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ARTICLE

The methylglyoxal-derived AGE tetrahydropyrimidine is increased in plasma of individuals with type 1 diabetes mellitus and in atherosclerotic lesions and is associated with sVCAM-1

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Abstract
Aims/hypothesis Methylglyoxal (MGO) is a major precursor for advanced glycation end-products (AGEs), which are thought to play a role in vascular complications in diabetes. Known MGO-arginine-derived AGEs are 5-hydro-5-methylimidazolone (MG-H1), argpyrimidine and tetrahydropyrimidine (THP). We studied THP in relation to type 1 diabetes, endothelial dysfunction, low-grade inflammation, vascular complications and atherosclerosis.

Methods We raised and characterised a monoclonal antibody against MGO-derived THP. We measured plasma THP with a competitive ELISA in two cohort studies: study A (198 individuals with type 1 diabetes and 197 controls); study B (individuals with type 1 diabetes, 175 with normoalbuminuria and 198 with macroalbuminuria [>300 mg/24 h]). We measured plasma markers of endothelial dysfunction and low-grade inflammation, and evaluated the presence of THP and N^{ε}-(carboxymethyl)lysine (CML) in atherosclerotic arteries.

Results THP was higher in individuals with type 1 diabetes than in those without (median [interquartile range] 115.5 U/μl [102.4–133.2] and 109.8 U/μl [91.8–122.3], respectively; $p=0.03$). THP was associated with plasma soluble vascular cell adhesion molecule 1 in both study A (standardised $\beta=0.48$ [95% CI 0.38, 0.58]; $p<0.001$) and study B (standardised $\beta=0.31$ [95% CI 0.23, 0.40]; $p<0.001$), and with secreted phospholipase A2 (standardised $\beta=0.26$ [95% CI 0.17, 0.36]; $p<0.001$) in study B. We found no association of THP with micro- or macrovascular complications. Both THP and CML were detected in atherosclerotic arteries.

Conclusions/interpretation Our results suggest that MGO-derived THP may reflect endothelial dysfunction among individuals with and without type 1 diabetes, and therefore may potentially play a role in the development of atherosclerosis and vascular disease.

Keywords Advanced glycation end-products · Atherosclerosis · Cardiovascular disease · Endothelial dysfunction · Macrovascular complications · Methylglyoxal · Microvascular complications · Soluble vascular cell adhesion molecule · Tetrahydropyrimidine · Type 1 diabetes mellitus

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Abbreviations

AGE	Advanced glycation end-product
CEL	N^{ε}-(Carboxyethyl)lysine
CML	N^{ε}-(Carboxymethyl)lysine
CVD	Cardiovascular disease
eGFR	Estimated GFR
HSA	Human serum albumin
hsCRP	High-sensitivity C-reactive protein
KLH	Keyhole limpet haemocyanin
MDRD	Modification of Diet in Renal Disease
MG-H1	5-Hydro-5-methylimidazolone
MGO	Methylglyoxal
Sβ	Standardised β
sICAM-1	Soluble intercellular adhesion molecule 1
sPLA2	Secreted phospholipases A2
sVCAM-1	Soluble vascular cell adhesion molecule 1
THP	Tetrahydropyrimidine
UPLC	Ultra performance liquid chromatography
vWF	von Willebrand factor

Introduction

The pathogenesis of vascular complications in type 1 diabetes is thought to involve damaging effects of advanced glycation end-products (AGEs) on vascular tissues [1, 2]. Methylglyoxal (MGO), which accumulates rapidly under hyperglycaemic conditions, has been demonstrated to be the most important precursor in the formation of AGEs [3, 4]. It is mainly formed by dephosphorylation and conversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. MGO is detoxified to D-lactate by the glyoxalase pathway [5, 6]. MGO primarily reacts with arginine residues in proteins to form three products: the non-fluorescent products 5-hydro-5-methylimidazolone (MG-H1) and tetrahydropyrimidine (THP), and the major fluorescent product, argpyrimidine [7]. Plasma concentrations of MGO [8], as well as MG-H1 [9] and argpyrimidine [10], are elevated in individuals with diabetes, and are associated with complications of diabetes [7, 11]. So far, such data on THP are lacking. THP is formed rapidly after incubation of MGO with arginine, following a similar pattern to MG-H1 [12, 13]. Since different MGO-derived AGEs may have different pathophysiological consequences, it is important to study the potential role of THP in type 1 diabetes and its complications.

MGO may exert detrimental effects on cellular function via intracellular modifications of proteins and changes in protein structure, function or activity [1]. We recently showed that MGO reduces endothelium-dependent vasodilatation in isolated arteries [14], providing a new mechanistic link between MGO and endothelial dysfunction. It has been demonstrated that the modification of proteins by MGO results in increased formation of reactive oxygen species [15–17]; increased expression of adhesion molecules (e.g. vascular cell adhesion molecule 1 [VCAM-1] and intracellular adhesion molecule 1 [ICAM-1]) and intra-growth factors; and sensitisation of cells to the effects of proinflammatory cytokines [18], i.e. early events in the initiation of atherosclerosis.

Although these studies [1, 3, 4, 7–10, 14–18] indicate that MGO is involved in the pathophysiology of vascular complications, the formation of MGO-modified proteins and their relationship in the development of complications require further investigation. Therefore, we raised and characterised an antibody against MGO-derived AGEs that preferentially binds THP over MG-H1 and argpyrimidine. Using the antibody, we first examined whether plasma levels of THP are elevated in type 1 diabetes. Second, we evaluated the association of plasma THP with markers of endothelial dysfunction and low-grade inflammation. Third, we examined if there was an association of THP with micro- and macrovascular complications in type 1 diabetes. Fourth, we used this antibody to evaluate the presence of THP in human coronary arteries.

Methods

Preparation of anti-MGO antibodies

MGO-modified keyhole limpet haemocyanin (MGO-KLH) was prepared by the reaction of MGO (10 mmol/l) with KLH for 7 days at 37°C and used as antigen for the immunisation of mice. MGO-KLH was emulsified in an equal volume of Freund's complete adjuvant; three mice were intradermally injected at multiple sites. These mice were boosted with the same amount of MGO-KLH emulsified in Freund's incomplete adjuvant 21 days later, and antisera obtained 14 days after the booster were tested. The booster was repeated twice. Ten days after the final booster, antisera were tested with MGO-albumin and the mouse with the highest titre was used for fusion. We obtained 40 positive clones as tested with MGO-albumin; one of them was further characterised. For the characterisation of the recognition epitope of the antibody, argpyrimidine, MG-H1 and THP were synthesised as described [7, 19].

Preparation of MGO-albumin

Human serum albumin (HSA) glycated by MGO was prepared by incubation of HSA (6.8 mg/ml) with MGO (0.5 mol/l) in PBS at 37°C for 0–8 days. After the incubations, the reaction mixtures were extensively dialysed against PBS at 4°C with three changes of solution. The reagents were divided into aliquots and stored at −20°C.

MGO-albumin ELISA

In a competitive ELISA, performed at room temperature, each well was coated with 1 μg minimally modified MGO-albumin in PBS for 1 h at room temperature. Minimally modified MGO-albumin was prepared by incubation of HSA (6.8 mg/ml) with MGO (0.5 mol/l) in PBS at 37°C for 2 days. The wells were washed twice with PBS. Each well was then blocked with 150 μl 1% BSA in PBS for 1 h. Wells were then washed three times with PBS containing 0.05% Tween 20 (PBS–Tween). To each well were added 50 μl of the anti-MGO antibody conjugated with biotin (1:2,000) and 50 μl of standard minimally modified MGO-albumin or a plasma sample to be tested diluted and incubated for 2 h. After three washes with PBS–Tween, the wells were incubated with streptavidin–horseradish peroxidase (CLB, Amsterdam, The Netherlands) for 1 h. Finally, the wells were washed five times with PBS–Tween, and the substrate was developed with 100 μl tetramethylbenzidine. The absorbance at 450 nm was measured with a multichannel spectrophotometer (SLT Microplate Reader, Wilten Bioteknika, Etten-Leur, The Netherlands). Plasma levels were expressed as MGO-albumin units (U/ml), and one U was defined as the antibody-reactive material equivalent to 1 μg of the MGO-albumin standard. The intra- and inter-assay variations were 5% and 8%, respectively.

Cohort study A

In 1998, a random sample of 199 men and women with type 1 diabetes aged 30–55 years was taken from the diabetes registers of five London hospitals. Type 1 diabetes was defined by age of onset ≤25 years and insulin treatment within 1 year of diagnosis. A random sample of 201 individuals from the general population, stratified to have a similar age and sex distribution to the individuals with diabetes, was drawn from the lists of two London general practices. It was confirmed that these controls had no clinical history of diabetes and were not receiving any treatment for diabetes. Individuals were included regardless of any history of heart disease. One participant (a woman with diabetes) had a history of angina; none had previously had a myocardial infarction. Pregnant women and individuals receiving renal replacement therapy were excluded. Retinopathy and neuropathy were self-reported via a standardised questionnaire. Details of this study have been described previously [20].

Laboratory methods Urinary albumin was measured with an immunoturbidimetric method (intra-assay CV 2.3%). Normoalbuminuria was defined as a urinary albumin excretion rate (AER) of <20 μg/min, microalbuminuria as an AER of 20–200 μg/min, and macroalbuminuria as an AER of >200 μg/min, in two 24 h urine collections. GFR was estimated (eGFR) according to the short Modification of Diet in Renal Disease (MDRD) equation: eGFR = 186 × [serum creatinine] (mg/dl)$^{-1.154}$ × age$^{-0.203}$ × 0.742 (if patient is female) [21]. High-sensitivity C-reactive protein (hsCRP), a marker of low-grade inflammation, was measured with a highly sensitive in-house ELISA, as described previously [22]. A commercially available ELISA kit was used to measure plasma soluble (s)VCAM-1 (R&D Systems Europe, Abingdon, UK). von Willebrand factor (vWF) activity, a marker of endothelial dysfunction, was measured in heparin plasma with a Shield vWF activity ELISA kit (Shield Diagnostics, Dundee, Scotland, UK) using IgG monoclonal antibodies, and expressed as percentage of vWF in pooled plasma of healthy volunteers. Levels of MGO-derived THP were measured in 198 individuals with and 197 without type 1 diabetes.

Cohort study B

In 1993, 199 individuals with type 1 diabetes and diabetic nephropathy, defined according to clinical criteria (i.e. persistent macroalbuminuria [>300 mg/24 h] in at least two out of three previous consecutive 24 h urine collections, in the presence of diabetic retinopathy, and in the absence of other kidney or urinary tract disease), and 192 individuals with type 1 diabetes and persistent normoalbuminuria (i.e. urinary AER <30 mg/24 h) were recruited from the outpatient clinic at Steno Diabetes Center for a prospective observational follow-up study. Details of the inclusion criteria and selection procedures have been described elsewhere [23].

Examination Diabetic retinopathy was assessed in all patients at baseline by fundus photography after pupillary dilatation and graded as nil, simplex or proliferative retinopathy. Any history of acute myocardial infarction or stroke was considered to be cardiovascular disease (CVD) at baseline.

Laboratory methods Urinary albumin concentration was measured by an enzyme immunoassay from 24 h urine collections. eGFR was estimated according to the MDRD equation [21]. Levels of hsCRP were determined by enzyme immunoassays (normal range 0.13–3.0 mg/l) as described previously [24]. Commercially available ELISA kits (R&D Systems Europe, Abingdon, UK) were used to measure markers of endothelial dysfunction: plasma sVCAM-1 (range for assay 538–1,286 ng/ml), sICAM-1 (range 98–647 ng/ml) and low-grade inflammation (IL-6, secreted phospholipase A2 [sPLA2] and total TGF-β1). The AGEs, N^{ε}-(carboxyethyl)lysine (CEL) and N^{ε}-(carboxymethyl)lysine (CML), were measured by ultra performance liquid chromatography tandem MS (UPLC–MS/MS), and pentosidine was measured by HPLC with fluorescence detection, as described previously [2]. Levels of the

MGO-derived AGE, THP, were measured in 175 individuals with normoalbuminuria and 198 individuals with macroalbuminuria and retinopathy. All measurements were performed on blood and urine samples collected at baseline.

Immunohistochemistry

Materials and processing of tissue specimens Histological specimens of coronary arteries were obtained from human autopsies of individuals who had died from non-cardiovascular causes in a hospital-based setting. We included 12 controls, three individuals with type 1 diabetes, and ten with type 2 diabetes. The specimens were routinely fixed with 4% formalin and subsequently embedded in paraffin. Serial paraffin-embedded vascular tissue sections (4 μm) were mounted on microscope slides and deparaffinised for 10 min in xylene at room temperature and rehydrated through descending concentrations of ethanol.

Immunohistochemical detection of THP, CML and CD68 in serial sections For staining with THP antibody, the sections were preincubated in 0.01 mol/l citrate, pH 6, at 37°C for 10 min. For CML and CD68 staining, the sections were preincubated in 0.1% pepsin with HCl. Thereafter, sections were incubated for 40 min with THP antibody (1:12.5 dilution), CML antibody [25] (1:4,000 dilution) and CD68 antibody specific for macrophages (dilution 1:1,000; Dako, Glostrup, Denmark) at room temperature. After being washed in PBS, sections were incubated for 40 min with labelled Polymer (Envision system K4007; Dako, Glostrup, Denmark) at room temperature and subsequently washed in PBS. Sections were then incubated for 5 min with liquid diaminobenzidine + substrate chromogen solution. Finally the sections were stained with haematoxylin, to visualise the cell nuclei.

Quantification of staining To quantify the amount of staining of THP and CML, two independent observers scored each specimen from 0 to 4:0 when there was no staining in the plaque or thickened intima; 4 when staining was abundant. The mean score of the two observers per specimen was used for analyses.

All studies were approved by the local ethics committee, and all participants gave their written informed consent [20, 23].

Statistical methods

Analyses were carried out with SPSS version 17 for Windows. Variables with a skewed distribution were log-transformed before further analyses. Comparisons of baseline characteristics between groups were performed with Student's t or χ^2 tests. All biomarkers were analysed by use of z scores (i.e. [individual values − sample's mean]/sample's SD). We used multiple linear regression analyses to evaluate the associations of THP and other AGEs with type 1 diabetes or markers of endothelial function and low-grade inflammation. Multiple logistic regression analysis was used to evaluate the associations of THP with microvascular complications, i.e. nephropathy, neuropathy, retinopathy and macrovascular complications. For analyses based on the immunohistochemical data, we used Pearson's χ^2 test to evaluate possible differences in percentage of stenosis between groups. We used the independent-samples Kruskall–Wallis test to evaluate possible differences in THP or CML staining between groups. A p value of <0.05 was considered significant.

Results

Characterisation of anti-MGO-derived AGEs

MGO-KLH was used as antigen for the immunisation of mice. We obtained 40 positive clones as tested with MGO-albumin, and one of them, a monoclonal antibody from the IgG1 subclass, was further characterised. This antibody reacted with MGO-modified albumin but not with glyoxal-modified albumin, 3-deoxyglucosone-modified albumin or well-known AGEs such as CML and pentosidine (Fig. 1a). The epitope of the anti-MGO antibody was studied using a competitive ELISA. Figure 1b shows the reactivity of the monoclonal anti-MGO antibody with THP with an at least 1,000-fold preference for THP over argpyrimidine or MG-H1. For further validation of our test system, comparable competition experiments were performed with two well-known specific monoclonal antibodies against argpyrimidine [7] and MG-H1 [9], which demonstrated that the epitopes of these monoclonal antibodies were indeed argpyrimidine (Fig. 1c) and MG-H1 (Fig. 1d), respectively, confirming the specificity of our test system. Our antibody was further characterised by immunoblotting. Analysis of MGO-derived arginine residues in minimally modified MGO-albumin showed that the THP epitope was detectable after 1 day, reached an apparent optimum at 2 days, and declined steadily thereafter, suggesting further chemical rearrangements that are less well recognised by our antibody (Fig. 1e). In the minimally modified MGO-albumin preparation, the argpyrimidine epitope was formed only after 12 h of incubation and further increased in time (Fig. 1f). MG-H1 formation occurred rapidly in the initial hours and attained a maximum value in the subsequent 18 h (Fig. 1g). Over the next 8 days, however, there was a further slow increase in MG-H1 concentration.

Association of type 1 diabetes with THP

Table 1 shows the general characteristics of cohort study A and B. In study A, type 1 diabetes was positively associated

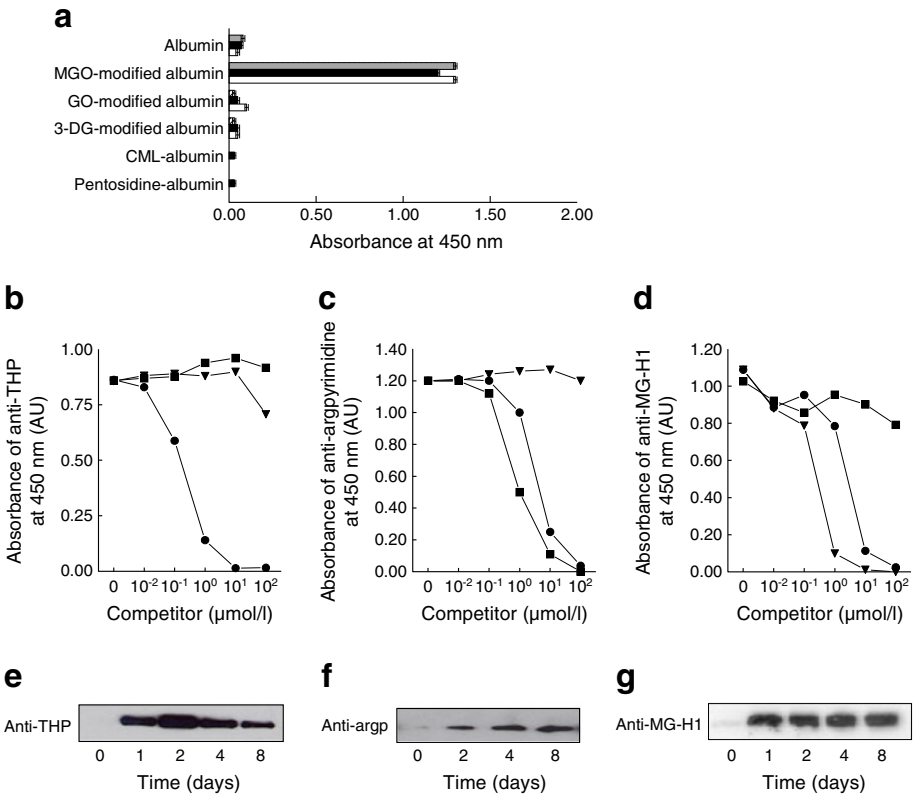

Fig. 1 Immunoreactivity and specificity of the MGO-albumin monoclonal antibody. (**a**) Microplate wells were coated with 1 μg non-modified albumin, MGO-modified albumin, glyoxal (GO)-modified albumin, 3-deoxyglucosone (3DG)-modified albumin, CML-albumin or pentosidine-albumin. MGO-modified albumin, GO-modified albumin and 3DG-modified albumin were prepared by incubation of albumin with 10 mmol/l MGO, GO and 3-DG, respectively, for 1 day (grey bars), 3 days (black bars) and 6 days (white bars). Detection was performed with the MGO-albumin monoclonal antibody. (**b**–**d**) Competitive ELISA demonstrating the specificity of the MGO-albumin monoclonal antibody for THP. MGO-modified albumin was used as the absorbed antigen, and the competition was performed with anti-THP (**b**), anti-argpyrimidine [7] (**c**) and anti-MG-H1 [9] (**d**) with the competitors THP (circles), argpyrimidine (squares) or MG-H1 (triangles). (**e**–**g**) Albumin was exposed to MGO (0.5 mmol/l) for different time points (0–8 days). Detection of THP (**e**), argpyrimidine (argp) (**f**) and MG-H1 (**g**) was performed by immunoblotting. This is a representative experiment from a series of three experiments with similar results

with THP (crude β=0.23 SD [95% CI 0.03, 0.43]; p=0.03). THP was associated with pack years of smoking and triacylglycerols, but not with age, sex, BMI, waist-to-hip ratio, LDL-cholesterol, HDL-cholesterol, AER, eGFR, HbA_{1c}, and systolic or diastolic blood pressure. Additional adjustment for age, sex, pack years of smoking and triacylglycerols slightly weakened the association of THP with type 1 diabetes (β=0.20 SD [95% CI 0.00, 0.40]; p=0.05). In study B, we found no significant association of THP with age, sex, macroalbuminuria, smoking, duration of diabetes, BMI, systolic or diastolic blood pressure, HbA_{1c}, AER, eGFR, LDL-cholesterol, HDL-cholesterol and triacylglycerols.

Association of THP with markers of endothelial dysfunction

THP was positively associated with sVCAM-1 in both cohort studies. Per SD increase in THP, sVCAM-1 increased by 0.51 SD in study A (Table 2, crude analysis) and by 0.29 SD in study B (Table 3, crude analysis). In both studies, additional adjustment for possible confounders did not materially change this association (Tables 2 and 3). We additionally investigated the association of the AGEs, CML, CEL and pentosidine, with sVCAM-1, which were available in cohort study B, and found that these AGEs were not independently associated with sVCAM-1 (Table 3). THP was not associated with vWF (study A: standardised β [sβ]=−0.07 [95% CI −0.17, 0.03]; p=0.19), nor with sICAM-1 (study B: sβ=0.04 [95% CI −0.06, 0.14]; p=0.47), in analyses adjusted for age and sex.

Association of THP with markers of low-grade inflammation

We found a significant positive association of THP with sPLA2 (study B: sβ=0.26 [95% CI 0.17, 0.36]; p<0.001). Additional adjustments for age, sex, macroalbuminuria, smoking, diabetes duration, HbA_{1c}, LDL-cholesterol, HDL-cholesterol, triacylglycerols, urinary AER, systolic and diastolic blood pressure, BMI and eGFR did not materially change this association. THP was not associated with

Table 1 Baseline characteristics of the study populations of the two cohort studies

Characteristic	Cohort study A			Cohort study B		
	Controls	T1DM	*p* value	T1DM normoalbuminuria	T1DM macroalbuminuria and retinopathy	*p* value
General						
Number	197	198		175	198	
Age (years)	37.8±3.7	37.9±4.3	0.92	42.7±9.7	40.9±9.5	0.07
Sex (number of males/females)	91/106	103/95	0.25	104/71	121/77	0.74
Diabetes duration (years)	–	23.4±7.7	–	27.7±8.2	27.9±7.8	0.84
HbA_{1c} (%)	5.31±0.41	8.79±1.54	<0.01	8.5±1.1	9.6±1.5	<0.001
HbA_{1c} (mmol/mol)	34.5±4.4	72.5±16.9	<0.01	69.8±12.1	80.9±16.8	<0.001
Smoking, former or current	96 (49)	88 (44)	0.39	62 (109)	68 (135)	0.23
Pack years of smoking for former or current smokers	9.3 (4.0–19.1)	9.5 (4.5–17.9)	0.85	–	–	–
BMI (kg/m^2)	25.3±4.7	25.4±3.5	0.83	23.7±2.5	24.0±3.3	0.32
Waist-to-hip ratio	0.86±0.08	0.87±0.08	0.35	–	–	–
Total cholesterol (mmol/l)	5.49±1.21	5.33±1.08	0.17	4.8±1.0	5.6±1.2	<0.001
HDL-cholesterol (mmol/l)	1.70±0.41	1.83±0.46	<0.01	1.6±0.5	1.5±0.5	0.09
LDL-cholesterol (mmol/l)	3.11±0.94	2.93±0.91	0.06	2.8±0.9	3.5±1.1	<0.001
Triacylglycerols (mmol/l)	1.08 (0.77–1.52)	1.01 (0.77–1.35)	0.08	0.77 (0.57–0.96)	1.22 (0.88–1.66)	<0.001
Systolic BP (mmHg)	117±14	124±14	<0.01	132±18	151±23	<0.001
Diastolic BP (mmHg)	73±10	74±9	0.33	76±10	86±13	<0.001
$eGFR_{MDRD}$ ($ml/min/1.73m^2$)	90.1±17.2	98.8±16.6	<0.001	93.1±14.9	66.5±28.1	<0.001
AGEs						
THP (U/ml)	109.8 (91.8–122.3)	115.5 (102.4–133.2)	0.03	116.2 (92.8–142.4)	109.9 (87.1–137.4)	0.62
CML (μmol/l)	–	–	–	3.73 (3.28–4.10)	3.27 (2.83–3.85)	<0.001
CEL (μmol/l)	–	–	–	0.93 (0.82–1.05)	0.94 (0.79–1.14)	0.19
Pentosidine (pmol/mg)	–	–	–	42.4 (36.6–49.9)	45.2 (32.6–62.6)	0.05
Markers of endothelial function						
sVCAM-1 (ng/ml)	975 (812–1,158)	1,164 (967–1,378)	<0.01	880 (753–1,020)	1,021 (859–1,221)	<0.001
vWF (%)	87.0 (65.0–110.0)	100.0 (74.0–127.0)	<0.01	–	–	–
sICAM-1 (ng/ml)	–	–	–	708±256	757±271	0.05
Markers of low-grade inflammation						
hsCRP (mg/l)	0.85 (0.43–1.79)	1.06 (0.46–2.79)	0.02	0.92 (0.36–2.06)	1.23 (0.56–30.7)	<0.01
sPLA2 (μg/ml)	–	–	–	4.00 (2.80–6.15)	4.55 (2.80–6.95)	0.12
IL-6 (pg/ml)	–	–	–	1.42 (0.95–2.10)	2.14 (1.29–3.64)	<0.001
TGF-β1 (pg/ml)	–	–	–	11.25 (8.10–18.42)	12.23 (8.77–22.97)	<0.01
Complications						
Albuminuria (normo/micro/macro) (%)	160/6/0 (96.4/3.6/0)	138/23/4 (83.6/13.9/2.4)	<0.001	–	–	–
AER (μg/min)	5.10 (3.47–7.94)	5.97 (4.25–12.28)	<0.001	–	–	–
AER (mg/24 h)	–	–	–	8 (5–13)	794 (342–2,050)	<0.001
Retinopathy, self-reported	–	34 (19.0)	–			
Retinopathy (no/NPDR/PDR)				61/95/19 (35/54/11)	0/61/137 (0/31/69)	<0.001
Neuropathy, self-reported	–	23 (12.0)	–	–	–	–
Prior CVD	–	–	–	3 (1.7)	21 (10.6)	<0.001

Data are presented as mean±SD, median (interquartile range) or number (percentage) unless otherwise indicated. T1DM, type 1 diabetes mellitus; $eGFR_{MDRD}$, estimated GFR by abbreviated MDRD equation; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy

Table 2 Association of THP with sVCAM-1 in individuals with and without type 1 diabetes (cohort study A)

Model	Adjustments	THP	
		Sβ (95% CI)	*p* value
	Crude	0.51 (0.41, 0.60)	<0.001
1	Age, sex and diabetes	0.49 (0.39, 0.58)	<0.001
2	Model 1 + additional adjustments[a]	0.48 (0.38, 0.58)	<0.001

Standardised β, the standardised regression coefficient obtained with linear regression analyses, indicates the change in sVCAM-1 (in SD) per 1 SD higher level in THP

[a] Model 2 is also adjusted for pack years of smoking, HbA_{1c}, LDL-cholesterol, HDL-cholesterol, triacylglycerols, eGFR, urinary AER, systolic and diastolic blood pressure, BMI and waist-to-hip ratio

hsCRP (study A: sβ=−0.004 [95% CI −0.10, 0.10], p=0.94; study B: sβ=0.02 [95% CI −0.08, 0.12], p=0.65), IL-6 (study B: sβ=−0.01 [95% CI −0.10, 0.10], p=0.93) and TGF-β1 (study B: sβ=0.04 [95% CI −0.06, 0.15], p=0.41), in analyses adjusted for age and sex.

Association of THP with micro- and macrovascular complications in type 1 diabetes

In study A, THP was associated with neither micro- or macroalbuminuria nor self-reported history of neuropathy or retinopathy in individuals with type 1 diabetes (Table 4). In study B, THP was not associated with macroalbuminuria, retinopathy or CVD at baseline (Table 4).

THP is present in atherosclerotic lesions

We determined whether THP is present in the atherosclerotic plaque of coronary arteries. Specimens were available in 12 controls, three individuals with type 1 diabetes, and ten with type 2 diabetes. Respectively, five, two and four individuals were male, and mean age at time of death was 67, 65 and 66. None of the individuals died from an acute myocardial infarction, but three had had a previous myocardial infarction, one in each group. The level of stenosis in the respective atherosclerotic coronary arteries was not significantly different between controls and individuals with type 1 or type 2 diabetes (p=0.55). In seven of the 12 controls, all three of the individuals with type 1 diabetes and four of the ten individuals with type 2 diabetes, coronary artery specimens showed >50% stenosis. In coronary arteries, staining for THP and CML was observed in atherosclerotic lesions (Fig. 2). In consecutive sections, THP and CML colocalised predominantly with macrophages (CD68-positive cells) (Fig. 2).

The amount of staining of THP and CML was not significantly different between controls, individuals with type 1 diabetes and individuals with type 2 diabetes. The median (range) score for THP staining was 2.3 (0.0–3.5) for controls, and 3.3 (2.0–3.5) and 0.5 (0.0–3.5) for individuals with type 1 and type 2 diabetes, respectively (p=0.12). The median (range) score for CML staining was 1.7 (0.0–3.0) for controls, and 3.0 (1.0–3.5) and 1.0 (0.0–2.5) for individuals with type 1 and type 2 diabetes, respectively (p=0.22).

Discussion

Rapid AGE formation from glucose-derived dicarbonyl precursors has attracted attention over the relatively slower non-enzymatic reactions between proteins and glucose [1]. MGO is believed to be the most potent glycating agent [3, 4]. The main findings of this study were fourfold. First, we developed a competitive ELISA for the MGO-derived AGE, THP, and showed increased plasma concentrations of THP in type 1 diabetes. Second, we found a strong association of plasma THP with sVCAM-1 in two, and sPLA2 in one type

Table 3 Association of THP, CEL, CML and pentosidine with sVCAM-1 in individuals with type 1 diabetes with and without macroalbuminuria (cohort study B)

Model	Adjustments	THP		CML		CEL		Pentosidine	
		Sβ (95% CI)	*p* value	Sβ (95% CI)	*p* value	Sβ (95% CI)	*p* value	Sβ (95% CI)	*p* value
	Crude	0.29 (0.19, 0.38)	<0.001	<0.01 (−0.10, 0.10)	0.99	0.08 (−0.02, 0.19)	0.11	0.16 (0.06, 0.26)	<0.01
1	Age, sex and macroalbuminuria	0.30 (0.21, 0.39)	<0.001	0.06 (−0.05, 0.16)	0.28	0.04 (−0.07, 0.13)	0.50	0.12 (0.02, 0.23)	0.02
2	Model 1 + additional adjustments[a]	0.31 (0.23, 0.40)	<0.001	−0.02 (−0.13, 0.08)	0.68	−0.09 (−0.19, 0.02)	0.10	−0.02 (−0.14, 0.10)	0.73

Standardised β, the standardised regression coefficient obtained with linear regression analyses, indicates the change in sVCAM-1 (in SD) per 1 SD higher level in AGE

[a] Model 2 is also adjusted for former or current smoking, diabetes duration, HbA_{1c}, LDL-cholesterol, HDL-cholesterol, triacylglycerols, urinary AER, systolic and diastolic blood pressure, BMI and eGFR

Table 4 Association of THP with micro- and macrovascular complications in individuals with type 1 diabetes

Study cohort	Complications	OR[a]	95% CI	*p* value
Cohort study A	Micro- or macroalbuminuria	0.92	0.52, 1.62	0.76
	Neuropathy (self-reported)	1.42	0.93, 2.16	0.10
	Retinopathy (self-reported)	1.25	0.87, 1.81	0.23
Cohort study B	Macroalbuminuria	0.95	0.77, 1.17	0.62
	Retinopathy	1.04	0.78, 1.39	0.78
	CVD	0.96	0.62, 1.49	0.87

[a] OR per SD increase in THP with 95% CI obtained with logistic regression analyses, adjusted for age, sex and pack years of smoking/former or current smoking in both cohorts, and triacylglycerols in study A, and macroalbuminuria, in the case of CVD, in study B

1 diabetes cohort study. Third, we could not find any association of plasma THP with micro- or macro-vascular outcomes. Fourth, however, we did show accumulation of THP in human atherosclerotic lesions.

We obtained a new murine monoclonal antibody that clearly distinguished MGO-modified proteins from non-modified proteins and from well-known AGEs such as CML and pentosidine, which can be used to further elucidate the role of MGO-derived AGEs in the development of diabetic complications. It appeared that this monoclonal antibody has a strong preference for THP over MG-H1 and argpyrimidine (Fig. 1). The formation of MGO-derived AGEs in MGO-modified albumin, as detected with antibodies, demonstrated that the formation of MG-H1 and THP was relatively rapid, mostly occurring within 24 h, and that the formation of argpyrimidine occurred at a later stage. This time frame is consistent with data obtained with MS [12, 13, 19]. Both the time frame and the competition experiments support the specificity of the antibody for THP. Comparable competition experiments with two well-known specific monoclonal antibodies against argpyrimidine [7] and MG-H1 [9] showed that the epitope recognition of these monoclonal antibodies are indeed argpyrimidine and MG-H1 and confirmed the specificity of our test system. Interestingly, a preliminary report about a novel monoclonal antibody against MGO-derived AGEs also found THP to be the dominant epitope [26]. Why THP is found be the dominant epitope in this and our study is unknown, but indicates that this MGO-derived AGE induces a highly antigenic epitope.

Although MGO-arginine modifications are also measurable with analytical techniques, such as UPLC–MS/MS, they are difficult to detect quantitatively. Because THP is not acid-stable, enzymatic digestion of proteins is essential for the detection of this MGO-derived AGE in proteins. However, proteins modified by AGEs may be incompletely digested [27] and may therefore affect outcomes of such analyses. By using immunological analysis, as we did in our study, the above limitations for the detection of THP are overcome.

Plasma concentrations of MGO [8], as well as MG-H1 [9] and argpyrimidine [10], have been shown to be elevated in individuals with diabetes. We have shown for the first time that THP is also elevated in individuals with type 1 diabetes.

We showed a strong, positive association of MGO-derived AGE THP with sVCAM-1 in individuals with and without type 1 diabetes. This is consistent with experiments that demonstrated that AGEs are able to induce the expression of sVCAM-1 [28, 29]. Both AGEs and sVCAM-1 have been shown to be elevated in type 1 diabetes [30–35]. sVCAM-1 is known to be a marker of endothelial dysfunction and is associated with atherosclerosis [36–38] and micro- and macrovascular complications in type 1 diabetes [39, 40]. In additional analyses, we investigated the association of the AGEs, CEL, CML and pentosidine, with sVCAM-1. We found that CEL, CML and pentosidine were not associated with sVCAM-1, after adjustment for possible confounders, whereas THP was. Therefore, it appears that THP is not a reflection of other AGEs such as CEL, CML and pentosidine. THP possibly reflects another pathophysiological pathway not involving these other, well-known, AGEs.

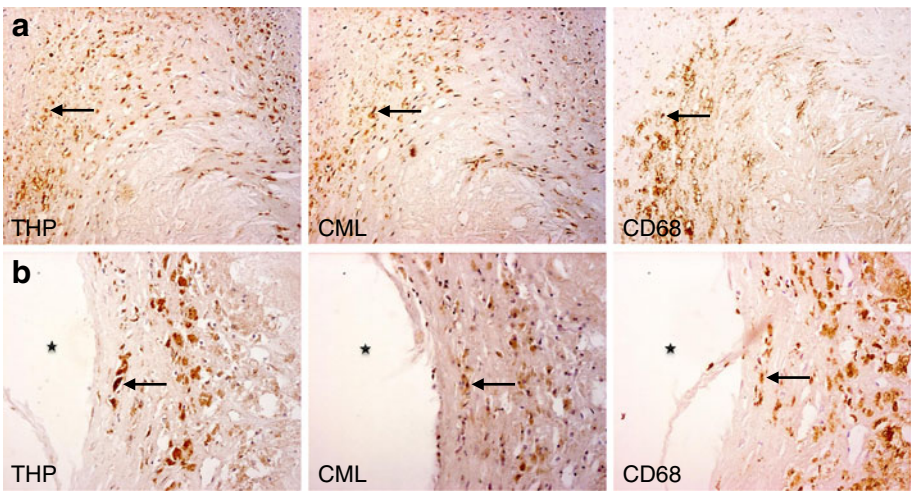

Fig. 2 Colocalisation of THP and CML with macrophages (CD68) in plaques of coronary arteries. Two representative immunohistochemical stainings of the coronary arteries studied, in serial sections, are shown. (**a**) and (**b**) show the colocalisation of THP and CML with macrophages (CD68). An anti-CD68 antibody was used as a marker for macrophages. The black arrows indicate examples of the colocalisation in macrophages; the asterisk indicates the lumen. Magnification ×200

The strong association of plasma THP with sVCAM-1 in two separate cohort studies may suggest that THP is involved in the development of micro- and macrovascular complications. We additionally show the presence of THP in macrophages in atherosclerotic coronary arteries. This further supports the hypothesis that THP is associated with vascular complications. In a previous study, we demonstrated that MGO-albumin did not bind or activate endothelial cells as measured by the expression of adhesion molecules, whereas, under the same conditions, TNF-α did [41]. In the same study we found binding of MGO-albumin to monocytes. Other studies have shown that MGO-derived AGEs are able to activate monocytes, thereby stimulating the production of certain cytokines [42, 43]. Therefore, endothelial cells may be indirectly activated by cytokines, which are induced and released by MGO-AGE-activated macrophages. In accordance with this mechanism, sPLA2, an enzyme expressed in activated macrophages and smooth muscle cells [44] which can be proatherogenic in both the circulation and the arterial wall [44, 45], was significantly associated with THP. Therefore, activation of macrophages by THP and the release of cytokines might be the mechanism by which THP is potentially associated with production of sVCAM-1. Since sVCAM-1 is associated with atherosclerosis, THP might be implicated in the pathogenesis of vascular complications in type 1 diabetes.

We did not find any association of THP with HbA_{1c} in both studies. These findings are consistent with many others studies that reported no association of plasma AGEs with HbA_{1c} [31, 46–49]. An explanation for this lack of association may be that AGEs can also be formed through pathways other than glucose metabolism, for example, lipid peroxidation. Moreover, HbA_{1c} and AGEs presumably reflect different pathways following hyperglycaemia and different time frames of hyperglycaemia.

Limitations of our study

Both our studies had a cross-sectional design; therefore we cannot draw any conclusion about causality in the association of THP with sVCAM-1. We developed an antibody against MGO-derived AGEs and demonstrated an at least 1,000-fold preference for THP over argpyrimidine or MG-H1. Despite this preference for THP, we cannot exclude the possibility that MGO-derived AGEs other than THP are detected in our analyses. Furthermore, we cannot rule out the possibility that plasma THP does not reflect intracellular glycation. This may imply that we underestimated the association of THP and vascular complications.

Since we do not have information about diet in both cohorts, we were not able to adjust our analyses for the possible influence of dietary AGEs on plasma AGE measurements. Since it is unknown if and how dietary AGEs are able to influence the levels of plasma AGEs measured in fasting plasma samples, this is a limitation of our study.

Since THP was detected in atherosclerotic arteries (Fig. 2), we expected to find an association of plasma THP with cardiovascular complications. We found that plasma levels of THP were associated with sVCAM-1, i.e. a marker of atherosclerosis, but we could not find any association of THP with vascular complications. Although we do not have a clear explanation so far for this finding, this might be due to the limited number of cases of prior CVD, i.e. the power to detect an association was low. In addition, retinopathy and neuropathy were evaluated via self-report in study A, which may have limited the power of the analyses.

Conclusion

Our results suggest that MGO-derived THP may reflect endothelial dysfunction and is present in atherosclerotic lesions in individuals with and without type 1 diabetes. This may mean that MGO-derived AGE THP plays a role in the pathophysiology of atherosclerosis in individuals with or without type 1 diabetes. Future studies are needed to elucidate the potential causal role of THP in the development of cardiovascular complications of diabetes.

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Contribution statement MGAE, MTS and CGS participated in the conception and design, analysed and interpreted the data, drafted the manuscript and provided final approval of the version to be published. HMC carried out study A, participated in the conception and design, analysed and interpreted the data, revised the paper for important intellectual content and provided final approval of the version to be published. NMJH analysed and interpreted the data, revised the paper for important intellectual content and provided final approval of the version to be published. HWMN and CDAS participated in the conception and design, analysed and interpreted the data, revised the paper for important intellectual content and provided final approval of the version to be published. LT, HHP and PR carried out study B, revised the paper for important intellectual content and provided final approval of the version to be published.

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