Short Communication

Effect of acute hyperglycaemia on selected plasma and urinary cytokine antagonists in Type 1 diabetes mellitus

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Abstract

Aims/hypothesis. Several cytokines have been implicated in the pathogenesis of diabetic nephropathy. Their ability to generate a biological response in vivo is modulated by specific antagonists and soluble receptors. The aims of the study were firstly, to measure the interleukin 1 receptor antagonist (IL-1ra) and tumour necrosis factor alpha soluble receptors p55 (TNFsr1) and p75 (TNFsr2) in plasma and urine, and secondly to test their response to acutely induced hyperglycaemia in Type 1 diabetes mellitus (DM 1).

Methods. Plasma concentrations and urinary excretions of IL-1ra, TNFsr1 and TNFsr2 were measured in two 90-min periods of glycaemic clamp-induced normoglycaemia and hyperglycaemia (5 and 12 mmol/l; study 1) and during time-controlled normoglycaemia (5 and 5 mmol/l; study 2) in 20 Type 1 diabetic patients with normal albumin excretion and normal glomerular filtration rate, and in 11 weight-, age- and sex-matched healthy control subjects.

Results. The plasma concentrations of IL-1ra, TNFsr1 and TNFsr2 were comparable in Type 1 diabetic patients and control subjects, and no significant changes during study 1 and study 2 were found. Urinary IL-1ra excretion measured during normoglycaemia was high-

er in Type 1 diabetic patients compared to control subjects ($p < 0.05$). In diabetic patients, it decreased in study 1 compared to study 2 ($p < 0.05$), while it did not change in control subjects. The urinary excretions of TNFsr1 and TNFsr2 during normoglycaemia were comparable in diabetic patients and controls. In diabetic patients, hyperglycaemia decreased TNFsr1 excretion (study 1 vs study 2; $p < 0.01$), while TNFsr1 excretion did not change in control subjects. Hyperglycaemia did not affect TNFsr2 excretion in diabetic patients, while it led to an increase in TNFsr2 excretion in control subjects (study 1 vs study 2; $p < 0.05$). Despite comparable renal haemodynamics, diabetic patients had lower fractional excretion of sodium compared to control subjects ($p < 0.01$). No significant relationships between cytokine antagonists and renal functions have been found.

Conclusion/interpretation. Type 1 diabetic patients with normal renal haemodynamics are associated with impaired regulation of renal IL-1ra, TNFsr1 and TNFsr2 production with a potential impact on local control of cytokine activity in the kidneys. [Diabetologia (2003) 46:470–474]

Keywords Cytokine antagonists, Type 1 diabetes mellitus, nephropathy, renal functions.

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Abbreviations: DM1, Type 1 diabetes mellitus; IL-1ra, interleukin 1 receptor antagonist; TNFsr1, tumour necrosis factor alpha soluble receptor p55; TNFsr2, tumour necrosis factor alpha soluble receptor p75; GFR, glomerular filtration rate; RPF, renal plasma flow; C_{in} , inulin clearance; C_{PAH} , para-amino-hippuric acid clearance; C_{Na} , sodium clearance; FE_{Na} , fractional excretion of sodium; IRI, plasma immunoreactive insulin.

Recent data have suggested that certain growth factors, proinflammatory cytokines and chemokines released from activated resident cells or infiltrating immune cells are involved in the pathogenesis of diabetic nephropathy [1, 2]. Being the mediators of immune and inflammatory reactions, interleukin-1 (IL-1) and tumour necrosis factor alfa (TNF α) have been shown to play a role in diabetic kidney disease [2, 3].

The ability of cytokines to generate a biological response in vivo is modulated by specific antagonists and soluble receptors. The bioactivity of TNF α is regulated by soluble TNF receptors (TNF1sr, TNF2sr); the biological responses of IL-1 are antagonised by an IL-1 receptor antagonist (IL-1ra) [4]. Experimental studies and association studies in humans indicate a potential role of TNFsr and IL-1ra in various kidney diseases such as nephrotoxic nephritis, chronic renal insufficiency [5, 6, 7] or acute rejection in kidney transplantation [8]. Furthermore, the elevation of urinary IL-1ra and TNFsr2, caused by cardiac surgery, correlated with renal proximal tubular dysfunction [9].

It is widely considered that hyperglycaemia is the primary causal factor in the development of diabetic nephropathy. However, describing exactly how high ambient glucose exerts its deleterious effect remains elusive. Recent experiments showed that high glucose and its metabolites, non-enzymatic glycation products, can stimulate the synthesis and release of a host of factors from residential renal cells [2, 3, 10].

To date, there have been no studies evaluating the concentrations of IL-1ra and TNFsr in the plasma and urine of Type 1 diabetic patients (DM 1) without signs of diabetic nephropathy, and designed to explore the effect of an acute, in vivo induced hyperglycaemia. To test the hypothesis that regulation of selected endogenous anti-inflammatory mediators is impaired in diabetes mellitus, plasma and urinary concentrations of IL-1ra, TNFsr1 and TNFsr2 and their responses to acute, in vivo, clamp-induced hyperglycaemia were measured in DM 1 and healthy subjects.

Subjects and methods

Subjects. We examined 20 DM 1 patients (mean age: 31.3 $\pm$ 6 years, BMI: 24.1 $\pm$ 2.4) with disease duration from 4 to 9 years. None had signs of nephropathy (i.e. normal albuminuria, blood pressure and serum creatinine concentration) or proliferative retinopathy as shown by routine fundoscopic examination, or clinical evidence of autonomic neuropathy. All patients were given four injections of human insulin per day (total daily insulin dose averaged 39 $\pm$ 9 IU, mean HbA_{1c} 8.6 $\pm$ 1.4%). Apart from insulin, none of the subjects received any drugs and none were on a sodium-restricted or low-protein diet. The control group (C) consisted of 11 healthy men matched in age (28.1 $\pm$ 5.1 years) and BMI (23.9 $\pm$ 1.7). The protocol was approved by the local ethics committee. The study was carried out in accordance with the Helsinki Declaration. Informed consent was obtained from all the individuals after the purpose, nature, and potential risks of the study had been explained.

Study protocol. Subjects were examined on two separate occasions: a normoglycaemic-hyperglycaemic clamp (study 1) was carried out first to be followed by time- and volume load-controlled normoglycaemic clamp (study 2) as a second test within two weeks. The investigations started in the morning at around 7:00 am after the subjects had fasted without coffee, tea and smoking, but with tap water available ad libitum, during the previous night. To ensure fasting blood glucose concentrations of up to 6 mmol/l, diabetic patients received their usual dose of bedtime insulin and additional 2 to 4 IU of short-acting insulin at 4:00 am according to blood glucose monitoring. If fasting blood glucose was above 6 mmol/l, the clamp study was postponed to another day.

Normoglycaemic-hyperglycaemic clamp (study 1). The responses of plasma and urinary IL-1ra, TNFsr1, TNFsr2 and renal haemodynamics to acute change from sustained clamp-induced normoglycaemia (period I) to sustained hyperglycaemia (period II) were measured. Each period lasted 90 min. Briefly, a teflon cannula (Venflon; Viggo, Helsingborg, Sweden) was inserted into an antecubital vein for the infusion of all test substances. A second cannula was inserted retrogradely into a wrist vein for blood sampling, and the hand was placed in a heated (65 C) box to achieve venous blood arterialisation. Insulin infusion was started at least 60 min before the study at a rate of between 0.3 and 0.8 IU per h to achieve and maintain normoglycaemia, and continued throughout the study periods in diabetic patients. Insulin was not administered in control subjects. The blood glucose concentration during period I was maintained at approx. 5 mmol/l and at 90 min, it was raised and maintained at approx. 12 mmol/l by continuous adjustment of the rate of infusion of 15% dextrose based on frequent blood glucose determinations. Furthermore, glomerular filtration rate (GFR) and renal plasma flow (RPF) were measured using the clearances of inulin (C_{in}) and para-amino-hippuric acid (C_{PAH}) calculated by standard formulas. Primed continuous infusions of polyfructosan (Inutest; Laevosan-Gesellschaft, Linz, Austria) and para-amino-hippurate (Nephrotest; Biologische Arbeitsgemeinschaft, Lich/Hessen, Germany) were started to maintain plasma concentrations of 350 and 20 mg/l, respectively. After at least 45 min equilibration, two 90-min urine collections (periods I and II) were made. All subjects drank 500 ml of nitrite, nitrate-free water at 6:00 am and 150 ml before periods I and II to ensure adequate diuresis. The subjects remained supine throughout the test, standing only to void urine. Blood samples for measuring IL-1ra, TNFsr1, TNFsr2, PAH, inulin, haematocrit, osmolality and plasma sodium were drawn at 90 and 180 min. Immunoreactive insulin (IRI) concentrations were measured at -45, 0, 45, 60, 75, 90, 94, 96, 100, 120, 150, 165, and 180 min. The concentrations of IL-1ra, TNFsr1, TNFsr2, glucose, inulin, PAH, and sodium were measured in the urine collected during periods I and II.

Time-controlled normoglycaemic clamp (study 2). To avoid the effects of time and volume load the normoglycaemic clamps using an identical protocol for urine collection and blood sampling was carried out. Blood glucose concentrations were maintained at approx. 5 mmol/l during both periods I and II. The volume of the oral water load was carefully adjusted in each subject to match individual volume load during study 1. Sodium concentration in drinking water was negligible.

Laboratory methods. Plasma glucose concentrations were measured using glucose oxidase method on a Beckman Analyzer (Beckman Instruments, Fullerton, Calif., USA). IRI was examined by radioimmunoassay using an Immunotech Insulin IRMA kit (Immunotech, Prague, Czech Republic). HbA_{1c} was

Table 1. Plasma glucose, IRI, glomerular filtration (C_{in}), renal plasma flow (CPAH) and cytokine antagonists excretions during the normoglycaemic- hyperglycaemic clamp (Study 1) and time-controlled normoglycaemia (Study 2)

		Diabetic patients (n=20)		Control subjects (n=11)	
		Period I	Period II	Period I	Period II
Plasma glucose (mmol/l)	Study 1	4.51±0.32	11.23±0.56 ^f	4.79±0.27	11.9±0.5 ^f
	Study 2	4.41±0.28	4.43±0.57	4.53±0.68	4.33±0.55
IRI (uU/ml)	Study 1	15.12±7.16 ^c	12.35±6.67 ^{c,d}	6.13±1.85	45.13±19.77 ^f
	Study 2	21.47±11.67 ^c	21.00±10.86 ^c	7.91±2.24	7.23±1.03
C _{in} (mL/s)	Study 1	1.78±0.38	1.51±0.19 ^{b,c}	1.62±0.21	1.65±0.26
	Study 2	1.55±0.34	1.52±0.33 ^d	1.52±0.35	1.60±0.19
CPAH (mL/s)	Study 1	9.76±2.74	8.70±1.65	9.38±1.98	9.79±2.25
	Study 2	8.22±1.80	8.52±1.39	9.28±2.41	9.65±1.65
IL-1ra (pg·min ⁻¹)	Study 1	8259±8401 ^b	4488±4006 ^c	1586±1123	1966±1757
	Study 2	2626±2102	2622±1925	1589±966	1681±1426
TNFsr1 (pg·min ⁻¹)	Study 1	1957±1384	925±450 ^e	1714±1136	1447±896
	Study 2	1522±730	1263±618	1275±476	1117±270
TNFsr2 (pg·min ⁻¹)	Study 1	2827±1934	1761±766 ^d	2689±1495	4033±4362
	Study 2	2422±1147	2026±942 ^d	2510±854	1577±525

Statistical significance: ^a $p < 0.05$ vs control subjects; ^b $p < 0.01$ vs control subjects; ^c $p < 0.001$ vs control subjects; ^d $p < 0.05$ vs period I; ^e $p < 0.01$ vs period I; ^f $p < 0.001$ vs period I

Table 2. Plasma concentrations of IL-1ra, TNFsr1 and TNFsr2 and their changes during hyperglycaemia (Δ Study 1) and normoglycaemia (Δ Study 2) in DM1 (n=20) and control subjects (n=11)

	IL-1ra (pg/ml)		TNFsr1 (pg/ml)		TNFsr2 (pg/ml)	
	DM 1	Control subjects	DM 1	Control Subjects	DM 1	Control subjects
Basal value	215±98	194±59	838±207	794±311	2538±421	2447±378
Δ Study 1	+53±65	-8±39	+38±24	+58±39	-88±57	-219±76
Δ Study 2	+4±67	+39±62	+37±26	-58±61	-71±32	-154±56

Δ Studies 1 and 2 are calculated as the difference between values in period I and II of the studies

measured by a Bio-Rad Haemoglobin A_{1c} Column Test (Bio-Rad Laboratories; Munich, Germany). Inulin, PAH and sodium concentration in serum and urine were analysed using routine methods. Plasma and urinary IL-1ra, TNFsr1, TNFsr2 concentrations were measured by ELISA method according to the manufacturer's instructions using a Quantikine kit (R&D System; Minneapolis, Minn., USA). The detection limit for IL-1ra was 14 pg/ml with intra-assay and inter-assay coefficients of variation of 3.1 to 6.2% and 4.4 to 6.7%, respectively. The detection limit for TNFsr1 was 1.5 pg/ml with intra-assay and inter-assay coefficients of variation of 2.7 to 6.9% and 3.7 to 8.8%, respectively. The detection limit for TNFsr2 was 1.0 pg/ml with intra-assay and inter-assay coefficients of variation of 1.6 to 2.5% and 3.5 to 5.1%, respectively.

Statistical analysis. Student's two-tailed *t* test for unpaired data was used to compare data between diabetic patients and control subjects. When the data were not normally distributed, the Mann-Whitney rank-sum test for unpaired data was used. Analysis of variance (ANOVA) with repeated measures and grouping factor was applied for comparison of data between DM 1 and C subjects and, also, for determining the effect of acute hyperglycaemia. Significance of the differences between periods I and II within each group was tested by one-way ANOVA for repeated measures. A *p* value of less than 0.05 was considered statistically significant.

Results

The plasma glucose, IRI concentrations, and parameters of renal haemodynamics are summarised in Table 1. Mean plasma glucose concentrations were comparable in DM 1 and C during studies 1 and 2. Serum IRI concentrations were higher during normoglycaemia ($p < 0.001$) and lower during hyperglycaemia ($p < 0.001$) in DM 1 compared to C. C_{in} and C_{PAH} were comparable in DM 1 and C subjects, while urine excretion of sodium (0.31±0.13 vs 0.42±0.11 mmol/min; $p < 0.05$) and fractional excretion of sodium (1.84±0.75 vs 2.46±0.78%; $p < 0.05$) were lower in DM 1 patients. Renal functions were not affected by hyperglycaemia. There were no differences in diuresis, systolic and diastolic blood pressure and glycosuria between DM 1 and C, in study 1 and 2, respectively (data not shown).

IL-1ra excretion during normoglycaemia was higher in DM 1 compared to C ($p < 0.01$). In DM 1 subjects, it decreased during study 1 compared to study 2 ($p < 0.05$), while it did not change in C. The difference between the effect of hyperglycaemia in DM 1 and C subjects was statistically significant ($p < 0.01$; by ANOVA). Uri-

nary TNFsr1 excretion during normoglycaemia was comparable in DM 1 and C subjects. Hyperglycaemia led to a decrease in TNFsr1 excretion in DM 1 (study 1 vs study 2; $p < 0.01$), while the excretion rate did not change in C. Basal urinary excretion of TNFsr2 was comparable in DM 1 and C subjects. Hyperglycaemia did not affect TNFsr2 excretion in DM 1 patients; TNFsr2 comparably decreased during study 1 ($p < 0.05$) and study 2 ($p < 0.05$) in this group while, in C, hyperglycaemia led to an increase in urinary TNFsr2 excretion (study 1 vs study 2; $p < 0.05$). The difference between the effect of hyperglycaemia in DM1 and C subjects was statistically significant ($p < 0.05$; by ANOVA).

The plasma concentrations of IL-1ra, TNFsr1 and TNFsr2 were comparable in DM 1 and C subjects (Table 2), and they were not affected by hyperglycaemia, because there were no differences between changes during study 1 and study 2 in both groups.

No relationships between cytokine antagonists and renal functions were found.

Discussion

Our study provides the *in vivo* evidence of impaired regulation of selected endogenous anti-inflammatory mediators (IL-1ra, TNFsr) in kidneys in DM 1 patients without signs of diabetic nephropathy.

The study shows higher basal urinary excretion of IL-1ra and normal TNFsr1 and TNFsr2 excretions, as measured during normoglycaemia in DM 1 compared to healthy subjects.

The second main observation is that the response of urinary TNFsr1, TNFsr2 and IL-1ra to acute, *in vivo* induced hyperglycaemia, when compared to control isovolumic and normoglycaemic conditions, is impaired in diabetic patients. We have shown the absence of any response to glucose in DM 1 patients, where increased production was expected (TNFsr2); and decreased urinary excretion, where no changes were seen in healthy control subjects (TNFsr1, IL-1ra).

It has been shown that TNFsr and IL-1ra antagonise the effects of pro-inflammatory cytokines *in vitro* [4] and the balance between mediators with anti- and pro-inflammatory properties could be an important mechanism needed to maintain limited inflammatory processes within the glomerulus. The above findings support the hypothesis that IL-1ra and TNFsr could play a role in the pathogenesis of diabetic nephropathy. However, the clinical significance is rather speculative and the corresponding agonists have not been measured. Furthermore, previous investigations have shown that a 100- to 10 000-fold molar excess of recombinant human IL-1ra relative to IL-1 is necessary to completely inhibit the physiologic and haemodynamic effects of endogenous IL-1 infusion in experimental animals [4].

We did not observe any changes in systemic IL-1ra, TNFsr1 and TNFsr2 concentrations. The plasma concentrations of the cytokine antagonists were normal in DM 1 patients and were not affected by hyperglycaemia in any group. Therefore, the local production of mediators in the kidney is probably responsible for the changes in urinary excretion of cytokine antagonists.

Finally, no relationships between TNFrs and IL-1ra excretions and renal functions have been found in our study. As GFR and RPF were not altered in our DM1 patients, we can exclude the confounding effect of impaired renal function on systemic and urinary IL-1ra and TNFsr concentrations [5, 6, 9].

The studies were carefully carried out to assure a comparable water load, diuresis and blood glucose concentrations. Plasma insulin concentrations were not maintained at comparable concentrations throughout the studies. The DM 1 patients required higher concentrations of plasma insulin to maintain normoglycaemia while, on the other hand, C had increased insulin concentrations during hyperglycaemic periods due to stimulation of endogenous insulin secretion by hyperglycaemia. Although direct effects of insulin on the expression and production of TNFsr and IL-1ra have not been studied, we cannot exclude the potential confounding role of insulin in our results.

In summary, this study conducted in DM 1 patients without signs of diabetic nephropathy and in healthy subjects firstly, shows that DM 1 patients, despite no alterations of plasma concentrations, show higher basal urinary excretion of IL-1ra and normal TNFsr1 and TNFsr2 excretion, as measured during normoglycaemia when compared to control isovolumic and normoglycaemic conditions, and secondly, provides the first *in vivo* evidence of impaired regulation of IL-1ra, TNFsr1 and TNFsr2 in the kidney by acute, clamp-induced hyperglycaemia in DM 1 patients.

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