renal fibrosis-related proteins (Fig 5, E) and lactic acidosis by reducing pH and lactate secretion (Fig S3, A and B). Silencing LDHA expression was accompanied by reductions in TGF-β1-induced increases in the levels of fibrosis-related proteins such as fibronectin and α-SMA (Fig 5, F), which correlated with a significant reduction in extracellular lactate levels (Fig 5, G), suggesting a critical role for lactate production in TGF-β1-mediated renal fibrosis. Along with a significant increase in the number of abnormal mitochondria, electron microscopy (EM) images of TGF-β1-treated RMCs revealed increased mitophagy-mediated autophagosome accumulation; LDHA silencing ameliorated these metabolic alterations (Fig 5, H). Our results indicate that blocking lactate production via aerobic glycolysis potentially preserves mitochondrial morphology and attenuates abnormal biogenesis.

Lower panel, higher magnification images of glomeruli (blue) and tubules (yellow) (boxed areas in the images in the upper panel) (200×). (B) Changes in eGFR (P < 0.001) and the expression of proteins, namely, α-SMA (P < 0.001) and WT-1 (P < 0.001), in the glomeruli of participants with low (n = 13) and high (n = 46) LDHA expression levels. The expression scores were assessed based on their intensity and distribution, and they were divided into two groups: low (0–0.5) and high (>0.5). (C) Western blot analysis of LDHA, α-SMA, WT-1, MCT1, and MCT4 levels in kidney tissues from human subjects without or with diabetic CKD (upper panel) and quantification of the protein intensity in Western blots (lower panel). *P < 0.05. (D) IHC analysis and quantification of MCT-1 expression in kidney tissues from subjects with or without DKD (scale bar = 50 μm). (E) ICC analysis of 8-OHdG and LDHA levels in the glomeruli of participants with or without diabetic CKD (scale bar = 50 μm) and quantification of cells positive for 8-OHdG and LDHA staining. *P < 0.05. (F) Plasma HbA1c levels were measured in participants with low (n = 13) and high (n = 46) LDHA expression levels (the P value was determined using the t test). (G) The eGFR levels were calculated in participants with HbA1c levels <6.5% (nondiabetes) and HbA1c levels ≥6.5% (defined as diabetes) (the P value was determined using the t test). (H and I) The positive correlation between HbA1c and urinary lactate levels in participants with or without diabetic CKD. (J) The negative correlation of HbA1c levels with eGFR in participants with or without diabetic CKD. All data are presented as the means ± SEM. Data in H and I were analyzed using Pearson’s correlation analysis. Pearson’s coefficients (r) and P values are shown. Data in A, D, F, and G are presented in box plots (medians, IQRs), and the P value was determined using the t test. α-SMA, α-smooth muscle actin; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; ICC, immunocytochemistry; IQR, interquartile range; LDHA, lactate dehydrogenase A; WT-1, Wilms’ tumor-1. (Color version of figure is available online.)
Fig 3. Enhanced lactate production is related to aerobic glycolysis and renal dysfunction in diabetic CKD rats.

(A) A diagram of the changes in metabolites involved in central carbon and glucose metabolism in the kidneys
Next, we measured the OCR and the ECAR to investigate the effect of excess lactate production on renal fibrosis and mitochondrial dysfunction. The OCR indicates mitochondrial OXPHOS, and the ECAR is an indicator of lactate production and glycolysis. TGF-β1-treated cells exhibited low OCRs normalized to cell number, which was rescued by LDHA silencing (Fig 5, I). Additionally, blocking LDHA expression attenuated the significant decreases in the basal and ATP-linked OCRs and maximal respiratory capacities (Fig S3, C). Under the same conditions, the ECAR increased in TGF-β1-treated cells and was markedly attenuated by LDHA silencing (Fig S3, D). Collectively, these results revealed that anaerobic glycolysis mediated by LDHA limits mitochondrial OXPHOS in RMCs.

Increased lactate levels are associated with renal mitochondrial dysfunction in STZ-treated rats. The mitochondrial ATP level was significantly decreased in STZ-treated rats (Fig S4, A). In contrast, the lactate-to-pyruvate ratio, which indicates an alteration in cellular redox homeostasis, was substantially increased in STZ-treated rats (Fig S4, B). Accordingly, the ratio of NADH (reduced) to NAD+ (oxidized), which represents the levels of lactate and pyruvate, respectively, was also significantly increased in STZ-treated rats (Fig S4, C), suggesting that mitochondrial OXPHOS was impaired upon the inhibition of dehydrogenase reactions of the citric acid cycle, resulting in a reduction in the metabolic rate. The decrease in OXPHOS in STZ-treated rats was positively correlated with a significant decrease in the mitochondrial DNA content (Fig S4, D), which plays a vital role in respiratory activity and copy number, and with the reduction in COX2 and COX4 levels (Fig S4, E). A negative correlation was observed between urinary lactate levels and mitochondrial COX2 expression in STZ-treated rats (Fig S4, F).

Collectively, overproduction of lactate via aerobic or anaerobic glycolysis may alter other metabolic homeostasis pathways, which are vulnerable to renal fibrosis and dysfunction; however, their exact interrelated mechanisms are poorly understood. Therefore, we also examined the relationship between the overproduction of lactate and metabolites related to central metabolic pathways and the significant changes in kidney tissues of STZ-treated rats (Supplementary text and Figs S5–S14).

Altered carbon glycolytic metabolism and pathological features in the kidney tissues of STZ-treated rats. Interestingly, STZ-injected rats also showed renal histology and function similar to those of patients with DKD (Table S6, Fig S5). We employed capillary electrophoresis time-of-flight mass spectrometry to investigate the metabolic changes in the kidney tissues of the diabetic rat model and further delineate the mechanism of action of DKD (Table S7).

A significant increase in LDHA mRNA expression (Fig S6, A) was confirmed by performing immunohistochemical staining for LDHA in the glomeruli and tubular epithelial cells of STZ-treated rats (Fig S6, B). Additionally, double immunofluorescence staining showed that high LDHA expression in the glomeruli of STZ-treated rats correlated with an increase in the expression of the renal cell fibrosis marker α-SMA (Fig S6, C) and a reduction in the expression of the podocyte marker WT-1 (Fig S6, D). As expected, plasma TGF-β1 levels were substantially increased in STZ-treated rats (Fig S6, E) and positively correlated with increased LDHA expression in both glomeruli and tubular epithelial cells in kidney tissues (Fig S6, F).

In addition, a positive correlation was observed between an increased TGF-β1 concentration and urinary 8-OHdG efflux (Fig S6, G). Furthermore, increased urinary lactate levels were positively correlated with increased urinary 8-OHdG secretion (Fig S7, A). When urinary lactate levels were categorized into tertiles, the levels of urinary 8-OHdG, BUN, albumin, and urine output increased in a dose-dependent manner and correlated with the increase in tubular hypoxia-inducible factor α expression and the decrease in Ccr (Fig S7, B–D). Similarly, LDHA expression in glomeruli and tubular epithelial cells was also
Fig 4. TGF-β1-mediated lactic acidosis is related to renal fibrosis. (A) Comparison of the plasma lactate levels with changes in fasting insulin levels in STZ-treated rats (at 2 and 8 weeks). (B) LDHA expression in kidney.
positively correlated with a reduction in Ccr and increases in urinary albumin, BUN, and urine output levels (Fig S7, E and F). STZ-injected rats also showed similar TGF-β1-mediated fibrotic oxidative damage to patients with DKD (Fig S8).

A functional pathway analysis using the KEGG database and the Human Metabolome Database revealed significant enrichment of pathways associated with central carbon and glucose metabolism, urea cycle-related metabolism, lipid and amino acid metabolism, BCAA metabolism, and nucleotide metabolism in the kidney tissues of STZ-treated rats (Tables S8—10).

**Increased lactate levels are associated with altered urea cycle metabolism in kidney tissues of STZ-treated rats.** Consistently, urea metabolism was also noticeably altered in the diabetic renal dysfunction groups compared to the healthy controls (Figs S6 and S7). The relative peak area of urea, a uremic toxin derived from arginine, in the kidney tissues of STZ-treated rats was higher than that in control rats, whereas the levels of creatine and creatinine, which are correlated with a decrease in the level of ornithine derived from arginine, were lower in STZ-treated rats (Fig S7, A—C). The levels of citrulline, another arginine derivative and a urea cycle intermediate, and γ-aminobutyrate (GABA), an inhibitory neurotransmitter derived from aspartate-derived glutamate, were significantly increased in STZ-treated rats. In contrast, the levels of β-alanine, proline, hydroxyproline, asparagine, histidine, and putrescine were markedly reduced in kidney tissues. In STZ-induced diabetic kidneys, the urea cycle is altered by increasing the levels of uremic toxins. The changes in the metabolites present at increased or decreased levels positively or negatively correlated with the increase in lactate metabolite production in the kidney tissues (Fig S7, D).

**Alterations in lipid and amino acid metabolism in kidney tissues from STZ-treated rats.** Consistently, lipid and amino acid metabolism, including choline metabolism, the methylation cycle, ketone bodies, and carnitine metabolism, exhibited diabetic renal injury-specific differences (Fig S8). Despite the increase in choline levels, the levels of related metabolites derived from choline metabolism, such as betaine, N,N-dimethylglycine, and sarcosine, were lower in STZ-treated rats and correlated with the decrease in the levels of serine and methionine related to the methylation cycle (Fig S8, B). On the other hand, the levels of S-adenosyl methionine (SAM), which is synthesized from ATP and methionine through the action of methionine adenosyl transferase, were significantly increased, whereas S-adenosyl homocysteine, which is derived from SAM, was not detected. Since SAM is an alkylating agent that induces DNA damage, its increased levels suggest that it may be a crucial metabolite leading to STZ-induced renal fibrotic injury. Additionally, rats with diabetic renal injury presented lower levels of glycine, threonine, alanine, and 3-hydroxybutyrate, which are related to both ketone bodies and pyruvate metabolism and were correlated with a significant increase in lactate levels. The metabolism of carnitine, which is involved in transporting fatty acids across the mitochondrial membrane, was also markedly altered in STZ-treated rats compared to control rats. Carnitine levels were increased in STZ-treated rats, whereas the levels of acylcarnitine, O-acetylcarnitine, and 5-hydroxylysine were decreased. Correspondingly, despite the increase in carnitine, the expression of carnitine palmitoyltransferase 1 and peroxisome proliferator-activated receptor alpha, which are involved in mitochondrial β-oxidation, was significantly decreased in the kidney tissues of STZ-treated rats (Fig...
Fig 5. Inhibition of aerobic glycolysis attenuates TGF-β1-mediated renal fibrosis. (A) Scheme showing the role of enhanced aerobic glycolysis in TGF-β1-mediated renal fibrosis. Cells were treated with the glycolytic pathway inhibitors 2-DG and DCA, along with oxamate, an inhibitor of LDHA, in the presence or absence of TGF-β1. (B) Effects of the glycolytic pathway inhibitor 2-DG (10 mM) on TGF-β1-mediated renal fibrosis. (C) 2-DG cotreatment inhibits TGF-β1-induced acidification. (D) 2-DG treatment attenuates TGF-β1-enhanced lactate excretion into the culture media. (E) Effects of DCA (1 mM) and oxamate (50 mM) cotreatment on TGF-β1-
S8, C), suggesting that STZ-induced metabolic changes may be associated with mitochondrial dysfunction.

**Differences in the levels of branched-chain amino acids and nucleotides in the kidney tissues of STZ-treated rats.** Furthermore, the diabetic renal injury models exhibited significant changes in the levels of metabolites related to branched-chain (BCAA) and aromatic amino acid (Fig S9) and nucleotide (pyrimidine and purine) (Fig S10) metabolism. Specifically, tissue levels of metabolites such as leucine, isoleucine, and valine from BCAA metabolism were significantly decreased in STZ-treated rats (Fig S9, A and B). Since BCAAs are an important group of essential amino acids that function in crucial nutrient signaling pathways, including glucose, leptin, cell signaling, adiposity, and body weight regulation, the reduction in their concentrations might be associated with deleterious effects on energy metabolism. Additionally, the aromatic amino acids phenylalanine, tryptophan, and tyrosine, which are responsible for the production of the neurotransmitters dopamine, serotonin, and fumarate, respectively, and represent important intermediates in the TCA cycle, were significantly decreased in STZ-treated rats. In addition, levels of cytidine and uracil that are involved in pyrimidine metabolism and hypoxanthine and uric acid, an oxidized product of xanthine, involved in purine metabolism were significantly decreased in STZ-treated rats (Fig S10, C and D). In contrast, higher levels of UMP from pyrimidine metabolism and ADP, AMP, GMP, GDP, and guanosine from purine metabolism were observed in STZ-treated rats than in control rats.

**Increased lactate levels are associated with altered homeostasis of metabolic pathways in the kidney tissues of STZ-treated rats.** Interestingly, significant differences in ATP levels were not observed (Fig S11, A), whereas the levels of NAD$^+$ and NADP$^+$, the oxidized forms of nicotinamide adenine dinucleotide (NAD) and its close analog NAD phosphate (NADP)$^+$, respectively, were significantly increased in STZ-injected rats. Similar to the urea cycle, the changes in representative metabolites involved in lipid and amino acid, BCAA, nucleotide (pyrimidine and purine), and energy metabolism were also correlated with the increase in lactate metabolite production (Fig S11, B), as observed in a correlation matrix of Pearson’s correlation coefficients (Fig S11, C). Since the nucleotides NAD and NADP move readily from one enzyme to another and serve as electron carriers in many oxidation-reduction reactions, these two cofactors have specialized metabolic roles, such as the oxidation of fuels including pyruvate, fatty acids, and α-keto acids derived from amino acids in the mitochondrial matrix. Therefore, the impaired regulation of oxidized NAD$^+$ and NADP$^+$ in several oxidation-reduction reactions may be involved in STZ-mediated renal dysfunction. As expected, the ratio of AMP to ATP was higher in STZ-treated rats than in control rats (Fig S11, D), suggesting that STZ-induced metabolic changes may reduce energy metabolism.

**DISCUSSION**

In the present study, we show that the increases in urine lactate levels and LDHA expression levels in human kidneys are closely related to renal fibrosis and dysfunction. Our findings were confirmed in rat STZ-induced DKD models. Despite indications of a link between plasma lactate levels and CKD development, the optimal interpretation of the role of renal lactate metabolism in the context of renal fibrosis and dysfunction in individuals with DKD has not been thoroughly investigated. Furthermore, the cause of the remodeling of metabolism mediated by the bidirectional enzymatic reaction in individuals with DKD is difficult to determine. The possible involvement of LDHA in renal fibrosis has not been verified in human studies due to a scarcity of human kidney samples for DKD research. Using STZ-injected rats, this study illustrates the mediated renal fibrosis. Western blot (upper panel) showing the levels of fibrosis-related proteins and LDHA; the color of the culture media is shown (lower panel). (F) LDHA silencing using an siRNA reduced TGF-β1-mediated renal fibrosis. Left panel, Western blots showing levels of renal fibrosis-related proteins and LDHA (upper panel) and immunocytochemistry analysis of α-SMA expression (lower panel). Right panel, Quantification of the protein intensity on the Western blots. (G) The TGF-β1-induced increase in lactate excretion was attenuated by LDHA silencing. (H) Representative EM images of mitochondria in RMCs treated with TGF-β1 in the presence or absence of the LDHA siRNA. Bars, 0.5 μm; 8000×. Left panel, higher magnification images of boxed areas (20,000×); right panel, percentage of abnormal mitochondria. (I) OCR profile normalized to the number of TGF-β1-treated RMCs. The cells were sequentially treated with glucose, oligomycin (which inhibits ATP synthesis), FCCP (which uncouples OXPHOS), rotenone (Rot, which inhibits mitochondrial complex I [C-I]), and antimycin A (AA, which inhibits C-III). All data are presented as the means ± SEM. Data in C, D, and F–I were analyzed using 2-way repeated-measures ANOVA with Tukey’s post hoc multiple comparison test. α-SMA, α-smooth muscle actin; ANOVA, analysis of variance; DCA, dichloroacetic acid; FCCP, fluoro-carbonyl-cyanide phenylhydrazone; LDHA, lactate dehydrogenase A; OCR, oxygen consumption rate; TGF-β1, transforming growth factor-beta 1. (Color version of figure is available online.)
functional roles of the hypoxia-mediated increase in renal lactate generation as a deleterious regulator of the etiology of DKD as described below. (1) In patients with DKD, a decrease in the eGFR was linked to an increase in urine lactate levels and LDHA expression, as well as an increase in FBG and HbA1c levels. (2) Higher lactate efflux was associated with a substantial increase in the expression of the glycolytic enzyme LDHA, as well as higher TGF-β1 levels and urinary 8-OHdG efflux. (3) In the analysis of RMCs in vitro, hypoxia-induced lactic acidosis was related to increased fibrosis and decreased mitochondrial metabolic activity, and these effects were mitigated by inhibiting aerobic glycolysis and silencing LDHA. (4) Collectively, our findings imply that hypoxia-induced increases in renal lactate generation may play a role in the pathophysiology of progressive DKD. Furthermore, our findings imply that baseline urine lactate levels can be employed as an early predictor of DKD development without the need for established cardio-renal risk markers, such as eGFR or albuminuria.

Increased acid retention leading to renal fibrosis and injury is widely accepted as a common symptom of chronic kidney disease in patients. However, the decreased intracellular pH in patients with CKD is often attributed to chronic and systemic metabolic acidosis. The diseased kidneys are unable to sufficiently excrete endogenous acid, leading to the loss of the ability to maintain the systemic acid-base balance. In contrast, our results show that lactate-derived local acidosis is a potential mechanism contributing to DKD. Aerobic glycolysis and lactate production are often activated to reduce the blood glucose level and prevent hyperglycemia in patients with diabetes. In this regard, excess lactate retention in the kidneys is a potential disease-causing factor in individuals with DKD. Our data obtained using the STZ-induced DKD model showed that an increase in urinary lactate levels was consistent with an increase in intracellular lactate levels and a decrease in intracellular pH in kidney tissues (Fig 4, E–G). Lactate is transported in and out of the cell through MCT1 and MCT4, respectively. During lactate import, hydrogen ions (H+) are also transported into cells such that intracellular pH levels decrease. However, the expression of MCT1 and MCT4 did not change in the kidney tissues of patients with DKD (Fig 2, C and D) or rats with STZ-induced DKD (Fig 4, D), suggesting the bidirectional transport of lactate. In addition, experiments using the RMC model showed that TGF-β1 mediated lactic acidosis by increasing intracellular and extracellular lactate levels and was sufficient to induce renal fibrosis. Additionally, our results provide clear evidence that silencing LDHA expression and blocking the aerobic glycolysis pathway markedly reversed TGF-β1-mediated lactic acidosis, which was accompanied by the amelioration of mitochondrial dysfunction and renal fibrosis. Moreover, our hypothesis was further validated by the association between the eGFR and renal LDHA gene expression or secreted urinary lactate levels, but not serum lactate levels, in patients with DKD. Based on these results, lactic acidosis induced by LDHA-mediated lactate production might be related to renal dysfunction and fibrosis, independent of changes in the expression of MCT1 and MCT4.

Fasting plasma lactate levels are increased in patients with diabetes or obese patients with diabetes, and lactate production progressively increases during hyperinsulinemia, which is similar to the early stages of type 2 diabetes development. Recent metabolomics studies have shown that plasma or urinary lactate levels are increased in animal models of CKD or DKD. Nevertheless, the exact role and mechanism of lactate in the development of CKD or renal failure remain largely unknown. In fact, since plasma lactate concentrations represent an equilibrium between its production and metabolism, these levels may differ from the renal lactate concentration. Under normal physiological conditions, lactate is metabolized and cleared by the liver and kidneys through conversion to pyruvate via LDHB and subsequently to glycerogen or carbon dioxide. Lactate is also transported to the liver and is converted back into glucose through the TCA cycle, thus serving as an energy source. Although this bidirectional mechanism is complex when considered systemically, our data show that lactate metabolite levels were significantly increased in the kidney tissues of rats with STZ-induced DKD and correlated with the increases in LDHA gene expression and secreted urinary lactate levels but not with serum lactate levels in patients with DKD.

Increased lactate secretion in the blood and urine may be due to alterations in or inactivation of the systematic activation pathway associated with lactate clearance in DKD rats and patients. However, our data show that increased lactate production and urinary lactate levels were closely associated with increased LDHA expression following oxidative damage. In addition, rats and human subjects with high LDHA expression exhibited a reduced eGFR or WT-1 expression and increased α-SMA expression. Participants with high HbA1c levels exhibited a reduced eGFR and increased LDHA expression. Increased HbA1c levels and a reduced eGFR were positively correlated with increased urinary lactate levels, but not with serum lactate levels. These data suggest that increased LDHA expression and urinary lactate levels were strongly associated with progressive diabetes and an altered
glycolytic pathway, followed by local acidosis-mediated renal fibrosis and dysfunction.

In general, DKD is well characterized by several histopathological changes, including glomerular hypertrophy, mesangial matrix expansion, glomerular basement membrane thickening, and renal fibrosis. However, these pathophysiological features have several limitations. They cannot be used as early predictive markers because these features appear after the disease progresses rather than at the early stage of the disease. A financially beneficial and favorable approach for preventative health measures is to predict and diagnose DKD before its progressive development. In particular, the use of a more sensitive and specific marker would facilitate an earlier diagnosis, an appropriate response to DKD, and reduced risks of morbidity and mortality. Increased potassium secretion and angiogenic signaling were identified as early kidney responses in humans with diabetic nephropathy by detecting the single-cell transcriptomic landscape of early diabetic nephropathy in humans. In the same study, the authors documented the differentially expressed genes in human proximal convoluted tubule cells from patients with diabetic nephropathy. Thirteen glucose metabolism-related genes annotated within the KEGG glucose metabolism pathway, including LDHB, were differentially expressed in proximal convoluted tubule cells (Table S11). Neither LDHA nor MCT1 (the lactate transporter expressed in proximal convoluted tubule cells) was differentially expressed in the data. However, because the authors only employed nuclear RNA to quantify RNA expression levels, their results may not reflect RNA expression or protein levels at the total cell level. Nevertheless, our data show that both MCT1 and MCT4, which are lactate transporters, may not play critical roles in increasing urinary lactate levels in patients with DKD because significant changes in the expression of the MCT1 and MCT4 proteins were not observed in patients with DKD compared to healthy subjects (Fig 2, C and D), which was confirmed in STZ-injected rats (Fig 4, D) and TGF-β1-treated cells (Fig 4, J). Several traditional indicators, such as albuminuria, serum creatinine, UPCR, and eGFR, are currently used to diagnose CKD, and numerous mechanisms and intervention strategies targeting the control of the pathogenic features of CKD have also been proposed. However, successful clinical effectiveness and translation of drugs that prevent or treat the progressive development of CKD are still relatively limited. Therefore, the identification of novel metabolic target mediators for predicting the progression of DKD in the context of renal fibrosis and therapeutic target molecules to slow the decline in kidney function and limit extrarenal complications is urgently needed.

In conclusion, an increase in LDHA expression levels in kidney biopsy samples and urinary lactate levels in patients with DKD allowed us to comprehend the pivotal role of lactate metabolism in DKD progression; hypoxia-mediated lactic acidosis resulting in the induction of fibrosis and mitochondrial abnormalities were suggested to participate in the mechanism of DKD. Our experimental results partially reveal the effect of lactate on mitochondria-related pathological phenotypes. In our subsequent study, we plan to further explore how local lactic acidosis regulates mitochondrial function. Moreover, blocking LDHA expression markedly attenuated the resulting pathological features. Thus, renal lactate metabolism might soon be identified as a therapeutic target for DKD.

**DATA AVAILABILITY STATEMENT**

All data from this study are included within the manuscript, including the Supplemental Information.

**AUTHORS’ CONTRIBUTIONS**

W.H.K. and G.S.H. conceived the project and supervised, guided, and designed the research. D.Y.L., J.Y.K., G.H.K., J.J.P., and Y.J.L. constructed the animal models and obtained the data. M.K.S. and S.W.K. analyzed the clinical data. J.S.H., Y.A.J., and G.S.H. analyzed the metabolomics data. S.Y.H. and D.R.C. coordinated the Korean diabetic CKD patient registry and analyzed the clinical data. S.Y.H., D.R.C., J.H.K., and G.S.R. provided support for the technical methods and instruments, contributed to the discussion, and reviewed the manuscript. E.Y.A., D.Y.L., J.Y.K., J.S.H., D.R.C., G.S.H., and W.H.K. contributed to the discussion and wrote, reviewed, and edited the manuscript.

**ACKNOWLEDGMENTS**

Conflicts of Interest: All authors have read the journal’s policy on disclosure of potential conflicts of interest and have declared that no conflicts of interest exist.

This work was supported by research grants from the Korean National Institutes of Health (4845-302-210-13 and 2018-N1007-01), the Korea Basic Science Institute (C270200) and National Research Foundation of Korea (NRF) grants funded by the Korean government (2019M3A9D5A01102796 and 2020R1A2C2007835). All named authors have read the journal’s authorship agreement and that the manuscript has been reviewed by and approved by all named authors.
SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.celrep.2022.06.015.

REFERENCES


