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ORIGINAL ARTICLE

Adenosine/adenosine type 1 receptor signaling pathway did not play dominant roles on the influence of sodium–glucose cotransporter 2 inhibitor in the kidney of bovine serum albumin-overloaded streptozotocin-induced diabetic mice

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Keywords
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ABSTRACT

Aims/Introduction: Sodium–glucose cotransporter 2 inhibitors (SGLT2i) have been shown to display excellent renoprotective effects in diabetic kidney disease with macroalbuminuria/proteinuria. Regarding the renoprotective mechanism of SGLT2i, a sophisticated hypothesis was made by explaining the suppression of glomerular hypertension/hyperfiltration through the adenosine/adenosine type 1 receptor (A1R) signaling-mediated restoration of the tubuloglomerular feedback mechanism; however, how such A1R signaling is relevant for renoprotection by SGLT2i in diabetic kidney disease with proteinuria has not been elucidated.

Materials and Methods: Streptozotocin-induced diabetic CD-1 mice were injected with bovine serum albumin (BSA) and treated with SGLT2i in the presence/absence of A1R inhibitor administration.

Results: We found that the influences of SGLT2i are essentially independent of the activation of A1R signaling in the kidney of BSA-overloaded streptozotocin-induced diabetic mice. BSA-overloaded diabetic mice showed the trend of kidney damage with higher glomerular filtration rate (GFR) and the significant induction of fibrogenic genes, such as transforming growth factor-β2 and collagen type III. SGLT2i TA-1887 suppressed diabetes-induced GFR in BSA-overloaded diabetic mice was associated with the significant suppression of transforming growth factor-β2 and collagen type III; A1R-specific inhibitor 8-cyclopentyl-1,3-dipropylxanthine did not cancel the effects of TA-1887 on either GFR or associated gene levels. Both TA-1887 and 8-cyclopentyl-1,3-dipropylxanthine-treated BSA-overloaded diabetic mice showed suppressed glycated hemoglobin levels associated with the increased food intake. When analyzing the association among histological evaluation, GFR and potential fibrogenic gene levels, each group of mice showed distinct correlation patterns.

Conclusions: A1R signaling activation was not the dominant mechanism on the influence of SGLT2i in the kidney of BSA-overloaded diabetic mice.

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INTRODUCTION

The number of patients with type 2 diabetes mellitus has increased globally, with diabetic complications becoming the major causes of morbidity and mortality among patients with diabetes[1]. Diabetic kidney disease (DKD) has been the leading cause of end-stage kidney disease[2]; in particular, patients with overt proteinuria are recognized as being at high risk of progression to end-stage kidney disease[3,4].

Sodium–glucose cotransporter 2 inhibitor (SGLT2i) blocks the action of SGLT2, which is primarily responsible for the reabsorption of filtered glucose, thereby increasing urinary glucose excretion and lowering the levels of glucose[5]. Randomized controlled trials[6,7] have provided clinical evidence regarding the cardiorenal protective effects of SGLT2i. Among the randomized controlled trials, the Canagliflozin and Renal Events in Diabetes with Established Nephropathy Clinical Evaluation (CREDENCE) trial, in which only patients with macroalbuminuria were recruited, clearly showed the protective effects of SGLT2i in high-risk DKD[8]. In addition, in the Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients–Removing Excess Glucose (EMPA-REG OUTCOME) subanalysis data, SGLT2i showed remarkable and early renal protection in patients with type 2 diabetes mellitus having overt nephropathy compared with those with and without microalbuminuria[9].

SGLT2i showed hemodynamic-dependent and hemodynamic-independent renoprotective effects[5]. One of the hemodynamic-dependent renoprotective effects of SGLT2i is explained by the suppression of glomerular hypertension/hyperfiltration through the restoration of the tubuloglomerular feedback system through the appropriate delivery of sodium ions to the macra densa (Figure S1). SGLT2i essentially works on the S1 and S2 segments of the proximal tubule of the kidney. Inhibition of SGLT2 induces the delivery of glucose to the downstream tubules associated with theoretically higher sodium levels. Higher sodium levels in urine are monitored by the Na-K-2Cl cotransporter (NKCC2) in the macra densa (Figure S1). NKCC2 activation leads to adenosine production, and synthesized adenosine is released into glomerular capillaries. Adenosine induces the contraction of afferent arterioles and dilation of efferent arterioles through activation of adenosine A1 receptor (A1R) and A2R; as a consequence, glomerular hypertension/hyperfiltration could be suppressed (Figure S1)[10,11]. Cherney *et al.*[12] elegantly showed that SGLT2i empagliflozin attenuates hyperfiltration in type 1 diabetes patients, and Kidokoro *et al.*[13] showed that the activation of the adenosine/A1R signaling pathway induced by SGLT2i is involved in the amelioration of glomerular hypertension/hyperfiltration. However, it remains unclear whether SGLT2i can ameliorate renal damage in patients with diabetes with remarkable proteinuria associated with improved glomerular hyperfiltration through activation of the adenosine/A1R signaling pathway.

Fibrosis is the final common step of any kidney disease, including DKD. In the kidney fibrosis pathway in diabetes, transforming growth factor (TGF)-β signaling has been shown to play essential roles. There are three TGF-βs, TGF-β1, -β2 and -β3, in mammals, and each of them plays distinct physiological/pathological roles[14]. Most research has focused on the role of TGF-β1 in fibroproliferative diseases; however, recent evidence suggests that TGF-β2 is strongly correlated with organ fibrosis and stronger fibrogenic activity[15]. Indeed, the fibrogenic/mesenchymal program in endothelial cells is only mediated by TGF-β2[16]. Activation of TGF-βs influences extracellular matrix accumulation, including collagen accumulation. In kidney fibrosis, fibrillar collagens, such as collagen type I or type III, have been quoted as essential; however, recent evidence suggests that kidney resident fibroblast-derived collagen type I is essential for the preservation of kidney function[17].

Here, we analyzed whether SGLT2i exerts renoprotective effects in streptozotocin (STZ)-induced type 1 diabetic mice with bovine serum albumin (BSA) overload, an experimental animal model of overt proteinuric kidney disease, and whether such renoprotection by SGLT2i is associated with the A1R signaling pathway.

MATERIALS AND METHODS

Animal experiments

Eight-week-old male CD-1 mice (Sankyo Lab Service, Tokyo, Japan) were used in the present study. The mice received one intraperitoneal injection of STZ (200 mg/kg bodyweight [BW]) to induce diabetes, as previously described[18,19]. Blood glucose levels were measured 2 weeks thereafter, and a diagnosis of diabetes was defined as a blood glucose level of >220 mg/dL. Four weeks after the induction of diabetes, the diabetic mice were divided into three groups: (i) gavage with 0.5% hydroxypropyl methylcellulose and intraperitoneal injection of dimethyl sulfoxide (DMSO) in olive oil; (ii) SGLT2i, TA-1887 (30 mg/kg BW/day in hydroxypropyl methylcellulose , gavage)[20] and intraperitoneal injection of DMSO in olive oil; and (iii) TA-1887 + A1R-specific inhibitor 8-cyclopentyl-1,3-dipropylxanthine (DPCPX: 1 mg/kg BW/day in DMSO in olive oil, intraperitoneal)[21] for 2 weeks. Simultaneously, the mice received a free fatty acid-bound BSA intraperitoneal injection (0.3 g/30 g BW; Sigma-Aldrich, St. Louis, MO, USA) for 11 days of 2 weeks, as previously described[18,19]. BSA-overloaded non-diabetic mice treated with vehicle (hydroxypropyl methylcellulose and DMSO in olive oil) were used as controls. Blood pressure was measured using the tail cuff method using BP-98A (Softron Co., Beijing, China). Food intake and water intake were measured for 2 days, and are shown as the amount per day. At the end of the experimental periods, blood samples were collected, and the kidneys were dissected and stored at −80°C until analysis. STZ, BSA and DPCPX were purchased from Sigma. TA-1887 was obtained from the Mitsubishi Tanabe Pharma Corporation (Osaka, Japan).

Morphological analyses

All kidney samples were fixed in fresh 10% formaldehyde and embedded in paraffin, and 3-μm thick sections were used for

Masson's trichrome staining (MTS) and Sirius red staining (SR), as previously described[18,19]. SR was carried out using a picrosirius red staining kit (Cosmo Bio Co., Ltd, Philadelphia, PA, USA). For the semiquantitative evaluation of renal fibrosis through MTS in 10 randomly selected renal cortex areas per mouse, the percentages of the areas stained for fibrosis were graded as follows: 0, 0–5% staining; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%[22]. SR images were quantitatively analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) in 10 randomly selected renal cortex areas, as previously described[18,19].

Real-time polymerase chain reaction

Total ribonucleic acid was isolated from the renal cortex, and complementary deoxyribonucleic acid synthesis and quantitative real-time polymerase chain reaction were carried out, as previously described[18,19]. TaqMan probes for type 1 collagen a1 (COL1) and type 3 collagen a1 (COL3), TGF-β1 or 2 and CD73 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The data were normalized to the level of 18S messenger ribonucleic acid that was used as an internal control.

Inulin clearance measurement

As the GFR, inulin clearance was measured at the end of the experimental periods by using a Functional Immunoassay Technology™ (FIT™) GFR kit (INULIN) (BioPhysics Assay Laboratory [BioPAL™], Inc, Worcester, MA, USA) according to the manufacturer's instructions[23]. Briefly, mice were placed on a temperature-controlled operating table and restrained inside a 50-mL centrifuge tube with large air holes drilled in the tip. After ~20 μL of blood was collected in a heparinized capillary tube from the tail veins through venipuncture using a sharp razor blade, fluorescein isothiocyanate-inulin solution (1 mg/mL) was injected intraperitoneally at 5 mL/g BW. Thereafter, blood was sampled at 30, 60 and 90 min after the injection of fluorescein isothiocyanate-inulin. The quantification for inulin concentration in isolated plasma was carried out using an enzyme-linked immunosorbent assay. Inulin plasma clearance was verified with non-linear regression with a one-phase exponential decay formula ($y = Be^{-bx}$), and the GFR was calculated as (GFR = [(I) / (B/b)] / KW, where I is the dose of inulin delivered by the bolus injection, B is the y-intercept, b is the decay constant, x is time, and KW is kilo weight of mouse).

Biochemical measurements

Blood glucose levels were measured using a portable glucose meter (Antisense III; HORIBA, Ltd., Kyoto, Japan). Glycated hemoglobin (HbA1c) levels were measured using a DCA 2000 Analyzer (Siemens Medical Solutions Diagnostics, Tokyo, Japan) at the end of the experimental periods. Urine samples were collected using the metabolic cage. Urinary adenosine concentration was measured through the liquid chromatography-tandem mass spectrometry method (Shimadzu Techno-Research Inc., Kyoto, Japan), as described in the electronic Appendix S1 methods for details. Urinary creatinine levels were measured with a creatinine companion kit (Exocell Inc., Philadelphia, PA, USA). Urinary adenosine excretion is shown as the urinary adenosine : creatinine ratio.

Statistical analysis

The data are expressed as the mean with a scatter dot plot. All the obtained data were analyzed by the Kolmogorov–Smirnov test to validate a normal distribution. To determine the significance (i) for the normal distribution dataset (BW, kidney weight/BW, blood pressure, HbA1c, histological quantifications, CD73 level), one-way ANOVA followed by Tukey's test was used; and (ii) for the dataset without normal distribution (other than above) or limited sample number (inulin clearance), the Kruskal–Wallis test followed by uncorrected Dunn's test was used. For the association of two datasets, linear regression analysis was used. $P < 0.05$ was considered to be a significant difference.

RESULTS

Characteristics of experimental mice

The characteristics of the mice are shown in Figure 1. The whole BW of the BSA-overloaded STZ-induced diabetic mice was significantly lower than that of the BSA-overloaded non-diabetic mice at the end of the experiment (Figure 1a). There was no difference in the whole BW among the three groups of BSA-overloaded diabetic mice (Figure 1a). Kidney weight/BW was higher in all diabetic mice than in the non-diabetic mice (Figure 1b). Blood glucose and HbA1c levels were significantly higher in all groups of diabetic mice than in the non-diabetic mice (Figure 1c,d). Although randomly measured blood glucose levels among all diabetic mice showed no difference (Figure 1c), HbA1c levels in BSA-overloaded diabetic mice treated with both TA-1887 (SGLT2i) and DPCPX (A1R-specific inhibitor) were significantly lower than those in BSA-overloaded diabetes mice treated with vehicle (Figure 1d). The amount of water intake was significantly increased in all diabetic mouse groups compared with that in BSA-overloaded non-diabetic mice (Figure 1e). The food intake in BSA-overloaded diabetic mice and BSA-overloaded diabetic mice treated with both TA-1887 and DPCPX was greater than that in non-diabetic mice; DPCPX treatment increased food intake compared with BSA-overloaded mice treated with TA-1887 (Figure 1f). The systolic blood pressure of the four groups was not different (Figure 1g).

Influences of SGLT2i on the renal fibrogenic program in BSA-overloaded diabetic mice and the effect of an adenosine A1R inhibitor

In whole mice, MTS and SR were well correlated ($R^2 = 0.3453$, $P = 0.0010$, $y = 0.2729 \times x + 1.837$). MTS showed some trend of kidney fibrosis in BSA-overloaded diabetic mice compared with non-diabetic mice without significant difference by ANOVA

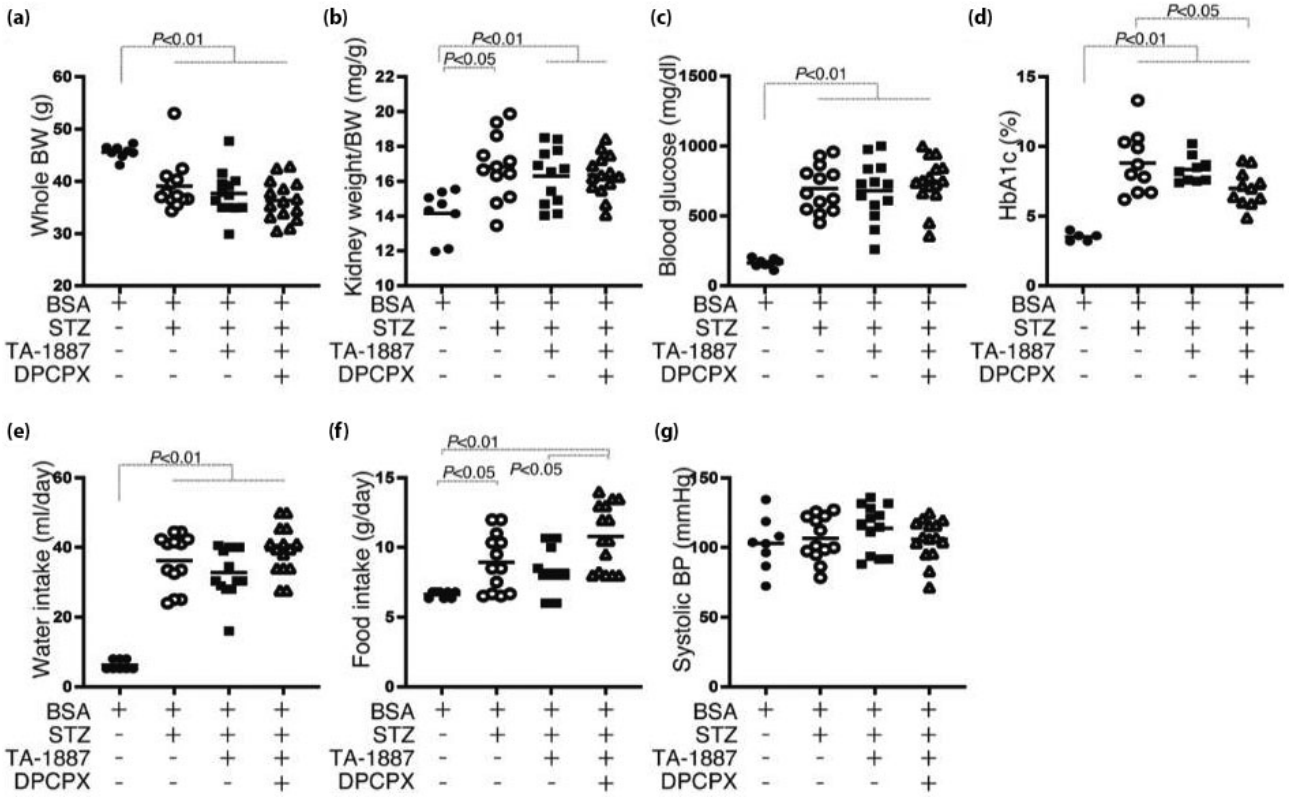

Figure 1 | Characteristics of the mice. (a) Whole bodyweight (BW) and (b) kidney weight/BW, (c) blood glucose levels, (d) glycated hemoglobin (HbA1c) levels at the end of the study, and amount of (e) water and (f) food intake over the entire study period. (g) Systolic blood pressure (BP) at the end of the experimental periods. The data are expressed as the mean with a scatter dot plot, and each dot represents one mouse.

(in two group comparisons: control vs vehicle-treated diabetic mice, $P = 0.0273$, Welch's *t*-test). TA-1887-treated mice, either with or without DPCPX, showed no remarkable difference (Figure 2a,b). By SR, some BSA-overloaded diabetic mice with vehicle and BSA-overloaded diabetic mice with both TA-1887 and DPCPX showed severe fibrosis; however, most of the animals remained mildly altered, and there were no significant differences (Figure 1c,d). The gene expression of TGF-β1 showed a higher trend in BSA-overloaded diabetic mice, but no significant difference (Figure 2e). The TGF-β2 level was significantly increased in vehicle-treated BSA-overloaded diabetic mice, and this induction of TGF-β2 in diabetic mice was significantly suppressed by TA-1887; DPCPX did not alter the TA-1887-suppressed TGF-β2 levels in BSA-overloaded diabetic mice (Figure 2f). The COL1 gene expression level was significantly higher in TA-1887-treated BSA-overloaded diabetic mice with or without DPCPX than in BSA-overloaded non-diabetic mice (Figure 2g). COL3 gene expression was significantly induced in vehicle-treated BSA-overloaded diabetic mice compared with BSA-overloaded non-diabetic mice; TA-1887 significantly suppressed COL3 gene levels either with or without DPCPX (Figure 2h). There was no correlation between MTS and TGF-βs or COLs (Figure S2). SR was well correlated with both TGF-β1 and TGF-β2; there was no significant association with COLs (Figure S2).

Effect of SGLT2i and adenosine receptor signaling on the GFR in BSA-overloaded diabetic mice

The GFR evaluated with inulin clearance was significantly higher in BSA-overloaded diabetic mice than in BSA-overloaded mice without diabetes (Figure 3a). TA-1887 significantly reduced GFR in BSA-overloaded diabetic mice; DPCPX treatment influenced some trend in the elevation of GFR in TA-1887-treated BSA-overloaded diabetic mice, but did not reach significance (Figure 3a). The messenger ribonucleic acid expression of CD73, the enzyme essential for the production of adenosine, was insignificantly decreased by ANOVA in the renal cortex of BSA-overloaded diabetic mice (in two group comparisons: control vs vehicle-treated diabetic mice, $P = 0.0314$, Welch's *t*-test; Figure 3b). Urinary adenosine excretion was highly variable and not significantly different among all the groups (Figure 3c). The interactions between GFR, CD73 and adenosine were not found in whole mice or individual groups (Figure S3).

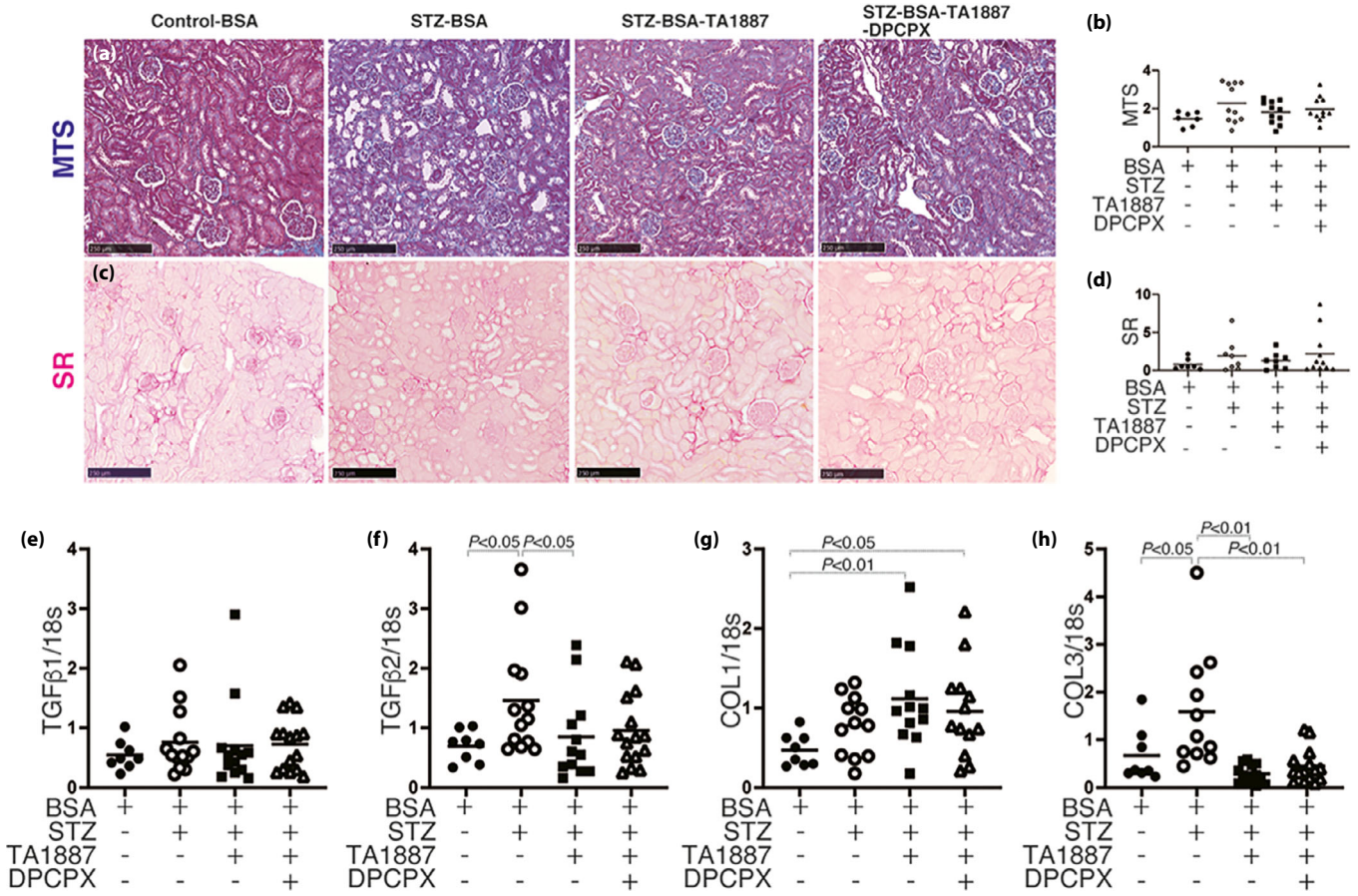

Figure 2 | Changes in renal fibrosis and the fibrogenic program. Representative image of histological evaluation. (a) Masson's trichrome staining (MTS) and (b) MTS-based semiquantitative renal fibrotic scores; (c) Sirius red and (d) quantitative analysis of Sirius red staining in the renal cortex area are shown (obtained by ImageJ software. Scale bar: 50 μm). (e–h) Quantitative polymerase chain reaction analysis of the messenger ribonucleic acid expression of (e) transforming growth factor (TGF)-β1, (f) TGF-β2, (g) COL1 and (h) COL3, expressed as each ratio to the 18S level. The data are expressed as the mean with a scatter dot plot, and each dot represents one mouse. The diabetic group is shown as STZ (streptozotocin) in the figures. BSA, bovine serum albumin.

Correlation between GFR and fibrogenic molecular markers

Alterations in GFR by diabetes or SGLT2i TA-1887 were expected; however, DPCPX administration to TA-1887-treated BSA-overloaded diabetic mice induced highly variable changes in GFR and no significant influences (Figure 3a). Therefore, we investigated whether individual GFRs in mice are associated with fibrogenic molecular markers. In the whole mice, GFR was associated with none of MTS, SR, TGF-β1, TGF-β2, COL1 or COL3 (not shown). Next, we carried out an association analysis between GFR and fibrogenic markers in each group. The histological evaluation by either MTS or SR was not associated with GFR levels in any of the groups (Figure 4a,b). Both TGF-β1 and TGF-β2 levels were negatively associated with GFR only in BSA-overloaded diabetic mice treated with TA-1887; DPCPX-treated mice showed a similar negative association, but no statistical significance (Figure 4c,d). COL1 and COL3 were not associated with GFR in any of the groups (Figure 4e,f).

Correlation between TGF-βs and COLs

TGF-β1 and TGF-β2 were well correlated in the whole mice and in each group (Figure 5a). COL1 and COL3 were not associated (Figure 5b). In whole mice, COL1 was well correlated with either TGF-β1 or TGF-β2; COL3 was not significantly associated with TGF-βs, yet with TGF-β2 it was marginally insignificant (P = 0.0549; Figure S4). In each group analysis, TGF-β1 and TGF-β2 were correlated with COL1 only in BSA-overloaded diabetic mice with both TA-1887 and DPCPX (Figure 5c,d). BSA-overloaded diabetic mice treated with vehicle showed a similar trend, but were marginally insignificant, with a P-value of 0.063 for TGF-β1 or 0.0561 for TGF-β2 (Figure 5c,d). Additionally, TGF-β1 and TGF-β2 were correlated with COL3 only in BSA-overloaded diabetic mice with TA-1887 (Figure 5c,d).

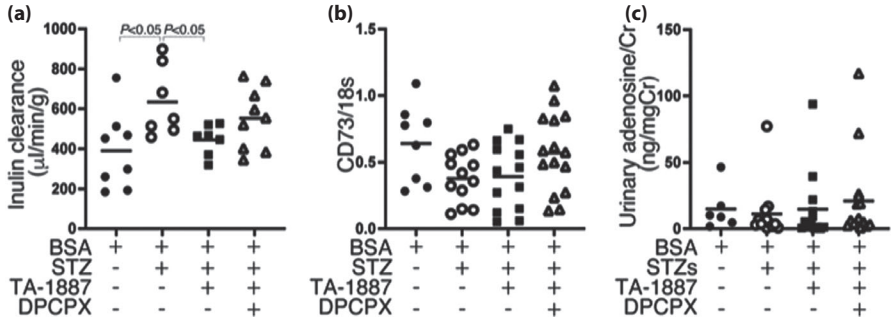

Figure 3 | Glomerular filtration rate, urinary adenosine excretion and renal CD73 expression. (a) The glomerular filtration rate evaluated by the inulin clearance. (b) Kidney messenger ribonucleic acid expression of CD73 expressed as each ratio to 18S level. (c) The urinary adenosine/creatinine (Cr) ratio. The data are expressed as the mean with a scatter dot plot, and each dot represents one mouse. Bovine serum albumin (BSA)-overloaded non-diabetic mice are shown as CONT in the graph. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

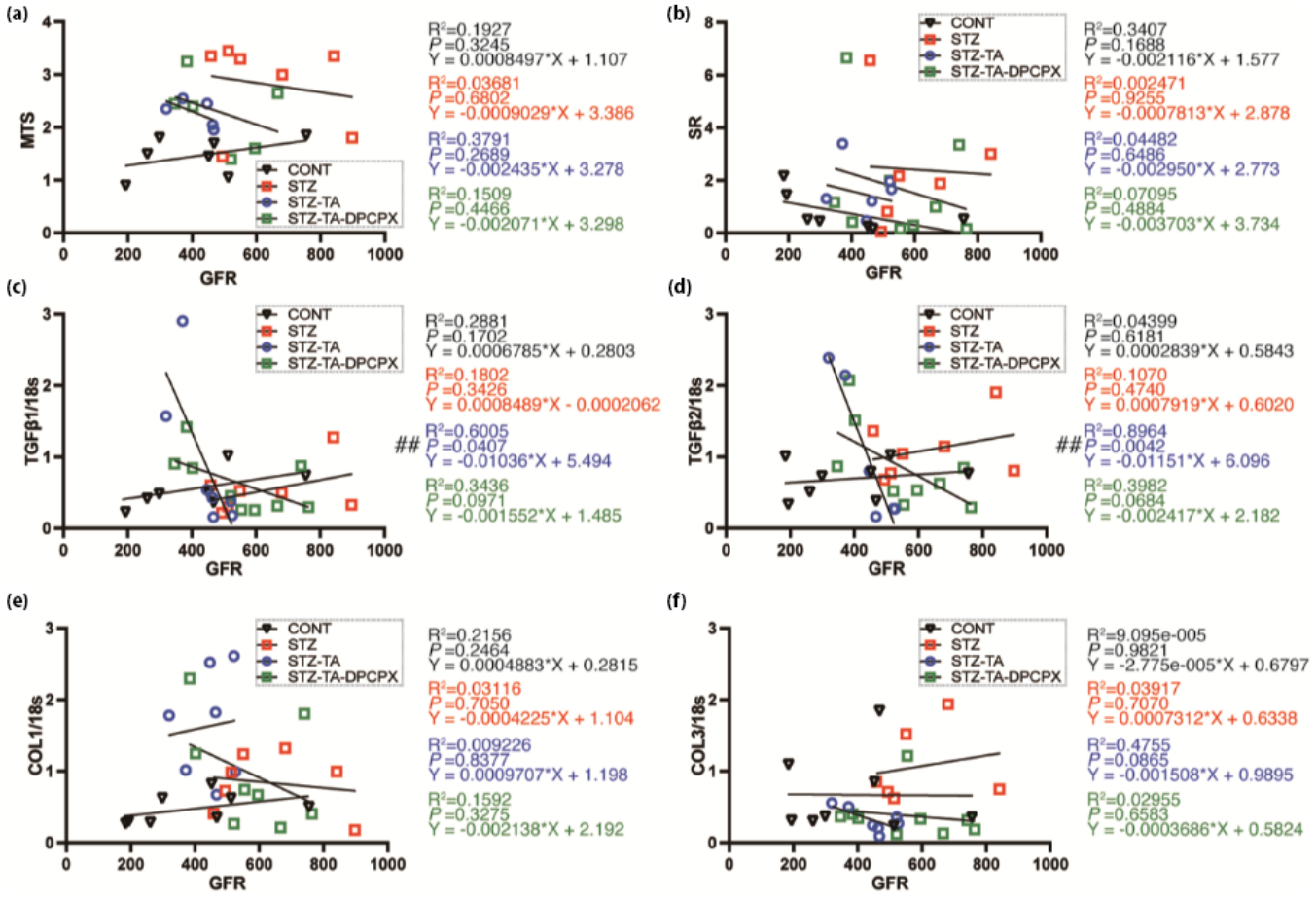

Figure 4 | The association of glomerular filtration rate (GFR) with histological score and fibrogenic gene expression. (a,b) Linear regression analysis of GFR to histological quantification ([a] Masson's trichrome staining [MTS], [b] Sirius red [SR]). (c–f) Linear regression analysis of GFR to gene expression levels of (c) transforming growth factor (TGF)-β1, (d) TGF-β2, (e) collagen type I (COL1) and (f) COL3 (f). The data are expressed as scatter dot plots, and each dot represents one mouse. The color used in symbol/statistical data showed the identical group. Bovine serum albumin (BSA)-overloaded non-diabetic mice are shown as CONT in the graph. The diabetic group is shown as STZ (streptozotocin) in the figures.

DISCUSSION

Proteinuria causes tubulointerstitial lesions, causing inflammation and fibrosis, in chronic kidney disease, including DKD[24], and glomerular hyperfiltration leads to glomerular cell damage, consequently leading to glomerulosclerosis[25]. Both of these pathological mechanisms are potential therapeutic targets of SGLT2i[5]. In the present study, we used a BSA-overloaded proteinuric mouse model and investigated whether SGLT2i exerts renoprotective effects in BSA-overloaded diabetic mice, and this renoprotection by SGLT2i is not dominantly regulated by the A1R signaling pathway. In brief, we showed here that: (i) SGLT2i TA-1887 influenced the kidney fibrogenic program associated with suppression of GFR in diabetic mice without alteration in blood glucose levels; (ii) the A1R-specific inhibitor, DPCPX, did not cancel the influences of TA-1887, either fibrogenic program or suppression of hyperfiltration; (iii) A1R inhibition by DPCPX influenced HbA1c levels and food intake; and (iv) among histological evaluation, GFR and potential fibrogenic gene levels, each group of mice showed distinct correlation patterns. Overall, the influences of SGLT2i on kidney of BSA-overloaded diabetic mice are not solely explained by the activation of A1R signaling.

Glomerular hyperfiltration is characteristically observed in the earlier stages of DKD. In addition, in the progressive stage of DKD, glomerular hyperfiltration as a compensative adaptation for nephron loss plays a crucial role in DKD progression[26]. In diabetes, defects in tubuloglomerular feedback mechanisms are attributed to glomerular hypertension/hyperfiltration, promoting DKD progression and the decline in renal function[27]. In diabetic conditions, urinary glucose filtered from glomerulus

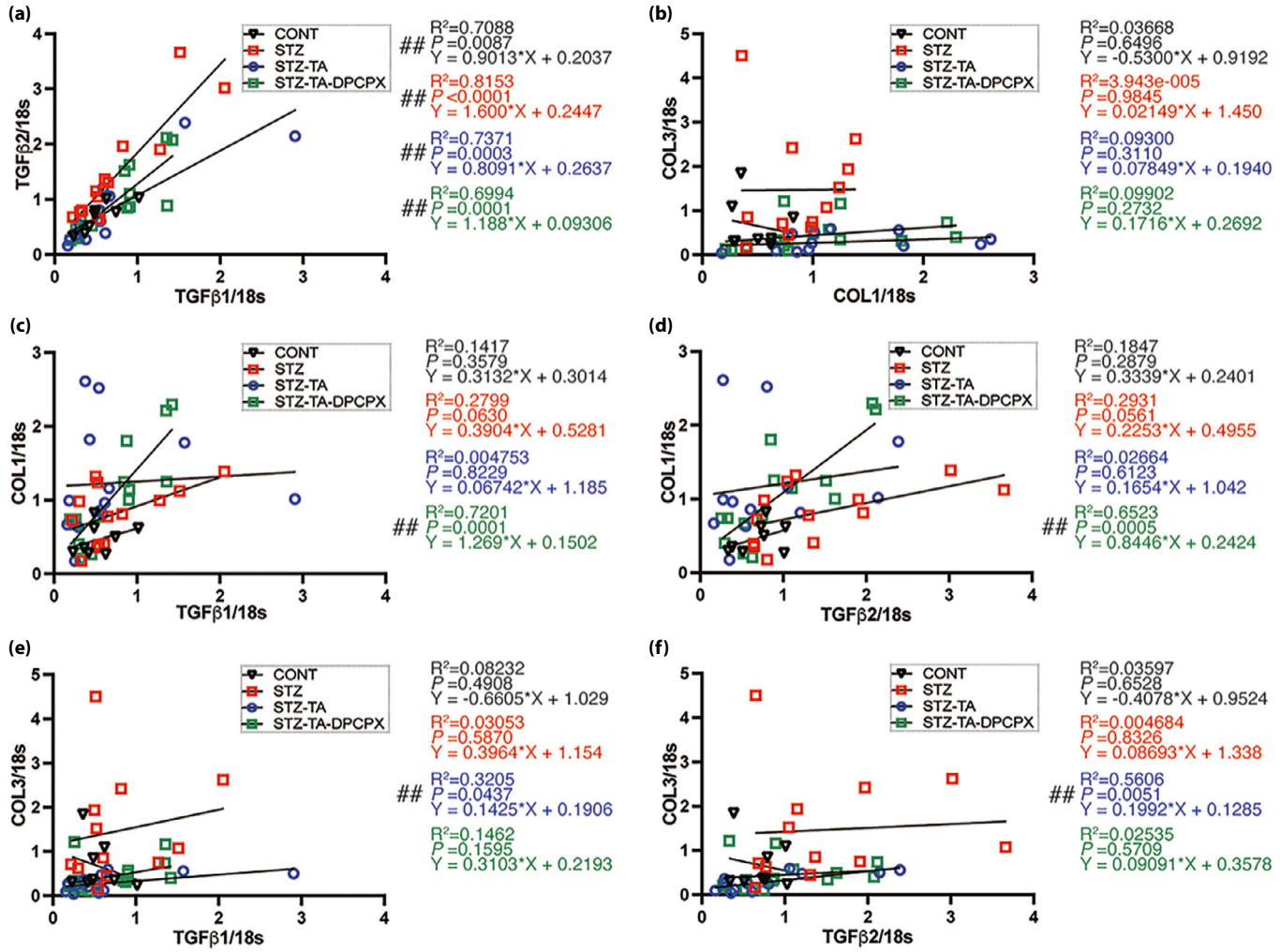

Figure 5 | The association of transforming growth factor (TGF)-βs and collagens (COLs) gene expression. Liner regression analysis of each gene. The label of *x*/*y*-axes showed each gene set. The data are expressed as scatter dot plots, and each dot represents one mouse. The color used in symbol/statistical data shows the identical group. BSA-overloaded non-diabetic mice are indicated as CONT in the graph. The diabetic group is indicated as STZ (streptozotocin) in the figures. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine.

increases, by which SGLT2 levels in the proximal tubule is induced and sodium uptake coupled with glucose transport is augmented (Figure S1). Subsequently, low-sodium urine delivered to lower tubular segments, macula densa, which is responsible for monitoring the sodium chloride levels in urine, resulted in afferent arteriole dilatation by the NKCC2–adenosine–A1R axis. However, low sodium levels in diabetic urine would underestimate the real glomerular filtration and, therefore, the NKCC2–adenosine–A1R axis might not be activated (Figure S1). Therefore, in the diabetic condition, possible roles of NKCC2–adenosine–A1R axis-mediated regulation of glomerular filtration were diminished, even in the condition with diabetes-induced glomerular hypertension and hyperfiltration (Figure S1).

Kidokoro *et al.*[13] showed that the adenosine/A1R signaling pathway plays a crucial role in the amelioration of the single-nephron GFR through the tubuloglomerular feedback mechanism in response to SGLT2i in type 1 diabetic Akita mice. Rajasekeran *et al.*[28] reported that urinary excretion of adenosine increases in response to empagliflozin in young type 1 diabetes patients, suggesting the involvement of the adenosine/A1R signaling pathway in hyperglycemia-induced glomerular hyperfiltration[27], yet there is no direct evidence regarding the role of the adenosine/A1R signaling pathway in SGLT2i effects on glomerular filtration in diabetes patients. Other than adenosine signals and vascular contraction-regulating mechanisms (such as endothelial nitric oxide synthase) might also be involved in a complex manner. Further detailed discussion regarding the hemodynamic effects of SGLT2i is found in Appendix S1.

The fibrogenic program is essential for tissue injury and tissue repair. TGF-βs have been crucial players in fibrogenic programs, including the kidney. Among the three TGF-β isoforms, TGF-β1 signaling has been extensively analyzed as a kidney fibrosis mechanism; TGF-β1 shows a higher threshold to be activated through mechanistic consequences of integrin binding, cleavage by protease or other mechanisms of latency-associated peptide release. In contrast, TGF-β2 shows a lower threshold with highly fibrogenic potentials[15]. In both idiopathic pulmonary fibrosis and non-alcoholic hepatitis, TGF-β2 was significantly induced, but TGF-β1 was not[15]. In our current analysis, both TGF-β1 and -β2 were significantly correlated with SR score, suggesting the potential rationale of our histological evaluation of even mild alterations of SR in each group. TGF-β2 was significantly induced in BSA-overloaded diabetic mice with vehicle, and TA-1887 treatment suppressed the elevation of TGF-β2, whereas such group-specific alterations were not found in TGF-β1. These data suggested that the elevation of TGF-β2 would play a fundamental fibrogenic role in our animal models, and that TA-1887 intervention could suppress kidney damage associated with suppression of TGF-β2 in BSA-overloaded diabetic mice either with or without DPCPX. Additional discussions about the fibrillar collagens are found in Appendix S1.

Additionally, the present data should be interpreted carefully with potential cofounders. Even though random blood glucose measurements were not different, unexpectedly, HbA1c levels were somewhat, but, significantly reduced in the TA-1887 with DPCPX-treated groups of mice. Regarding the role of A1R signaling in glucose homeostasis, the inhibition of A1R by BW1433 suppressed blood glucose levels with suppression of insulin in both lean and obese Zucker rats without altering the BW and percentage body fat[29], suggesting the amelioration of insulin resistance. We utilized an STZ-induced diabetes model; however, STZ administration could not completely diminish pancreatic insulin secretion; therefore, suppression of average glucose levels through suppression of insulin resistance in DPCPX-treated mice might influence GFR levels and fibrogenic programs. Additionally, food intake was more abundant in DPCPX-treated BSA-overloaded diabetic mice on TA-1887 with suppression of HbA1c. In A1R-deficient mice, improved glucose tolerance with suppression of insulin without altering food intake has been reported[30]. The difference could be explained by transient A1R suppression by chemical compounds or basal SGLT2 inhibition in the present model. Additionally, A1R could contribute to leptin release; therefore, DPCPX administration might increase food intake[31]. Furthermore, more food intake in DPCPX-treated mice without alteration in BW could suggest the blockade of A1R signaling on energy expenditure[32]. The complexity of A1R signaling in biology would make the data puzzling; therefore, the role of A1R in either GFR or other altering characteristics would require further investigation.

In conclusion, A1R signaling activation is not the dominant mechanism in the influence of SGLT2i in the kidney of BSA-overloaded diabetic mice.

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DISCLOSURE

Boehringer Ingelheim, Mitsubishi Tanabe Pharma, Taisho Pharmaceutical and Ono Pharmaceutical contributed to establishing the Division of Anticipatory Molecular Food Science and Technology. KK collaborated with Boehringer Ingelheim in both Kanazawa Medical University and Shimane University with a project not related to this manuscript. KK also collaborated with Taisho Pharma on a project not related to this manuscript. Shimane University was supported by funding from Boehringer Ingelheim, Mitsubishi Tanabe Pharma, Taisho Pharmaceutical, Ono Pharmaceutical and Kowa. KK received lecture

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The authors declare no conflict of interest.
Approval of the research protocol: N/A.
Informed consent: N/A.
Approval date of registry and the registration no. of the study/trial: N/A.
Animal studies: The animal experiment was approved by the institutional animal care and use committee of Kanazawa Medical University (protocol number 2017-70, 2020-50).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Hypothetical influence of diabetes and sodium–glucose cotransporter 2 inhibitor on tubuloglomerular feedback mechanisms. The sodium load on macra densa would be key to understand the influence of diabetes and/or SGLT2 inhibitors on tubuloglomerular feedback mechanisms. Furthermore, SGLT1 on macra densa would play roles in the synthesis of nitric oxide (NO), by which afferent arterioles would be dilated. Regarding the net effects of both enhanced sodium delivery and glucose to the macula densa, the two players would have the potential opposite influence on vascular tone, and the condition with SGLT2 inhibitor administration has not been clearly shown yet and further investigation is required. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate.

Figure S2 | The association between histological quantification and fibrogenic gene expression. Linear regression analysis of (a,b) Masson's trichrome staining and (c,d) Sirius red staining association with (a,c) transforming growth factor-β2 and (b,d) collagens. The data are expressed as scatter dot plots, and each dot represents one mouse. The color used in symbol/statistical data indicated identical group. Bovine serum albumin-overloaded non-diabetic mice are shown as CONT in the graph. The diabetic group is shown as STZ (streptozotocin) in the figures.

Figure S3 | The association between glomerular filtration rate (GFR) and *CD73* gene levels and adenosine. Linear regression analysis of (a) GFR-CDC73, (b) adenosine-CD73 and (c) GFR-adenosine. The data are expressed as scatter dot plots, and each dot represents one mouse. The color used in the symbol/statistical data shows the identical group. Bovine serum albumin-overloaded non-diabetic mice are shown as CONT in the graph. The diabetic group is shows as STZ (streptozotocin) in the figures.

Figure S4 | The association between transforming growth factor-βs (TGF-β) and collagen (COL) gene levels in whole mice. Linear regression analysis of (a) TGF-β1-COLs and (b) TGF-β2-COLs. The data are expressed as scatter dot plots, and each dot represents one mouse. The color used in symbol/statistical data shows the identical group.