The effect of aliskiren on urinary cytokine/chemokine responses to clamped hyperglycaemia in type 1 diabetes

David Z. I. Cherney · Heather N. Reich ·
James W. Scholey · Denis Daneman · Farid H. Mahmud ·
Ronnie L. H. Har · Etienne B. Sochett

Received: 16 April 2013 / Accepted: 28 June 2013 / Published online: 28 July 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract

Aims/hypothesis Acute clamped hyperglycaemia activates the renin–angiotensin–aldosterone system (RAAS) and increases the urinary excretion of inflammatory cytokines/chemokines in patients with uncomplicated type 1 diabetes mellitus. Our objective was to determine whether blockade of the RAAS would blunt the effect of acute hyperglycaemia on urinary cytokine/chemokine excretion, thereby giving insights into potentially protective effects of these agents prior to the onset of clinical nephropathy.

Methods Blood pressure, renal haemodynamic function (inulin and *para*-aminohippurate clearances) and urinary cytokines/chemokines were measured after 6 h of clamped euglycaemia (4–6 mmol/l) and hyperglycaemia (9–11 mmol/l) on two consecutive days in patients with type 1 diabetes mellitus ($n=27$) without overt nephropathy. Measurements were repeated after treatment with aliskiren (300 mg daily) for 30 days.

Results Before aliskiren, clamped hyperglycaemia increased filtration fraction (from 0.188 ± 0.007 to 0.206 ± 0.007 , $p=0.003$) and urinary fibroblast growth factor-2 (FGF2), IFN- $\alpha 2$ and macrophage-derived chemokine (MDC) ($p<0.005$). After aliskiren, the filtration fraction response to hyperglycaemia was abolished, resulting in a lower filtration fraction after aliskiren under clamped hyperglycaemic conditions ($p=0.004$), and none

of the biomarkers increased in response to hyperglycaemia. Aliskiren therapy also reduced levels of urinary eotaxin, FGF2, IFN- $\alpha 2$, IL-2 and MDC during clamped hyperglycaemia ($p<0.005$).

Conclusions/interpretation The increased urinary excretion of inflammatory cytokines/chemokines in response to acute hyperglycaemia is blunted by RAAS blockade in humans with uncomplicated type 1 diabetes mellitus.

Keywords Clamped hyperglycaemia · Type 1 diabetes mellitus · Urine cytokines/chemokines · Urine proteomic analysis

Abbreviations

DRI	Direct renin inhibition
ERPF	Effective renal plasma flow
FGF-2	Fibroblast growth factor-2
GM-CSF	Granulocyte–monocyte colony stimulating factor
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MIP-1 α	Macrophage inflammatory protein-1 α
PAH	<i>para</i> -Aminohippurate
PDGF-AB/BB	Platelet-derived growth factor-AB/BB
RAAS	Renin–angiotensin–aldosterone system
sCD40K	sCD40ligand
SGLT2	Sodium-glucose cotransport-2

Introduction

Glycaemic excursions may contribute to microvascular end-organ injury in patients with diabetes mellitus, independent of long-term glycaemic control [1]. From a haemodynamic perspective in the kidney, the influence of hyperglycaemia manifests as a hyperfiltration response characterised by a rise

D. Z. I. Cherney (✉) · R. L. H. Har
Division of Nephrology, University Health Network – Toronto
General Hospital, Banting and Best Diabetes Centre, University of
Toronto, 585 University Ave, 8N-845,
Toronto, ON, Canada M5G 2N2
e-mail: david.cherney@uhn.on.ca

H. N. Reich · J. W. Scholey
Division of Nephrology, University Health Network – Toronto
General Hospital, Toronto, ON, Canada

D. Daneman · F. H. Mahmud · E. B. Sochett
Division of Pediatric Endocrinology, Hospital for Sick Children,
University of Toronto, Toronto, ON, Canada

in filtration fraction [2–6], which may cause renal injury through shear-stress effects [7–9]. The haemodynamic effects of hyperglycaemia are attributed, in part, to activation of the renin–angiotensin–aldosterone system (RAAS) [6].

In addition to haemodynamic effects, hyperglycaemia activates inflammatory pathways in the kidney, leading to further injury. We have recently demonstrated that acute clamped hyperglycaemia in a separate cohort of young patients with type 1 diabetes mellitus increases the urinary excretion of factors implicated in the initiation and progression of diabetic nephropathy, including eotaxin, fibroblast growth factor-2 (FGF-2), granulocyte–monocyte colony stimulating factor (GM-CSF), IFN- α 2, IL-2, IL-12, monocyte chemoattractant protein (MCP)-3, macrophage-derived chemokine (MDC), macrophage inflammatory protein-1 α (MIP-1 α), platelet-derived growth factor-AB/BB (PDGF-AB/BB), sCD40ligand (sCD40K) and TNF- β [10]. We have further demonstrated that urinary cytokine/chemokine levels are associated with protein excretion within the normal range in children, suggesting a role for renal inflammation in the early pathogenesis of diabetic nephropathy [11].

As inflammation and immune-mediated injury induced by intrarenal cytokine/chemokine expression are implicated in both the initiation and progression of diabetic nephropathy it is important to determine the relationship between existing pharmacotherapeutic agents and measures of renal inflammatory factors [12–14]. One of the pleiotropic protective effects of RAAS blockade on the kidney in patients with overt diabetic nephropathy is anti-inflammatory activity [15]. The ability of clinicians to suppress renal inflammation may have particularly important implications for patients with uncomplicated type 1 diabetes mellitus, in whom urinary inflammatory markers may be the only sign of kidney disease prior to the onset of microalbuminuria or declining kidney function [11]. New non-invasive biomarkers could therefore be used to target high-risk patients with otherwise clinically silent disease for earlier therapies [16].

Although it is known that RAAS blockade is more effective under conditions of dietary sodium depletion, RAAS inhibitors are still effective in both diabetic and non-diabetic sodium-replete individuals [17–20]. These observations suggest persistent intrarenal RAAS activation despite high dietary sodium intake. As a consequence, in sodium-replete individuals RAAS inhibitors exert important renal haemodynamic and blood-pressure effects, and in patients with established diabetic nephropathy these agents suppress urinary levels of inflammatory mediators [17, 21–25]. In patients with uncomplicated type 1 diabetes mellitus who would not otherwise be candidates for RAAS inhibition, the effect of these agents on urinary biomarker excretion or biomarker responses to acute clamped hyperglycaemia remain unknown. It is important to establish the effect of RAAS inhibitors on urinary markers of

inflammation under sodium-replete conditions, as this would most closely mimic most typical clinical settings.

Accordingly, our goal was to examine whether RAAS blockade would mitigate the effect of acute hyperglycaemia on renal inflammatory pathways. We hypothesised that RAAS inhibition would reduce urinary cytokine/chemokine excretion and would also blunt the increased urinary excretion of these factors in response to clamped hyperglycaemia. We included young patients with uncomplicated type 1 diabetes mellitus who would not otherwise be treated with RAAS inhibitors, and studied participants in a sodium-replete state to replicate typical North American dietary conditions. Our goal was to better understand the effect of these agents on renal inflammatory pathways early in the natural history of the disease when clinical risk factors such as albuminuria may not identify those at elevated risk of renal disease progression.

Methods

Patients with uncomplicated type 1 diabetes mellitus ($n=27$) were recruited. Inclusion criteria were: duration of type 1 diabetes ≥ 5 years; age ≥ 18 years; blood pressure $<140/90$ mmHg; no history of renal disease or macrovascular disease; and not taking any regular medications other than insulin, thyroid replacement therapy or inhalers to treat asthma. Patients who were normoalbuminuric were recruited based on a spot urine albumin-to-creatinine ratio <2.8 mg/mmol for women and <2.1 mg/mmol for men. All participants were subsequently shown to be normoalbuminuric on a 24 h urine collection on the day prior to all physiological assessments (<30 mg per 24 h).

We aimed to study women during the early follicular phase of the menstrual cycle, determined by cycle day and measurement of 17 β -oestradiol levels. None was using oral contraceptive medication. The local Research Ethics Board at the University Health Network (Toronto, ON, Canada) approved the protocol and all participants gave informed consent.

Experimental design The experimental design is summarised in Fig. 1. In order to suppress endogenous RAAS activity and avoid the effect of a high-dietary-protein diet on renal function, participants adhered to a high-sodium (>140 mmol/day) and moderate-protein (<1.5 g kg $^{-1}$ day $^{-1}$) diet during the 7 day period before renal haemodynamic testing and collection of the urine sample, as described previously [26]. The high-sodium diet was also used to avoid confounding effects of having inter- and intra-individual differences in sodium intake before and after direct renin inhibition (DRI), and to keep experimental conditions close to typical North American clinical conditions, in terms of diet. First, patients were studied after clamped euglycaemic (4–6 mmol/l) conditions had been

Fig. 1 Study outline. FF, filtration fraction; MAP, mean arterial pressure; o.d., once daily; p.o., by mouth

maintained for approximately 6 h, a period of time previously demonstrated to be sufficient to influence vascular function and urinary cytokines/chemokines [8]. This level of ambient glycaemia was maintained during all investigations.

In all phases of the experiment, blood glucose was maintained by a modified glucose-clamp technique, as described previously [8]. In brief, a 16-gauge peripheral venous cannula was inserted into the left antecubital vein for infusion of glucose and insulin and a second cannula was inserted for blood sampling more distally. Blood glucose was measured approximately every 10 min and the insulin infusion was adjusted to maintain euglycaemia. All experiments were performed in the same warm (25°C), temperature-controlled room and in a dark, quiet environment in the supine position. The following day, identical sets of experiments were performed during clamped hyperglycaemia (blood glucose 9–11 mmol/l).

Renal and urinary biomarker studies were performed at baseline and then repeated in an identical fashion after 4 weeks of aliskiren therapy (300 mg orally per day).

Assessment of renal variables Following maintenance of the euglycaemic clamp, a third intravenous line was inserted into

the right arm and was connected to a syringe infusion pump for administration of inulin and *para*-aminohippurate (PAH). After collecting blood for inulin and blank, a priming infusion containing 25% inulin (60 mg/kg) and 20% PAH (8 mg/kg) was administered. Thereafter, inulin and PAH were infused continuously at a rate calculated to maintain their respective plasma concentrations constant at 2.0 and 0.15 mg/l, respectively. After a 90 min equilibration period, blood was collected for inulin, PAH and haematocrit. Two blood samples were further collected at 30 min intervals over 60 min for inulin and PAH. GFR and effective renal plasma flow (ERPF) were estimated from steady-state infusion of inulin and PAH, respectively. Filtration fraction was derived as GFR/ERPF [8].

Sample collection and analytical methods Blood samples collected for inulin and PAH determinations were immediately centrifuged at 96 g for 10 min at 4°C. Plasma was separated, placed on ice and then stored at –70°C before the assay. Inulin and PAH were measured in serum by colorimetric assays using anthrone and *N*-(1-naphthyl)ethylenediamine, respectively [27–29]. The mean of two baseline clearance periods

represent GFR and ERPF, expressed per 1.73 m^2 body surface area [27, 29].

On arrival at the renal physiology laboratory, participants were asked to void and this final volume completed the 24 h urine collection. Participants then voided again mid-morning at approximately 10:00 hours to empty the bladder of urine that was produced while the euglycaemic clamp was being stabilised. The subsequent spot urine sample that was used for the analysis was collected prior to the first set of renal haemodynamic variables. This 50 ml mid-stream sterile urine specimen was used to measure levels of cytokines/chemokines using a Millipore Cytokine/Chemokine Panel Luminex Assay (Eve Technologies, Calgary, AB, Canada) corrected for urine creatinine concentration [10, 11]. Immediately after collection, urine was centrifuged at $1,500 \text{ g}$ for 15 min to remove cells, then separated into 1 ml aliquots and frozen at -80°C . Urine was then thawed at 4°C 1 day prior to use. As the protocol for handling urine specimens was designed to avoid protein degradation, this analysis included spot urines and not the timed collections that were carried out before the study visit. Furthermore, a priori we only included analytes that we have observed either to increase in response to hyperglycaemia or that were associated with the highest tertile of normoalbuminuria in our previous work [10, 11]. Our analysis therefore included eotaxin, FGF-2, GM-CSF, IFN- $\alpha 2$, IL-2, IL-12, MCP-1, MCP-3, MDC, MIP-1 α , TNF- β , sCD40K and PDGF-AB/BB. We limited our analysis to these factors to maintain statistical power, to minimise false-positive results and to further elucidate mechanisms that may link high intraglomerular pressure with factors that may promote the initiation of renal disease. The accuracy and precision of the urinary cytokine/chemokine assay is available from the manufacturer's website (www.millipore.com/userguides/tech1/proto_mpxhcyto-60k). The detection limits of our assays have also been published previously [11]. The investigator performing data analysis was blinded to all study variables.

Urinary albumin excretion rate was determined from a 24 h urine collection by immunoturbidimetry. HbA_{1c} was measured by high-performance liquid chromatography and plasma renin concentration and plasma insulin were measured using previously described methods [8, 30].

Statistical analysis Descriptive statistics were used to compare baseline clinical and demographic characteristics. Based on our previous work with MCPs, to be able to detect a 16 unit or greater change in response to RAAS blockade (80% power, α 0.05, standard deviation 20 units), a minimum of 24 patients were required [31]. Within-subject responses to hyperglycaemia and RAAS inhibition were determined by repeated measures analysis of variance (ANOVA), followed by Bonferroni's correction for multiple comparisons using a two-tailed test and $p \leq 0.005$ to indicate

statistical significance (SPSS 20.0 software package, IBM, Armonk, NY, USA).

Results

Baseline characteristics Table 1 describes the clinical characteristics of the cohort ($n=27$). Participants were normotensive, normoalbuminuric young men and women with type 1 diabetes mellitus. None of the participants had a history of retinopathy, neuropathy or macrovascular disease. Participants adhered to the controlled sodium and protein diet, and these dietary variables were similar before and after DRI therapy. Ambient glycaemia was in the desired range (Fig. 2), and plasma insulin levels were similar on each study day (Table 1). Fractional sodium-excretion rates were also similar at baseline during clamped euglycaemia and hyperglycaemia. None of the participants experienced clinical adverse effects such as hyperkalaemia or dizziness. In this analysis, MIP-1 α and TNF- β were undetectable in the majority of samples and therefore we did not consider these analytes further.

Baseline response to clamped hyperglycaemia At baseline, GFR increased in response to clamped hyperglycaemia while ERPF remained stable, resulting in an increase in filtration fraction (Table 1). As expected, clamped hyperglycaemia was associated with increases in urinary cytokines/chemokines. Changes in FGF-2, IFN- $\alpha 2$ and MDC remained significant after correcting for multiple comparisons with a conservative threshold of $p=0.005$ (Fig. 3), while effects on GM-CSF ($p=0.038$), IL-2 ($p=0.007$), MCP-1 ($p=0.038$) and MCP-3 ($p=0.023$) were no longer significant after correcting for multiple comparisons (Fig. 3). Trends for eotaxin, IL-12, PDGF-AB/BB and sCD40K were not significant. Baseline circulating levels of plasma renin were similar during clamped euglycaemia and hyperglycaemia (6.7 ± 0.7 vs $5.7 \pm 0.7 \text{ pg/ml}$, $p=0.244$).

Effects of RAAS blockade on haemodynamics and urinary cytokines/chemokines After treatment with aliskiren for 30 days, plasma renin increased during clamped euglycaemia (from 6.7 ± 0.7 to $164.5 \pm 37.1 \text{ pg/ml}$, $p < 0.0001$) and hyperglycaemia (from 5.7 ± 0.7 to $84.5 \pm 26.3 \text{ pg/ml}$, $p=0.007$). The filtration fraction response to hyperglycaemia was reversed compared with baseline effects, resulting in a lower filtration fraction after vs before aliskiren under clamped hyperglycaemic conditions (unadjusted $p=0.02$, Table 1, Fig. 4) but this was not significant after correcting for multiple comparisons. Fractional sodium excretion remained unchanged in response to aliskiren (Table 1). Compared with pre-aliskiren hyperglycaemic conditions, post-aliskiren clamped euglycaemic levels of urinary

Table 1 Demographic characteristics at baseline and after aliskiren therapy in patients with uncomplicated type 1 diabetes

Characteristic	Baseline	Aliskiren
Male:female	13:14	
Age (years)	25.6±2.1	–
Diabetes duration (years)	17.2±1.2	–
HbA _{1c} (%)	8.8±1.0	–
HbA _{1c} (mmol/mol)	73±3	–
BMI (kg/m ²)	24.4±0.6	–
Retinopathy history	None	–
Neuropathy history	None	–
Albumin excretion rate (mg/day)	12±3	9±2
24 h Na ⁺ intake (mmol/day)	171±6	174±6
Estimated protein intake (g kg ⁻¹ day ⁻¹)	1.03±0.01	0.92±0.01
Oestrogen in women (pmol/l)	188±42	176±78
Systolic blood pressure (mmHg)	113±2	108±3***
Diastolic blood pressure (mmHg)	64±2	59±2***
Pulse (beats per minute)	57±6	63±4
Plasma insulin (pmol/l)		
Euglycaemia	188±53	201±98
Hyperglycaemia	175±68	232±136
Plasma glucose (mmol/l)		
Euglycaemia	4.79±0.10	4.44±0.21
Hyperglycaemia	11.33±0.15	11.00±0.22
Fractional Na ⁺ excretion		
Euglycaemia	1.21±0.12	1.19±0.12
Hyperglycaemia	1.17±0.11	1.20±0.10
Renal haemodynamic function		
GFR (ml min ⁻¹ 1.73 m ⁻²)		
Euglycaemia	136±6	140±5
Hyperglycaemia	143±5 [†]	146±7 [†]
Effective renal plasma flow (ml min ⁻¹ 1.73 m ⁻²)		
Euglycaemia	718±26	763±37
Hyperglycaemia	710±30	816±40 [‡]
Filtration fraction		
Euglycaemia	0.188±0.007	0.193±0.011
Hyperglycaemia	0.206±0.007 [§]	0.183±0.008 [¶]

Data are mean ± SD

*** $p<0.001$ for blood-pressure responses to aliskiren; [†] $p=0.015$ for GFR vs pre-aliskiren during euglycaemia vs GFR pre-aliskiren during hyperglycaemia; $p=0.029$ for GFR vs post-aliskiren during euglycaemia vs GFR post-aliskiren during hyperglycaemia; [‡] $p=0.004$ vs ERPF during clamped hyperglycaemia at baseline; [§] $p=0.003$ vs filtration fraction during baseline clamped euglycaemia; [¶] $p=0.004$ vs filtration fraction during clamped hyperglycaemia at baseline pre-aliskiren

eotaxin, FGF-2, GM-CSF, IFN- α 2, IL-2, IL-12, MCP-3 and MDC were reduced ($p\leq 0.019$), but only differences for FGF-2, GM-CSF, IFN- α 2, IL-2 and MDC remained significant after correcting for multiple comparisons (Fig. 3).

Compared with levels during baseline clamped hyperglycaemia, RAAS blockade similarly reduced the urinary levels of eotaxin, FGF-2, IFN- α 2, IL-2, MCP-3, MDC, PDGF-AB/BB and MCP-1 under clamped hyperglycaemic conditions (Fig. 3). Differences in each of these mediators except for MCP-3 ($p=0.015$), PDGF-AB/BB ($p=0.022$) and MCP-1 ($p=0.006$) were significant after correcting for multiple comparisons (Fig. 3). In addition, RAAS blockade reversed cytokine/chemokine responses to clamped hyperglycaemia, such that none of the ten biomarkers increased in response to clamped hyperglycaemia.

Renal hyperfiltration, RAAS blockade and urinary cytokines/chemokines In a post hoc analysis, we examined the effect of DRI on urinary cytokine/chemokine excretion levels according to whether or not patients had underlying renal hyperfiltration ($n=10$, GFR ≥ 135 ml min⁻¹ 1.73 m⁻²) or normofiltration ($n=17$, GFR <135 ml min⁻¹ 1.73 m⁻²), as hyperfiltration can influence urinary cytokine/chemokine levels [32]. The effect of DRI on urinary cytokines/chemokines was generally similar in the two groups, and between-group effects were not significant (data not shown), except for MCP-1. Urinary MCP-1 declined to a greater extent in hyperfilterers compared with normofilterers during clamped hyperglycaemia (ANOVA interaction, $p=0.002$, Fig. 5). In contrast, between-group differences for the effect of DRI on MCP-1 during clamped euglycaemia were not significant ($\Delta -6.6\pm 7.1$ pmol/mmol creatinine in hyperfilterers vs $\Delta 6.0\pm 6.3$ pmol/mmol creatinine in normofilterers, $p=0.201$).

Discussion

The goal of this study was to determine whether the effect of acute clamped hyperglycaemia on urinary cytokines/chemokines could be reversed with RAAS inhibition in patients with uncomplicated type 1 diabetes mellitus. Previous work in animals and humans has demonstrated that pathways that are upregulated by hyperglycaemia, including RAAS and oxidative stress pathways, produce increased systemic and renal levels of cytokines/chemokines [21, 33]. These deleterious effects have been attributed to chronic hyperglycaemia-induced angiotensin II and aldosterone activity [34–36], RAAS blockade is thought to be renal protective in part by abrogating these inflammatory responses. We have recently demonstrated that inflammatory urine biomarkers also increase in response to acute clamped hyperglycaemia; however, the role of the RAAS in mediating this response and the reversibility of this phenomenon was unknown. We hypothesised that blockade of the RAAS would mitigate the effect of clamped hyperglycaemia on urinary cytokines/chemokines [37–40].

Our first major finding was that in patients with uncomplicated type 1 diabetes mellitus, the effect of clamped

Fig. 2 Capillary blood glucose readings on each of the 4 study days before and after aliskiren treatment. Data shown are mean $\pm$ SD. Squares, euglycaemia pre-DRI; circles, hyperglycaemia pre-DRI; triangles, euglycaemia post-DRI; inverted triangles, hyperglycaemia post-DRI

hyperglycaemia on urinary cytokines/chemokines was inhibited by RAAS blockade. Similar to our previous observations in an independent cohort, factors that are involved in either chemotaxis or inflammatory kidney disease initially increased in response to clamped hyperglycaemia, prior to any RAAS-modulating therapy. We have previously hypothesised that the increase in urinary cytokine/chemokine excretion occurred because of increased tubular secretion, although it is also possible that the urinary

Fig. 4 The effect of aliskiren on filtration fraction response to clamped hyperglycaemia. Data shown are mean $\pm$ SD. Unadjusted $p=0.020$ (not significant after correcting for multiple comparisons) for the filtration fraction response to hyperglycaemia before vs after aliskiren. FF, filtration fraction

excretion of these factors could reflect systemic ‘spillover’ into the urine. Regardless of the responsible mechanism, previous studies have demonstrated that renoprotective medications, including protein kinase C- β inhibitors [13, 31, 41] and ACE inhibitors and angiotensin-receptor blockers [22], reduce urinary cytokines/chemokines in diabetes mellitus patients with established nephropathy. Less was known about the urinary excretion of inflammatory biomarkers in response to RAAS inhibition in humans with uncomplicated type 1 diabetes mellitus, or the effect of these agents on urinary biomarker responses to clamped hyperglycaemia [42]. To our knowledge this is the first study to demonstrate potentially positive effects of RAAS inhibition on urinary biomarkers in patients with

Fig. 3 The effect of aliskiren and clamped hyperglycaemia on urinary chemokines/cytokines (pmol/mmol): (a) eotaxin; (b) FGF-2; (c) GM-CSF; (d) IFN- α 2; (e) IL-2; (f) IL-12; (g) MCP-3; (h) MDC; (i) PDGF-AB/BB; (j) sCD40K; and (k) MCP-1. Data shown are mean $\pm$ SD. $^{\dagger}p<0.005$ for the effect of clamped hyperglycaemia vs euglycaemia pre-

aliskiren; $^{\ddagger}p<0.0001$ for the level of urinary cytokine/chemokine excretion during clamped euglycaemia post-aliskiren compared with pre-aliskiren hyperglycaemic levels; $^{\S}p\leq 0.002$ for the effect of aliskiren on the analyte under clamped hyperglycaemic conditions (hyperglycaemia pre-aliskiren vs hyperglycaemia post-aliskiren)

Fig. 5 The effect of aliskiren on MCP-1 in patients with type 1 diabetes mellitus and either renal hyperfiltration ($\text{GFR} \geq 135 \text{ ml min}^{-1} 1.73 \text{ m}^{-2}$) or normofiltration ($\text{GFR} < 135 \text{ ml min}^{-1} 1.73 \text{ m}^{-2}$). $^{\dagger}p=0.002$ for the between-group change in urinary MCP-1 in response to aliskiren

uncomplicated type 1 diabetes mellitus under different conditions of clamped euglycaemia and hyperglycaemia.

While the factors responsible for the effect of RAAS blockade on urinary cytokines/chemokines cannot be determined from this study, it is perhaps important that aliskiren also blocked the effect of hyperglycaemia on filtration fraction, which is often used as a surrogate for intraglomerular pressure in humans [6]. The decline in filtration fraction occurred because of a predominant increase in ERPF without affecting GFR responses, suggesting similar afferent compared with efferent vasodilatory effects. As reductions in glomerular stretch and shear-stress mechanical forces suppress renal inflammation in animals, it is tempting to speculate that the effect of RAAS blockade on urine cytokines/chemokines was caused by a decline in intraglomerular pressure [43]. Interestingly, the effect of DRI on MCP-1 was more pronounced in patients with renal hyperfiltration under clamped hyperglycaemic conditions. As hyperfiltration is associated with high intraglomerular pressure, the exaggerated response in hyperfiltering individuals may highlight a possible physiological benefit of earlier RAAS blockade in this group [7]. Alternatively, changes in urinary factors may have been on the basis of known ‘non-haemodynamic’ effects of RAAS blockade on critical pro-inflammatory pathways, including nuclear factor- $\kappa\beta$ [44]. Ultimately, the clinical importance of our observations will have to be clarified in long-term prospective trials in humans to assess whether RAAS blockade exerts protective effects on early markers of renal injury, including albuminuria and declining renal function, and to determine if certain patient subgroups such as hyperfiltering individuals derive additional benefit.

Hyperglycaemia is necessary but not sufficient for the initiation and progression of diabetic nephropathy. It is instead the individual response to hyperglycaemia that is the critical determinant of diabetic complications. The acute effect of glucose on renal markers of inflammation and fibrosis is therefore important because our work may give insight into pathways that permit the initiation of nephropathy in some patients and not others. Moreover, in light of the observations in the present study, these hyperglycaemia-

inducible pathways are sensitive to existing pharmacological agents. As ‘untargeted’ primary-prevention trials in type 1 diabetes mellitus have failed, it is now more important than ever to translate observations from experimental animal models and from pre-clinical patient studies into the clinical trial arena. A better understanding of the clinical significance of urinary cytokines/chemokines and their ability to reflect biological effects of experimental and therapeutic interventions in patients with type 1 diabetes mellitus may lead to a more targeted, personalised approach to primary-prevention strategies. Our observations suggest that non-invasive early biomarkers that may contribute to progressive kidney injury over time are dynamic and responsive to RAAS blockade. Future work should define the time course of this response, and should also correlate these factors with the development of proteinuria and declining kidney function. Finally, as the effects of RAAS blockade on urinary cytokines/chemokines was greatest under conditions of clamped hyperglycaemia, future studies should consider taking into account the level of ambient glycaemia at the time biomarkers are measured.

It is also important to recognise that our observations were made under conditions of a relatively high-sodium diet that are common in the North American clinical context. As we studied patients under these conditions of high dietary sodium, the magnitude of our results may be less than would be expected under conditions of salt depletion, when the RAAS is activated and the response to RAAS blockers is enhanced [45]. The effect of sodium intake may be of particular importance in light of recent clinical trial data from the Reduction in End points in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study and the Irbesartan Diabetic Nephropathy Trial (IDNT) that demonstrated enhanced renal protective effects of RAAS inhibition in participants with the two lowest tertiles of dietary sodium intake [20]. Whether the protective effect observed with low-sodium diet and RAAS inhibition in this post hoc analysis was due to greater haemodynamic effects, enhanced anti-inflammatory effects or both, is unknown and requires further study. Nevertheless, as previously reported by our group [17–19] and by other investigators [46], RAAS inhibitors still exert prominent haemodynamic effects on the intrarenal RAAS in the presence of relative RAAS suppression induced by a high-salt diet in diabetic patients. Our results extend this previous haemodynamic-focused body of work by suggesting that RAAS inhibition during salt repletion also reduces inflammatory urinary cytokines/chemokines.

Although we have focused on neurohormonal RAAS-related factors that have been implicated in the pathogenesis of renal haemodynamic dysfunction and renal inflammation, it is also important to recognise that tubuloglomerular feedback mechanisms have been implicated in the initiation and progression of diabetic nephropathy [47]. In brief, increased

proximal sodium–glucose cotransport via sodium–glucose cotransport-2 (SGLT2) reduces delivery of sodium to the macula densa, thereby leading to afferent vasodilatation through tubuloglomerular feedback [47]. Perhaps as a result of upregulated SGLT2 activity in diabetes mellitus, animals and humans with type 1 diabetes mellitus exhibit a phenomenon call the ‘salt paradox of diabetes mellitus’, whereby sodium restriction leads to exaggerated renal haemodynamic responses (hyperfiltration and afferent vasodilatation) despite blunted levels of circulating RAAS mediators [48, 49]. This again suggests that under conditions of sodium restriction when the RAAS is activated and tubuloglomerular feedback mechanisms may play a more prominent role, pharmacological agents such as RAAS inhibitors or novel SGLT2 inhibitors (that may influence tubuloglomerular feedback), may exert more complete protective effects on renal haemodynamic dysfunction, intraglomerular pressure and renal inflammation [50]. However, as fractional sodium excretion did not change in response to aliskiren, alterations in tubuloglomerular feedback may not have contributed significantly to the declines in urinary cytokines/chemokines in this cohort.

Our study has limitations. We attempted to minimise the effect of the small sample size by using homogeneous study groups and by using a careful pre-study preparation phase with a focus on known factors that influence neurohormonal activation, including dietary sodium intake. We also scheduled studies in women to coincide with the early follicular phase of the menstrual cycle to avoid the confounding effects of oestrogen on vascular function. In addition, we decreased variability and increased statistical power by using a study design that allowed each participant to act as his/her own control. Next, we are unable to comment about the relative effect of direct renin inhibitors compared with ACE inhibitors or angiotensin-receptor blockers. While this was not the goal of this study, based on comparable haemodynamic effects we would expect similar findings to be observed using these older classes of agents. Finally, it is important for future studies to determine whether changes in urinary cytokine/chemokine excretion are systemically derived (i.e. ‘spillover’) or due to renal production. In light of the exaggerated effects of DRI on MCP-1 in hyperfiltering patients in our post hoc analysis, future adequately powered trials should determine whether hyperfiltering patients exhibit exaggerated urinary cytokine/chemokine responses, as this may identify a group that could benefit from earlier primary-prevention strategies.

In conclusion, RAAS blockade for 30 days reduces the effect of acute clamped hyperglycaemia on filtration fraction and urinary cytokines/chemokines. Our results suggest that RAAS inhibitors may result in a beneficial urinary cytokine/chemokine physiological profile in patients with type 1 diabetes mellitus prior to the clinical onset of diabetic nephropathy.

Acknowledgements The authors would like to thank Paul Yip and Jenny Cheung in the Special Testing Laboratory at the University Health Network (Toronto, ON, Canada) for their invaluable assistance with the biochemical assays included in this work. Finally, the authors are grateful to the study participants whose time and effort are critical to the success of our research program.

Funding This work was supported by operating grants from the Canadian Institutes of Health Research, Heart Stroke Foundation (to DZIC), a University of Toronto Dean’s Fund Grant and by a grant from the JDRF to DD, FHM and EBS. DZIC was also supported by a Kidney Foundation of Canada Scholarship, a Canadian Diabetes Association-KRESCENT Program Joint New Investigator Award and a University of Toronto Dean’s Fund Award. RH is supported by a Banting and Best Graduate Award and an Institute of Medical Science Open Fellowship Award. JWS is the Canadian Institutes of Health Research (CIHR)/Amgen Canada Kidney Research Chair at the University Health Network, University of Toronto (ON, Canada).

Duality of interest DZIC is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The results presented in this paper have not been published previously in whole or in part. The authors declare that there is no duality of interest associated with this manuscript

Contribution statement DZIC and RH were involved in study design, and acquisition and interpretation of data. JWS, DD, FHM, EBS and HNR were involved in the interpretation of data. All authors revised the manuscript and approved the final version.

References

1. Brownlee M, Hirsch IB (2006) Glycemic variability: a hemoglobin A1c-independent risk factor for diabetic complications. *JAMA* 295:1707–1708
2. Gordin D, Ronnback M, Forsblom C, Heikkilä O, Saraheimo M, Groop PH (2007) Acute hyperglycaemia rapidly increases arterial stiffness in young patients with type 1 diabetes. *Diabetologia* 50:1808–1814
3. Esposito K, Nappo F, Marfella R et al (2002) Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. *Circulation* 106:2067–2072
4. Stegenga ME, van der Crabben SN, Delsing MC et al (2008) Effect of acute hyperglycaemia and/or hyperinsulinaemia on proinflammatory gene expression, cytokine production and neutrophil function in humans. *Diabet Med* 25:157–164
5. Gordin D, Forsblom C, Ronnback M et al (2008) Acute hyperglycaemia induces an inflammatory response in young patients with type 1 diabetes. *Ann Med* 1–7
6. Miller JA (1999) Impact of hyperglycemia on the renin angiotensin system in early human type 1 diabetes mellitus. *J Am Soc Nephrol* 10:1778–1785
7. Cherney DZ, Scholey JW, Miller JA (2008) Insights into the regulation of renal hemodynamic function in diabetic mellitus. *Curr Diabetes Rev* 4:280–290
8. Cherney DZ, Miller JA, Scholey JW et al (2008) The effect of cyclooxygenase-2 inhibition on renal hemodynamic function in humans with type 1 diabetes. *Diabetes* 57:688–695
9. Cherney DZI, Scholey JW, Sochett EB (2013) Sex differences in renal responses to hyperglycemia, L-arginine and L-NMMA in

- humans with uncomplicated type 1 diabetes mellitus. *Diabetes Care* 36:1290–1296
10. Cherney DZ, Scholey JW, Sochett E, Bradley TJ, Reich HN (2011) The acute effect of clamped hyperglycemia on the urinary excretion of inflammatory cytokines/chemokines in uncomplicated type 1 diabetes: a pilot study. *Diabetes Care* 34:177–180
  11. Cherney DZ, Scholey JW, Daneman D et al (2012) Urinary markers of renal inflammation in adolescents with type 1 diabetes mellitus and normoalbuminuria. *Diabet Med* 29:1297–1302
  12. Sanchez-Nino MD, Bozic M, Cordoba-Lanus E et al (2012) Beyond proteinuria: VDR activation reduces renal inflammation in experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 302:F647–F657
  13. Merchant ML, Perkins BA, Boratyn GM et al (2009) Urinary peptidome may predict renal function decline in type 1 diabetes and microalbuminuria. *J Am Soc Nephrol* 20:2065–2074
  14. Nguyen TQ, Tarnow L, Andersen S et al (2006) Urinary connective tissue growth factor excretion correlates with clinical markers of renal disease in a large population of type 1 diabetic patients with diabetic nephropathy. *Diabetes Care* 29:83–88
  15. Amann B, Tinzmann R, Angelkort B (2003) ACE inhibitors improve diabetic nephropathy through suppression of renal MCP-1. *Diabetes Care* 26:2421–2425
  16. Mauer M, Zinman B, Gardiner R et al (2009) Renal and retinal effects of enalapril and losartan in type 1 diabetes. *N Engl J Med* 361:40–51
  17. Cherney DZ, Scholey JW, Jiang S et al (2012) The effect of direct renin inhibition alone and in combination with ACE inhibition on endothelial function, arterial stiffness, and renal function in type 1 diabetes. *Diabetes Care* 35:2324–2330
  18. Cherney DZ, Lai V, Scholey JW, Miller JA, Zinman B, Reich HN (2010) Effect of direct renin inhibition on renal hemodynamic function, arterial stiffness, and endothelial function in humans with uncomplicated type 1 diabetes: a pilot study. *Diabetes Care* 33:361–365
  19. Sochett EB, Cherney DZ, Curtis JR, Dekker MG, Scholey JW, Miller JA (2006) Impact of renin angiotensin system modulation on the hyperfiltration state in type 1 diabetes. *J Am Soc Nephrol* 17:1703–1709
  20. Lambers Heerspink HJ, Holtkamp FA, Parving HH et al (2012) Moderation of dietary sodium potentiates the renal and cardiovascular protective effects of angiotensin receptor blockers. *Kidney Int* 82:330–337
  21. Duprez DA (2006) Role of the renin-angiotensin-aldosterone system in vascular remodeling and inflammation: a clinical review. *J Hypertens* 24:983–991
  22. Song JH, Cha SH, Lee HJ et al (2006) Effect of low-dose dual blockade of renin-angiotensin system on urinary TGF-beta in type 2 diabetic patients with advanced kidney disease. *Nephrol Dial Transplant* 21:683–689
  23. Esmatjes E, Flores L, Inigo P, Lario S, Ruilope LM, Campistol JM (2001) Effect of losartan on TGF-beta1 and urinary albumin excretion in patients with type 2 diabetes mellitus and microalbuminuria. *Nephrol Dial Transplant* 16(Suppl 1):90–93
  24. Song JH, Cha SH, Hong SB, Kim DH (2006) Dual blockade of the renin-angiotensin system with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers in chronic kidney disease. *J Hypertens* 24(Suppl):S101–S106
  25. Song JH, Lee SW, Suh JH et al (2003) The effects of dual blockade of the renin-angiotensin system on urinary protein and transforming growth factor-beta excretion in 2 groups of patients with IgA and diabetic nephropathy. *Clin Nephrol* 60:318–326
  26. Cherney DZ, Reich HN, Jiang S et al (2012) Hyperfiltration and the effect of nitric oxide inhibition on renal and endothelial function in humans with uncomplicated type 1 diabetes mellitus. *Am J Physiol Regul Integr Comp Physiol* 303:R710–R718
  27. Davidson WD, Sackner MA (1963) Simplification of the anthrone method for the determination of inulin in clearance studies. *J Lab Clin Med* 62:351–356
  28. Jung K, Klotzek S, Schulze BD (1990) Refinements of assays for low concentrations of inulin in serum. *Nephron* 54:360–361
  29. Florijn KW, Barendregt JN, Lentjes EG et al (1994) Glomerular filtration rate measurement by ‘single-shot’ injection of inulin. *Kidney Int* 46:252–259
  30. Cherney DZ, Miller JA, Scholey JW et al (2010) Renal hyperfiltration is a determinant of endothelial function responses to cyclooxygenase 2 inhibition in type 1 diabetes. *Diabetes Care* 33:1344–1346
  31. Cherney DZ, Konvalinka A, Zinman B et al (2009) Effect of protein kinase Cbeta inhibition on renal hemodynamic function and urinary biomarkers in humans with type 1 diabetes: a pilot study. *Diabetes Care* 32:91–93
  32. Har R, Scholey JW, Daneman D et al (2013) The effect of renal hyperfiltration on urinary inflammatory cytokines/chemokines in patients with uncomplicated type 1 diabetes mellitus. *Diabetologia* 56:1166–1173
  33. Touyz RM, Tabet F, Schiffrin EL (2003) Redox-dependent signaling by angiotensin II and vascular remodelling in hypertension. *Clin Exp Pharmacol Physiol* 30:860–866
  34. Touyz RM (2005) Molecular and cellular mechanisms in vascular injury in hypertension: role of angiotensin II. *Curr Opin Nephrol Hypertens* 14:125–131
  35. Ruperez M, Ruiz-Ortega M, Esteban V et al (2003) Angiotensin II increases connective tissue growth factor in the kidney. *Am J Pathol* 163:1937–1947
  36. Duprez DA (2004) Angiotensin II, platelets and oxidative stress. *J Hypertens* 22:1085–1086
  37. Chobanian AV, Haudenschild CC, Nickerson C, Drago R (1990) Antiatherogenic effect of captopril in the Watanabe heritable hyperlipidemic rabbit. *Hypertension* 15:327–331
  38. Schieffer B, Bunte C, Witte J et al (2004) Comparative effects of AT1-antagonism and angiotensin-converting enzyme inhibition on markers of inflammation and platelet aggregation in patients with coronary artery disease. *J Am Coll Cardiol* 44:362–368
  39. Proudfoot JM, Croft KD, Puddey IB, Beilin LJ (2003) Angiotensin II type 1 receptor antagonists inhibit basal as well as low-density lipoprotein and platelet-activating factor-stimulated human monocyte chemoattractant protein-1. *J Pharmacol Exp Ther* 305:846–853
  40. Fliser D, Buchholz K, Haller H (2004) Antiinflammatory effects of angiotensin II subtype 1 receptor blockade in hypertensive patients with microinflammation. *Circulation* 110:1103–1107
  41. Tashiro K, Koyanagi I, Saitoh A et al (2002) Urinary levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), and renal injuries in patients with type 2 diabetic nephropathy. *J Clin Lab Anal* 16:1–4
  42. Pelikanova T, Simkova R, Tesar V, Jirsa M (2003) Effect of acute hyperglycaemia on selected plasma and urinary cytokine antagonists in type 1 diabetes mellitus. *Diabetologia* 46:470–474
  43. Riser BL, Cortes P, Heilig C et al (1996) Cyclic stretching force selectively up-regulates transforming growth factor-beta isoforms in cultured rat mesangial cells. *Am J Pathol* 148:1915–1923
  44. Hernandez-Presa M, Bustos C, Ortego M et al (1997) Angiotensin-converting enzyme inhibition prevents arterial nuclear factor-kappa B activation, monocyte chemoattractant protein-1 expression, and macrophage infiltration in a rabbit model of early accelerated atherosclerosis. *Circulation* 95:1532–1541
  45. Fisher ND, Jan Danser AH, Nussberger J, Dole WP, Hollenberg NK (2008) Renal and hormonal responses to direct renin inhibition with aliskiren in healthy humans. *Circulation* 117:3199–3205
  46. Hollenberg NK, Fisher ND, Nussberger J, Moukarbel GV, Barkoudah E, Danser AH (2011) Renal responses to three types of renin-angiotensin system blockers in patients with diabetes mellitus on a high-salt diet: a need for higher doses in diabetic patients? *J Hypertens* 29:2454–2461

47. Vallon V, Thomson SC (2012) Renal function in diabetic disease models: the tubular system in the pathophysiology of the diabetic kidney. *Annu Rev Physiol* 74:351–375
48. Vallon V, Blantz RC, Thomson S (2003) Glomerular hyperfiltration and the salt paradox in early [corrected] type 1 diabetes mellitus: a tubulo-centric view. *J Am Soc Nephrol* 14:530–537
49. Miller JA (1997) Renal responses to sodium restriction in patients with early diabetes mellitus. *J Am Soc Nephrol* 8:749–755
50. Thomson SC, Rieg T, Miracle C et al (2012) Acute and chronic effects of SGLT2 blockade on glomerular and tubular function in the early diabetic rat. *Am J Physiol Regul Integr Comp Physiol* 302:R75–R83