Sodium-Glucose Linked Cotransporter-2 Inhibition Does Not Attenuate Disease Progression in the Rat Remnant Kidney Model of Chronic Kidney Disease

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Abstract

Pharmacological inhibition of the proximal tubular sodium-glucose linked cotransporter-2 (SGLT2) leads to glycosuria in both diabetic and non-diabetic settings. As a consequence of their ability to modulate tubuloglomerular feedback, SGLT2 inhibitors, like agents that block the renin-angiotensin system, reduce intraglomerular pressure and single nephron GFR, potentially affording renoprotection. To examine this further we administered the SGLT2 inhibitor, dapagliflozin, to 5/6 (subtotally) nephrectomised rats, a model of progressive chronic kidney disease (CKD) that like CKD in humans is characterised by single nephron hyperfiltration and intraglomerular hypertension and where angiotensin converting enzyme inhibitors and angiotensin receptor blockers are demonstrably beneficial. When compared with untreated rats, both sham surgery and 5/6 nephrectomised rats that had received dapagliflozin developed substantial glycosuria. Nephrectomised rats developed hypertension, heavy proteinuria and declining GFR that was unaffected by the administration of dapagliflozin. Similarly, SGLT2 inhibition did not attenuate the extent of glomerulosclerosis, tubulointerstitial fibrosis or overexpression of the profibrotic cytokine, transforming growth factor-β1 mRNA in the kidneys of 5/6 nephrectomised rats. While not precluding beneficial effects in the diabetic setting, these findings indicate that SGLT2 inhibition does not have renoprotective effects in this classical model of progressive non-diabetic CKD.

Introduction

The progressive nature of kidney disease, almost regardless of aetiology, suggests common responses to injury that may be likewise amenable to common therapeutic strategies. Consistent with this observation and in conjunction with experimental findings in rodents, Brenner and colleagues formulated a unifying hypothesis to explain the progressive nature of CKD [1]. According to this haemodynamic-based theory, injury-mediated focal nephron loss leads to hypertrophy, hyperfiltration and increased pressure among remaining glomeruli. These...
responses, serving to increase GFR in remaining nephrons, whilst initially beneficial are ultimately detrimental, increasing glomerular work and causing barotrauma.

Proof-of-concept studies that seem to confirm the hemodynamic hypothesis of CKD progression were founded, in part, on studies in the remnant kidney model where the increase in single nephron glomerular filtration rate (SNGFR) among remaining glomeruli arises from a proportionally greater increase in efferent versus afferent arteriolar resistance [2]. The resultant increase in intraglomerular pressure (P_GC), leads in turn, to intraglomerular hypertension whereby barotrauma induces glomerulosclerosis, proteinuria and declining filtration. Although not associated with nephron loss, a similar scenario is thought to apply to diabetes where glomerular hyperfiltration occurs as a consequence of relative afferent arteriolar dilatation in individuals with recent onset diabetes [3] and rats with streptozotocin-induced diabetes [4]. As a corollary, strategies that reduce intraglomerular pressure should be renoprotective in both non-diabetic and diabetic kidney disease. Indeed, blocking angiotensin II’s predilection for preferentially increasing efferent arteriole tone and thereby elevating intraglomerular pressure [5] has placed angiotensin converting enzyme inhibitors and angiotensin receptor blockers at the cornerstone of renal medicine in most CKD settings.

In addition to lessening angiotensin II-dependent efferent arteriolar constriction, intraglomerular pressure may also be reduced by increasing afferent arteriolar tone. Indeed, these latter changes can be induced by modulating tubuloglomerular feedback (TGF) function where the physiological response to excessive delivery of NaCl to the distal nephron includes afferent arteriolar constriction and consequent reductions in both SNGFR and P_GC [6]. Accordingly, reducing proximal tubular Na⁺ reabsorbtion by blocking the activity of sodium-glucose co-transporter 2 (SGLT2), leads to a reduction in GFR in humans with diabetes-associated hyperfiltration [7] and in both total and single nephron GFR in rodents with diabetes [8]. These data have led to the suggestion that SGLT2 inhibitors, by reducing hyperfiltration and by also possibly lowering P_GC may, akin to agents that block the RAS, be renoprotective [9–11].

While studies with SGLT2 inhibitors have been conducted almost entirely in the diabetes, this drug class lowers tubular glucose reabsorption sufficiently to induce glycosuria in both animal models and human subjects without hyperglycaemia [12–16]. As such, by modulating TGF, SGLT2 inhibition’s effects on SNGFR and P_GC should also apply to the non-diabetic setting, the other differences between diabetic and non-diabetic kidney disease notwithstanding. Accordingly, like the original experiments by Brenner and colleagues with ACE inhibition in both the remnant kidney and diabetic rat models [17, 18], we postulated that SGLT2 inhibition might be similarly renoprotective in the non-diabetic setting.

Materials and Methods

Animals

Fifty-three male Sprague-Dawley rats (Charles River, Montreal, Quebec), aged 10 weeks were randomly assigned to undergo subtotal nephrectomy or sham surgery. Subtotal (5/6) nephrectomy (SNX) was performed in a one-step procedure, as previously described [19] whereby animals were under 2.5% isoflurane anaesthesia the right kidney was excised and infarction of approximately two thirds of the left kidney was achieved via selective ligation of 2 out of the 3 or 4 branches of the renal artery. Sham surgery consisted of laparotomy and manipulation of both kidneys before wound closure. Rats were maintained at the St. Michael’s Hospital Animal Research Vivarium in a temperature-controlled (22°C) room with ad libitum access to commercial standard rat chow. All animal studies were approved by the St. Michael’s Hospital Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).
One week after surgery, sham and nephrectomised animals were randomly assigned to receive dapagliflozin (0.5 mg/kg, twice/day, Shanghai Sun-shine chemical Technology Co., Ltd.) or vehicle (5% 1-methyl-2-pyrrolidinone, 20% polyethylene glycol, and 20 mmol/l sodium diphosphate) by gavage and followed for a total of 12 weeks.

Biochemistry and function

Body weight was measured monthly. Systolic blood pressure was measured every four weeks in conscious rats using an occlusive tail-cuff plethysmograph attached to a pneumatic pulse transducer (Powerlab, AD Instruments, Colorado Springs, CO), as previously described [20]. Similarly, every four weeks, animals were housed in individual metabolic cages for 24 hours in order to determine their daily water intake, urine output and urine protein excretion using the benzethonium chloride method and urinary glucose excretion by glucose oxidase technique (Eton Biosciences, San Diego, CA).

Glomerular filtration rate (GFR) was assessed at 8 weeks and 12 weeks post surgery, using a modified FITC-inulin plasma clearance assay, as previously reported [21]. In brief, FITC-inulin (3.74 μL/g body weight) was injected via the tail vein and venous blood sampled at various time points subsequently. Fluorescence was detected using a Fluoroscan Ascent FL machine (Thermo Scientific, Rockford, IL) with 485 nm excitation and 538 nm emission settings and GFR was calculated using the following two phase, exponential decay curve with non-linear regression statistics, as previously described [21], whereby GFR = I/(A/α + B/β), where I is the amount of FITC-inulin injected, A and B are the y-intercept values for the two decay rates, and α and β are the decay constants for the distribution and elimination phases. As a further marker of GFR, creatinine was measured in plasma and urine by autoanalyzer (Beckman Instruments, Palo Alto, CA), as previously reported [22], and the creatinine clearance calculated.

Tissue preparation and histochemistry

At the end of the study, 12 weeks after SNX surgery, rats were anesthetized with inhaled isoflurane 2.5%. The left renal artery was clamped and the remnant kidney removed, decapsulated, and sliced transversely before immersion fixation in 10% neutral buffered formalin (NBF), embedding in cryostat matrix (Tissue-Tek, Sakura, Kobe, Japan), or flash frozen in liquid nitrogen. Formalin-fixed tissues were routinely processed, embedded in paraffin, and sectioned before staining with Periodic acid-Schiff (PAS) or Masson’s trichrome, as previously reported [23].

Glomerulosclerosis and interstitial fibrosis

Microscopy was performed by observers who were masked to the study group from which the kidney sections were derived. Glomerulosclerosis was assessed in 4 μm thick sections using a semi-quantitative technique, as previously described [19]. In brief, the extent of sclerosis was graded subjectively in each glomerulus on a scale of 0–4: Grade 0, normal; Grade 1, sclerotic area up to 25% (minimal); Grade 2, sclerotic area 25–50% (moderate); Grade 3, sclerotic area 50–75% (moderate to severe) and Grade 4, sclerotic area 75–100% (severe). The glomerulosclerotic index (GSI) was then calculated using the formula: GSI = (1 x n₁ + 2 x n₂ + 3 x n₃ + 4 x n₄)/(n₀ + n₁ + n₂ + n₃ + n₄), where nᵢ is the number of glomeruli in each grade of glomerulosclerosis. The extent of interstitial fibrosis was quantified in 4 μm kidney sections stained with Masson’s trichrome. Stained sections were scanned with the Aperio Ultra-Resolution Digital Scanning System (Aperio Technologies Inc., Vista, CA), and images were analyzed with Aperio ImageScope software. An area of blue in cortex of the kidney was selected for its colour range and the proportional area of the selected colour range was then
quantified, based on the method adapted from Lehr et al. [24, 25]. Ten randomly selected non-overlapping 10x fields for each animal was analysed in a masked fashion. Data were expressed as percentage change per area.

**Real-Time quantitative RT-PCR**

Quantitative real time PCR was performed using SYBR green with the abundance of transcript expressed relative to that of the housekeeping gene Rpl13a. In brief, frozen kidney tissue that had been stored at -80°C, was homogenized (Polytron, Kinematica Gmbh, Littau, Switzerland) and total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY). Total RNA (4 μg) was treated with RQ1 DNAse (1U/μl) (Promega, Madison, WI) to remove genomic DNA. RNA was reverse transcribed with 1μl of random hexamers (2μg/μl) and 8μl of DEPC-treated water and incubated for 5min at 70°C. After cooling on ice, 5μl of 5x AMV reaction buffer, 2.5μl of 10mmol/L dNTP mix, 0.5μl RNase inhibitor (40U/μl) (Roche, Indianapolis, IN), 0.5μl AMV reverse transcriptase (25U/μl) (Roche) and 4.5μl DEPC water were added. The reaction mixture was incubated for 60 minutes and the cDNA samples were stored at -20°C. Real-time PCR was performed on an ABI Prism 7900HT Fast PCR System (Applied Biosystems, Foster City, CA) using sequence specific primers for TGF-ß1, α1 (IV) collagen, α1 (I) collagen and RPL13a were obtained from Applied Biosystems (Foster City, CA). Experiments were performed in triplicate and data analysis was performed using the Comparative C_T method (Sequence Detection Systems 2.0; Applied Biosystems).

**Immunohistochemistry**

In addition to examining TGF-ß1 mRNA we also assessed activation of the TGF-ß signalling pathway by quantifying nuclear expression of phosphorylated Smad2 using a rabbit antiphospo-Smad2 antibody (Cell Signalling Technology, Boston, MA, USA) that detects endogenous Smad2 only when dually phosphorylated at Ser465 and Ser467. In brief and as previously described [26, 27], sections were immunostained according to the manufacturer’s instructions and the number of immunolabelled nuclei quantified in 5 random non-overlapping fields per kidney by an observer who was masked to the identity of the experimental group.

Macrophages were identified by the monoclonal rat macrophage marker (ED-1; Serotec, Raleigh, NC), as previously reported [28] and quantified as described above for phospho-Smad2. Type I collagen was examined immunohistochemically using an anti-type I collagen antibody (Southern Biotechnology Associates Inc, Birmingham, Ala), as previously reported [29] with the extent of its immunolabelling quantified as the proportional area in 5 random non-overlapping fields per kidney.

**Statistics**

Data are expressed as means ± SEM. Between group differences were analysed by one way ANOVA with Tukey’s post hoc test for multiple comparisons. Survival analysis was conducted according to the method of Kaplan and Meier. All statistics were performed using GraphPad Prism 5 for Mac OS X (GraphPad Software Inc., San Diego, CA). A p value of <0.05 was considered statistically significant.

**Results**

**Animal data**

Dapagliflozin, commenced one week after sham surgery or SNX induced substantial glycosuria contrasting its near absence in vehicle-treated animals (Table 1). Urine output was higher in
dapagliflozin-treated rats when compared with their untreated counterparts. Body weight was lower in SNX rats compared with shams.

Kidney function and mortality

When compared with animals that had undergone sham surgery, subtotal nephrectomised rats developed substantial proteinuria and hypertension (Fig 1A and 1B). No difference in either blood pressure or the magnitude of proteinuria was evident between animals that had received dapagliflozin or vehicle. At eight weeks post surgery, GFR, as measured by FITC-inulin clearance was markedly impaired in rats that had undergone subtotal nephrectomy and to a similar extent in dapagliflozin and vehicle-treated groups (Fig 1C). At 12 weeks, GFR was slightly lower in SNX rats that had received dapagliflozin when compared with those that had received vehicle (Fig 1D) when measured by FITC-inulin clearance. These differences were, however, no longer evident when GFR was assessed by creatinine clearance. Mortality was increased in subtotal nephrectomised rats but was similar in those that received dapagliflozin or vehicle (Fig 2).

Histopathology

By 12 weeks post surgery, examination of PAS-stained kidney sections from subtotal nephrectomised rats revealed substantial and widespread glomerulosclerosis (Fig 3). The extent of the glomerulosclerosis was unaffected by dapagliflozin where the extent of injury was similar to that found in vehicle-treated rats. Marked interstitial fibrosis was also evident in subtotal nephrectomised animals, the extent of which was similar in treated and untreated rats (Fig 4). In sharp contrast, animals that had undergone sham surgery showed no evidence of fibrosis in either their glomeruli or tubulointerstitium, regardless of whether they had been randomised to receive vehicle or dapagliflozin.

Gene expression

Overexpression of transforming growth factor-ß1 has been repeatedly implicated in the pathogenesis of kidney fibrosis. As expected, TGF-ß1 mRNA was markedly and similarly increased in the kidneys of animals that had undergone subtotal nephrectomy whether they randomised to receive vehicle or dapagliflozin when compared with sham surgery animals. In parallel with these changes in TGF-ß1 gene expression, type IV collagen mRNA was also increased in the kidneys of SNX rats in both dapagliflozin and vehicle-treated groups (Fig 5). Collagen I mRNA was similar in all groups with exception of dapagliflozin-treated SNX rats where expression was increased (Fig 5).

| Table 1. Animal characteristic and kidney function parameters. |
|---------------------------------------------------------------|
| sham + vehicle | sham + dapa | SNX + vehicle | SNX + dapa |
| Body weight (g) | 707.5 ± 10.7 | 659 ± 21.9 | 631.1 ± 25.8 | 568.5 ± 21.3* |
| Urine output (ml/day) | 33.1 ± 2.4 | 64 ± 4.2* | 43 ± 2.4 | 71.9 ± 3.5*† |
| Urine glucose (mg/day) | 13.9 ± 8.6 | 1569.7 ± 107.3* | 2.3 ± 0.5 | 1061.4 ± 267.9**† |

* p < 0.05 vs. sham + vehicle group;
† p < 0.05 vs. SNX + vehicle group.

doi:10.1371/journal.pone.0144640.t001
Immunohistochemistry

Macrophages infiltration, as assessed by the number of ED-1 positive cells, was increased in SNX rats but was unaffected by dapagliflozin (Fig 6). These changes were more marked in dapagliflozin-treated SNX animals where the increased abundance reached statistical significance.

When compared with sham nephrectomised rats, the kidneys of those that had undergone SNX showed a dramatic increase in the abundance of nuclei immunolabelled for phospho-Smad2 consistent with the activation of the TGF-β signalling pathway (Fig 7). No difference in the proportion of labelled nuclei was found between vehicle and dapagliflozin-treated animals.

Minimal type I collagen was detected in sham animals while in SNX rat kidneys it was noted in areas of marked tubulointerstitial fibrosis and in the arterial adventitia. There was, however, no difference in abundance between those animals that had received dapagliflozin and those that had not (Fig 8).
Discussion

Reduction in single nephron hyperfiltration and intraglomerular hypertension are fundamental pathophysiological changes that not only underlie the progression of CKD but also provide the rationale for therapeutic strategies to attenuate it. Indeed, blockade of the renin-angiotensin system with ACE inhibitors and ARBs, ostensibly as a consequence of their ability to lower intraglomerular pressure, have been repeatedly shown to reduce the rate of GFR decline in both diabetic and non-diabetic settings [30, 31], placing them along with blood pressure control at the forefront of CKD management. SGLT2 inhibition also leads to an acute reduction in SNGFR in animals [8] and lowering of global GFR in individuals with diabetes [32], akin to the acute reduction in inulin clearance reported with the SGLT1/2 inhibitor, phlorizin, in healthy non-diabetic subjects [33]. SGLT2 inhibition, in the present study, however, did not provide any evidence of renoprotection in the subtotally nephrectomised rat model of non-diabetic CKD.

The hemodynamic hypothesis of CKD progression, posits a vicious cycle whereby hyperfiltration leads to sclerosis that results in a further increase in SNGFR among remaining glomeruli [34]. To examine the ostensibly renoprotective effects of SGLT2 inhibition, we used the subtotally nephrectomized rat, a model of chronic kidney disease, relevant to both diabetic and non-diabetic settings [34] and which develops a decline in GFR, the sine qua non of chronic kidney disease. Despite its ability to reduce single nephron and global hyperfiltration in both the animal and human diabetic and non-diabetic settings [7, 8, 32], SGLT2 inhibition in the present study did not affect disease progression in the remnant kidney model. The lack of kidney protection with SGLT2 inhibition in this model contrast the numerous studies showing beneficial effects with agents that block the RAS [17, 19, 35].

A number of very recent studies have addressed the possibility that SGLT2 inhibitors may afford renoprotection in animal models of diabetic nephropathy. A notable caveat for most rodent models of diabetes is that they do not develop a reduced GFR or severe histopathological injury, rather GFR is frequently increased, associated with mild mesangial expansion and modest albuminuria [36, 37]. As such, they mostly resemble early diabetes-associated kidney changes and not the later stages of human diabetic nephropathy where patients present with low GFR, heavy proteinuria and hypertension in conjunction with advanced glomerulosclerosis and marked interstitial fibrosis. Notably, some of the diabetic animal studies that examined
the effects of SGLT2 inhibition on GFR, showed a reduction in an elevated GFR rather than a slowing in the progression of CKD [38–41]. Indeed, the reduction in hyperfiltration observed with SGLT2 inhibition in these studies, along with improvements in glycaemic control may well be sufficient to explain the improvements in albuminuria that were also reported.
**Fig 4. Interstitial fibrosis.** Representative cortical tubulointerstitial images (A-D) and with quantitative analysis (E) of Masson’s trichrome-stained kidney sections. When compared with animals that had undergone sham surgery (A, B), kidney sections from SNX rats (C, D) show substantial interstitial fibrosis (blue) that was similar in vehicle (C) and dapagliflozin-treated rats (D). Original magnification x 100. Scale bar: 200 μm. *p < 0.05 vs. sham-operated animals.

doi:10.1371/journal.pone.0144640.g004
While RAS blockade and SGLT2 inhibition both reduce hyperfiltration via their hemodynamic actions, angiotensin II, the primary effector molecule of the RAS, also exerts several other effects that contribute to the ability of ACE inhibitors and ARBs to reduce interstitial fibrosis, tubular atrophy and glomerulosclerosis [19, 42, 43]. Notably, acting via its AT₁ receptor, angiotensin II potently induces expression of the pro-fibrotic and pro-apoptotic growth factor, transforming growth factor-ß (TGF-ß) in a range of kidney cell types [44, 45]. Concor-dant with these cell culture studies, TGF-ß overexpression is seen in both the glomerular and tubulointerstitial compartments in the 5/6 nephrectomised and diabetic rats where studies also showed that both ACE inhibitors and ARBs were effective at reducing TGF-ß and disease progression [19, 46]. Consistent with these animal experiments, studies in human diabetic nephropathy showed that the ACE inhibitor, perindopril was able to reduce TGF-ß mRNA in a sequential renal biopsy study [47] and the ARB, losartan, was shown to lower urinary TGF-ß excretion [48]. In contrast to these studies of RAS blockade, SGLT2 inhibition, in the present study, did not affect the expression of TGF-ß or the downstream activation of its canonical Smad signalling pathway. Moreover, expressions of the matrix proteins, collagens I and IV were not improved by the administration of dapagliflozin either. Consistent with these findings SGLT2 inhibition did not affect the extent of glomerulosclerosis, interstitial fibrosis or macrophage infiltration in SNX rats. While dapagliflozin administration led to an increase in collagen I mRNA in SNX rat kidneys, this was not accompanied by a commensurate change in immunostainable collagen that was similarly increased in both treated and untreated SNX rats. The mechanisms underlying the differences between collagen I transcription and protein expression are speculative but could include differences in translation as well as the activities of the collagen I degrading matrix metalloproteinases.

As in the diabetic setting, SGLT2 inhibition has been shown to induce substantial glycosuria in the absence of hyperglycaemia as well [12–16, 49]. As might be expected, rats that were randomised to receive dapagliflozin in the present study developed marked glycosuria and attendant osmotic diuresis with a substantial increase in urinary glucose and comparatively lower body weight. Consistent with GFR as a determinant of SGLT2-induced glycosuria [50], the 24 hour urinary excretion of glucose was less in dapagliflozin treated SNX rats when compared with animals that had only undergone sham surgery. Given the volume contraction that accompanies SGLT2 inhibition [51], there might likely have been a pre-renal component to the reduced GFR seen in dapagliflozin-treated animals that could potentially have masked any improvement in GFR that might have otherwise been detected.
Although single nephron hyperfiltration is a feature of both diabetic and non-diabetic CKD, other aspects of their pathogenesis are substantially different. Indeed, even the genesis of single nephron hyperfiltration is different in the two disease states. In the 5/6 nephrectomized rat, for instance, remaining nephrons hyperfilter to compensate for nephron loss while in diabetes single nephron hyperfiltration may be more a consequence of the proximal tubular growth and generalized kidney enlargement seen early in the disease process [52]. Accordingly, the lack of

![Fig 6. Macrophage infiltration. Kidney sections from SNX rats revealed abundant ED-1 labelled macrophages, predominantly localized to the interstitium that were more common in SNX rats that had received dapagliflozin. Original magnification x 400. Scale bar: 50 μm. * p < 0.05 vs. sham-operated animals.](https://example.com/image)

doi:10.1371/journal.pone.0144640.g006
Fig 7. Phosphorylated Smad2. Kidney sections from SNX rats showed abundant nuclear staining for phosphorylated Smad2 in both vehicle and dapagliflozin-treated animals contrasting the dearth of immunopositive cells in sham nephrectomised rats. Original magnification x 160. Scale bar: 100 μm. * p < 0.05 vs. sham-operated animals.

doi:10.1371/journal.pone.0144640.g007
Fig 8. Type I collagen. Representative sections stained with anti-collagen I antibody revealing minimal immunolabeling in sham rats (A, B) while those from SNX animals (C, D) displayed increased deposition surrounding Bowman’s capsule and in bands of interstitial fibrosis. No difference in immunolabelling was evident between those rats that had or had not received dapagliflozin. Original magnification x 100. Scale bar: 200 μm.

doi:10.1371/journal.pone.0144640.g008
demonstrable renoprotection in the subtotal nephrectomised rat model, as described in the present study, should not be extrapolated to the diabetic setting where protection from kidney injury has been noted in many [38, 39, 41] but not all studies [53]. As such, the absence of renoprotection in our study should not be viewed as precluding a favourable outcome in kidney disease in individuals with diabetes where a range of non-hemodynamic effects afforded by SGLT2 inhibition may underlie their potential beneficial effects. Indeed, a large multi-centre clinical trial, CREDENCE (The Evaluation of the Effects of Canagliflozin on Renal and Cardiovascular Outcomes in Participants With Diabetic Nephropathy, clinicaltrials.gov identifier: NCT02065791) to address the possible renoprotective properties of SGLT2 inhibition, is currently being conducted in patients with diabetic kidney disease.

Acknowledgments
The authors are grateful for the excellent technical assistance of Jennifer Switzer. Disclosure: RG has been the recipient of research grants to his institution, has provided consultative advice, received travel grants to scientific meetings and received honoraria for CME events from Astra Zeneca, Janssen and Boehringer-Ingelheim/Eli Lilly and Co.

Author Contributions
Conceived and designed the experiments: REG YZ. Performed the experiments: KT DMK. Analyzed the data: REG YZ. Wrote the paper: REG.

References
1. Brenner BM, Meyer TW, Hostetter TH. Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. N Engl J Med. 1982; 307 (11):652–9. PMID: 7050705.
2. Toke A, Meyer TW. Hemodynamic effects of angiotensin II in the kidney. Contrib Nephrol. 2001;(135):34–46. PMID: 11705290.
3. Wiseman MJ, Saunders AJ, Keen H, Viberti G. Effect of blood glucose control on increased glomerular filtration rate and kidney size in insulin-dependent diabetes. N Engl J Med. 1985; 312:617–21. PMID: 3883162.
4. Hayashi K, Epstein M, Loutzenhiser R, Forster H. Impaired myogenic responsiveness of the afferent arteriole in streptozotocin-induced diabetic rats: role of eicosanoid derangements. J Am Soc Nephrol. 1992; 2(11):1578–86. PMID: 1610978.
5. Davalos M, Frega NS, Saker B, Leaf A. Effect of exogenous and endogenous angiotensin II in the isolated perfused rat kidney. Am J Physiol. 1978; 235(6):F605–10. PMID: 736144.
6. Munger KA, Kost CK, Brenner BM, Maddox DA. The Renal Circulations and Glomerular Ultrafiltration. In: Taal MW, editor. Brenner and Rector's The Kidney. 1.9th ed. Philadelphia: Elsevier; 2012. p. 94–137.
7. Cherney DZ, Perkins BA, Soleymanlou N, Maione M, Lai V, Lee A, et al. Renal hemodynamic effect of sodium-glucose cotransporter 2 inhibition in patients with type 1 diabetes mellitus. Circulation. 2014; 129(5):587–97. doi: 10.1161/CIRCULATIONAHA.113.005081 PMID: 24334175.
8. Thomson SC, Rieg T, Miracle C, Mansoury H, Whaley J, Vallon V, et al. Acute and chronic effects of SGLT2 blockade on glomerular and tubular function in the early diabetic rat. Am J Physiol Regul Integr Comp Physiol. 2012; 302(1):R75–83. doi: 10.1152/ajpregu.00357.2011 PMID: 21940401; PubMed Central PMCID: PMC3349378.
9. Cherney DZ, Perkins BA. Sodium-glucose cotransporter 2 inhibition in type 1 diabetes: simultaneous glucose lowering and renal protection? Can J Diabetes. 2014; 38(8):356–63. doi: 10.1016/j.jcjd.2014.05.006 PMID: 25192954.
10. De Nicola L, Gabbai FB, Liberti ME, Sagliocca A, Conte G, Minutolo R. Sodium/glucose cotransporter 2 inhibitors and prevention of diabetic nephropathy: targeting the renal tubule in diabetes. Am J Kidney Dis. 2014; 64(1):16–24. doi: 10.1053/j.ajkd.2014.02.010 PMID: 24673844.
11. Gilbert RE. Sodium-glucose linked transporter-2 inhibitors: potential for renoprotection beyond blood glucose lowering? Kidney Int. 2014; 86(4):693–700. doi: 10.1038/ki.2013.451 PMID: 24257692.
12. Komoroski B, Vachharajani N, Boulton D, Kornhauser D, Geraldes M, Li L, et al. Dapagliflozin, a novel SGLT2 inhibitor, induces dose-dependent glucosuria in healthy subjects. Clin Pharmacol Ther. 2009; 85(5):520–6. doi: 10.1038/clpt.2008.251 PMID: 19129748.

13. Sha S, Devineni D, Ghosh A, Polidori D, Chien S, Wexler D, et al. Canagliflozin, a novel inhibitor of sodium-glucose co-transporter 2, dose dependently reduces calculated renal threshold for glucose excretion and increases urinary glucose excretion in healthy subjects. Diabetes Obes Metab. 2011; 13(7):669–72. doi: 10.1111/j.1463-1326.2011.01406.x PMID: 21457428.

14. List JF, Whaley JM. Glucose dynamics and mechanistic implications of SGLT2 inhibitors in animals and humans. Kidney Int Suppl. 2011; (120):S20–7. doi: 10.1038/kid.2010.512 PMID: 21358698.

15. Kaschayanula S, Liu X, Lacreta F, Griffen SC, Boulton DW. Clinical pharmacokinetics and pharmacodynamics of dapagliflozin, a selective inhibitor of sodium-glucose co-transporter type 2. Clinical pharmacokinetics. 2014; 53(1):17–27. doi: 10.1007/s40262-013-0104-3 PMID: 24105299.

16. Fujimori Y, Katsuno K, Ojima K, Nakashima I, Nakano S, Ishikawa-Takemura Y, et al. Sergliflozin etabonate, a selective SGLT2 inhibitor, improves glycemic control in streptozotocin-induced diabetic rats and Zucker fatty rats. Eur J Pharmacol. 2009; 609(1–3):148–54. doi: 10.1016/j.ejphar.2009.03.007 PMID: 19281809.

17. Anderson S, Rennke HG, Brenner BM. Therapeutic advantage of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in the rat. J Clin Invest. 1986; 77(6):1993–2000. doi: 10.1172/JCI112528 PMID: 3011863; PubMed Central PMCID: PMC370560.

18. Zatz R, Dunn BR, Meyer TW, Anderson S, Rennke HG, Brenner BM. Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. J Clin Invest. 1986; 77(6):1925–30. doi: 10.1172/JCI112521 PMID: 3011862; PubMed Central PMCID: PMC370553.

19. Wu L, Cox A, Roe C, Dziadek M, Cooper ME, Gilbert RE. Transforming growth factor β1 and renal injury following subtotal nephrectomy in the rat: Role of the renin-angiotensin system. Kidney Int. 1997; 51:1553–67. PMID: 9150473.

20. Advani A, Kelly DJ, Advani SL, Cox AJ, Thai K, Zhang Y, et al. Role of VEGF in maintaining renal structure and function under normotensive and hypertensive conditions. Proc Natl Acad Sci U S A. 2007; 104(36):14448–53. PMID: 17726104.

21. Yuen DA, Connelly KA, Zhang Y, Advani SL, Thai K, Kabir G, et al. Early outgrowth cells release soluble endocrine antifibrotic factors that reduce progressive organ fibrosis. Stem Cells. 2013; 31(11):2408–19. doi: 10.1002/stem.1502 PMID: 23922321.

22. Gilbert RE, Wu LL, Kelly DJ, Cox A, Wilkinson-Berka JL, Johnston CI, et al. Pathological expression of renin and angiotensin II in the renal tubule after subtotal nephrectomy—Implications for the pathogenesis of tubulointerstitial fibrosis. American Journal of Pathology. 1999; 155(2):429–40. PMID: 10433936.

23. Gilbert RE, Zhang Y, Williams SJ, Zammit SC, Stapleton DI, Cox AJ, et al. A purpose-synthesised antifibrotic agent attenuates experimental kidney diseases in the rat. PLoS One. 2012; 7(10):e47160. doi: 10.1371/journal.pone.0047160 PMID: 23071743; PubMed Central PMCID: PMC3468513.

24. Lehr HA, Mankoff DA, Corwin D, Santeusianio G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. J Histochem Cytochem. 1997; 45(11):1559–65. PMID: 9358857.

25. Lehr HA, van der Loos CM, Teeing P, Gown AM. Complete chromogen separation and analysis in double immunohistochemical stains using photoshop-based image analysis. J Histochem Cytochem. 1999; 47(1):119–26. PMID: 9857219.

26. Kelly DJ, Zhang Y, Gow R, Gilbert RE. Tranilast attenuates structural and functional aspects of renal injury in the remnant kidney model. J Am Soc Nephrol. 2004; 15(10):2619–29. PMID: 15466266.

27. Kelly DJ, Zhang Y, Cox AJ, Gilbert RE. Combination therapy with tranilast and angiotensin-converting enzyme inhibition provides additional renoprotection in the remnant kidney model. Kidney Int. 2006; 69(11):1954–60. PMID: 16557218.

28. Kelly DJ, Chanle A, Gow RM, Zhang Y, Gilbert RE. Protein kinase C beta inhibition attenuates osteopontin expression, macrophage recruitment, and tubulointerstitial injury in advanced experimental diabetic nephropathy. J Am Soc Nephrol. 2005; 16(6):1654–60. PMID: 15643473.

29. Connelly KA, Kelly DJ, Zhang Y, Prior DL, Advani A, Cox AJ, et al. Inhibition of protein kinase C-beta by ruboxistaurin preserves cardiac function and reduces extracellular matrix production in diabetic cardiomyopathy. Circ Heart Fail. 2009; 2(2):129–37. PMID: 19808326. doi: 10.1111/J.CIRCHEARTFAILURE.108.765750.

30. The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. Lancet. 1997; 349:1857–63. PMID: 9217756.
31. Lewis EJ, Hunsicker LG, Bain RP, Rohde RD, for the Collaborative Study Group. The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. N Engl J Med. 1993; 329:1456–62. PMID: 8413456

32. DeFronzo RA, Hompesch M, Kasichayanula S, Liu X, Hong Y, Pfister M, et al. Characterization of renal glucose reabsorption in response to dapagliflozin in healthy subjects and subjects with type 2 diabetes. Diabetes Care. 2013; 36(10):3169–76. doi: 10.2337/dc13-0387 PMID: 23753727; PubMed Central PMCID: PMC3781504.

33. Shannon JA, Smith HW. The Excretion of Inulin, Xylose and Urea by Normal and Phlorizinized Man. J Clin Invest. 1935; 14(4):393–401. doi: 10.1172/JCI100680 PMID: 16694313; PubMed Central PMCID: PMC424694.

34. Brenner BM. Hemodynamically mediated glomerular injury and the progressive nature of kidney disease. Kidney Int. 1983; 23(4):647–55. PMID: 6336299.

35. Benigni A, Zoja C, Noris M, Corna D, Benedetti G, Bruzzi I, et al. Renoprotection by nitric oxide donor and lisinopril in the remnant kidney model. Am J Kidney Dis. 1999; 33(4):746–53. PMID: 10196019.

36. Tesch GH, Allen TJ. Rodent models of streptozotocin-induced diabetic nephropathy. Nephrology (Carlton). 2007; 12(3):261–6. doi: 10.1111/j.1440-1797.2007.00796.x PMID: 17498121.

37. Allen TJ, Cooper ME, Lan HY. Use of genetic mouse models in the study of diabetic nephropathy. Curr Diab Rep. 2004; 4(6):435–40. PMID: 15539008.

38. Terami N, Ogawa D, Tachibana H, Hatanake T, Wada J, Nakatsuka A, et al. Long-term treatment with the sodium glucose cotransporter 2 inhibitor, dapagliflozin, ameliorates glucose homeostasis and diabetic nephropathy in db/db mice. PLoS One. 2014; 9(6):e100777. doi: 10.1371/journal.pone.0100777 PMID: 24960177; PubMed Central PMCID: PMC4069074.

39. Gembardt F, Bartaun C, Jarzebska N, Mayoux E, Todorov VT, Hohenstein B, et al. The SGLT2 inhibitor emagliflozin ameliorates early features of diabetic nephropathy in BTBR ob/ob type 2 diabetic mice with and without hypertension. Am J Physiol Renal Physiol. 2014; 307(3):F317–25. doi: 10.1152/ajprenal.00145.2014 PMID: 24944269.

40. Vallon V, Gerasimova M, Rose MA, Masuda T, Satriano J, Mayoux E, et al. SGLT2 inhibitor emagliflozin reduces renal growth and albuminuria in proportion to hyperglycemia and prevents glomerular hyperfiltration in diabetic Akita mice. Am J Physiol Renal Physiol. 2014; 306(2):F194–204. doi: 10.1152/ajprenal.00520.2013 PMID: 24226524; PubMed Central PMCID: PMC3920018.

41. Nagata T, Fukuzawa T, Takeda M, Fukazawa M, Mori T, Nihei T, et al. Tofogliflozin, a novel sodium-glucose co-transporter 2 inhibitor, improves renal and pancreatic function in db/db mice. Br J Pharmacol. 2013; 170(3):519–31. doi: 10.1111/bph.12269 PMID: 23751087; PubMed Central PMCID: PMC3791991.

42. Cordonnier DJ, Pinel N, Barro C, Maynard C, Zaoui P, Halimi S, et al. Expansion of cortical interstitium is limited by converting enzyme inhibition in type 2 diabetic patients with glomerulosclerosis. Journal of the American Society of Nephrology. 1999; 10(6):1253–63. PMID: 10361863

43. Ruster C, Wolf G. Angiotensin II as a morphogenic cytokine stimulating renal fibrogenesis. J Am Soc Nephrol. 2013; 24(1):1152/ajprenal.00520.2013 PMID: 24944269.

44. Kagami S, Border WA, Miller DE, Noble NA. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. J Clin Invest. 1994; 93(6):1072–80. PMID: 8200978.

45. Wolf G, Mueller E, Stahl RAK, Ziyadeh FN. Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-ß. J Clin Invest. 1993; 92:1366–72. PMID: 7690779.

46. Gilbert RE, Cox A, Wu LL, Allen TJ, Hultgren L, Jerums G, et al. Expression of transforming growth factor-beta1 and type IV collagen in the renal tubulointerstitium in experimental diabetes: effects of angiotensin converting enzyme inhibition. Diabetes. 1998; 47:414–22. PMID: 9519748.

47. Langham RG, Kelly DJ, Gow RM, Zhang Y, Cordonnier DJ, Pinel N, et al. Transforming growth factor-beta in human diabetic nephropathy: effects of ACE inhibition. Diabetes Care. 2006; 29(12):2670–5. PMID: 17130203.

48. Houlihan CA, Akdeniz A, Tsalamandris C, Cooper ME, Jerums G, Gilbert RE. Urinary transforming growth factor-beta excretion in patients with hypertension, type 2 diabetes, and elevated albumin excretion rate: effects of angiotensin receptor blockade and sodium restriction. Diabetes Care. 2002; 25(6):1072–7. PMID: 12032117.

49. Komoroski B, Vachharajani N, Feng Y, Li L, Konhauser D, Pfister M. Dapagliflozin, a novel, selective SGLT2 inhibitor, improved glycemic control over 2 weeks in patients with type 2 diabetes mellitus. Clin Pharmacol Ther. 2009; 85(5):513–9. doi: 10.1038/clpt.2008.250 PMID: 19129749.
50. Gilbert RE. SGLT-2 inhibition in patients with kidney disease. Diabetes Metab. 2014; 40(6 Suppl 1): S23–7. doi: 10.1016/S1262-3636(14)72692-8 PMID: 25554068.

51. Lambers Heerspink HJ, de Zeeuw D, Wie L, Leslie B, List J. Dapagliflozin a glucose-regulating drug with diuretic properties in subjects with type 2 diabetes. Diabetes Obes Metab. 2013; 15(9):853–62. doi: 10.1111/dom.12127 PMID: 23668478; PubMed Central PMCID: PMC3906841.

52. Seyer-Hansen K. Renal hypertrophy in experimental diabetes. Kidney Int. 1983; 23:643–6. PMID: 6571419

53. Gangadharan Komala M, Gross S, Mudaliar H, Huang C, Pegg K, Mather A, et al. Inhibition of kidney proximal tubular glucose reabsorption does not prevent against diabetic nephropathy in type 1 diabetic eNOS knockout mice. PLoS One. 2014; 9(11):e108994. doi: 10.1371/journal.pone.0108994 PMID: 25369239; PubMed Central PMCID: PMC4219667.