Research paper

Impacts of Diabetes and an SGLT2 Inhibitor on the Glomerular Number and Volume in db/db Mice, as Estimated by Synchrotron Radiation Micro-CT at SPring-8

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Abstract

Background: Recent large-scale clinical studies demonstrate that sodium-glucose cotransporter 2 (SGLT2) inhibitors protect the diabetic kidney. However, clinical and animal studies have not shown the changes of the total glomeruli in the whole kidney treated with SGLT2 inhibitors.

Methods: We performed computed tomography (CT) imaging on mice using synchrotron radiation to investigate the impact of luseogliflozin, an SGLT2 inhibitor, on the number and volume of glomeruli in the whole kidney.

Findings: We did not observe a significant difference in the total glomerular number (Nglom) among mice. Luseogliflozin redistributed the number of glomeruli in different regions, accompanied by the normalization of diabetes-augmented renal volume (Vkll). Diabetic db/db mice had a larger glomerular volume in the mid-cortex than did control db/m mice, and luseogliflozin increased the glomerular volume in all renal cortical zones of the whole kidney in db/db mice. According to the multivariate regression analysis, hemoglobin A1c level was the most relevant determinant of Vkll, not Nglom or mean glomerular volume (Vglom), indicating that hyperglycemia induced renal (tubular) hypertrophy, but not glomerular enlargement. Luseogliflozin increased hypoxia in the juxamedullary region, sustained upregulated renal renin expression and plasma renin activity, and failed to decrease albuminuria by downregulating megalin in db/db mice.

Interpretation: Based on our findings, SGLT2 inhibitors may alter glomerular distribution and size in addition to their glucose-lowering effects, presumably by affecting oxygen metabolism and humoral factors.

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1. Introduction

Diabetic nephropathy (DN) is now a leading cause of end-stage renal failure, and DN therefore constitutes a major proportion of progressive kidney disease cases. For the past several decades, DN was postulated to be a primarily glomerular disease, rather than a disease based on tubular interstitial lesions, and glomerular hypertrophy is pathophysiologically associated with glomerular sclerosis [1]. Although reversible glomerular hypertrophy is defined as the remission of the renal disease [2], glomerular enlargement leads to glomerular sclerosis,
by performing a renal biopsy of patients with diabetes who are treated with SGLT2 inhibitors. However, in an in vivo study, empagliflozin failed to improve albuminuria and glomerulosclerosis in diabetic \( db/db \) mice [12]. In contrast, an SGLT2-selective inhibitor, JNJ 39933673, prevented increases in urinary albumin excretion in \( db/db \) mice [13]. This change was associated with a decrease in mesangial expansion in \( db/db \) mice, as determined by periodic acid-Schiff (PAS) staining, but had no impact on glomerular size [13]. Therefore, the impacts of SGLT2 inhibitors on the glomeruli in vivo also remain controversial.

However, conventional methodologies have limitations that prevent a precise representation of glomerular structure [14–18]. Because the glomerulus, when open, forms a glomerular capillary tuft, the intracapillary contrast agent must accurately visualize the size of the open glomerulus. Thus, synchrotron radiation microcomputed tomography (micro-CT) in SPring-8 (Super Photon ring-8 G electron volts, the power output of the ring) [19] has been proposed as an alternative method for counting tiny glomeruli throughout the kidney without destroying it. Here, we show the impacts of diabetes and luseogliflozin, an SGLT2 inhibitor, on glomerular number and volume in whole kidneys from mice that were intravenously injected with contrast medium using synchrotron radiation micro-CT.

2. Materials and methods

2.1. Animal study

All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Research Center for Animal Life Science at Asahikawa Medical University. The male \( db/db \) (Lepr\( ^{db/db} \)) mice used as a model of type 2 diabetes [20] and \( db/m \) mice (on a C57BLKS\( ^{j} \) background) used as controls were purchased from the Institute for Animal Reproduction (Ibaraki, Japan). The mice were housed on a 12-h light/dark cycle and provided regular chow, namely, MF ( Oriental Yeast Co, Tokyo, Japan). All mice were assessed for glomerular morphology, body weight (BW), blood pressure, and hemoglobin A1c (HbA1c) levels. Systolic blood pressure (SBP) and mean blood pressure (MBP) were measured in conscious mice using an automated tail-cuff manometer system (MK–2000ST; Muromachi Kikai, Tokyo, Japan). The average of 10 consecutive measurements from each mouse was calculated. Glucose levels in whole blood extracted from the tail were quantified using a One Touch glucose analyzer (LifeScan Inc., Milpitas, CA, USA). HbA1c levels were measured using the DCA 2000 analyzer (Siemens Medical Solutions Diagnostics, Tokyo, Japan). Mice in each group were placed in metabolic balance cages for 24-h urine collections to measure urine samples. Plasma and renal cortical renin activities were measured using the fluorometric renin assay kit from Abcam (Ab138875, Abcam Inc., Cambridge, MA, USA) with a Tide Fluor™ 3 (TF3)/Tide. Quencher™ 3 (TQ3) fluorescence resonance energy transfer (FRET) peptide [21]. The fluorescent signal was measured using a GloMax® Discover multimode microplate reader (Promega, Madison, WI, USA). Renal function was assessed by measuring urinary albumin excretion (UAЕ) and plasma cystatin C levels, using ELISA kits for UAЕ (Exocell, Philadelphia, PA, USA) and plasma cystatin C (R&D Systems, Minneapolis, MN, USA).

Eight-week-old male \( db/db \) mice received luseogliflozin (0.01% in chow; 15 mg/kg/day) in a regular rodent diet (MF, Oriental Yeast, Tokyo, Japan) for 8 to 14 weeks to examine the effects of an SGLT2 inhibitor on the diabetic kidney. Following the treatment with luseogliflozin, we performed three series of studies. First, we measured the glomerular number and volume in mice using synchrotron micro CT-imaging at SPring-8. Second, we performed histological and biochemical studies, including the detection of hypoxia, immunohistochemical staining, western blots for megalin and electron microscopy of mouse tissue sections. Third, confocal microscopy was performed to determine albumin endocytosis in vivo. Luseogliflozin was provided by Taisho Pharmaceutical (Tokyo, Japan).
Fig. 1. Detection of glomeruli in synchrotron radiation micro-CT images. (a) Synchrotron radiation micro-CT imaging at SPring-8. (b) Images of glomerular tufts filled with contrast medium (gray-colored granules) (left panel, scale bar, 10 μm, right panel, scar bar, 2 mm). A representative axial image of the kidney of mice injected with contrast medium from the micro-CT dataset (right panel). (c) The two panels show magnified images of the sections identified by the squares in the left and right panels. The dimensions of the glomerulus measured in PAS-stained kidney sections (red circles) are similar to those measured in the CT image (white spots). Scale bars in PAS-stained sections, 300 μm. Scale bar in the left CT image, 2 mm. (d) The reconstructed data were analyzed with a 3D counting algorithm to identify individual glomeruli using the program Amira (left and middle panels). The glomeruli in 100 slices of CT images are displayed (right panel). Glomerular number and individual glomerular volume (e), kidney volume (left panel, scale bar, 2 mm) (f), and the distribution of glomeruli in 10 layers from the centroid (G) of the kidney to the surface (g) were assessed by using the program Amira.
Fig. 2. Glomerular number and volume estimated using synchrotron radiation micro-CT at SPring-8. (a) The glomerular number (Nglomer) in kidneys. (b) The mean glomerular volume (Vglomer) in kidneys. (c) The renal volume (Vkidney). (d) The ratio of the total glomerular volume to the kidney volume (Rglomer/kidney). (e) The normalized Vglomer divided by body weight (BW) (Vglomer/BW). (f) The normalized Vglomer divided by BW (Vglomer/BW). (g) The normalized Nglomer divided by BW (Nglomer/BW). (h) The normalized Nglomer divided by Vkidney (Nglomer/Vkidney). (i) The normalized Vglomer divided by Vkidney (Vglomer/Vkidney). (j) CV (SD/mean of Vglomer). (k) The histograms of glomerular volume in mice. (l) The number of glomeruli within each of the 10 spatial layers created by normalizing the distance from the surface to the centroid of the kidney. (m) The difference in mean glomerular volumes (Vglomer) in the spatial distribution (from the surface to the juxtamedullary region). Black circles: db/m (n = 14); red triangles: db/db (n = 14); blue triangles: luseogliflozin-treated db/db mice (n = 6).

Values are presented as means ± standard deviation (SD). One-way (a-j) and two-way (k-m) repeated-measures ANOVAs with Bonferroni's multiple comparison post hoc tests, **p < 0.01, and ***p < 0.001.
2.2. Kidney sample preparation for the analysis of glomerular morphology using synchrotron radiation micro-CT

The contrast medium was prepared using the protocol published by Toyota et al. [22]. Briefly, the contrast medium consisted of 40% wt/vol (in distilled water) BaSO4 and 8% wt/vol gelatin. Mice were anesthetized with pentobarbital [55 mg/kg of body weight, intraperitoneal injection]. The abdomen and thorax were cut via a midline incision extending from the symphysis pubis to the jugulum. Three hundred units of heparin were injected in the beating left ventricle of the heart using a 24-gauge catheter. The right atrium was then cut, and the mouse was perfused first with 42 °C prewarmed heparinized saline (100 IU/1 ml heparin in 0.9% NaCl) and then with the contrast medium (42 °C). Immediately after perfusion, cold saline (<4 °C) was gently poured into the abdominal cavity. The mouse was carefully placed in an ice bucket for 10 min to solidify the contrast medium. Thereafter, the kidneys, which were filled with contrast medium, were removed and stored in a freezer.

2.3. Imaging of kidney samples

Samples were visualized using the synchrotron micro-CT system in BL20B2 at SPring-8 [19]. The synchrotron radiation beam is produced by deflecting the electron beam with a bending magnet, and the flux is much higher than laboratory X-ray sources. The X-ray was monochromatized with a Si (111) double crystal monochromator, and the beam then irradiated the samples. As the X-ray beam is approximately parallel, each horizontal line corresponded to a slice position along the rotation axis, and images of multiple slices (the slice pitch was equal to the pixel size, namely, the “cubic voxel”) were easily obtained in one rotation (3D-CT).

A high-resolution image detector (Beam monitor 5 and ORCA-Flash4.0; Hamamatsu Photonics, Hamamatsu, Japan) was used for radiographic imaging [23]. The format of the 3D image was 2048 × 2048 × 1320 pixels, with cubic voxels of 15.53 μm3 (3.72 × 10−6 mm3). The scintillator, which converts the X-ray beam to visible light, was a 25-μm layer of GADOX (Gd2O2S: Tb±, P43). The X-ray energy was 37.6 or 35 keV, and the exposure time per projection was 100 ms.

For each sample, 1800 radiographic images were acquired over an angular range of 0–180° within 3 min. In this study, the kidney sample, on the rotating stage was placed in an insulated chamber with double Kapton and foamed styrol walls and then scanned while cryogenic temperatures were maintained using dry ice. After scanning, we reconstructed the CT images from the radiographic images by using a conventional tomography algorithm and convolution back projection.

2.4. Segmentation of glomeruli and kidneys

When enhanced with contrast agents, glomeruli have higher CT values than other tissues. We therefore conducted segmentation based on a region-growing algorithm with multiple seed points to count the glomeruli within the whole kidney. Segmentation was conducted with constraints on anisotropy and the volume of the objects to filter out noise and remove unwanted objects. The anisotropy $\psi$ was defined as follows:

$$\psi = 1 - \frac{\lambda_{\text{min}}}{\lambda_{\text{max}}}$$  (1)

Table 1

| Mouse | $N_{\text{glom}}$ | $V_{\text{glomer}}$ (mm$^3$) | $N_{\text{glomer}}/V_{\text{kidney}}$ | $V_{\text{glomer}}/V_{\text{kidney}}$ | Mean (SD) | CV | Mean (SD) |
|-------|-------------------|-----------------------------|------------------------------------|------------------------------------|-----------|----|-----------|
| $db/m$| Mean 9546.29      | 322.22                      | 29.68                               | 0.81%                              | 2.70E-04  | 1.27E-04 | 4.72E-01  | 8.42E-07  | 3.97E-07  |
| n = 14| SD 1912.79        | 79.13                       | 5.78                                | 0.18%                              | 3.98E-04  | 1.55E-04 | 4.15E-01  | 7.79E-07  | 4.07E-07  |
| $db/db$| Mean 9871.71      | 233.98                      | 25.44                               | 0.75%                              | 2.99E-04  | 1.55E-04 | 4.15E-01  | 7.79E-07  | 4.07E-07  |
| n = 14| SD 1434.65        | 79.13                       | 5.78                                | 0.18%                              | 3.98E-04  | 1.55E-04 | 4.15E-01  | 7.79E-07  | 4.07E-07  |
| $SGLT_2i$| Mean 10,168.5     | 304.66                      | 34.11                               | 1.22%                              | 3.48E-04  | 1.61E-04 | 4.68E-01  | 1.21E-06  | 5.54E-07  |
| n = 6 | SD 1210.24        | 51.98                       | 6.08                                | 0.44%                              | 8.73E-05  | 3.49E-05 | 2.81E-02  | 4.78E-07  | 1.90E-07  |

$N_{\text{glomer}}$: glomerular number; $V_{\text{glomer}}$: glomerular volume; $V_{\text{kidney}}$: kidney volume; $R_{\text{glomer/kidney}}$: ratio of the total glomerular volume to kidney volume; CV: coefficient of variation (SD/mean).
Fig. 3. The characteristics of type 2 diabetic model mice, and the effects of luseogliflozin on the laboratory parameters in db/db mice. (a) FBS levels. (b) HbA1c levels. (c) BW. (d) SBP. (e) Mean blood pressure (MBP). (f) Food intake for 24 h. (g) Water intake for 24 h. (h) Urinary volume for 24 h. (i) Urinary albumin levels per day. (j) Plasma cystatin C levels. Black circles: db/m mice (n = 7); red triangles: db/db mice (n = 7); blue triangles: luseogliflozin-treated db/db mice (n = 3). Values are presented as means±standard deviation (SD). One-way repeated-measures ANOVA with Bonferroni’s multiple comparison post hoc tests, *p < 0.05, **p < 0.01, and ***p < 0.001.
where \( \lambda_{\text{max}} \) and \( \lambda_{\text{min}} \) are the maximum and minimum eigenvalues of the object, respectively. The monochromatic X-ray used the absorption edge of the contrast media (barium), and the X-ray energy was 37.6 keV. In addition, CT imaging was conducted with an X-ray energy level of 35 keV when renal vessels neighboring the glomeruli-related streak artifacts were observed in 37.6 keV images, and these artifacts were substantially reduced in the 35 keV images. We introduced the following correction coefficient to compensate for the glomerular volume measurements conducted between the two energy levels of the beam:

\[
V' = cV
\]

where \( V \) and \( V' \) are the volumes of glomeruli before and after the correction, respectively, and \( c \) is the correction coefficient. We imaged the samples with both 37.6 keV and 35 keV, and compared the volume of glomeruli at the same position to determine the correction coefficient (Supplemental Fig. 1).

Good linearity was observed between the glomerular volumes measured using the images obtained at 35 keV and those obtained at 37.6 keV. We applied a linear regression analysis using Eq. (2) that had no intercept to the data, and this calculation provided the correction coefficient \( c = 2.5966 \). The object volume was determined from the number of voxels in the object and the correction coefficient. Objects with an anisotropy of 0–0.75 and a corrected volume of 43,826–631,932 \( \mu \text{m}^3 \) yielded an equivalent spherical diameter of 53–129 \( \mu \text{m} \) and were regarded as glomeruli. Finally, the spatial distribution of glomeruli within the kidney was assessed. For this experiment, the kidney was divided into 10 layers according to the normalized distance from the centroid of the kidney to the surface, and the number of glomeruli in each layer was counted.

### 2.5. Quantification of morphology

We repeated the experiment without contrast media (\( \text{db}/\text{m}, \text{db}/\text{db}, \text{luseogliozin-treated db}/\text{m} \) or \( \text{db}/\text{db} \) mice; \( n = 5–6 \) mice per group) and the kidneys were fixed by perfusion as described in a previous study to confirm the histological findings [24]. We performed periodic acid–Schiff (PAS) staining on these sections using previously described methods to evaluate the changes in renal structure [24,25]. Using coronal sections of the kidney, 15 consecutive glomeruli per mouse were examined to evaluate the glomerular mesangial expansion. The mesangial expansion index was defined as the ratio of the mesangial area to the glomerular tuft area. The mesangial area was determined by assessing the PAS-positive and nucleus-free areas in the mesangium using ImageJ software (Image Pro Plus v. 6.0 for Windows, Media Cybernetics; Silver Spring, MD, USA). The semiquantitative morphometric analysis of tubular injury was performed using the paraffin sections stained with PAS. Tubular damage was scored by evaluating the percentage of injured tubules showing dilation or atrophy, brush border loss, cell lysis, and cast formation in the corticomedullary junction and outer medulla. Armanni–Ebstein lesions were also evaluated by determining the percentage of injured tubules appearing empty or full of PAS-positive material. Then, grading on a scale from one to five, where one indicated lack of injury, two indicated that <10% of tubules were injured, three indicated <10% and <25% of tubules were injured, four indicated ≥25% and <50% of tubules were injured, and five indicated that >50% of tubules were injured in 15 randomly selected fields of view (original magnification, 400×) in each section per kidney. Morphometry was conducted in a blinded manner by two experienced nephrologists.

#### 2.6. Immunohistochemistry

Immunohistochemistry was performed with a rabbit polyclonal anti-renin antibody (1:400) (ab 180,608, Abcam Inc.), mouse polyclonal anti-megalin antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal anti-aquaporin 1 antibody (1:100) (Novus Biologicals, Littleton, CO, USA). Immunohistochemical staining was performed as previously described [25]. For the analysis, the total renin-immunoreactive area per kidney section was related to the number of visible glomeruli in the sections. For each kidney used for the morphometric analysis, 10 highly visible afferent arterioles were selected for measurement. Since 5–6 kidneys from mice in each experimental group were analyzed, 50–60 afferent arterioles were measured in each experimental group. The total renin-immunoreactive area multiplied by its mean intensity per kidney section was measured using ImageJ software. The immunoreactive score for megalin expression was determined by the extent of positive immunoreactivity: one for negative, two for positive immunoreactivity <10%, three for ≥10% and <25%, four for ≥25% and ≤30%, and five for >30% of megalin-positive section.

#### 2.7. Electron microscopy

Parts of the removed kidneys were cut into small tissue blocks (1 mm\(^3\)) and fixed with 2.0% glutaraldehyde and 2.0% paraformaldehyde in 0.1 mol/l phosphate buffer at 4 °C. After fixation with 2% osmium tetroxide, the tissues were dehydrated in a series of ethanol solutions, and ethanol was then substituted with propylene oxide. The tissues were then embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead. Sections were examined under an electron microscope (JEM1200EX, JEOL, Tokyo, Japan) at 80 kV.

#### 2.8. Western blot analysis

Total cellular extracts from mouse kidneys were prepared, and Western blotting was performed using a denaturing 3–8% Tris-acetate gel (Invitrogen, Carlsbad, CA, USA) under reducing conditions, as previously described [25]. Membranes were blocked and incubated with a primary rabbit anti-megalin antibody (kindly provided by Prof. Akihiko Saito, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan) [26] for 2 h at room temperature, followed by an incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. For loading controls, the same membranes were stripped and reprobed with a \( \beta \)-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) system according to the manufacturer’s instructions (Amersham). Immunoblots were quantified using National Institutes of Health ImageJ software. The ratio between the intensities of megalin
and β-actin bands was used to normalize megalin expression in each sample.

2.9. Detection of renal hypoxia

For the detection of hypoxic conditions in mice, pimonidazole (60 mg/kg) was injected intraperitoneally 1 h before the animals were euthanized [27]. The kidneys were fixed by perfusion to eliminate artificial staining, as recommended in a previous study [27]. Staining was performed using Hydroxyprobe (Pharmacia International, Belmont, MA, USA), according to the manufacturer’s instructions [25].

2.10. Confocal microscopy analysis of albumin endocytosis in vivo

Anesthetized mice were intravenously injected with 10 μg/g body weight of Texas Red (TR) conjugated-albumin (Molecular Probes, Eugene, OR, USA) 30 min before euthanasia [28]. TR-albumin was visualized using a confocal laser scanning biological microscope (Olympus Fluoview FV 1000, Olympus, Tokyo, Japan).

2.11. Statistics

The sample sizes for the animal studies were determined according to a previous publication [22] that studied the heterogeneity in
Table 3
Multivariate linear regression analysis of the determinants of \( \log(e) \) transformed glomerular number or mean glomerular volume in the kidney using a stepwise forward method.

| Model | \( N_{\text{glomer}} \) | B   | SEM  | Standardized | \( \beta \) | p-value |
|-------|------------------|-----|------|-------------|----------|--------|
| \( r^2 = 0.533 \) | (Constant) | 7.183 | 1.402 | -0.001 | -0.01 | -0.001 |
|       | CV              | -1.105 | 0.234 | -0.639 | -0.001 | -0.001 |
|       | \( V_{\text{kidney}} \) | 0.641 | 0.139 | 0.614 | -0.001 | -0.001 |
|       | \( V_{\text{glomer}} \) | 0.312 | 0.130 | 0.300 | 0.019 | 0.019 |

\( r^2 = 0.667 \)

| Model | \( V_{\text{glomer}} \) | B   | SEM  | Standardized | \( \beta \) | p-value |
|-------|------------------|-----|------|-------------|----------|--------|
| (Constant) | -7.927 | 1.045 | -0.001 | -0.001 | -0.001 |
| \( \text{UAE} \) | 0.046 | 0.013 | 0.418 | 0.002 | 0.002 |
| \( \text{Water Intake} \) | 0.324 | 0.063 | 0.954 | -0.001 | -0.001 |
| \( \text{Food Intake} \) | -0.204 | 0.048 | -0.678 | -0.001 | -0.001 |
| \( \text{HbA1c} \) | -0.553 | 0.121 | -0.351 | -0.001 | -0.001 |
| \( N_{\text{glomer}} \) | 0.267 | 0.104 | 0.278 | 0.016 | 0.016 |

\( r^2 = 0.515 \)

| Model | \( V_{\text{kidney}} \) | B   | SEM  | Standardized | \( \beta \) | p-value |
|-------|------------------|-----|------|-------------|----------|--------|
| (Constant) | 6.003 | 0.335 | -0.001 | -0.001 | -0.001 |
| \( \text{Body weight} \) | -0.426 | 0.121 | -0.335 | -0.001 | -0.001 |
| \( \text{HbA1c} \) | 0.478 | 0.086 | 1.317 | 0.001 | 0.001 |

A multivariate linear regression model with the stepwise forward method was applied to test the ability of the \( \log(e) \) transformed glomerular number (\( N_{\text{glomer}} \)) to predict the mean glomerular volume (\( V_{\text{glomer}} \)) in the kidney and vice versa. The kidney volume (\( V_{\text{kidney}} \)), coefficient variation (CV) of glomerular volume, HbA1c levels, body weight (BW), urinary volume, urinary albumin excretion (UAE), plasma cystatin C levels, systolic blood pressure, mean blood pressure, food intake, and water intake (all \( \log(e) \) transformed) were used as the dependent variables in the model. Only the models that were ultimately selected are shown. The analysis was performed with SPSS software (ver. 24; SPSS, Chicago, IL). P-values <0.05 were considered significant.

3. Results

3.1. Glomerular number, volume, and distribution

We performed CT imaging on nondiabetic control db/m mice, type 2 diabetic model db/db mice [20] and luseogliflozin-treated db/db mice at 22 weeks of age with the medical imaging beamline BL20B2 at SPring-8 (Fig. 1a). The glomeruli filled with contrast medium were detected using synchrotron radiation micro-CT as described in a previous study using punched-out renal tissue specimens (column) [22] (Fig. 1b). Additional observations were performed on kidney samples after completing the micro-CT visualization to validate the quality of the synchrotron radiation micro-CT images. The histopathological findings of an optional cross-section were compared with CT images in the same layer. Corresponding glomerular images were consistent with the histological findings (Fig. 1c). Fig. 1d shows the results obtained from an automated 3D segmentation algorithm to identify individual glomeruli. Identified glomeruli are arbitrarily colored in slices of the 3D images of excised kidneys of contrast medium-injected mice (Fig. 1d). Then, the glomerular number (Fig. 1e), individual glomerular volume (Fig. 1e), kidney volume (Fig. 1f), and glomerular distribution in the kidney (Fig. 1g) were assessed using Amira software.

3.2. Glomerular number and volume estimated by synchrotron radiation micro-CT at SPring-8

We did not observe a difference in the total glomerular number (\( N_{\text{glomer}} \)) among the mice (Fig. 2a). The mean glomerular volume (\( V_{\text{glomer}} \)) did not significantly increase in diabetic db/db mice compared to the control db/m mice, because of the wide distribution (Fig. 2b and Table 1). Interestingly, db/db mice treated with the SGLT2 inhibitor luseogliflozin (SGLT2i group) exhibited an increased \( V_{\text{glomer}} \) compared with db/m mice (\( p = 0.01 \), one-way repeated-measures ANOVA with Bonferroni’s multiple comparison post hoc tests), similar to db/db mice (\( p = 0.05 \)) (Fig. 2b and Table 1). Consistent with the reduction in blood glucose levels (Fig. 3a, b and Supplemental Table 1), the SGLT2i group exhibited a reduction in whole kidney volume (390 ± 57.2 mm\(^3\) in db/db mice vs. 304.7 ± 52.08 mm\(^3\) in SGLT2i mice, \( p = 0.001 \) (Fig. 2c), but still showed glomerular hypertrophy (Fig. 2b and Table 1). Because the glomeruli constitute <2% of the total kidney volume (Fig. 2d and Table 1), these changes in renal volume were largely attributed to tubular volume, suggesting that the renal tubules are the susceptible lesions in diabetic db/db mice. The glomerular or renal hypertrophy observed in db/db mice was attributed to a decrease in the volume of the glomeruli or kidney normalized to the body weight (Fig. 2e and f), suggesting that either glomerular or renal sizes, but not \( N_{\text{glomer}} \) (Fig. 2g), might be partially affected by body weight. The density of glomeruli per kidney (\( N_{\text{glomer}}/V_{\text{kidney}} \)) was increased in the SGLT2i group, due to the attenuation of renal hypertrophy (\( p < 0.01 \), Fig. 2h). The mean glomerular volume normalized to the renal volume (\( V_{\text{glomer}}/V_{\text{kidney}} \)) was not different among the mice, because of the wide distribution (Fig. 2i). A larger coefficient of variation (CV; SD/mean) of glomerular volume was calculated for db/db mice than db/m mice (0.472 ± 0.048 for db/m mice vs. 0.519 ± 0.044 for db/db mice, \( p < 0.001 \)), and luseogliflozin restored this variation (Fig. 2j).

3.3. Spatial or regional heterogeneity of the glomeruli in diabetic mice

Next, we analyzed the distribution of the volume of individual glomeruli in the kidney (Fig. 2k). We identified an interaction between the distribution of glomerular volume and the group of mice using two-way ANOVA (\( p < 0.001 \)). The glomerular volume was shifted rightward (larger volume) in diabetic db/db mice, particularly in luseogliflozin-treated db/db mice, compared to the db/m mice (Fig. 2k). We also analyzed the spatial distribution of the glomeruli in the ten segmented layers from the centroid to the surface of the kidney (Fig. 2l, m and Supplemental Table 2). We identified a weak interaction in the distribution of glomerular number between db/db and luseogliflozin-treated db/db mice (\( p = 0.050 \), two-way ANOVA, Fig. 2l). Luseogliflozin decreased the glomerular number in the juxtamedullary region by one-half compared with the db/m and db/db mice, accompanied by an increase in the number of glomeruli in the superficial cortex (Fig. 2l and Supplemental Table 2a). In addition, we observed a significant correlation in the distribution of glomerular volume (\( V_{\text{glomer}} \)) between db/m and db/db mice (\( p = 0.004 \), two-way ANOVA, Fig. 2m and Supplemental Table 3). The db/db mice had significantly larger glomeruli in the mid-cortex compared with db/m mice (\( p < 0.01 \) at layer 0.7 and \( p < 0.05 \) at layer 0.8, post hoc independent and unpaired t-test, Fig. 2m and Supplemental Tables 2b and 3).
Fig. 5. Representative images of PAS-stained kidney sections from each group of mice. (a) Mesangial index. (b) Tubular damage score. Bar, 100 μm. (c) Armanni-Ebstein lesions. Arrows highlight glycogen accumulation in the distal tubules, such as the thick ascending limb of Henle’s loop (TAL). Black circles: db/m mice (n = 6); blue circles: luseogliflozin-treated db/m mice (n = 5); red triangles: db/db mice (n = 6); blue triangles: luseogliflozin-treated db/db mice (n = 6). Bars, 300 μm in the upper panel and 30 μm in the middle and lower panels. Values are presented as means±standard deviation (SD). Kruskal-Wallis test with the unpaired, non-parametric Mann-Whitney U test as a post hoc analysis, *p < 0.05.
Interestingly, FBS level was strongly correlated with $V_{\text{glomerulus}}$ in normoglycemic $db/m$ mice ($r = 0.8717, p = 0.0001$) and diabetic $db/db$ mice ($r = 0.8186, p = 0.0006$) (Fig. 4a). However, no correlation between FBS level and $V_{\text{glomerulus}}$ was observed in luseogliozin-treated $db/db$ mice ($r = -0.7171, p = 0.0667$) (Fig. 4a). The lack of a correlation between HbA1c levels and $V_{\text{glomerulus}}$ may result from the narrow range of HbA1c levels measured in any of the groups of mice (Fig. 4b). Thus, blood glucose levels per se do not determine the glomerular hypertrophy. $V_{\text{kidney}}$ was positively correlated with FBS levels ($r = 0.7005, p < 0.0001$) (Fig. 4c) and HbA1c level ($r = 0.042, p < 0.001$) (Fig. 4d) in all mice, indicating that renal tubular volume, which occupies >90% of the renal volume, might depend on blood glucose level. $V_{\text{glomerulus}}$ was significantly correlated with UAE ($r = 0.5067, p = 0.001$) (Fig. 4e), but $V_{\text{kidney}}$ was not correlated with UAE ($r = 0.3133, p = 0.0712$) (Fig. 4f).

According to the results from the multivariate regression analysis with the stepwise forward method, the values measured by synchrotron micro-CT, such as CV, $V_{\text{glomerulus}}$ and $V_{\text{glomerulus}}$ were the most relevant determinants of $N_{\text{glomerulus}}$ (Table 3). However, UAE, water intake, food intake and $V_{\text{glomerulus}}$ and $N_{\text{glomerulus}}$ were the determinants of $V_{\text{glomerulus}}$, suggesting that hemodynamic or metabolic factors might change the glomerular volume (Table 3). Interestingly, body weight and HbA1c levels were determinants of $V_{\text{glomerulus}}$, indicating that renal hypertrophy is a characteristic of obesity or hyperglycemia (Table 3).

### 3.6. Luseogliozin-induced tubular hypoxia and renin expression in the kidneys of diabetic mice

Although luseogliozin failed to attenuate glomerular hypertrophy (Fig. 2b) or albuminuria (Fig. 3i, Supplemental Figs. 2 and 3, and Supplemental Table 1), luseogliozin significantly ameliorated mesangial expansion, tubular injury and glycogen accumulation in the distal tubules (Armanni-Ebstein lesions) compared to non-treated $db/db$ mice (Fig. 5a, b, and c). The renal proximal tubules are susceptible to diabetes-induced hypoxia [25], and phlorizin induces hypoxia in the juxtamedullary regions [33]. Therefore, we examined the presence of hypoxia in the kidneys of luseogliozin-treated mice by staining with pimonidazole to explore the effects of luseogliozin on hypoxia (Fig. 6a). Luseogliozin significantly increased pimonidazole staining of the S3 segment of the proximal tubular cells (Fig. 6a), which were immunostained with aquaporin-1 (Fig. 6b), in the outer stripe of the kidney of $db/db$ mice. In addition, luseogliozin also induced hypoxia in the juxtaglomerular apparatus (JGA) (Fig. 6a, inset). Electron microscopy images showed an attenuation of the thickening of the basement membrane in luseogliozin-treated $db/db$ mice (Fig. 6c). Intriguingly, luseogliozin markedly increased the number of large and confluent vesicles with a low density in juxtaglomerular (JG) cells (Fig. 6d), suggesting that the renin-producing cells were strongly stimulated for a prolonged period of time, and that SGLT2 inhibition may be involved in the activation of the renin-angiotensin system (RAS). Accordingly, renin immunoreactivity was still observed in the JGA of the luseogliozin-treated $db/db$ mice (Fig. 6e, lower left panel). In addition, luseogliozin did not decrease diabetes-increased plasma renin activity (Fig. 6e, lower middle panel). A significant difference in cortical renin activity was not observed among mice (Fig. 6e, lower right panel).

### 3.7. Luseogliozin inhibited albumin uptake by decreasing megalin expression

Finally, we assessed the pathological mechanism underlying the sustained albuminuria in luseogliozin-treated $db/db$ mice. Intriguingly, luseogliozin reduced the numbers of recycling vesicles and endosomes in $db/db$ mice (Fig. 7a), as previously observed in megalin knock-out mice [34]. Megalin was expressed at higher levels in the cortex of $db/m$ mice than in $db/db$ mice (Fig. 7b, upper panel). We confirmed that $db/db$ mice exhibited a significant decrease in the levels of the megalin protein in the kidneys using Western blotting, as shown in Fig. 7c.
Fig. 6. Luseogliflozin induced renal hypoxia and renin expression in db/db mice. (a) Immunohistochemistry for the hypoxia marker pimonidazole. The inset shows positive pimonidazole staining in the macula densa and JG cells. Bars, 300 μm. Black circles: db/m mice (n = 6); blue circles: luseogliflozin-treated db/m mice (n = 5); red triangles: db/db mice (n = 6); blue triangles: luseogliflozin-treated db/db mice (n = 6). (b) Immunolocalization of aquaporin-1 and pimonidazole in the OSOM. Arrows show the colocalization of both proteins in the same tubular cells. Stars show the lack of staining of both proteins in the same tubular cells. Insets show higher magnification images of the selected areas. Bars, 300 μm in the upper panel and 30 μm in the middle and lower panels. OSOM: the outer stripe of the outer medulla; ISOM: the inner stripe of the outer medulla; IM: the inner medulla. (c) Representative electron microscopy images of the glomeruli of mice. Bars, 1 μm. (d) The vesicles of JG cells (arrow). Bars, 5 μm. (e) Immunohistochemistry for renin. Red arrows show the macula densa. Bars, 30 μm. Renin immunoreactivity (lower left panel); Plasma renin activity (lower right panel); Cortical renin activity (lower middle panel). Black circles: db/m mice (n = 5–6); blue circles: luseogliflozin-treated db/m mice (n = 3–5); red triangles: db/db mice (n = 6); blue triangles: luseogliflozin-treated db/db mice (n = 6). Values are presented as means±standard deviation (SD). Kruskal-Wallis test with the unpaired, non-parametric Mann-Whitney U test as a post hoc analysis, *p < 0.05 and **p < 0.01.
Fig. 6 (continued).
(p < 0.05, one-way repeated-measures ANOVA with Bonferroni’s multiple comparison post hoc tests). Luseoglioflozin also decreased megalin expression in db/db mice (Fig. 7b, lower panel, and 7c), and decreased TR-albumin uptake (Fig. 7d), suggesting that luseoglioflozin induced albuminuria in db/db mice by inhibiting megalin expression.

4. Discussion

Our study is the first to reveal the impacts of diabetes and an SGLT2 inhibitor on Nglomer and Vglomer throughout the kidney, which was accomplished by developing an imaging and analysis system using synchrotron micro-CT. We observed glomerular enlargement in the mid-cortex of kidneys in diabetic db/db mice compared with control mice, but the difference in mean glomerular volume of total glomeruli was not significant because of the wide variation in a kidney and among mice (Fig. 2b and Table 1). In addition, luseoglioflozin markedly ameliorated hyperglycemia (Fig. 3a, b, and Supplemental Table 1), but not glomerular hypertrophy (Fig. 2b and Table 1) in db/db mice. Based on these results, the glomerular volume might be determined by complicated mechanisms and not simply by the plasma glucose level.

A significant difference in the distribution of the glomerular number throughout the kidney was not observed between db/m and db/db mice, although db/db mice displayed diabetic renal injury with albuminuria. Traditionally, diabetic nephropathy has been classified as a glomerular disease. However, the histological predictor of the progression of patients with diabetic nephropathy is tubulointerstitial lesions, but not glomerular lesions [35,36]. Taken together, these findings indicate that glomerular loss is not involved in the pathological mechanism of diabetic nephropathy in db/db mice. Intriguingly, luseoglioflozin redistributed and expanded the glomeruli, accompanied by the normalization of diabetes-induced renal hypertrophy, suggesting that the renal architecture was remodeled. Furthermore, the multivariate regression analysis with the stepwise forward method showed that HbA1c levels are the most relevant determinant of Vglomer, but not Nglomer and Vglomer. Indicating that chronic hyperglycemia induces renal hypertrophy but does not affect the glomeruli.

The luseoglioflozin treatment increased the number of vesicles with a low density (Fig. 6d), which have also been observed following strong, prolonged stimulation with isoproterenol [37]. The luseoglioflozin treatment also induced renin protein accumulation in JG cells and plasma renin activity in db/db mice (Fig. 6e), consistent with previous reports [12,13]. During hypoxia, the β-adrenergic receptor signaling pathway increases renin release from JG cells [38]. In the present study, luseoglioflozin induced hypoxia in the JGA of db/db mice (Fig. 6a, inset). Hypoxia stimulates renin secretion and renin gene expression in rats [39]. Therefore, in addition to volume depletion [40], SGLT2 inhibitor-induced hypoxia in the JGA might trigger the activation of the RAS.

SGLT2 inhibition induces intrarenal RAS activity in diabetic mice [12,13], and increases circulating levels of RAS mediators in patients with type 1 diabetes [41,42]. The efferent arterioles (EA) are 10–100 times more sensitive to the vasoconstrictive properties of angiotensin (Ang) II than are the afferent arterioles (AA) [43,44]. Accordingly, the activation of the RAS by SGLT2 inhibitors might induce the vasoconstrictive action of EA rather than AA. Indeed, in Dahl salt-sensitive diabetic rats, luseoglioflozin alone fails to prevent diabetic renal injury, but exerts renoprotective effects in combination with ACE inhibitor [45]. Although further studies are required to verify these conclusions, luseoglioflozin might fail to ameliorate glomerular enlargement due to the lack of favorable effects on EA (Fig. 2b). Intriguingly, the ACE inhibitor enalapril was shown to normalize glomerular pressure and glomerular sclerotic lesions, but not glomerular hypertrophy [46]. Thus, two renoprotective inhibitors targeting ACE and SGLT2 induced glomerular hypertrophy without glomerular injury. Collectively, the glomerular size does not correlate with the severity and the percentage of glomerulosclerosis or the severity of tubulointerstitial lesions.

SGLT2 is located in the S1 and S2 segments of proximal tubules, whereas SGLT1 is located in the S3 segment, which is susceptible to hypoxia [47]. SGLT2 inhibitors force SGLT1 to reabsorb glucose at its maximum capacity [48,49]. Thus, SGLT2 inhibitors enhanced hypoxia in the juxtamedullary region, which includes the S3 segment of proximal tubules (Fig. 6a and b). Although an SGLT2 inhibitor might protect the diabetic kidney by inducing hypoxia in the outer medulla, which may enhance erythropoiesis [50,51], SGLT2 inhibitors potentially lead to hypoxic renal injury. Therefore, we recommend the avoidance of the concomitant administration of non-steroidal anti-inflammatory drugs in radiocontrast studies [52,53]. Chronic hypoxia induces tubulointerstitial fibrosis, which in turn could decrease the GFR [54]. In this regard, cortical hypoxia predicts a progressive decrease in renal function in patients with chronic kidney disease [55,56]. In contrast, SGLT2 inhibitors induced hypoxia in the outer stripe of the outer medulla (OSOM), but not in the cortex. These data, along with the amelioration of diabetic tubular injury by luseoglioflozin, showed that SGLT2 inhibitor-induced hypoxia in the OSOM might exert less of an effect on the decrease in GFR.

Unexpectedly, we observed decreased activity of the endocytic apparatus and decreased cortical megalin expression in luseoglioflozin-treated db/db mice (Fig. 7a, b, and c). In addition, we discovered that luseoglioflozin reduced the uptake of TR-conjugated albumin in the proximal tubules of db/db mice (Fig. 7d). Based on these results, luseoglioflozin decreased albumin uptake in the proximal tubules by reducing megalin expression and not by damaging the tubules, resulting in albuminuria. These effects are similar to the beneficial effects of a Nrf2 activator bardoxolone methyl [57]. Because megalin-mediated endocytic handling of substances filtered by the glomeruli is involved...
Fig. 7. Luseogliflozin reduced the activity of the endocytic apparatus, megalin expression and albumin uptake in renal proximal tubules from \(db/db\) mice. (a) The numbers of recycling vesicles and endosomes (E) in the renal proximal tubules. Bars, \(1 \mu m\). (b) Images of megalin immunohistochemistry. Values are presented as means±standard deviation (SD). Kruskal-Wallis test with the unpaired, non-parametric Mann-Whitney \(U\) test as a post hoc analysis. (c) Western blot analysis of megalin expression in the mouse kidney. Values are presented as means±standard deviation (SD). One-way repeated-measures ANOVA with Bonferroni’s multiple comparison post hoc tests, \(*p < 0.05\). Black circles: \(db/m\) mice (\(n = 4\)); blue circles: luseogliflozin-treated \(db/m\) mice (\(n = 3\)); red triangles: \(db/db\) mice (\(n = 6\)); blue triangles: luseogliflozin-treated \(db/db\) mice (\(n = 6\)). (d) Representative confocal microscopy images of TR-conjugated albumin fluorescence in the proximal tubules of mice. These mice showed similar levels of albuminuria with different FBS levels, as shown in Supplemental Fig. 3. Lower panels show higher magnification images of the proximal cells presented in the upper panels. Bar, \(20 \mu m\) in the upper panels. Red: TR-albumin.
Fig. 7 (continued).
in tubular injury [58,59], luseogliozfizin might exert its renoprotective effects by inhibiting the expression of the megalin protein. Notably, increased mitochondrial oxygen consumption results in renal hypoxia and induces nephropathy without the complications of hyperglycemia or oxidative stress [60]. Based on the effects of luseogliozfizin on the glomeruli and megalin, which are independent of its glucose-lowering effects, the renoprotective effects observed in the current study prompted the hypothesis that SGLT2 inhibitors ameliorate diabetic nephropathy by directly reducing oxygen consumption and avoiding decreased energy levels in renal proximal tubules by inhibiting the uptake of glucose, sodium and albumin and restoring intracellular hypoxia in cortical proximal tubular cells, which express SGLT2 and megalin. In an attempt to corroborate our hypothesis, an investigation of effects of luseogliozfizin on mitochondrial oxygen consumption and hypoxia in the cortical proximal tubular cells is ongoing in our laboratories.

In conclusion, the current observations present the effects of SGLT2 inhibitors on the glomerular number, size and renal volume in diabetic mice. These effects of SGLT2 inhibitors are at least partially independent of their glucose-lowering actions, presumably by affecting oxygen metabolism and humoral factors, and these findings warrant further study.

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Conflicts of interests

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Authors' contributions

Y.T., T.S. and M.N. conceived and designed the project and wrote the manuscript. Y.T., T.S., T.Y., M.K., R.B., T.T., H.K., H.S., and D.F. performed the experiments. M.H. and K.U. assisted with the synchrotron X-ray imaging experiments at SPring-8. Y.T., T.S., M.N., K.I., and Y.S. analyzed the data and interpreted the results with N.Y. T.O. contributed to the discussion and reviewed the manuscript. M.H., Y.F., Y.M., and A.A. staffed proxy for outpatient during the experiments at SPring-8. All authors approved the final version of the manuscript. Y.T. is responsible for the integrity of the work as a whole.

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Appendix A. Supplementary data

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