ARTICLE

High prevalence of low plasma thiamine concentration in diabetes linked to a marker of vascular disease

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Abstract

Aims/hypothesis To assess thiamine status by analysis of plasma, erythrocytes and urine in type 1 and type 2 diabetic patients and links to markers of vascular dysfunction.

Methods Diabetic patients (26 type 1 and 48 type 2) with and without microalbuminuria and 20 normal healthy control volunteers were recruited. Erythrocyte activity of transketolase, the concentrations of thiamine and related phosphorylated metabolites in plasma, erythrocytes and urine, and markers of metabolic control and vascular dysfunction were determined.

Results Plasma thiamine concentration was decreased 76% in type 1 diabetic patients and 75% in type 2 diabetic patients: normal volunteers 64.1 (95% CI 58.5–69.7) nmol/l, type 1 diabetes 15.3 (95% CI 11.5–19.1) nmol/l, $p <$ 0.001, and type 2 diabetes 16.3 (95% CI 13.0–9.6) nmol/l, $p<0.001$. Renal clearance of thiamine was increased 24-fold in type 1 diabetic patients and 16-fold in type 2 diabetic patients. Plasma thiamine concentration correlated negatively with renal clearance of thiamine ($r=-0.531$, $p<0.001$) and fractional excretion of thiamine ($r=-0.616$, $p<0.001$). Erythrocyte transketolase activity correlated negatively with urinary albumin excretion ($r=-0.232$, $p<0.05$). Thiamine transporter protein contents of erythrocyte membranes of type 1 and type 2 diabetic patients were increased. Plasma thiamine concentration and urinary excretion of thiamine correlated negatively with soluble vascular adhesion molecule-1 ($r=-0.246$, $p<0.05$, and -0.311, $p<0.01$, respectively).

Conclusions/interpretation Low plasma thiamine concentration is prevalent in patients with type 1 and type 2 diabetes, associated with increased thiamine clearance. The conventional assessment of thiamine status was masked by increased thiamine transporter content of erythrocytes.

Keywords Adhesion molecules · Microalbuminuria · Thiamine · Type 1 diabetes · Type 2 diabetes

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Abbreviations

ARB	angiotensin receptor blocker
$Cl_{Thiamine}$	renal clearance of thiamine
$FE_{Thiamine}$	fractional excretion of thiamine
MNL	mononuclear leucocyte
RFC-1	reduced folate carrier-1
sVCAM-1	soluble vascular adhesion molecule-1
THTR-1	thiamine transporter-1
THTR-2	thiamine transporter-2
TK	transketolase
TMP	thiamine monophosphate
TPP	thiamine pyrophosphate

Introduction

Diabetes is increasing in incidence in the UK and elsewhere to epidemic proportions. The major health concerns of diabetes are achieving good control of blood glucose, BP and lipids to suppress the development of associated vascular complications—microvascular complications (nephropathy, retinopathy and peripheral neuropathy) and macrovascular complications (cardiovascular disease and stroke) [1, 2]. This is not always achievable because of limitations of current drug therapy, patient compliance and other factors linked to the development of vascular complications [3]. Secondarily to the global epidemic of diabetes, therefore, is a global burden of vascular complications [4]. The significant residual risk of development of microvascular and macrovascular complications in prospective clinical trials where best efforts have been made to optimise glycaemic control [5, 6], and the failure of major genetic susceptibility factors for diabetic complications to emerge from intensive studies [7] (although some may be found with further study [8, 9]), encourage the search for other metabolic and nutritional factors that may predispose to the development of vascular complications in diabetes.

Vascular complications develop progressively from 5–40 years after the onset of diabetes, although they may appear earlier in type 2 diabetes when associated with a period of undiagnosed diabetes and impaired glucose tolerance. A strategy to counter multiple pathways of biochemical dysfunction linked to the development of vascular complications emerged from recent advances in understanding of the cell biology of diabetic complications: high-dose therapy with thiamine and related derivatives such as benfotiamine. High-dose thiamine therapy prevented the development of microvascular complications in experimental diabetes without improvement of metabolic control, as reviewed in [10]. Experimental diabetes was also associated with tissue-specific thiamine deficiency characterised by a marked decrease of plasma thiamine concentration and decreased activity of the thiamine-dependent enzyme of transketolase (TK) and decreased levels of TK protein in renal glomeruli linked to a profound increase in renal clearance of thiamine ($Cl_{Thiamine}$) [11]. Thiamine deficiency was not detected by the conventional indicator of thiamine status, the erythrocyte 'thiamine effect' (the percentage unsaturation of TK with thiamine pyrophosphate [TPP] cofactor) [12]. The prevalence of similar low plasma thiamine concentrations and impaired renal handling of thiamine in clinical diabetes has not been addressed previously.

In this study, we sought to establish whether there is a disturbance of thiamine homeostasis prevalent in type 1 and type 2 diabetic patients in the UK and if this is linked to incipient nephropathy and markers of vascular dysfunction and early stage renal dysfunction.

Methods

Patients and normal healthy volunteers Diabetic patients were recruited from patients attending the Diabetes Clinic at Colchester General Hospital, and normal healthy control volunteers from partners and friends of the patients and investigators. Inclusion criteria were: diabetic patients with normoalbuminuria (AER <30 mg/24 h) and microalbuminuria (AER 30–300 mg/24 h); matched for age and sex (18–65 years); diabetes duration of ≥5 years; HbA_{1c} <10%; and BMI 19–40 kg/m^2. Exclusion criteria were: patients with end-stage renal disease; severe excess alcohol consumption (>50 U per week; 1 U is 8 g alcohol); significant comorbidities; known allergy or intolerance to thiamine; use of thiamine supplements or goldenseal (*Hydrastis canadensis*, a multipurpose herbal remedy); participating in an intervention study within 30 days; recipients of renal and/or pancreatic transplants; and women who were pregnant or breastfeeding or of child-bearing potential not using adequate contraceptive precautions. Twenty-four hour urine collections and blood samples (fasting) were taken with informed consent of the patients and volunteers. Plasma, erythrocyte and mononuclear leucocyte (MNL) fractions were prepared immediately and stored at −80°C until analysis. Ethical approval for the study was given by the local ethics committee (North and Mid-Essex Local Research Ethics Committee).

Assay of thiamine and phosphorylated metabolites Thiamine status was assessed by measurement of thiamine, thiamine monophosphate (TMP) and TPP, determined by HPLC with fluorimetric detection (pre-column derivatisation to thiachromes) [13]. The retention times, limits of detection, interbatch CV values and recoveries for these metabolites were: thiamine 13.1 min, 36 fmol, 1.1 and 97%; TMP 6.0 min, 52 fmol, 2.1 and 92%; and TPP 4.5 min, 51 fmol, 2.9 and 94%. There was no interference from glucose in the assay and validations were not significantly different in analysis of samples from normal healthy volunteers and diabetic patients. Sample storage studies indicated that analyte content was stable for plasma, erythrocytes and urine stored at −80°C for at least 6 weeks and storage of plasma, erythrocyte haemolysate and urine and related de-proteinised extracts for 8 h. Stock solutions of thiamine, TMP and TPP were calibrated by spectrophotometry assuming molar extinction coefficients of $\varepsilon_{233}=$ 14.2, $\varepsilon_{247}=15.3$ and $\varepsilon_{247}=13.0$ (mmol/l)$^{-1}$ cm^{-1}, respectively [14]. Shewhart analysis assessing the stability of analyte estimates in samples every day over a period of ten consecutive days indicated the analysis had acceptable quality control (all estimates within mean±2 SD) [15]. The concentration of thiamine metabolites was determined in plasma, erythrocytes and urine; $Cl_{Thiamine}$ and fractional excretion of thiamine ($FE_{Thiamine}$) were deduced.

Other biochemical measurements Erythrocyte TK activity and percentage unsaturation with TPP (thiamine effect) were determined. Erythrocyte membrane content of thiamine transporter-1 (THTR-1) [16] and reduced folate carrier-1 (RFC-1), a transporter of TMP [17], were determined by SDS-PAGE electrophoresis and western blotting, normalising band intensities to β-actin and preparing erythrocyte membrane protein extracts using the optimised procedure described in [18], and commercial antibodies to THTR-1 and RFC-1 (Alpha Diagnostics, San Antonio, CA, USA).

Markers of metabolic control were determined: plasma glucose, HbA$_{1c}$, total cholesterol, HDL-cholesterol, triacylglycerol, systolic and diastolic BP and GFR assessed by Cockcroft–Gault-corrected creatinine clearance. Markers of vascular and metabolic dysfunction (plasma plasminogen activator inhibitor-1; von Willebrand factor and soluble vascular adhesion molecule-1 [sVCAM-1]; and MNL protein kinase C) were determined by commercial ELISAs (Technoclone, Dorking, UK; R&D Systems, Abingdon, UK; and Stressgen, Ann Arbor, MI, USA, respectively).

Statistical analysis The significance of differences between mean and median analyte concentrations was determined using Student's *t* test and the Mann–Whitney *U* test, respectively, and correlation analysis was performed by calculating Spearman's ρ statistic, as indicated in the text. Data presented are mean±SD or median (minimum–maximum).

Results

Patient characteristics The characteristics of type 1 and type 2 diabetic patients and normal healthy control volunteers recruited for this study are given in Table 1. The diabetic patients had moderate glycaemic control; mean HbA$_{1c}$ was 8.6–8.7%. GFR was within the normal range, indicative of normal renal function for creatinine

clearance. Fifty diabetic patients (17 type 1 and 33 type 2) had urinary albumin excretion within the normal range (median 11.5, 2.3–29.1 mg/24 h) and 24 had urinary albumin excretion characteristic of microalbuminuria and incipient nephropathy (median 46.1, 30.6–297.3 mg/24 h). Diabetic patients with and without microalbuminuria were of similar age (60±14 vs 56±15 years), duration of diabetes (17±11 vs 16±12 years) and HbA$_{1c}$ (8.9±1.9 vs 8.5± 1.5%). Nine type 1 diabetic patients and 33 type 2 diabetic patients were receiving therapy with ACE inhibitors or angiotensin receptor blockers (ARBs).

Thiamine status of patients with type 1 and type 2 diabetes Surveying the thiamine status in the study patients and normal volunteer groups, we found the plasma concentration of thiamine was decreased 76% and 75% in type 1 and type 2 diabetic patients, respectively. The plasma concentration of thiamine (mean±SD) was: normal volunteers 64.1±12.0 nmol/l, type 1 diabetes 15.3±9.6 nmol/l and type 2 diabetes 16.3±11.5 nmol/l, *p*<0.001 (Student's *t* test) (Fig. 1a). There was no significant difference between plasma thiamine concentration of type 1 and type 2 diabetic patients or between diabetic patients with and without microalbuminuria (14.4±8.9 vs 16.7±11.6 nmol/l, *p*>0.05). The urinary excretion of thiamine was increased fourfold and threefold in type 1 and type 2 diabetic patients with respect to normal volunteers. The urinary excretion of thiamine (median, minimum–maximum) was: normal volunteers 0.31 (0.23–2.45) μmol/24 h; type 1 diabetes 1.36 (0.39–7.65) μmol/24 h; and type 2 diabetes 1.04 (0.08– 6.85) μmol/24 h, *p*<0.001 (Mann–Whitney *U* test). The urinary excretion of thiamine was higher in type 1 than in type 2 diabetic patients (*p*<0.05) but was not significantly different in diabetic patients with and without microalbuminuria [1.10 (0.08–7.65) vs 1.23 (0.33–6.85) μmol/ 24 h, *p*>0.05]. Cl$_{Thiamine}$ was increased 24-fold in type 1 diabetic patients and 16-fold in type 2 diabetic patients. Cl$_{Thiamine}$ (median, minimum–maximum) was: normal volunteers 3.7 (2.6–26.2) ml/min; type 1 diabetes 86.5 (12.8–228.4) ml/min; and type 2 diabetes 59.8 (1.4–256.6)

Table 1 Characteristics of normal control volunteers and diabetic patients recruited for this study

Participant type	*n*	Sex M/F	Age (years)	Duration of diabetes (years)	BMI (kg/m^2)	GFR (ml/min)	Fasting plasma glucose (mmol/l)	HbA$_{1c}$ (%)	Systolic BP (mmHg)	Diastolic BP (mmHg)	ACE inhibitor/ ARB therapy
Control volunteers	20	10/10	53±10	–	27±4	89±18	5.6±0.8	5.0±0.2	ND	ND	–
Type 1 diabetes	26	10/16	48±15	22.1±13.3	28±5	93±28	9.2±1.7*	8.7±1.2*	131±21	73±11	9
Type 2 diabetes	48	29/19	62±12	13.0±8.9**	31±6	92±30	9.1±2.2*	8.6±1.8*	141±22	77±9	33

Data are mean±SD.
ND Not determined
**p*<0.001 with regard to normal volunteers
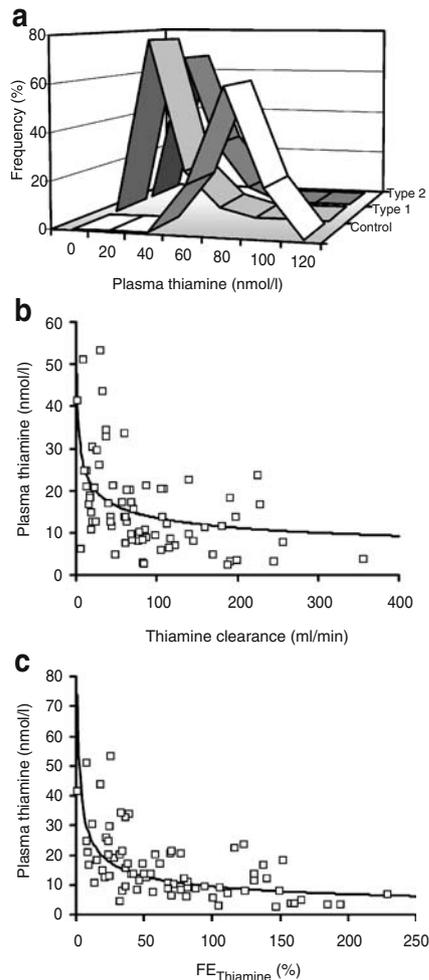***p*<0.01 with regard to type 1 diabetes (Mann–Whitney *U* test)

Fig. 1 Thiamine status of diabetic patients and link to sVCAM-1. **a** Frequency ribbon graph of plasma thiamine concentration. **b** Inverse relationship between plasma thiamine concentration and thiamine clearance. Nonlinear regression equation: $[\text{Thiamine}]_{\text{Plasma}} = 47.7 \times \text{Cl}_{\text{Thiamine}}^{-0.274}$, $p < 0.001$. **c** Inverse relationship between plasma thiamine concentration and FE$_{\text{Thiamine}}$. Nonlinear regression equation: $[\text{Thiamine}]_{\text{Plasma}} = 74.0 \times \text{FE}_{\text{Thiamine}}^{-0.448}$, $p < 0.001$

ml/min, $p < 0.001$ (Mann–Whitney U test). There was no significant difference in Cl$_{\text{Thiamine}}$ between diabetic patients with and without microalbuminuria (72.9 [1.4–355.7] vs 66.3 [5.8–245.3] ml/min). FE$_{\text{Thiamine}}$ was increased 25-fold in type 1 diabetic patients and 15-fold in type 2 diabetic patients. FE$_{\text{Thiamine}}$ (median, minimum–maximum) was: normal volunteers 2.8% (1.4–17.2%); type 1 diabetes 71.2% (7.6–165.9%); and type 2 diabetes 41.6% (1.1–228.9%), $p < 0.001$ (Mann–Whitney U test). There was no significant difference in FE$_{\text{Thiamine}}$ between diabetic patients with and without microalbuminuria (57.7% [8.0–228.9%] vs 43.4% [1.1–165.9%]). Plasma thiamine concentration correlated negatively with Cl$_{\text{Thiamine}}$ ($r = -0.531$, $p < 0.001$; Spearman) and FE$_{\text{Thiamine}}$ ($r = -0.616$, $p < 0.001$; Spearman) (Fig. 1b,c). There was no significant difference

in these thiamine-related variables between diabetic patients with and without ACE or ARB therapy.

Thiamine deficiency is assessed conventionally by assay of TK activity of erythrocytes [12]. Erythrocyte activity of TK was not changed significantly in diabetic patients, with regard to normal volunteers. TK activity was: normal volunteers 1.09±0.06 mU/mg Hb; type 1 diabetes 1.18±0.25 mU/mg Hb; and type 2 diabetes 1.01±0.17 mU/mg Hb. Erythrocyte activity of TK was not significantly different in diabetic patients with and without microalbuminuria (1.03±0.24 vs 1.08±0.21 mU/mg Hb), although it correlated negatively with urinary albumin excretion ($r = -0.232$, $p < 0.05$; Spearman). All patients were not thiamine deficient by the definition of the 'thiamine effect'; they had a thiamine effect of <15%. The concentration of thiamine in erythrocytes was not changed significantly in type 1 and type 2 diabetic patients, with respect to normal volunteers. The concentration of thiamine in erythrocytes (mean±SD) was: normal volunteers 0.318±0.028 pmol/mg Hb; type 1 diabetes 0.345±0.025 pmol/mg Hb; and type 2 diabetes 0.328±0.137 pmol/mg Hb.

Masking of the clinical thiamine deficiency in erythrocytes by increased levels of thiamine transporter proteins We sought evidence to understand how erythrocytes of diabetic patients maintained a normal concentration of thiamine whilst the plasma concentration of thiamine was severely decreased. The transport of thiamine and TMP from plasma to the interior of erythrocytes is mediated by the transporters THTR-1 and RFC-1, respectively (Fig. 2a). Western blotting of erythrocyte membrane proteins from diabetic patients showed increased contents of these transporters compared with membrane-associated housekeeping protein β-actin (Fig. 2b). Thiamine transporter: β-actin blot intensity ratios (median, minimum–maximum) were: normal volunteers ($n = 10$), THTR-1 0.69 (0.12–1.43) and RFC-1 0.042 (0.012–0.093); type 1 diabetes ($n = 12$), THTR-1 1.06 (0.64–2.02) (+54%, $p < 0.05$) and RFC-1 0.146 (0.021–0.902) (+248%, $p < 0.01$); and type 2 diabetes ($n = 12$), THTR-1 1.20 (0.62–2.39) (+74%, $p < 0.01$) and RFC-1 0.206 (0.038–1.382) (+390%, $p < 0.001$) (Mann–Whitney U tests).

Low plasma thiamine concentration and markers of metabolic control and vascular dysfunction We screened markers of metabolic control and vascular dysfunction in diabetes for linkage to the low plasma thiamine concentration in diabetes. The only significant correlations were of plasma thiamine concentration with plasma sVCAM-1 ($r = -0.246$, $p < 0.05$; Spearman) (Fig. 3a) and urinary excretion of thiamine with plasma sVCAM-1 ($r = -0.311$, $p < 0.01$) (Fig. 3b).
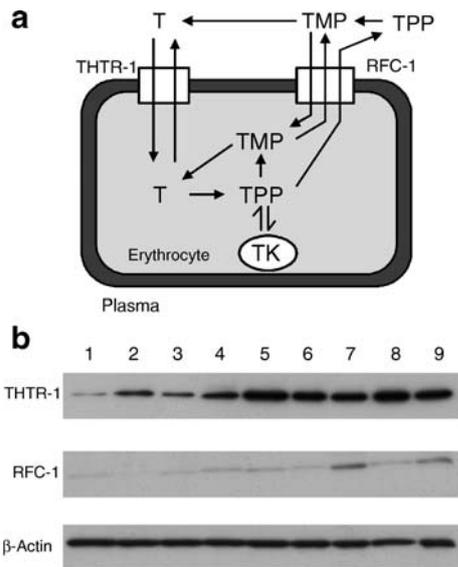
Fig. 2 **a** Schematic diagram of thiamine membrane transport and metabolism in erythrocytes. **b** Representative Western blot of THTR-1, RFC-1 and housekeeping protein β-actin of erythrocyte membranes. *Lanes 1–3*, normal volunteers; *lanes 4–6*, type 1 diabetic patients; and *lanes 7–9*, type 2 diabetic patients. *T*, Thiamine

Discussion

In this study we found clinical diabetes was associated with a marked deficiency of thiamine in blood plasma linked to increased $Cl_{Thiamine}$ and $FE_{Thiamine}$. The low plasma thiamine concentration in diabetes was linked inversely to plasma sVCAM-1.

The strengths of this study were the use of a validated assay for thiamine and phosphorylated metabolites, a comprehensive assessment of thiamine-related variables (including thiamine transporter content of erythrocyte membranes, urinary excretion, $Cl_{Thiamine}$ and $FE_{Thiamine}$) and investigation of the association of thiamine-related variables with markers of metabolic and vascular dysfunction. The decreased plasma thiamine concentration in clinical diabetes was probably not due to a deficiency of dietary input of thiamine. The urinary excretion of thiamine of diabetic patients and normal healthy volunteers was within the normal range (>0.20 μmol/24 h) [19], except for one type 2 diabetic patient with a urinary thiamine excretion of 0.08 μmol/24 h. Urinary excretion of thiamine accounts for a minor part of normal thiamine turnover: pyrimidine and thiazole degradation products of thiamine are also excreted in the urine and account for the major component of thiamine turnover [20]. Rather, low plasma thiamine concentration was linked to a profound increase in $Cl_{Thiamine}$ and $FE_{Thiamine}$. The molecular mass of thiamine is <500 Da and hence it is filtered from plasma in renal glomeruli [10]. Increased $Cl_{Thiamine}$ is probably due to

decreased re-uptake of thiamine in renal proximal tubules. Thiamine clearance was dysfunctional in diabetic patients with normal GFR, as assessed by creatinine clearance. This study suggests renal mishandling of thiamine in diabetic patients is an early marker of renal dysfunction in diabetes and, linked to the locus of renal thiamine re-uptake, particularly relates to proximal tubule dysfunction.

Re-uptake of thiamine occurs in the proximal tubules by thiamine transporters THTR-1 and THTR-2 via a sodium-independent, proton antiport mechanism with regulation by Ca^{2+}/calmodulin [21]. The expression of the genes encoding THTR-1 and THTR-2 (and also RFC-1) transporters are regulated via SP1 promoter elements [22–24]. SP1 signalling in the tubular epithelium is impaired in hyperglycaemia associated with diabetes by increased *O*-glycosylation of the SP1 via enhanced hexosamine pathway activity [25]. Re-uptake of thiamine by the tubular epithelium in experimental diabetes [26] and clinical diabetes (this work) may be impaired by hexosamine pathway-linked decreased expression of the genes encoding THTR-1 and THTR-2, inhibition of these thiamine transporters by dicarbonyl glycation [27] and acidification of the tubular lumen [28]. This deserves further investigation.

A weakness of the study was the finding of no strong link to a recognised clinical endpoint such as incipient nephropathy judged by microalbuminuria, although there was a weak negative correlation of erythrocyte TK activity
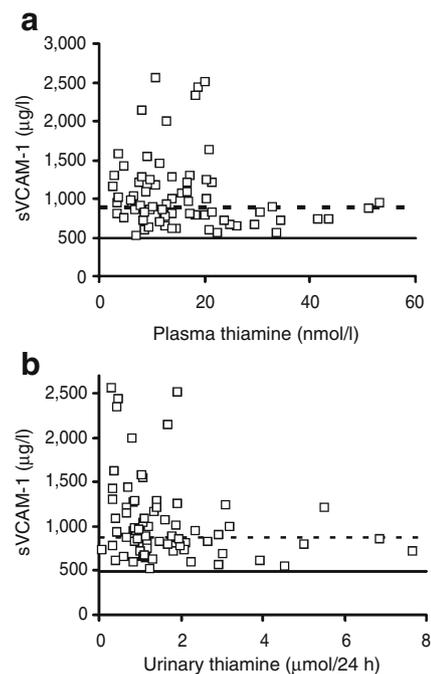

Fig. 3 Correlation of sVCAM-1 with plasma thiamine concentration (**a**) and urinary thiamine excretion (**b**). *Solid horizontal line*, reference mean of sVCAM-1 of normal volunteers; *broken horizontal line*, upper limit of sVCAM-1 of normal volunteers

with urinary albumin excretion. It is becoming increasingly evident, however, that urinary albumin excretion may not be a reliable marker of early stage decline in renal function in diabetes [29]. Future studies would be probably better addressed to investigation of the link between changes in thiamine homeostasis and early decline in GFR assessed by cystatin C measurement [30].

Decreased plasma thiamine concentration has been reported in diabetic patients previously in studies with small patient numbers [31, 32] but $Cl_{Thiamine}$ and $FE_{Thiamine}$, thiamine and TMP transporter content of erythrocyte membranes and plasma sVCAM-1 were not evaluated and hence links to these variables were not disclosed. Thiamine deficiency is conventionally assessed by measuring the percentage unsaturation of erythrocyte TK with TPP cofactor [12]. Herein we found, however, that plasma deficiency of thiamine in diabetes was masked in erythrocytes by increased levels of THTR-1 and RFC-1 transporter protein. As erythrocytes lack protein synthetic capability, increased protein levels of these transporters was produced by increased expression of the genes encoding THTR-1 and RFC-1 in the erythrocyte precursors, reticulocytes and erythroblasts. Indeed, increased levels of THTR-1 and RFC-1 transporter proteins were also found in MNLs in these diabetic patients (data not shown). Increased thiamine transporter gene expression and protein level is a response to thiamine deficiency [21, 33] and is consistent with the low plasma thiamine concentration found in diabetic patients herein. This explains why a low plasma thiamine concentration in clinical diabetes has hitherto gone unrecognised. This study indicates that the measurement of the 'thiamine effect' based on erythrocyte TK activities is an inadequate assessment of clinical thiamine status as it can be masked by changes in thiamine transporter protein levels.

Low plasma thiamine concentration in diabetes may be of limited significance if tissues can upregulate the gene expression and protein levels of thiamine transporters and maintain normal TK activity. Although this occurs in the normoglycaemic state [21, 33], our recent studies of experimental diabetes indicated it does not occur in renal glomeruli in the diabetic state [26] where enhanced hexosamine signalling may block increased expression of the genes encoding THTR-1, THTR-2 and RFC-1 and their protein levels. In diabetic glomeruli, TK activity and level of TK protein was decreased 60% [26], and similar impairment of thiamine uptake and metabolism may occur in the diabetic retina and peripheral nerve [34, 35]. Indeed, RFC-1 has impaired gene expression and protein levels in the diabetic retina [35].

Decreased availability of thiamine in vascular cells in diabetes exacerbates metabolic dysfunction in hyperglycaemia. Increased plasma sVCAM-1 is a marker of endothelial dysfunction [36] and increased risk of atherosclerosis [37].

sVCAM-1 was increased in diabetic patients with normal renal function [38] and was linked to microvascular and macrovascular complications in diabetes [39–41], although the link to glycaemic control is less certain [39, 42, 43]. Low plasma thiamine concentration may be a confounding factor linked to increased sVCAM-1 in diabetes.

This study indicates that type 1 and type 2 diabetic patients in the UK exhibit low plasma thiamine concentration. The conventional indicator of thiamine sufficiency, erythrocyte TK activity, is masked in clinical diabetes by increased protein levels of thiamine and TMP transporters, THTR-1 and RFC-1. The deficiency of thiamine in clinical diabetes may increase the fragility of vascular cells to the adverse effects of hyperglycaemia and thereby increase the risk of developing microvascular complications. Correction of the low plasma thiamine concentration with thiamine supplements may decrease the risk of microvascular complications in diabetes.

Important areas for future study are: (1) confirmation of low plasma thiamine concentrations in diabetic populations of other countries independent of local dietary and culinary practice; (2) the evaluation of thiamine and thiamine derivatives to correct low plasma thiamine concentration in diabetes, reverse vascular dysfunction and prevent vascular complications; and (3) investigation of the mechanism of increased $Cl_{Thiamine}$ in diabetes. These studies are either ongoing or in preparation.

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