CLINICAL STUDY

The tumour necrosis factor (TNF)- α system is activated in accordance with pulse pressure in normotensive subjects with type 1 diabetes mellitus

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Abstract

Objective: Pulse pressure (PP) and inflammation are important predictors of cardiovascular disease (CVD), even in the normotensive. The age-related increase in PP can be diagnosed up to 20 years earlier in subjects with type 1 diabetes mellitus (T1DM) than in the general population. Some evidence suggests that PP can stimulate inflammation. Our aim was to study the relationship between PP and plasma inflammatory proteins in normotensive subjects with T1DM.

Design: This was a cross-sectional study of a group of normotensive (<140/80 mmHg) subjects diagnosed with T1DM 14 years before. None of them had clinically proven CVD or inflammatory conditions or were on antiplatelet, antihypertensive, anti-inflammatory or lipid-lowering treatment.

Methods: The following information was recorded: sex, age, body-mass index (BMI), waist-to-hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), PP, mean blood pressure (MBP), smoking, alcohol intake, insulin dose, lipid profile, HbA1c, microvascular complications, and plasma concentrations of soluble receptor types 1 and 2 of tumour necrosis factor (TNF)- α (sTNFR1 and sTNFR2, respectively), interleukin-6, C-reactive protein, adiponectin and leptin.

Results: A total of 112 subjects were evaluated (aged 27.4 ± 6.6 years, 52.7% women, BMI: 20.4 ± 2.7 kg/m², WHR: 0.82 ± 0.09 , SBP: 112 ± 12 mmHg, DBP: 68 ± 9 mmHg, PP: 45 ± 9 mmHg, MBP: 82 ± 9 mmHg, HbA1c: 8.2% (7.3–9.0%), 41.1% microvascular complications). After adjusting for potential confounders, only inflammatory markers of the TNF- α system correlated significantly with PP (Pearson correlation coefficient between sTNFR1 and PP: $r = 0.215$, $P = 0.030$; and between PP and sTNFR2: $r = 0.238$, $P = 0.020$).

Conclusion: In normotensive subjects with T1DM after 14 years of diagnosis, the activation of the TNF- α system is positively associated with PP levels. This finding might suggest a pathogenic role of the TNF- α system in the development of cardiovascular disease in T1DM.

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Introduction

Subjects with type 1 diabetes mellitus (T1DM), even those younger than 30 years, have an extra risk of cardiovascular disease (CVD) (1, 2). Inflammation has recently been recognized as a predictor of CVD (3), and CVD has also been associated with the score of three inflammatory markers (tumour necrosis factor (TNF)- α , interleukin-6 (IL-6) and C-reactive protein (CRP)) in subjects with T1DM (4). In addition, pulse pressure (PP), which increases with ageing as a consequence of arterial stiffness (5), is another predictor of CVD (6). In particular, it is a predictor of CVD mainly over the age of 50 years (7), even in normotensive

subjects (8). In subjects with T1DM, the age-related increase in PP levels can be diagnosed up to 20 years before, even in subjects without nephropathy (9), and it is also associated with CVD (10). In addition, in young subjects with T1DM, an increase in arterial stiffness has also been described (11, 12), suggesting a pathogenic role in the early increase of PP in T1DM.

There is some evidence that an increase in PP may stimulate inflammation, suggesting that one of the mechanisms underlying the link between elevated PP and increased CVD risk may be inflammation (13–15). If the link between PP and CVD risk is indeed mediated by inflammation and operates in subjects with T1DM, one would expect to see positive

associations between PP and markers of systemic inflammation that are predictive of CVD risk, such as TNF- α , IL-6 or CRP. To gain some insight into the role of inflammation and PP in the pathogenesis of CVD in T1DM, we thought it worthwhile to analyse their relationship in a group of normotensive subjects with T1DM after 14 years of evolution. In addition to the above-mentioned inflammatory markers previously associated with CVD, we also explored the relationship between PP and other plasma proteins, which have more recently been associated positively (leptin) (16) or negatively (adiponectin) with CVD (17).

Subjects and methods

Subjects

A group of normotensive (<140/80 mmHg) subjects with T1DM, all having been diagnosed 14 years before (18), were recruited. None of them had any condition that increased plasma inflammatory markers (such as acute or chronic inflammatory, or infectious, diseases) or had received antiplatelet, antihypertensive, anti-inflammatory or lipid-lowering treatment. None of them had clinically proven CVD (angina pectoris, myocardial infarction, intermittent claudication, amputation or stroke). The study protocol was approved by the local ethics committee and was conducted according to the principles of the Declaration of Helsinki. All subjects gave their informed consent before participating in the study and were evaluated after an overnight fast and before administering the morning insulin injection. Venous blood samples were drawn, and aliquots of plasma and serum were stored at -70°C until processing. The following information was collected with a predefined, standardized form: sex, age, body-mass index (BMI), waist-to-hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), PP, mean blood pressure (MBP), smoking, alcohol intake, insulin dose, lipid profile, HbA1c, microvascular complications, and plasma concentrations of soluble receptor types 1 and 2 of TNF- α (sTNFR1 and sTNFR2 respectively), IL-6, CRP, adiponectin and leptin.

Anthropometric measurements

Height was measured to the nearest 0.5 cm and body weight to the nearest 0.1 kg. BMI was calculated as weight (in kg)/height (in m^2). Waist circumference was measured midway between the lower rib margin and the iliac crest. Hip circumference was determined as the widest circumference measured over the greater trochanter. WHR was then calculated. After subjects rested for at least 5 min, blood pressure was measured twice in a sitting position with a mercury sphygmomanometer. SBP was recorded at phase I and DBP at phase V of Korotkoff sounds. The mean of the two recordings was used in the study. PP was calculated

as the difference between SBP and DBP, and MBP as one-third of SBP plus two-thirds of DBP.

Analytical methods

HbA1c was determined in capillary blood by immunoassay (DCA 2000, Bayer AG, Zurich, Switzerland). Total serum cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol were measured by standard enzymatic methods. Low-density lipoprotein (LDL) cholesterol was estimated by the Friedewald formula (19). Plasma sTNFR1 and sTNFR2 were determined by a solid-phase, enzyme-amplified, sensitivity immunoassay (Medgenix sTNFR1-EASIA, sTNFR2-EASIA; BioSource Europe, Fleurus, Belgium); plasma IL-6 by an ultrasensitive, solid-phase enzymeimmunoassay (Biosource, Nivelles, Belgium); plasma CRP by a highly sensitive immunonephelometry kit (Dade Behring, Marburg, Germany); and plasma adiponectin and leptin by RIA (Linco Research, St Charles, MO, USA).

Assessment of microvascular complications

The assessment of all microvascular complications was blind to the clinical characteristics of the subjects. Retinal photographs of the macular field were taken of each eye with a non-mydratic retinal camera (CR6-45NM; Canon, Lake Success, NY, USA) by an experienced endocrinologist. An ophthalmologist assessed all these photographs and classified them into three categories according to the degree of retinopathy in the worst eye: no retinopathy, non-proliferative retinopathy and proliferative retinopathy. Proliferative retinopathy was defined as the presence of new vessels, fibrous proliferations, preretinal haemorrhage, vitreous haemorrhage or photocoagulation scars. Any other lesion was classified as a non-proliferative retinopathy. The level of urinary albumin excretion was evaluated as previously recommended (20), using urinary samples obtained during the study. Subjects with a ratio greater than 30 mg albumin/g creatinine were considered to have diabetic nephropathy.

To establish the presence of diabetic peripheral neuropathy, we used a two-step protocol. The first step consisted of a 15-item MNSI questionnaire (21). The second step consisted of a quantitative neurological examination of the lower extremities (22), including assessment of the following: 1. pressure perception using a 5.07/10 g Semmes-Weinstein monofilament; 2. vibratory perception using a 128 Hz tuning fork with the on-off method; 3. superficial pain sensation with a sterile tip; 4. ankle and knee reflexes. The presence of diabetic peripheral neuropathy was defined by a MNSI questionnaire score of >7 plus an alteration in any of these four neurological examinations. Cardiovascular autonomic neuropathy was studied by the following three tests: 1. heart rate variation in response to deep breathing; 2. heart rate variation in response to

the Valsalva manoeuvre; 3. SBP changes on standing after resting in the horizontal position for at least 5 min (23). The response to each of these tests was categorized as normal, borderline, or abnormal by a portable computerized system (Cardionomic; Medimatica, Martinsicuro, Italy) (24). Cardiovascular autonomic neuropathy was defined as an abnormality in at least one of these three tests.

Statistical analyses

To improve skewness and kurtosis, variables not normally distributed were log transformed. Multiple linear regression analyses were carried out to assess the relationships between blood pressure indexes and inflammatory markers, adjusting for potential confounders. The SPSS/PC + statistical program (Version 11.5 for Windows; Chicago, IL, USA) was used. All *P* values were two-sided, and a *P* value of <0.05 was considered statistically significant.

Results

A total of 112 subjects were evaluated, 46 of them with one or more microvascular complications. Their main clinical and analytical characteristics are shown in Table 1. Table 2 shows the unadjusted and adjusted

Table 1 Clinical characteristics of the subjects with type 1 diabetes mellitus (T1DM) included in the study.

	Subjects with T1DM (<i>n</i> = 112)
Sex (% of women)	52.7
Age (years)	27.4±6.6
BMI (kg/m ²)	20.4±2.7
Waist-hip ratio	0.82±0.09
Systolic blood pressure (mmHg)	112±12
Diastolic blood pressure (mmHg)	68±9
Pulse pressure (mmHg)	45±9
Median blood pressure (mmHg)	82±9
Current smokers (%)	68.9
Current alcohol intake > 20 g/day (%)	21.4
Insulin dose ((IU/day)	49.7±16.3
Cholesterol (mmol/l)	4.92±0.8
Triglycerides (mmol/l)	1.46±0.38
HDL cholesterol (mmol/l)	0.84 (0.62–1.17)
LDL-cholesterol (mmol/l)	3.02±0.69
HbA1c (%)	8.2 (7.3–9.0)
Retinopathy	16.1
Nephropathy	3.6
Peripheral neuropathy	25.0
Cardiovascular autonomic neuropathy	6.3
sTNFR1 (ng/ml)	2.09±0.69
sTNFR2 (ng/ml)	4.27±1.14
IL-6 (pg/ml)	0.84 (0.60–1.25)
hsCRP (mg/dl)	0.95 (0.46–2.49)
Adiponectin (mg/l)	11.11 (8.47–15.92)
Leptin (ng/ml)	10.85±7.44

All values are given as percentages, means±s.d. or medians (interquartile range).

correlations between blood pressure indexes and plasma inflammatory proteins. Of all the evaluated inflammatory proteins, only those associated with the activation of the TNF- α (sTNFR1 and sTNFR2) were associated with some of the evaluated blood pressure indexes. In particular, after adjusting for potential confounders, we found a positive relationship between sTNFR1 and PP ($r = 0.215$; $P = 0.030$). This relationship would be explained mainly through the positive relationship between sTNFR1 and SBP, since SBP and PP were obviously highly correlated ($r = 0.656$; $P = 0.001$). In addition, we also found a positive relationship between sTNFR2 and PP ($r = 0.238$; $P = 0.020$), although in this case this association would not be explained by a positive relationship between sTNFR2 and SBP ($r = 0.091$; $P = 0.372$).

Discussion

To our knowledge, this is the first study showing that PP is associated with activation of the TNF- α system in normotensive subjects with T1DM. This association remains unchanged after adjusting for potential confounders, such as microvascular complications (including nephropathy). In addition, no other pro-inflammatory (IL-6, CRP, leptin) or anti-inflammatory (adiponectin) plasma proteins are associated with any of the evaluated blood pressure indexes in these subjects.

The main finding of the present study is that PP is associated with an activation of the TNF- α system, measured through the plasma concentrations of sTNFR1 and sTNFR2. It is thought that sTNFR1 and sTNFR2 may better reflect longer-term average levels of TNF- α than TNF- α itself (25, 26). In contrast to sTNFR1, sTNFR2 is more closely associated with the metabolic effects of TNF- α (27). In this study, both sTNFR1 and sTNFR2 were positively associated with PP levels. The association between sTNFR1 and PP would be explained mainly through the systolic component of PP, because a positive relationship was also found between sTNFR1 and SBP. However, this was not the case in the association between sTNFR2 and PP, because no significant relationship was found between sTNFR2 and SBP. There is some evidence that increases in PP may stimulate inflammation. In particular, an increase in PP is associated with elevated levels of reactive oxygen species (13), which in turn may stimulate inflammatory signalling pathways (14, 15). In addition, higher levels of PP are associated with greater flow reversals during diastole (28), and flow reversals can increase the expression of adhesion molecules (29), which would promote the inflammatory atherosclerotic process.

Apart from CRP, no other inflammatory proteins have previously been evaluated in relation to PP. TNF- α stimulates the synthesis of CRP (30). In apparently healthy adults, elevated non-high-sensitivity CRP plasma concentrations are associated with an increase in PP,

Table 2 Pearson correlation coefficients between pressure indexes (SBP, DBP, PP and MBP) and evaluated inflammatory markers (sTNFR1, sTNFR2, IL-6, CRP, adiponectin and leptin), unadjusted (first line of each cell), adjusted only for age and sex (second line of each cell) and adjusted for age, sex, BML, smoking alcohol intake, insulin dose, HDL cholesterol, HbA1c, retinopathy, albumin excretion rate, diabetic peripheral neuropathy and cardiovascular autonomic neuropathy (third line of each cell). Only sTNFR1 and sTNFR2 were positively associated with PP levels.

	SBP	DBP	PP	MBP	sTNFR1	sTNFR2	IL-6	CRP	Adiponectin	Leptin
SBP	1.0	0.500*	0.634*	0.788*	0.207	0.232	-0.137	-0.133	-0.261	-0.093
		0.565*	0.647*	0.827*	0.153	0.096	-0.039	-0.058	0.009	0.121
		0.523*	0.656*	0.808*	0.219	0.091	-0.026	-0.075	0.090	0.028
DBP		1.0	0.334 [†]	0.927*	-0.049	-0.014	0.100	0.054	-0.145	0.043
			-0.249 [†]	0.931*	0.016	-0.110	0.087	0.004	-0.016	0.120
			-0.285*	0.924*	0.042	-0.141	0.153	-0.007	-0.013	0.058
PP			1.0	0.034	0.259	0.273 [†]	-0.206	-0.170	-0.132	-0.086
				0.114	0.175	0.224 [†]	-0.112	-0.064	0.034	0.049
				0.094	0.215 [†]	0.238 [†]	-0.148	-0.075	0.127	0.004
MBP				1.0	0.055	0.089	0.010	-0.021	-0.219	-0.017
					0.075	-0.034	0.043	-0.023	-0.009	0.132
					0.124	-0.058	0.095	-0.038	0.028	0.049
sTNFR1					1.0	0.534*	0.014	0.029	0.098	-0.013
						0.384*	0.122	0.160	0.049	0.047
						0.349*	0.138	0.162	-0.014	0.138
sTNFR2						1.0	0.113	0.064	0.110	0.000
							0.128	0.152	0.152	-0.004
							0.138	0.176	0.135	0.048
IL-6							1.0	0.432*	-0.180	0.045
								0.413*	-0.252 [†]	0.139
								0.414*	-0.224	0.162
CRP								1.0	-0.119	0.115
									-0.133	0.077
									-0.104	0.031
Adiponectin									1.0	0.106
										-0.006
										0.163
Leptin										1.0

* $P < 0.01$; [†] $P < 0.05$.

regardless of SBP and DBP (31). However, we did not find such an association. Our study was performed in a group of subjects with T1DM with little heterogeneity regarding their atherosclerotic burden (14 years of evolution, normotensive, no clinical CVD). Under these circumstances, any possible association between PP and inflammation would tend to be underestimated. However, we cannot exclude that our results may reflect real pathophysiological differences in how these inflammatory proteins relate to CVD in early stages of the atherosclerotic process. Nevertheless, the activation of the TNF- α system seems to be a risk factor for CVD (3, 32, 33) and several pathogenic mechanisms have been suggested in this respect (33–35).

Although the findings of the present study suggest that an increase in PP may activate the TNF- α system, other explanations are possible. Firstly, the present study was based on observational data, and confounding from factors that were not controlled for, and/or residual confounding from factors that we did control for but measured inaccurately, may be an alternative explanation for our results. Secondly, the cross-sectional design of the present study makes it impossible to determine the temporal ordering of the association that we observed between PP and the activation of the TNF- α

system. Although the above-mentioned evidence suggests that increases in PP would enhance inflammation, we cannot rule out inflammation as a mechanism to increase PP, as has been previously suggested (36). Nevertheless, the present study suggests that mechanisms linking PP and inflammation already operate in normotensive subjects with T1DM, and that the inflammatory mechanism mainly implicated is the activation of the TNF- α system.

In summary, in normotensive subjects with T1DM 14 years after diagnosis, an increase in PP levels is associated with an activation of the TNF- α system. This finding might specifically suggest a pathogenic role of TNF- α system in CVD in T1DM, and argues for future prospective studies to assess the involvement of PP and the TNF- α system in the pathogenesis of CVD in T1DM.

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