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Title: Sodium-glucose co-transporter 2 inhibition does not improve the acute pressure natriuresis response in rats with Type 1 diabetes

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Running Title: SGLT2 inhibition during acute pressure natriuresis in T1DM

Abstract: Type 1 diabetes mellitus (T1DM) leads to serious complications including premature cardiovascular and kidney disease. Hypertension contributes importantly to these adverse outcomes. The renal pressure natriuresis (PN) response, a key regulator of blood pressure (BP), is impaired in rats with T1DM as tubular sodium reabsorption fails to down-regulate with increasing BP. We hypothesized that sodium-glucose co-

transporter 2 (SGLT2) inhibitors, which reduce cardiovascular risk in kidney disease, would augment the PN response in T1DM rats. Non-diabetic or T1DM (35-50 mg/kg streptozotocin IP) adult male Sprague-Dawley rats were anesthetized (thiopental 50mg/kg IP) and randomized to receive either dapagliflozin (10 mg/kg IV) or vehicle. Baseline sodium excretion was measured and then BP was increased by sequential arterial ligations to induce the PN response. In non-diabetic animals, the natriuretic and diuretic response to increasing BP was not augmented by dapagliflozin. Dapagliflozin induced glycosuria but this was not influenced by BP. In T1DM rats the PN response was impaired. Dapagliflozin again increased urinary glucose excretion but did not enhance PN. Inhibition of SGLT2 does not enhance the PN response in rats, either with or without T1DM. SGLT2 makes only a minor contribution to tubular sodium reabsorption and does not contribute to the impaired PN response in T1DM.

New Findings: Sodium-glucose co-transporter 2 (SGLT2) inhibitors reduce cardiovascular risk in patients with both diabetic and non-diabetic kidney disease, the mechanism responsible is currently unknown.

We investigated whether SGLT2 inhibition could improve renal pressure natriuresis (PN), an important mechanism for long-term blood pressure control, which is impaired in type 1 diabetes mellitus (T1DM). SLGT2 inhibitor dapagliflozin did not enhance the acute *in vivo* PN response in either healthy or T1DM Sprague Dawley rats. Our data suggest that the mechanism underpinning the clinical benefits of SGLT2 inhibitors on health are unlikely to be due to an enhanced natriuretic response to increased blood pressure.

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Sodium-glucose co-transporter 2 inhibition does not improve the acute pressure natriuresis response in rats with Type 1 diabetes

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Abstract

Type 1 diabetes mellitus (T1DM) leads to serious complications including premature cardiovascular and kidney disease. Hypertension contributes importantly to these adverse outcomes. The renal pressure natriuresis (PN) response, a key regulator of blood pressure (BP), is impaired in rats with T1DM as tubular sodium reabsorption fails to down-regulate with increasing BP. We hypothesized that sodium-glucose co-transporter 2 (SGLT2) inhibitors, which reduce cardiovascular risk in kidney disease, would augment the PN response in T1DM rats. Non-diabetic or T1DM (35-50 mg/kg streptozotocin IP) adult male Sprague-Dawley rats were anesthetized (thiopental 50mg/kg IP) and randomized to receive either dapagliflozin (10 mg/kg IV) or vehicle. Baseline sodium excretion was measured and then BP was increased by sequential arterial ligations to induce the PN response. In non-diabetic animals, the natriuretic and diuretic response to increasing BP was not augmented by dapagliflozin. Dapagliflozin induced glycosuria but this was not influenced by BP. In T1DM rats the PN response was impaired. Dapagliflozin again increased urinary glucose excretion but did not enhance PN. Inhibition of SGLT2 does not enhance the PN response in rats, either with or without T1DM. SGLT2 makes only a minor contribution to tubular sodium reabsorption and does not contribute to the impaired PN response in T1DM.

Introduction

The prevalence of Type 1 diabetes mellitus (T1DM) in children and adolescents is approximately 1 in 300 in the USA [1] and the incidence is increasing worldwide [1,2]. T1DM decreases life-expectancy by ~13 years [3], in part due to macrovascular and microvascular complications causing premature cardiovascular (CV) disease and nephropathy [4]. Increased renal tubular sodium reabsorption [5] and sodium retention [6] are early hallmarks of clinical and experimental T1DM. Since renal regulation of extracellular fluid volume by modifying sodium reabsorption is a major determinant of long-term blood pressure (BP) [7], dysfunction within the kidney impairs its ability to stabilize BP. Both hypertension and hyperglycaemia are major risk factors for CV disease and nephropathy [2,8–10], and so restoration of the normal renal regulation of sodium balance is a therapeutic goal in order to reduce CV risk and further renal injury. Pressure natriuresis (PN) is the positive relationship between BP, renal perfusion pressure, and sodium excretion. This relationship, often attenuated in experimental hypertension, is hypothesized as a key regulator of long-term BP [7,11], although this remains controversial [12]. Experimentally, PN largely reflects reduced sodium reabsorption in the proximal tubule due to inactivation of major sodium transport proteins [7]. We have recently shown that the PN response is severely impaired in Sprague Dawley rats with streptozotocin (STZ)-induced T1DM [13]. This dysfunction occurs because renal tubular sodium reabsorption does not down-regulate following ramps in BP [13]. The molecular basis of this impairment is not known.

The sodium-glucose co-transporter 2 (SGLT2) is the major route for glucose and sodium reabsorption in the proximal tubule, while SGLT1 in the S3 segment is responsible for a much smaller amount of reabsorption [14]. Acute inhibition of SGLT2 causes glycosuria, diuresis, and natriuresis [15,16]. Chronically, SGLT2 inhibitors have been shown to reduce hyperglycaemia in patients with T1DM and Type 2 diabetes mellitus (T2DM). They promote weight loss, prevent albuminuria, and reduce BP [17,18], thus leading to fewer CV events and kidney failure in clinical trials [19–21]. The reduction in CV and renal risk appears to be independent of the level of hyperglycaemia in patients [21], and following meta-analysis of clinical trial data, the United States Food and Drug Administration (FDA) have recently approved the use of

SGLT2 inhibitors in non-diabetic patients with heart failure and reduced ejection fraction [22]. The mechanisms underpinning these clinical benefits remain elusive. Experimentally, SGLT2 inhibition is renoprotective, reducing markers of tubular and glomerular injury in the urine of diabetic rodent models [23,24]. Many anti-hypertensive agents are able to stabilize extracellular fluid volume, reduce BP, and protect glomeruli from an excessive hemodynamic load, and these effects are associated with an enhanced PN response [7,25,26]. This raises the possibility that SGLT2 inhibitors enhance the acute PN response, and thereby reduce long-term renal injury and CV risk.

We hypothesized that SGLT2 inhibition would restore the normal PN response in a rat model of T1DM. In this study, we recorded the natriuretic response after acute ramps in BP in anesthetized healthy and T1DM rats and examined the effect of concurrent SGLT2 inhibition.

Experimental materials and methods

Animals and husbandry

Experiments were performed in accordance with the UK's Animals (Scientific Procedures) Act under a UK Home Office Project License. All protocols were reviewed by the University of Edinburgh's Animal Welfare and Ethics Review Board prior to experimentation.

For all studies, adult male Sprague Dawley rats (250-300 g) were purchased from Charles River UK and were maintained on standard chow (0.25% sodium), water *ad libitum*, and were housed in rooms with a 12-hour light cycle (lights 07.00–19.00 h) at 21±1°C and 50% humidity.

Induction of T1DM

T1DM was induced by intraperitoneal (IP) injection of streptozotocin (STZ; 35 mg/kg in 0.1 M citrate buffer, pH 4.5; Sigma-Aldrich, Gillingham, UK). Rats had free access to food and drinking water was supplemented with 10% sucrose to prevent initial hypoglycaemia. A blood glucose measurement 48 hours later of >12 mmol/L on glucometer (Accu-Chek Aviva; Roche Diagnostics Limited, Burgess Hill, UK; maximum blood glucose reading of 32 mmol/L) was required to confirm T1DM; rats that did not reach this threshold received a second injection of 15 mg/kg. Blood glucose was again measured at day 7 and immediately prior to the experimental procedure to confirm sustained hyperglycaemia. Non-T1DM control rats received citrate vehicle alone by IP injection. All experiments were performed 2–3 weeks after the first STZ injection.

In vivo pressure natriuresis protocol

PN experiments were carried out as previously described [13] with all procedures beginning at around 10am. Briefly, non-recovery anaesthesia was induced with barbiturate anaesthetic agents. In the T1DM rats, sodium thiobutabarbital was used (Inactin; 120 mg/kg IP; Sigma Aldrich, UK). Supply issues necessitated a change of anaesthetic and for the non-T1DM rats and sodium thiopental was used (50 mg/kg IP; Archimedes Pharma, Reading, UK). The right jugular vein was cannulated for

intravenous (IV) infusion, a tracheotomy was performed, and the right carotid artery cannulated. The arterial line was used for intermittent blood sampling and otherwise was connected to a calibrated BP transducer and multi-channel data acquisition system (Powerlab; ADInstruments, Oxford, UK) for real-time mean BP (MBP) measurement. Physiological saline (pH 7.4; 1 mL/h/100 g body weight), containing 2% bovine serum albumin and FITC-inulin (both Sigma-Aldrich), was infused through the venous line. General anaesthesia was maintained through this line by 20–30 µL injections of the barbiturate anaesthetic.

After a post-surgical equilibration period of 30 minutes, the SGLT2 inhibitor dapagliflozin (1 mg/kg, Selleckchem, Munich, Germany) or vehicle (2% DMSO in 0.9% saline, Sigma-Aldrich) was injected through the venous line in a blinded fashion. After a 30 min baseline period, PN was induced by sequential arterial ligation of the celiac and cranial mesenteric arteries (Period 1), followed by a second ligation of the distal aorta (Period 2). During baseline and both periods of increased MBP, urine was collected for 30 min and glomerular filtration rate (GFR) was measured by FITC-inulin clearance.

Electrolyte analysis was carried out using a Spotchem EL SE-1520 analyser (Arkray, Kyoto, Japan). Plasma and whole blood glucose were measured using the Accu-Chek Aviva glucose meter (Roche). Glucose concentration in urine was measured by an enzymatic UV test using the hexokinase method (Beckman Coulter, High Wycombe, UK).

Statistical analysis

Statistical comparisons were made with GraphPad Prism 8 (San Diego, CA). Data are presented as individual measurements or mean ± standard deviation (SD). The study was designed to obtain a power >80% if group sizes were six rats and dapagliflozin reduced the suppression of urinary sodium excretion during PN in T1DM rats by 50% (12±6 µmol/min/g kw) [13]. Additional rats were included in each group to account for anticipated dropouts. Overall, there was an experimental mortality rate of 6.25%, data and samples from these animals were not used in the subsequent analysis, resulting in final cohort sizes of n=7 for T1DM rats and n=8 for non-diabetic rats. Because the anaesthetic agent had to be changed, comparisons were only made within groups

(T1DM v T1DM, and non-T1DM v non-T1DM) with two-way analysis of variance (ANOVA), to avoid potential confounding effects of the anaesthetic. The fixed factors of BP ramps and dapagliflozin (dapa), and their interaction, generated three P values per comparison. Significance was explored further with Tukey's *post hoc* tests. Regression analysis plotted dependent variables from vehicle- and SGLT inhibitor-treated rats against MBP (independent variable). An extra sum-of-squares F test was used to determine whether one curve fitted both data sets. For all tests, $P<0.05$ was considered significant.

Results

Pressure natriuresis and SGLT2 inhibition in non-diabetic Sprague Dawley rats

The PN response was measured in 16 male adult Sprague Dawley rats. Eight rats received vehicle (weight, 399.6±14.9 g; blood glucose, 7.5±0.9 mM), and eight received dapagliflozin (weight, 397±31.3 g; blood glucose, 7.4±0.7 mM). MBP (Figure 1A) increased in both groups ($P<0.001$) by a similar extent ($P=0.813$), with ramps of ~20 mmHg (period 1) and ~45 mmHg (period 2) from baseline. GFR values increased after ligation ($P<0.001$) in a comparable manner ($P=0.087$), doubling from baseline in period 2 but still remaining within a range suggestive of effective autoregulation (Figure 1B). From similar baseline urine flow rates (vehicle, 4.7±3.4 µL/min/g kw; dapa, 12.1±9.6 µL/min/g kw; $P=0.073$), increases in BP induced a diuresis ($P<0.001$) that was comparable between the two groups ($P=0.287$, Figure 2A). Dapagliflozin increased urinary glucose excretion at all time points (all $P<0.001$, Figure 2B). Urinary sodium excretion rates were also similar between groups at baseline (vehicle, 0.3±0.3 µmol/min/g kw; dapa, 0.5±0.7 µmol/min/g kw; $P=0.879$) and both increased by ~40-fold when BP was increased (vehicle, 20.4±7.5 µmol/min/g kw; dapa, 20.8±11.2 µmol/min/g kw; effect of BP ramps, $P<0.001$; effect of dapagliflozin, $P=0.799$; interaction, $P=0.825$; Figure 2C).

The fractional excretions of sodium and glucose were then calculated to determine whether the natriuretic/glycosuric responses were due to a reduction in tubular reabsorption. As expected, dapagliflozin increased the fractional excretion of glucose ($P<0.001$) but the magnitude varied according to the clearance period ($P<0.001$, Figure 3A). Fractional excretion of sodium mirrored the increases in urine flow rates and sodium excretion rates with eightfold increases ($P<0.01$) from baseline that were unaffected by dapagliflozin (effect of dapa, $P=0.997$; interaction, $P=0.636$; Figure 3B). Similar results were also seen for potassium and chloride (Figure 3C and D).

The relationships of urine flow rate, urinary sodium excretion rate, and GFR with MBP were all curvilinear (Figure 4A, B, and C). For each parameter, data sets from vehicle- and dapagliflozin-treated rats could be fitted with a single curve (urine flow rate, $P=0.081$; urinary sodium excretion, $P=0.521$; GFR, $P=0.163$). A regression line could

not be fitted to either urinary glucose dataset, but all values from dapagliflozin-treated rats were greater than those from vehicle-treated rats (P<0.001, Figure 5D).

In contrast to the urine, plasma concentrations of glucose and electrolytes were relatively stable. When MBP increased, there were small reductions in plasma glucose of ~2 mmol/L (P<0.001) and potassium of ~0.2 mmol/L (P=0.040) but these were unaffected by dapagliflozin (glucose: effect of dapa, P=0.102; interaction, P=0.946; potassium: effect of dapa, P=0.598; interaction, P=0.620; Figure 5A and B). Similarly, plasma chloride (P=0.441) and sodium (P=0.740) were unaffected by increases in MBP and did not differ between groups (chloride: effect of dapa, P=0.087; interaction, P=0.355; sodium: effect of dapa, P=0.231; interaction, P=0.715; Figure 5C and D).

Pressure natriuresis and SGLT2 inhibition in T1DM Sprague Dawley rats

T1DM was induced by STZ injection in 14 Sprague Dawley rats, and, 14 days later, they were randomly allocated to receive either vehicle (n=7; weight, 376±29 g; blood glucose, 29.6±4.8 mmol/L) or dapagliflozin (n=7; weight, 339±33 g; blood glucose, 24.0±7.7 mmol/L).

MBP increased in both groups by a similar extent (effect of BP ramps, P<0.001; effect of dapa, P<0.001; interaction, P= 0.949; Figure 6A), with ramps of ~18 mmHg (period 1) and ~35 mmHg (period 2) from baseline. Overall, MBP was ~13 mmHg lower in dapagliflozin-treated rats compared to vehicle (P=0.003) but there were no differences between the groups during individual clearance periods. There was no difference in baseline GFR between groups (P=0.383) and no measurable effect on GFR of increasing MBP (effect of ligations P=0.448; interaction P=0.054; Figure 6B).

Increases in MBP induced a diuresis (P<0.001) that was comparable between the dapagliflozin- and vehicle-treated groups (effect of dapa, P=0.119; interaction, P=0.083; Figure 7A) at either time-point. Dapagliflozin treatment induced glycosuria, but vehicle did not (effect of dapa, P<0.0001; interaction, P=0.705; Figure 7B) but there was no effect on glucose excretion from increasing MBP in either group (P=0.728). Urinary sodium excretion rates were very similar between groups at baseline (vehicle, 0.2±0.2 µmol/min/g kw; dapa, 0.2±0.2 µmol/min/g kw; P=0.383). Natriuresis was also induced by the MBP ramps. However, despite increases in sodium excretion of up to ~10-fold (period 1) and ~30-fold (period 2) (P<0.001) in both groups, the overall natriuretic

effect was reduced by dapagliflozin (effect of dapa, P=0.004; interaction, P=0.059; Figure 7C).

Fractional excretion of glucose was higher in the dapagliflozin-treated group than vehicle-treated controls throughout the protocol and remained stable (effect of BP ramps, P=0.452; effect of dapa, P<0.0001; interaction, P=0.476; Figure 8A). Fractional excretion of sodium was ~0.3% at baseline in both vehicle and dapagliflozin-treated rats (Figure 8B). Acutely elevated BP increased fractional excretion of sodium in both groups (P=0.0001), indicating a reduction in tubular sodium reabsorption, but the effect was blunted by dapagliflozin compared with vehicle (effect of dapa, P=0.005; interaction P=0.016).

The relationships of urine flow rate and urinary sodium excretion rate with MBP were curvilinear (Figure 9A and B). For both parameters, data sets from vehicle- and dapagliflozin-treated rats could be fitted with a single curve (urine flow rate, P=0.225; urinary sodium excretion, P=0.531). A regression line could not be fitted to either GFR or urinary glucose dataset (Figure 9C and D). There was considerable overlap of both GFR datasets.

Discussion

The main finding of our study is that acute inhibition of sodium and glucose transport via SGLT2 with dapagliflozin does not enhance the experimental PN response. Of translational clinical importance, and contrary to our hypothesis, SGLT2 inhibition does not restore normal PN in T1DM rats. We conclude that SGLT2 makes only a minor contribution to overall tubular sodium reabsorption and does not contribute to the abnormal PN response in T1DM.

PN describes the renal response to acutely elevated BP; the rapid reduction in tubular sodium transport along the entire nephron can be measured as increased fractional excretion of sodium [7]. *In vivo*, micropuncture confirms that due to autoregulation, the contribution that GFR makes to natriuresis is minor, whereas the largest contribution can be localized to inhibited sodium transport in the proximal tubule [27,28]. SGLT2 is also located within the proximal tubule, and when SGLT2 is inhibited and MBP maintained, glycosuria is accompanied by a ~20% reduction in proximal tubular sodium reabsorption [29]. Therefore, we anticipated that our first PN experiment, in non-T1DM rats, would demonstrate positive relationships not only between MBP and sodium excretion, but also MBP and glycosuria when SGLT2 was inhibited with dapagliflozin. Since we have previously identified an impaired PN response in pre-nephropathy T1DM rats [13] that model sodium and water retention observed in people with T1DM [6,30,31], we also used dapagliflozin in a separate PN experiment to try to restore these positive relationships in T1DM.

In both experiments marked increases in MBP were achieved and increases in fractional excretion of sodium were consistent with induction of PN. However, regardless of diabetic status, there was no positive relationship between MBP and urinary glucose, even during SGLT2 blockade. The absence of glycosuria in the T1DM rats receiving vehicle may have reflected a dip in blood glucose levels below the glycaemic threshold under anaesthesia, or increased SGLT2 activity. However, when SGLT2 was blocked, the impaired PN response we have observed in T1DM rats [13], still did not resolve. Therefore, overall sodium excretion and MBP are tightly linked, and this relationship is independent of sodium and glucose co-transport in the PT. In the context of the renal

response to increased BP, the contribution of SGLT2-mediated transport appears to be negligible.

The failure of blocking SGLT2 to enhance natriuresis in this PN model is worthy of further consideration because sodium and water retention contributes to cardiovascular risk in T1DM [32]. All rats treated with dapagliflozin had glycosuria, demonstrating that glucose reabsorption distal to SGLT2 is low. By contrast, overall sodium excretion did not increase, consistent with a compensatory increase in sodium transport within the nephron, downstream of SGLT2. Such a compensatory response is reported with other agents that interfere with sodium transport in the proximal tubule [33,34], but this not been reported previously after acute SGLT2 blockade. Instead, increased rather than decreased urine output and sodium excretion have been described [35]. This discrepancy might be explained by our study design, in that, unlike previous studies, MBP was acutely increased rather than maintained at a baseline level. This should activate an integrated PN response that switches off sodium transporters along the nephron [7]. An additional natriuretic response would not occur if any one of these transporters remained active after SGLT2 blockade. Identifying the transporter responsible was beyond the scope of this study and would require a combination of micropuncture and selective inhibition of sodium transport distal to the site of SGLT2 in the proximal tubule. However, compensatory reabsorption was also evident following dapagliflozin administration to T1DM, even when T1DM, itself, had already suppressed PN. Neither MBP nor GFR was reduced during individual clearance periods or on regression analysis, raising the possibility that reabsorption is driven by the sodium or glucose content of the tubular fluid or osmotic forces. This is already known to reduce the tubuloglomerular feedback signal in T1DM rats [36,37], and therefore would be predicted to occur distal to the macula densa where tubuloglomerular feedback is initiated. We do not believe our results reflect RAAS activation because urinary aldosterone excretion is not increased in this pre-nephropathy T1DM model [13], and in T1DM patients, angiotensin II and plasma renin activity are reduced [6,38]. However, we do believe that our study may provide some explanation as to why sustained natriuresis or reductions in plasma volume are not observed in longer term clinical studies in people receiving SGLT2 inhibitors [15,39,40]. Furthermore, since patients

with T1DM already have difficulty in excreting an acute sodium load, our data do not support the use of SGLT2 inhibitors to improve this.

According to the Guyton hypothesis, sodium and water excretion are key determinants of BP [41], so our data are also consistent with clinical trials that have demonstrated only a modest reduction in BP with SGLT2 inhibitors, despite inducing glycosuria [42], and a failure to promote natriuresis in T2DM patients over two weeks [39]. However, care should be taken when placing our data within a clinical context. SGLT2 inhibitors do show promise in providing nephroprotection to T1DM patients [20] suggesting that even if SGLT2 blockade fails to improve natriuresis, the clinical consequences are not deleterious. We also did not specifically address the effects of chronic administration of an SGLT2 inhibitor on the acute PN response and therefore did not take into account the effect of long-term effects of SGLT2 blockade on the renal transcriptome, which can be profound, even inducing a phenotype that mimics fasting [43]. At a functional level, in rats with congestive heart failure, where there is a drive to sodium and water retention, four-weeks of empagliflozin enhanced excretion of an acute sodium load by downregulating proximal tubule NHE3-activity [44], overwhelming any compensatory response downstream. This might be expected in people, since sustained dapagliflozin treatment enhances lithium clearance [45], a marker of reduced proximal tubular sodium reabsorption, in T2DM. Therefore, to help determine the clinical implications from our work, PN experiments after long-term administration of dapagliflozin are justified.

A further limitation of our study is that all the experimental rats used were exclusively male. Differences in the contribution of renal blood flow to PN are known to exist between male and female rats [46,47] and so extrapolation of our data to female rats should be made with caution. This is of clinical relevance since the CV benefits of SGLT2 inhibitors may be less in females than in males [48].

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Data availability

All supporting data are included within the main article or are available upon request by contacting the corresponding author.

Figures

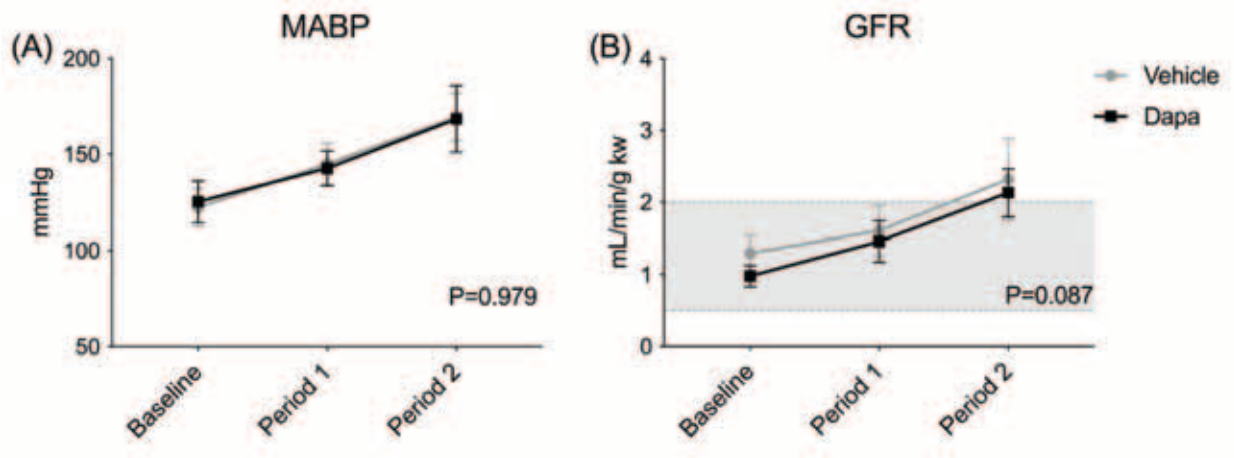

Figure 1. Changes in mean arterial blood pressure (MABP) and glomerular filtration rate (GFR) during the pressure natriuresis protocol. Sprague Dawley rats were treated with a bolus IV injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=8) or dapagliflozin (dapa, 1 mg/kg, n=8) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal aorta (period 2). (A) MABP averaged over the 30-minute periods and (B) GFR measured using FITC-inulin. The shaded area represents the normal autoregulatory range of GFR in Sprague Dawley rats [49]. Data are mean±standard deviation, statistical comparisons were made by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph.

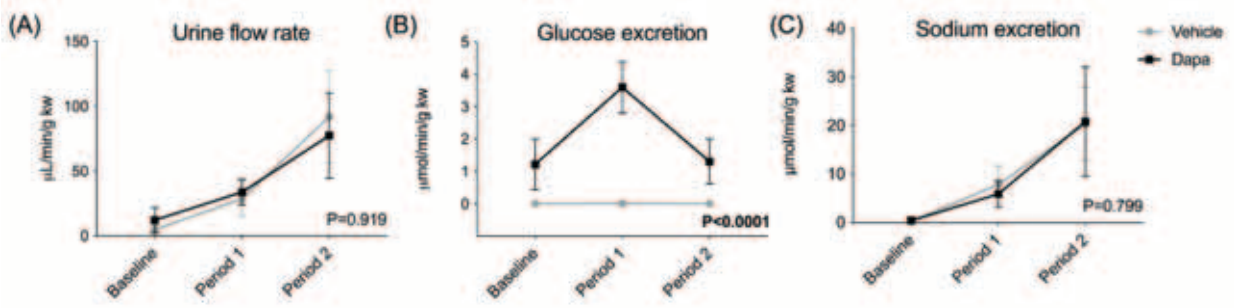

Figure 2. Changes in urine flow rate and urinary excretion of glucose and sodium. (A) Urine flow rate, as well as (B) glucose and (C) sodium excretion, was measured in Sprague Dawley rats treated with an IV bolus injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=8) or dapagliflozin (dapa, 1 mg/kg, n=8) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal

aorta (period 2) to increase blood pressure under terminal anaesthesia. Data are mean±standard deviation, statistical comparisons were made by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph.

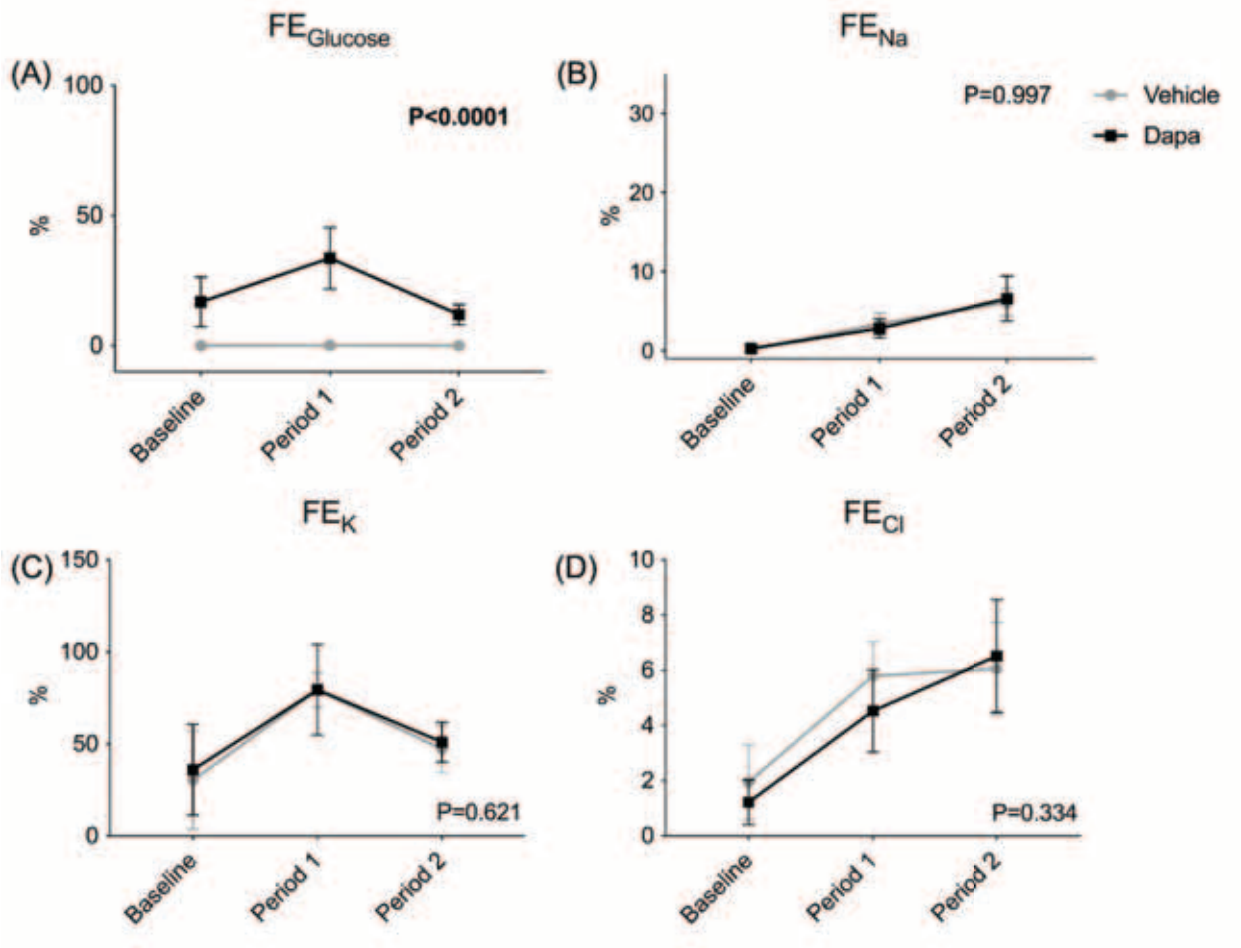

Figure 3. Fractional excretion (FE) of glucose, sodium, potassium, and chloride. Urinary and plasma levels of (A) glucose, (B) sodium, (C) potassium, and (D) chloride were measured in Sprague Dawley rats treated with an IV bolus injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=8) or dapagliflozin (dapa, 1 mg/kg, n=8) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal aorta (period 2) to increase blood pressure under terminal anaesthesia. The fraction of these molecules that was excreted rather than reabsorbed was calculated. Data are mean±standard deviation, statistical comparisons were made

by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph

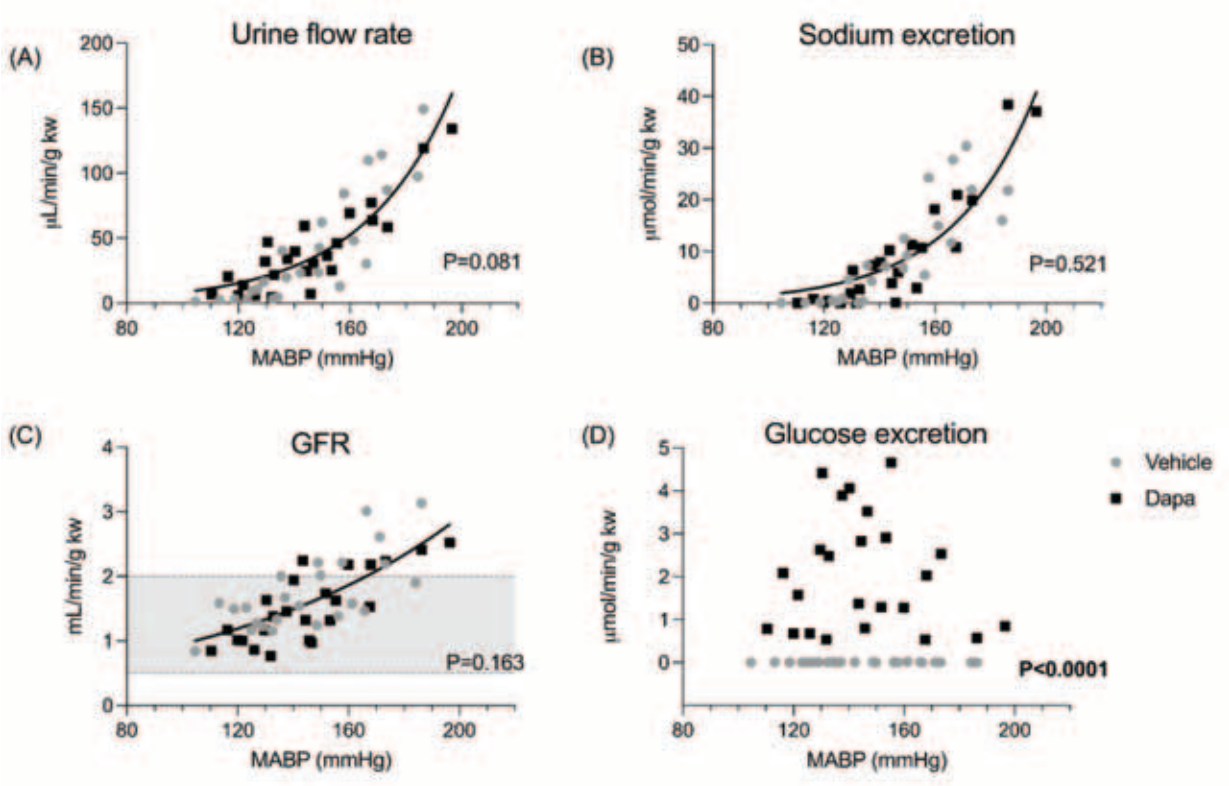

Figure 4. Pressure natriuresis response in Sprague Dawley rats. Rats were treated with an IV bolus injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=8) or dapagliflozin (dapa, 1 mg/kg, n=8) prior to stepwise arterial ligations to increase mean arterial blood pressure (MABP) under terminal anaesthesia. MABP plotted against (A) urine flow rate, (B) urinary sodium excretion, (C) urinary glucose excretion, and (D) glomerular filtration rate (GFR). Data analysed by a non-linear fit curve with the null hypothesis of one curve fitting both data sets. Shaded area shows normal autoregulatory values for GFR in Sprague Dawley rats [49]. P values shown on each graph.

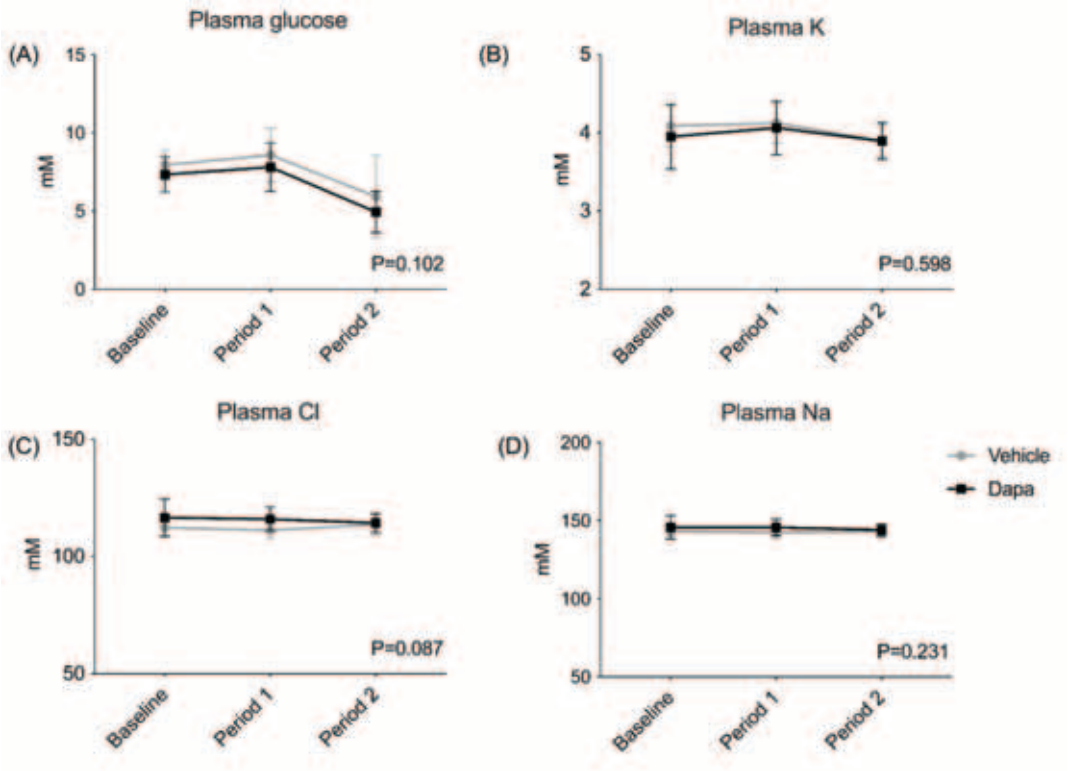

Figure 5. Plasma levels of glucose and electrolytes. Sprague Dawley rats treated with an IV bolus injection of either vehicle (2% DMSO in 0.9% saline, n=8) or dapa (1 mg/kg, n=8) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal aorta (period 2) to increase blood pressure under terminal anaesthesia. (A) Glucose, (B) sodium, (C) chloride, and (D) potassium were measured in plasma at baseline and at each period. Data are mean±standard deviation, statistical comparisons were made by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph

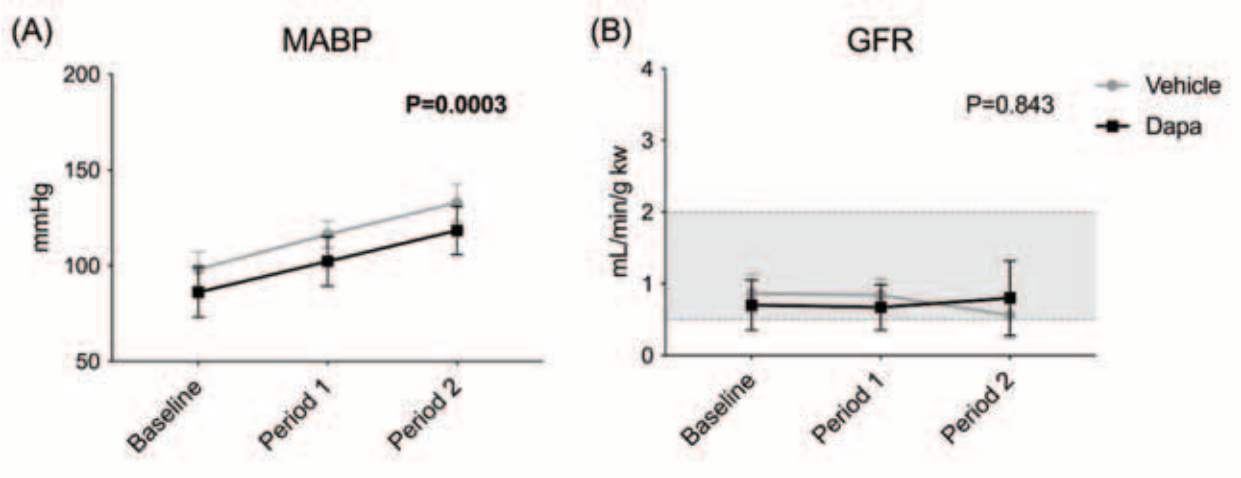

Figure 6. Mean arterial blood pressure (MABP) and glomerular filtration rate (GFR) during the pressure natriuresis protocol. Sprague Dawley rats were treated with streptozotocin to induce type 1 diabetes and, two weeks later, were then treated with a bolus IV injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=7) or dapagliflozin (dapa, 1 mg/kg, n=7) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal aorta (period 2). (A) MABP averaged over the 30-minute periods and (B) GFR measured using FITC-inulin. The shaded area represents the normal autoregulatory range of GFR in Sprague Dawley rats [49]. Data are mean±standard deviation, statistical comparisons were made by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph.

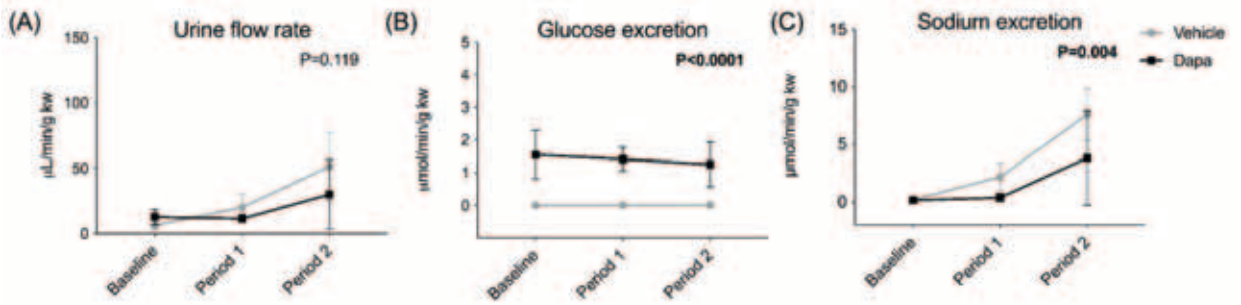

Figure 7. Urine flow and urinary excretion of glucose and sodium in type 1 diabetic rats. (A) Urine flow rate, as well as (B) urinary glucose excretion and (C) sodium excretion, was measured in streptozotocin treated Sprague Dawley rats then injected with an IV bolus of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=7) or dapagliflozin

(dapa, 1 mg/kg, n=7) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal aorta (period 2) to increase blood pressure under terminal anaesthesia. Data are mean±standard deviation, statistical comparisons were made by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph.

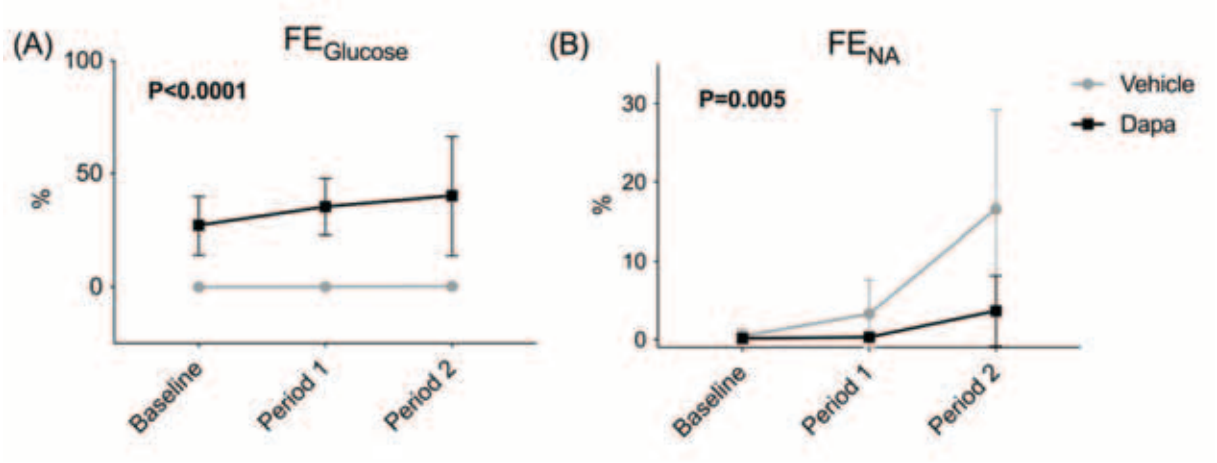

Figure 8. Fractional excretion (FE) of glucose and sodium. Urinary and plasma levels of (A) glucose and (B) sodium were measured in streptozotocin treated type 1 diabetic Sprague Dawley rats treated with an IV bolus injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=7) or dapa (1 mg/kg, n=7) prior to stepwise arterial ligations of the coeliac and cranial mesenteric arteries (period 1) and the distal aorta (period 2) to increase blood pressure under terminal anaesthesia to determine fraction of these molecules that was excreted rather than reabsorbed. Data are mean±standard deviation, statistical comparisons were made by 2-way analysis of variance (ANOVA) where the main effects of dapagliflozin treatment are shown on each graph.

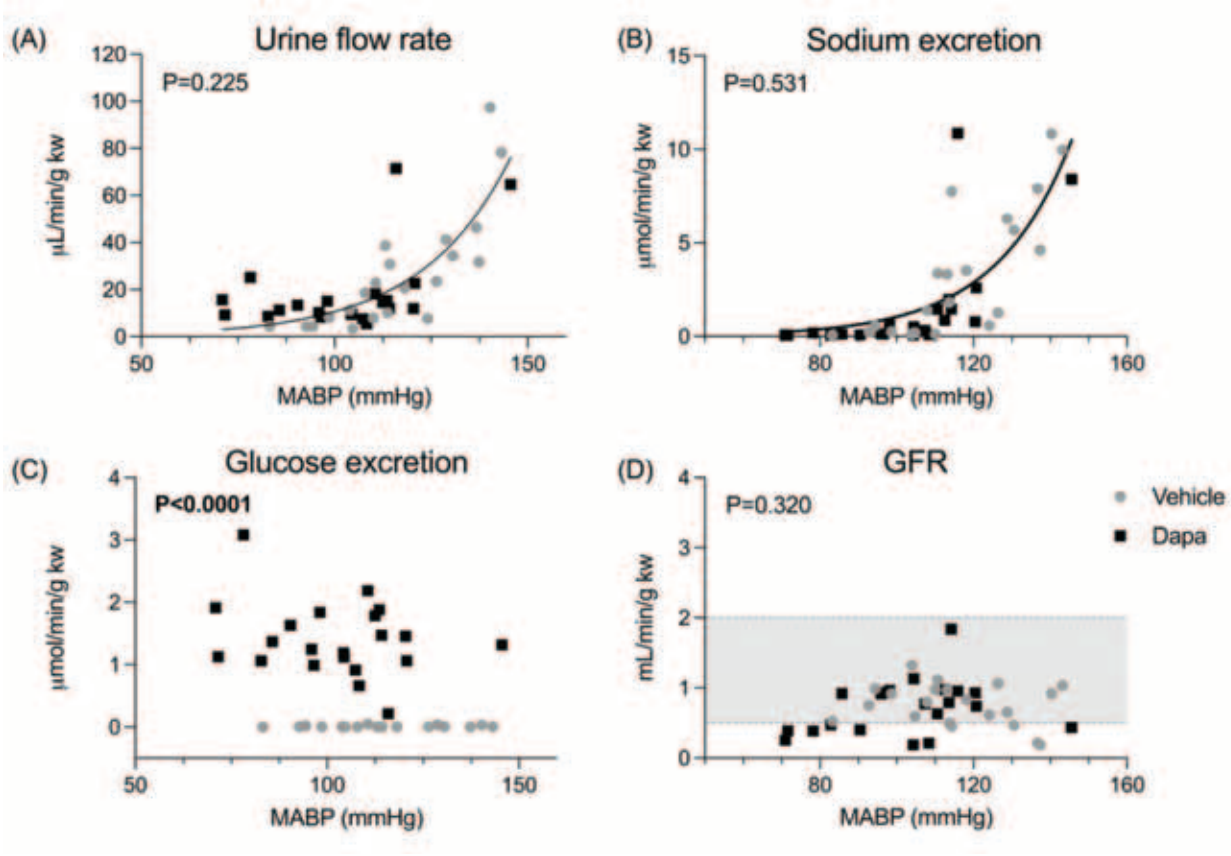

Figure 9. Pressure natriuresis response in type 1 diabetic Sprague Dawley rats. Diabetes was induced by streptozotocin injection 2 weeks prior to an IV bolus injection of either vehicle (2% dimethyl sulfoxide in 0.9% saline, n=7) or dapagliflozin (dapa, 1 mg/kg, n=7). Stepwise arterial ligations were carried out to increase mean arterial blood pressure (MABP) under terminal anaesthesia. MABP plotted against (A) urine flow rate, (B) urinary sodium excretion, (C) urinary glucose excretion, and (D) glomerular filtration rate (GFR). Data analysed by a non-linear fit curve with the null hypothesis of one curve fitting both data sets. Where curves could not be fitted, they were excluded from the graphs. Shaded area shows normal autoregulatory values for GFR in Sprague Dawley rats [49]. P values shown on each graph.