Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1639 (2003) 121–132
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Increased formation of methylglyoxal and protein glycation, oxidation and nitrosation in triosephosphate isomerase deficiency

Naila Ahmed[a], Sinan Battah[a], Nikolaos Karachalias[a], Roya Babaei-Jadidi[a], Margit Horányi[b], Klára Baróti[c], Susan Hollan[b,c], Paul J. Thornalley[a,*]

[a] *Department of Biological Sciences, University of Essex, Central Campus, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK*
[b] *National Medical Center, Budapest, H-1113, Hungary*
[c] *National Blood Transfusion Service, Budapest, H-1113, Hungary*

Received 25 March 2003; received in revised form 16 July 2003; accepted 12 August 2003

Abstract

Triosephosphate isomerase deficiency is associated with the accumulation of dihydroxyacetonephosphate (DHAP) to abnormally high levels, congenital haemolytic anaemia and a clinical syndrome of progressive neuromuscular degeneration leading to infant mortality. DHAP degrades spontaneously to methylglyoxal (MG)—a potent precursor of advanced glycation endproducts (AGEs). MG is detoxified to D-lactate intracellularly by the glyoxalase system. We investigated the changes in MG metabolism and markers of protein glycation, oxidation and nitrosation in a Hungarian family with two germline identical brothers, compound heterozygotes for triosephosphate isomerase deficiency, one with clinical manifestations of chronic neurodegeneration and the other neurologically intact. The concentration of MG and activity of glyoxalase I in red blood cells (RBCs) were increased, and the concentrations of D-lactate in blood plasma and D-lactate urinary excretion were also increased markedly in the propositus. There were concomitant increases in MG-derived AGEs and the oxidative marker dityrosine in hemoglobin. Smaller and nonsignificant increases were found in the neurologically unaffected brother and parents. There was a marked increase (15-fold) in urinary excretion of the nitrosative stress marker 3-nitrotyrosine in the propositus. The increased derangement of MG metabolism and associated glycation, oxidative and nitrosative stress in the propositus may be linked to neurodegenerative process in triosephosphate isomerase deficiency.

Keywords: Triosephosphate isomerase; Dihydroxyacetonephosphate; Methylglyoxal; Advanced glycation endproduct; Glyoxalase; Neuromuscular degeneration; Oxidative stress

1. Introduction

Triosephosphate isomerase (EC 5.3.1.1, TPI) deficiency is a rare autosomal recessive disorder. The clinical syndrome of homozygous or compound heterozygous deficiency is characterised by extraordinary low activities of TPI in many tissues and is associated with chronic hemolytic anemia and neuromuscular disorders originating from focal degenerative processes of groups of neurons in different areas of the brain. Onset occurs in childhood (< 2 years old) and the majority of cases die within 6 years. The cellular and physiological characteristics of the TPI deficiency syndrome involve loss of neurones from the dentate hypothalamus and medulla oblongata and hyaline deposits throughout the hypothalamus and cerebellar cortex, degeneration of vascular endothelial cells and pericytes, and muscle fibre atrophy and regeneration. Recurrent infections, cardiomyopathy and severe and progressive neuromuscular disease lead to death [1].

TPI is coded by a single gene, TPI1, on chromosome 12p13. Thirteen mutations have been reported—the most common occurring at Glu-104 (GAG:Glu → GAC:Asp) [2]. Recently, one of us began a study of two Hungarian brothers

Abbreviations: AGE, advanced glycation endproduct; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate; CE, collision energy; CEL, N_ε-(1-carboxyethyl)lysine; CML, N_ε-carboxymethyllysine; DHAP, dihydroxyacetonephosphate; FL, N_ε-fructosyl-lysine; G-H1, glyoxal-derived hydroimidazolone N_δ-(5-hydro-4-imidazolon-2-yl)ornithine; GA3P, glyceraldehyde-3-phosphate; GOLD, glyoxal-derived lysine dimer; 1,3-di(N^ε-lysino)imidazolium salt; Hb, hemoglobin; HbA_1, glycated hemoglobin; LC-MS/MS, liquid chromatography–triple quadrupole mass spectrometry; MetSO, methionine sulfoxide; MG, methylglyoxal; MG-H1, methylglyoxal-derived hydroimidazolone N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N^ε-lysino)-4-methylimidazolium salt; RBC, red blood cell; TPI, triosephosphate isomerase

* Corresponding author. Tel./fax: +44-1206-873010.
E-mail address: thorp@essex.ac.uk (P.J. Thornalley).

0925-4439/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2003.08.002

with severe TPI deficiency. Molecular analysis revealed that both brothers had identical genotypes and were compound heterozygotes for two previously unknown mutations. One of the mutations was at position 240 with Phe replacement by Leu (TTC: Phe → CTC: Leu), creating a thermolabile protein. The other mutation decreased the abundance of TPI mRNA 10–20 fold [3]. This was later shown to be a nonsense mutation, 145 (GAG:Glu → TAG:Stop) [4]. The brothers are of interest in the mechanism of development of the complications of TPI deficiency since one brother has the expected neurological clinical syndrome and continuing survival into his third decade whereas the other is neurologically asymptomatic. The red blood cells (RBCs) of both siblings had < 3% of normal TPI activity and a 30–50-fold increased concentration of dihydroxyacetonephosphate (DHAP) in RBCs [5,6].

The most profound biochemical feature of TPI deficiency is the dramatic increase in the cellular concentration of DHAP. The high concentrations of DHAP, or a derivative of it, for many years has been viewed as the cause of morbidity in TPI deficiency [7,8]. This hallmark of the TPI deficiency syndrome, and recent evidence of depletion of

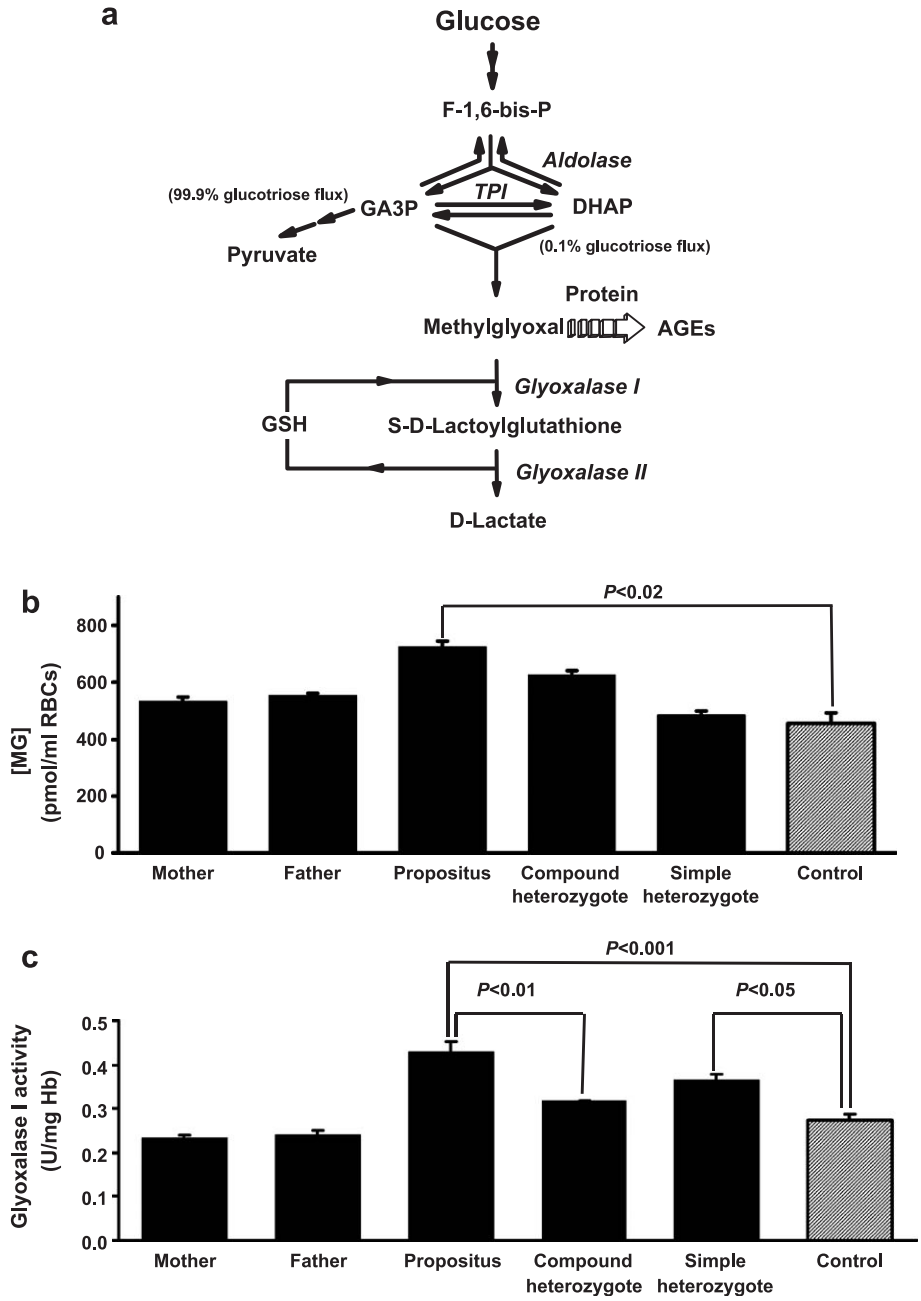

Fig. 1. (a) Triosephosphate metabolism to the formation and the formation and metabolism of MG. (b) Concentration of MG and (c) activity of glyoxalase I in RBCs of TPI deficient family members. Data are mean ± S.E. ($n = 3$ replicates) except control value (hatched bar) is the mean ± S.E. ($n = 21$ subjects).

reduced glutathione and oxidative stress [9], suggested to us that abnormal accumulation of methylglyoxal (MG) may be associated with the clinical TPI deficiency syndrome. MG is formed by the spontaneous degradation of DHAP—and also from glyceraldehyde-3-phosphate (GA3P) [10]—and is detoxified by conversion to D-lactate catalysed by the glutathione-dependent glyoxalase system [11]—Fig. 1a. Increased concentrations of DHAP and decreased cellular reduced glutathione are conditions ripe for MG accumulation [12]. MG is a potent glycating agent of proteins and precursor of advanced glycation endproducts (AGEs) [13,14]. MG-derived AGEs are the hydroimidazolone N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine (MGH1), N_{ε}-(1-carboxyethyl)lysine (CEL), MG-derived lysine dimer (MOLD) and argpyrimidine. Oxidative stress in TPI deficiency may increase the formation of glyoxal which is also detoxified by the glyoxalase system [11]. Glyoxal-derived AGEs are the hydroimidazolone N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), N_{ε}-carboxymethyl)lysine (CML) and glyoxal-derived lysine dimer (GOLD); CML may also be formed by the degradation of fructosyl-lysine, other ketoamines and ascorbic acid [15].

We investigated the involvement of MG in TPI deficiency by studying the RBC activity of glyoxalase I and II and the concentrations of MG, MG-derived AGEs and other protein biomarkers, and D-lactate in the RBCs, plasma and urine of the TPI deficient brothers, a further unaffected brother (simple heterozygote), their unaffected parents and normal healthy controls. There were derangement of MG metabolism and indications that oxidative and nitrosative stress are involved in the neurodegenerative symptoms of the TPI deficiency syndrome.

2. Materials and methods

2.1. Materials

High-purity MG was prepared by acid hydrolysis of methylglyoxal dimethylacetal and purified by fractional distillation under reduced pressure [16]. Glyoxal solution was purchased from Sigma. N_{ε}-Fructosyl-lysine (FL), MG-derived AGEs (MG-H1, CEL, MOLD and argpyrimidine), glyoxal-derived AGEs (G-H1, CML and GOLD), pentosidine, the markers of oxidative stress, methionine sulfoxide (MetSO) and dityrosine, the marker of nitrosative stress, 3-nitrotyrosine, and 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) were prepared as described [15,17–19]. [*guanidino*-$^{15}N_2$]-MG-H1 and [*guanidino*-$^{15}N_2$]-G-H1 were prepared from [*guanidino*-$^{15}N_2$]-L-arginine after conversion to the N_{α}-t-BOC-derivative [20]. [$^{13}C_6$]-CEL and [$^{13}C_6$]-CML were prepared from [$^{13}C_6$]-L-lysine, and [$^{2}H_8$]-MOLD, [$^{2}H_8$]-GOLD and [$^{2}H_4$]-fructosyl-lysine were prepared from [4,4,5,5-$^{2}H_4$]-L-lysine after conversion to the N_{α}-formyl derivative [21]. Synthetic methods for the preparation, purification and characterisation of these AGE calibration standards were as described for their non-isotopically substituted analogues [15]. [*methyl*-$^{2}H_3$]-MetSO was prepared from [*methyl*-$^{2}H_3$]-L-methionine, and [$^{2}H_6$]-dityrosine and [$^{2}H_3$]-3-nitrotyrosine were prepared from *ring*-[$^{2}H_4$]-L-tyrosine, by the methods described [17–19]. Isotopically substituted standard amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Familial human subjects in the Hungarian family with triosephosphate isomerase deficiency

The characteristics of the Hungarian family with TPI deficiency studied have been described [5,22]. Briefly, the TPI substitutions are: mother 240 Phe → Leu, father 145 Glu → Stop, propositus and compound heterozygote brother have both substitutions, and simple heterozygote son 240 Phe → Leu. The parents and the simple heterozygote son have RBC TPI activity around 50% of the normal range. The propositus and compound heterozygote have the same level of well-compensated hemolytic anemia but only the propositus has characteristic neurological symptoms (severe extrapyramidal disorder). Blood samples and 24-h urine samples from family and control subjects were taken with informed consent. Venous blood samples were taken with heparin anticoagulant and blood cells sedimented by centrifugation ($2000 \times g$, 10 min). The plasma and buffy coat were removed. Aliquots (0.25 ml) of plasma and packed RBCs for α-oxoaldehyde analysis were acidified with 1 volume of 500 mM acetic acid. Acidified and non-acidified samples (for glyoxalase activity, D-lactate and protein biomarker assays) were snap-frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ prior to analysis, with intervening shipment between collaborating laboratories on cardice. Normal healthy control subjects had a median age of 26 years, range 24–47 years ($n = 21$).

2.3. Glyoxalase activity and metabolite assays

The activities of glyoxalase I and glyoxalase II in red blood lysates were determined as described [23], referencing activity to sample content haemoglobin determined by the Drabkin method. The concentration of D-lactate in blood plasma and urine was determined by endpoint enzymatic assay with D-lactic dehydrogenase in neutralised perchloric acid extracts [24]. MG and glyoxal contents of plasma and RBCs were determined by derivatisation with 1,2-diamino-4,5-dimethoxybenzene and HPLC of the resulting quinoxaline adducts with fluorimetric detection. Stock solutions of MG and glyoxal stock solutions were calibrated by endpoint enzymatic assay with glyoxalase I [25].

2.4. Protein biomarker determination by LC-MS/MS

Biomarkers of early-stage protein glycation (fructosyl-lysine FL) and advanced-stage protein glycation (AGEs), oxidative stress and nitrosation were determined in enzymatic

hydrolysates of hemoglobin and free in blood plasma and urine. Analytes were measured by liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS), calibrated by stable isotope substituted standards referenced to authentic analyte calibration curves. N_ε-Fructosyl-lysine (FL), MG-derived AGEs (MG-H1, CEL and MOLD), glyoxal-derived AGEs (G-H1, CML and GOLD), the markers of oxidative stress, MetSO and dityrosine, and the marker of nitrosative stress, 3-nitrotyrosine, were determined. Free protein markers in blood plasma and urine were determined by assay of analytes in ultrafiltrate (12 kDa filter cut-off, 50 μl aliquot) of plasma and urine. Protein biomarkers in hemoglobin were determined in enzymatic digests of hemoglobin of RBC lysates. Lysate was diluted with water to 10 mg/ml haemoglobin and an aliquot (500 μl) was washed by three cycles of concentration and dilution by ultrafiltration. A portion (100 μg) was then hydrolysed exhaustively by consecutive incubation with pepsin, pronase E, and finally aminopeptidase and prolidase, as described [15]. All steps at pH 7.4 were performed under carbon monoxide to prevent heme-catalysed oxidations during the hydrolysis.

Samples were assayed by LC-MS/MS using Waters 2690 Separation module with a Quattro Ultima triple quadrupole mass spectrometric detector (Waters-Micromass, Manchester, UK). AGE analytes were determined without derivatisation since for analytes MG-H1, G-H1, CML and CEL derivatisation led to transfer of the α-oxoaldehyde-derived moiety in the isotopically labelled standard to unlabelled lysine and arginine in the sample and compromise of the stable isotope calibration. Samples were also derivatised with AQC prior to analysis since this provided required increased sensitivity for dityrosine. For underivatised analytes, two 5-μm Hypercarb™ columns (Thermo Hypersil Ltd., Runcorn, UK) in series were used: 2.1 × 50 mm (column 1) and 2.1 × 250 mm (column 2). The mobile phase was 26 mM ammonium formate, pH 3.8, with a gradient of 0–50% acetonitrile from 17 to 30 min and isocratic 50% acetonitrile from 30 to 40 min; the column was washed with 30% isopropanol from 40 to 50 min and re-equilibrated with initial mobile phase from 50 to 60 min. The flow rate was 0.2 ml/min. The flow was diverted from column 2 at 17 min to facilitate elution of hydrophobic biomarkers and column washing, and diverted back through columns 1 and 2 after 56 min to complete column washing and re-equilibration. Flow from the column in the interval 4–40 min was directed to the MS/MS detector. For AQC derivatised dityrosine, the column was a SymmetryTM 3.5-μm ODS, 2.1 × 100 mm. The mobile phases were: A—10 mM ammonium acetate with 5% methanol, pH 4.8, and B—10 mM ammonium acetate with 95% methanol, pH 4.8. The elution profile was: 0–12 min, isocratic 10% B; 12–20 min, linear gradient of 10–40% B; 20–38 min, isocratic 40% B; 38–48 min, 100% B (column washing); and 48–55 min, re-equilibration with 10% B. The flow rate was 0.2 ml/min and flow from the column in the interval 4–38 min was directed to the detector.

Biomarkers were detected by electrospray positive ionisation-mass spectrometric multiple reaction monitoring. The ionisation source temperature was 120 °C and the desolvation gas temperature 350 °C. The cone gas and desolvation gas flow rates were 150 and 550 l/h, respectively. The capillary voltage was 3.55 kV and the cone voltage 80 V. Argon gas (2.7×10^{-3} mbar) was in the collision cell and programmed molecular ion, fragment ion and collision energies optimised to ±0.1 Da and ±1 eV for multiple reaction monitoring detection and are given with neutral fragment losses in Table 1. Amounts of internal standard used were: 10 nmol for amino acids, 250 pmol for FL, and 10–50 pmol for glycation, oxidation and nitrosation bio-

Table 1
Chromatographic retention times and mass spectrometric multiple reaction monitoring detection of protein biomarkers

Analyte	R_t (min)	Parent ion (Da)	Fragment ion (Da)	Neutral fragment loss(es)	Internal standard	Isotopic purity (%)	Parent ion (Da)	Fragment ion (Da)	CE (eV)
Lysine	5.0	147.1	84.3	H_2CO_2, NH_3	[$^{13}C_6$]-Lysine	83.9 (0.7)	153.1	89.3	15
CML	6.7	204.9	130.1	$NH_2CH(CH_3)CO_2H$	[$^{13}C_6$]-CML	68.6 (1.4)	210.9	136.1	12
MetSO	6.8	166.1	102.2	CH_3-SOH	[methyl-$^2H_3$]-MetSO	99.5 (0.5)	169.1	102.2	14
CEL	7.0	219.2	130.1	$NH_2CH(CH_3)CO_2H$	[$^{13}C_6$]-CEL	82.5 (2.4)	225.2	136.1	13
FL	7.0	291.0	84.3	H_2CO_2, fructosylamine	[$^2H_4$]-FL	90.5 (0.6)	295.0	88.3	31
Arginine	10.9	175.2	70.3	H_2CO_2, $NH_2C(=NH)NH_2$	[$^{15}N_2$]-Arginine	96.4 (3.6)	177.2	72.3	15
GOLD	23.6	327.1	198.3	$NH_2CH(CO_2H)CH_2CH_2CH=CH_2$	[$^2H_8$]-GOLD	84.3 (3.1)	335.1	202.3	21
MG-H1	23.6 and 24.0	229.2	114.3	$NH_2CH(CO_2H)CH_2CH=CH_2$	[$^{15}N_2$]-MG-H1	96.1 (3.1)	231.2	116.3	14
MOLD	24.4	341.2	212.3	$NH_2CH(CO_2H)CH_2CH_2CH=CH_2$	[$^2H_8$]-MOLD	83.0 (0.3)	349.2	216.3	21
G-H1	25.0	215.0	100.2	$NH_2CH(CO_2H)CH_2CH=CH_2$	[$^{15}N_2$]-G-H1	94.5 (2.7)	217.0	102.2	14
Tyrosine	26.9	182.1	136.2	H_2CO_2	[*ring*-$^2H_4$]-Tyrosine	94.0 (0.5)	186.1	140.2	13
Dityrosine	26.9	361.2	315.3	H_2CO_2	[*ring*-$^2H_6$]-Dityrosine	80.9 (1.8)	367.2	321.3	15
Methionine	8.2	150.0	104.2	H_2CO_2	[*methyl*-$^2H_3$]-Methionine	99.1 (0.5)	153.0	107.2	11
3-Nitrotyrosine	22.1	227.1	181.2	H_2CO_2	[*ring*-$^2H_3$]-3-Nitrotyrosine	95.7 (0.1)	230.1	184.2	13
AQC-derivatised analyte:									
Dityrosine	30.7	701.3	531.4	8-Isocyanatoquinoline	[*ring*-$^2H_6$]-Dityrosine	93.4 (0.3)	707.3	537.4	23

The two epimeric forms of MG-H1 were resolved and hence the R_t values for both are given.

markers. Limits of detection were 0.2–1 pmol, interbatch coefficients of variation 2–16% ($n=6$) and analyte recoveries in the enzymatic hydrolysis >90% (except 71% for FL and 54% for G-H1), depending on the analyte.

2.5. Assay of pentosidine and argpyrimidine by HPLC detection of intrinsic fluorescence

The pentosidine contents of hemoglobin, plasma and urine were determined without derivatisation by HPLC with a Waters 2475 fluorescence detector. The column was of 5-μm particle size, 50 × 2.1 mm Hypercarb (Thermo-Hypersil-Keystone, Runcorn, UK). The mobile phase was 0.1% trifluoroacetic acid with 10% acetonitrile, a linear gradient to 50% acetonitrile at 15 min and isocratic 50% acetonitrile thereafter. For pentosidine and argpyrimidine, the retention times were 32.8 and 31.0 min and the limits of detection 30 and 400 fmol, respectively.

3. Results

3.1. Increased RBC MG concentration in triosephosphate isomerase deficiency

Increased concentration of DHAP in TPI deficiency may give rise to increased formation of MG [10]. We measured the concentrations of MG and glyoxal in RBCs of the Hungarian family with the TPI deficiency trait. The concentration of MG in RBCs was increased significantly in the propositus (59%, $P<0.01$), with respect to normal healthy controls; it was increased in the neurologically unaffected brother, mother, father and simple heterozygote but not significantly from the normal control value. The concentration of MG in RBCs was higher in the propositus than in the compound heterozygote ($P<0.05$)—Fig. 1b. There concentration of glyoxal in RBCs was not increased significantly in any family member, with respect to normal controls. The MG and glyoxal concentrations in RBC of normal control subjects were 445 ± 177 and 826 ± 504 pmol/ml RBCs, respectively ($n=21$). The concentrations of MG and glyoxal in blood plasma were not increased significantly in any family member, with respect to that of normal control subjects.

MG and glyoxal are substrates for glyoxalase I. RBC glyoxalase I activity was increased significantly in the propositus (58%, $P<0.001$) and simple heterozygote (33%, $P<0.05$) with respect to the glyoxalase I activity of normal healthy controls—Fig. 1c. The RBC glyoxalase I activity in normal controls was 0.273 ± 0.069 U/mg Hb ($n=21$). There was no significant change in RBC glyoxalase II activity in any family members. The RBC glyoxalase II activity in normal controls was 0.062 ± 0.009 U/mg Hb ($n=21$).

The major metabolic fate of MG is conversion to D-lactate catalysed by the glyoxalase system [11]. We therefore investigated the concentrations of D-lactate in blood plasma and urine of family members and normal control subjects. Plasma D-lactate was increased markedly in the propositus (250%, $P<0.001$) and moderately in the compound heterozygote (182%, $P<0.001$), with much smaller increases in the mother (64%, $P<0.01$) and father (69%, $P<0.01$), with respect to the normal control levels. Plasma D-lactate concentration was higher in the propositus than in the compound heterozygote ($P<0.01$)—Fig. 2a. The concentration of D-lactate in blood plasma of normal healthy control subjects was 12.5 ± 5.4 μM ($n=21$). A similar effect was observed for 24-h urinary D-lactate where the D-lactate elimination was increased 325% in the propositus ($P<0.001$), 75% in the compound heterozygote ($P<0.05$) and not increased significantly in both parents, with respect to value in normal controls—Fig. 2b. Urinary D-lactate excretion in normal, healthy human subjects was 27.9 ± 13.6 μmol/24 h (range 9.0–52.5).

3.2. Accumulation of MG-derived AGEs and biomarkers of oxidative and nitrosative stress in triosephosphate isomerase deficiency

The prolonged accumulation of MG in RBCs of the propositus may give rise to increased formation of AGEs which may, in turn, reflect the severity of increased MG exposure of the 6–8 weeks prior to sampling. These and markers of protein oxidative damage (MetSO and dityrosine) and nitrosative damage (3-nitrotyrosine) were assayed in hemoglobin and free in blood plasma and urine of family members and normal control subjects—Figs. 3–6. Specimen analytical chromatograms for analytes and stable isotope-substituted standards are given in Fig. 4a–h.

For MG-derived protein biomarkers, MG-H1 in hemoglobin was increased in all family members but only significantly in the propositus (49%), compound heterozygote (48%) and simple heterozygote (35%), with respect to normal control subjects. MG-H1 was significantly higher in the propositus ($P<0.01$) and compound heterozygote ($P<0.05$) than in the simple heterozygote—Fig. 5a. The concentration of MG-H1 (mean ± S.D.) in normal control subjects was 1.47 ± 0.49% Hb ($n=11$). CEL was increased significantly in all family members ($P<0.001$): fivefold in the mother, threefold in the father, sevenfold in the propositus, fivefold in the compound heterozygote and fourfold in the simple heterozygote—Fig. 5b. The concentration of CEL (mean ± S.D.) in normal control subjects was 0.24 ± 0.16% Hb ($n=11$). MOLD was also increased in all family members, and was highest in the propositus at 0.083% Hb—Fig. 5c. The concentration of MOLD in normal healthy human subjects was 0.024 ± 0.023% Hb ($n=11$). The concentration of argpyrimidine was below the limit of detection (<0.35% Hb) in all family members and control subjects. Surprisingly, the concentration of FL was decreased in the propositus and the compound heterozygote (3.35 and 4.09% Hb, $P<0.001$, respectively) with respect to the control value (7.66 ± 0.62%

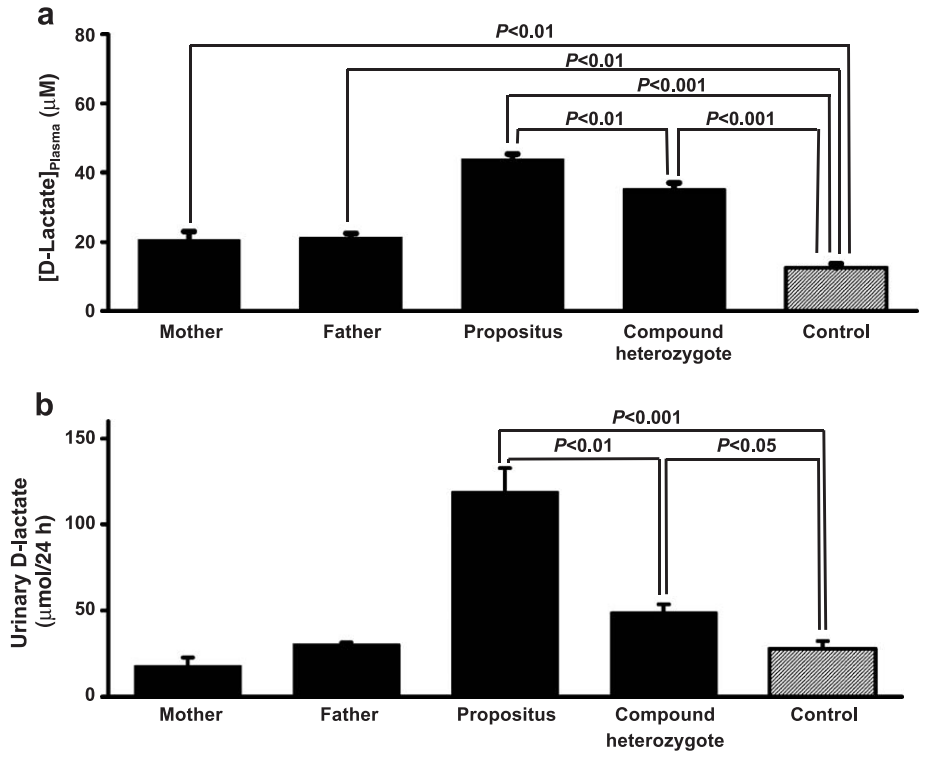

Fig. 2. Plasma and urinary D-lactate of TPI-deficient family members. (a) Plasma D-lactate, and (b) 24 h urinary D-lactate. Data are mean ± S.E. ($n = 3$ replicates) except control value (hatched bar) is the mean ± S.E. ($n = 21$ subjects).

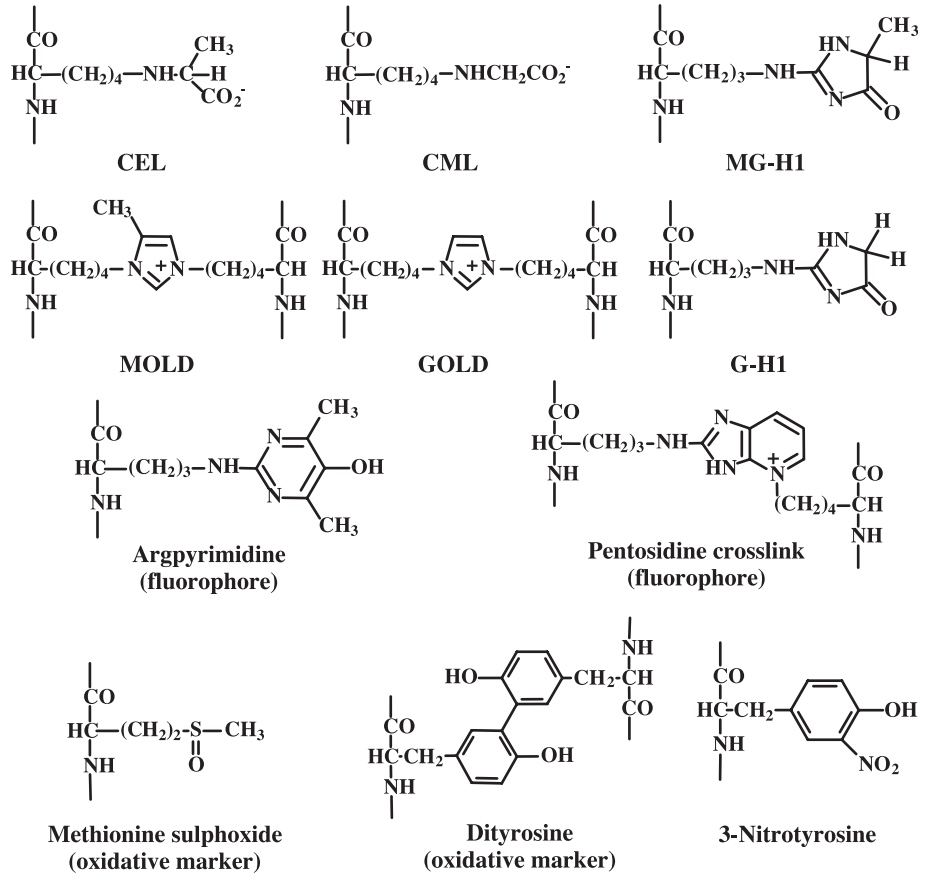

Fig. 3. Protein biomarkers of glycation, oxidation and nitrosation.

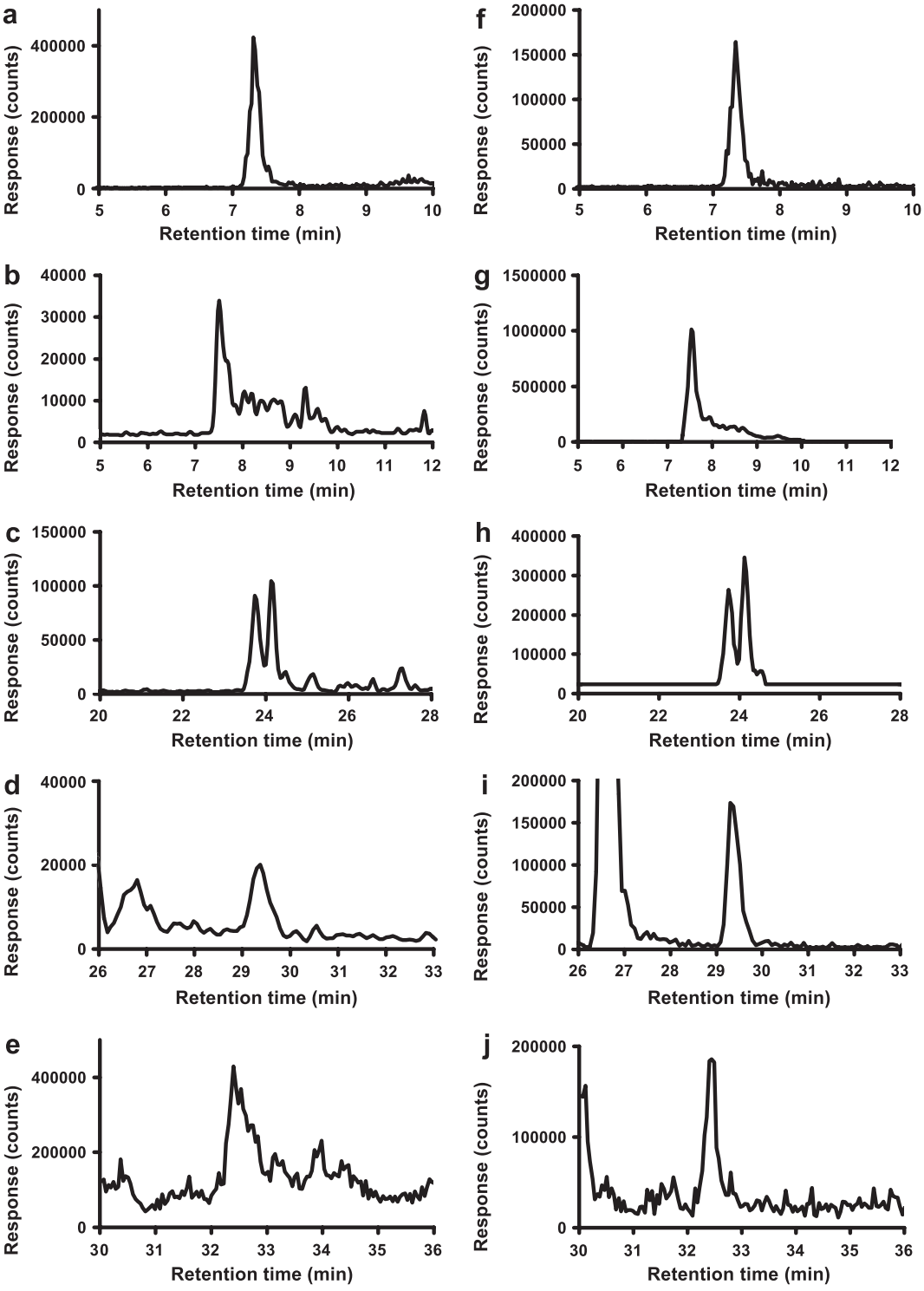

Fig. 4. Analytical chromatograms in the determination of protein biomarkers by LC-MS/MS in the propositus. Protein biomarkers: (a) CEL, Rt 7.3 min; (b) fructosyl-lysine, Rt 7.5 min (extensive peak tailing was observed); (c) MG-H1, Rt 23.7 and 24.2 min (two epimers resolved); (d) Dityrosine, Rt 29.4; (e) 3-Nitrotyrosine, Rt 32.4 min; (f) [$^{13}C_6$]-CEL (5 pmol); (g) [$^{4}H_2$]-fructosyllysine (125 pmol); (h) [$^{15}N_2$]-MG-H1 (25 pmol); (i) [$^{6}H_2$]-dityrosine (12.5 pmol); and (j) [$^{3}H_2$]-3-nitrotyrosine (5 pmol). Chromatographic conditions were described in Section 2.

Hb) and other family members—Fig. 5d. Glyoxal-derived AGEs, CML, G-H1 and GOLD, were also analysed. CML was 0.34 ± 0.10% Hb in the control subjects ($n = 11$) and was not changed significantly in any family member. G-H1 and GOLD were below the limits of detection in all samples (< 0.3% Hb and < 0.2% Hb, respectively). The AGE pentosidine, often viewed as a general marker of advanced glycation, accounted for 0.096 ± 0.029% Hb in the normal

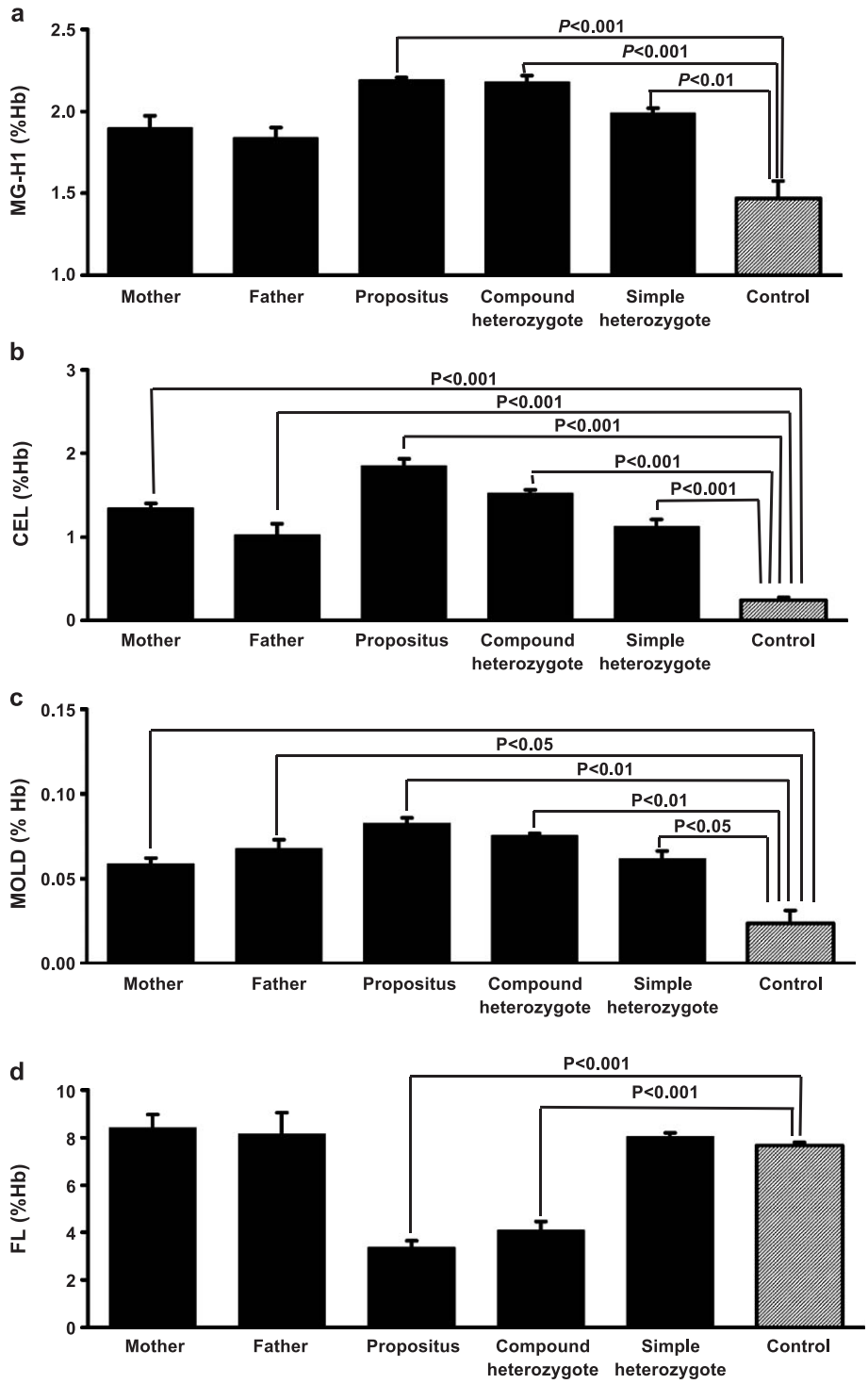

Fig. 5. Concentration of protein glycation biomarkers in hemoglobin of TPI deficient family members. (a) MG-H1, (b) CEL, (c) MOLD and (d) FL. Data are mean ± S.E. ($n=3$ replicates) except control value (hatched bar) is the mean ± S.E. ($n=11$ subjects).

control subjects ($n=11$) and was not changed significantly in any family member. The biomarkers of oxidative damage, MetSO and dityrosine were also investigated. MetSO was ca. 1.06 ± 0.15% Hb ($n=11$) in normal healthy controls and was not changed significantly in any family member. In contrast, dityrosine was 0.42% Hb in the normal healthy control subjects and was increased significantly (61%) in the propositus only—Fig. 6a.

We investigated the concentrations of free protein biomarkers in plasma since these may be released by protein turnover from tissues suffering metabolic derangement in TPI deficiency. Such measurements are complicated, how-

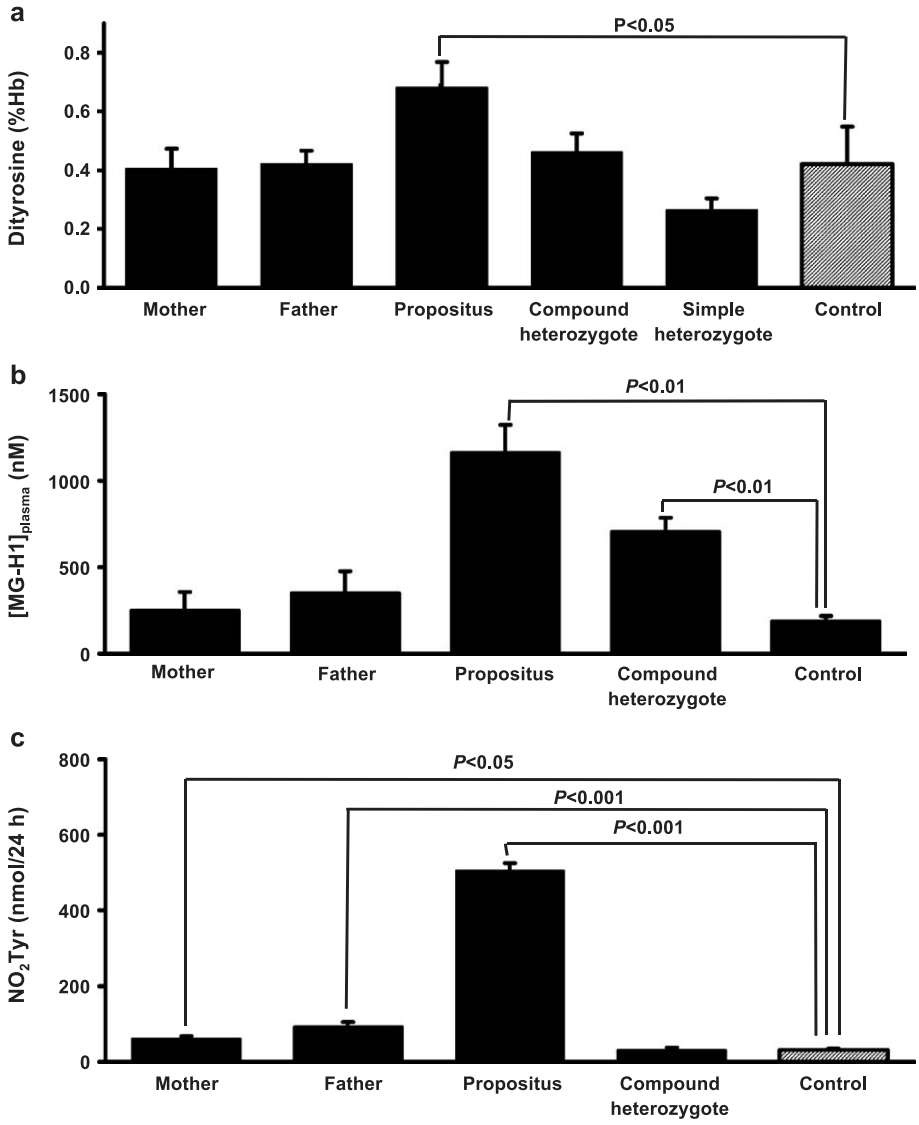

Fig. 6. Protein biomarkers of oxidation, glycation and nitrosation. (a) Concentration of dityrosine in hemoglobin, (b) plasma MG-H1 and (c) urinary 3-nitrotyrosine of TPI deficient family members. Data are mean ± S.E. ($n = 3$ replicates) except control value (hatched bar) is the mean ± S.E. ($n = 11$ subjects).

ever, by effects of metabolic transit of biomarkers from food and renal clearance of biomarkers. Biomarker concentrations were generally within the range found in normal healthy controls except for MG-H1 which was increased fivefold in the propositus ($P < 0.01$) and threefold in the compound heterozygote ($P < 0.01$)—Fig. 6b. Similarly, the urinary output of biomarkers of family members was generally within the range of normal healthy controls except that 3-nitrotyrosine was increased 15-fold in the propositus—Fig. 6c. This marked increase of urinary 3-nitrotyrosine in the propositus was retained when estimates were normalised to urinary creatinine. Urinary 3-nitrotyrosine (nmol/mmol creatinine): mother 4.58 ± 0.97, father 4.78 ± 1.03, propositus 35.6 ± 2.5, compound heterozygote 2.80 ± 0.94 and control 5.16 ± 0.67. 3-Nitrotyrosine was also determined in blood plasma. The concentration was 9.4 ± 0.4 nM in controls and was not changed significantly in any family member.

4. Discussion

The spontaneous degradation of triosephosphates is considered to be the major source of formation of MG in vivo in normal healthy subjects [10,11]. In TPI deficiency there was the expectation, therefore, that MG formation may be increased and this would produce increased concentrations of MG and MG-derived AGEs. The concentrations of DHAP in RBCs of the propositus and compound heterozygote were typically 70- and 45-fold higher than normal, and in the parents ca. one- to twofold higher than normal. The increase in the concentration of MG in RBCs of the propositus was relatively modest, and increases in other family members were generally within the highest quartile of the normal range. Normally ca. 40–67% of MG is formed from GA3P and 33–60% from DHAP, depending on the DHAP/GA3P molar ratio (4–12). In TPI deficiency, the concentration of

GA3P declines and the concentration of DHAP increases markedly. The specific reactivity towards fragmentation of DHAP to MG decreases because it hydrates fully. The minimum expected increase in MG formation (for a 45-fold increase in DHAP concentration, a DHAP/GA3P molar ratio = 4 and attainment of DHAP hydration equilibrium) is ca. eightfold. The metabolism of D-lactate may increase twofold such that the remaining urinary D-lactate is predicted to increase fourfold, as observed. The derangement of glyoxalase metabolites found in the propositus may be in keeping with increased degradation of DHAP to MG. MG concentration was only increased modestly because of the efficiency of its detoxification by the glyoxalase system; the assay methodology used to measure MG herein measures the sum of MG in free solution and bound reversibly to protein (mainly on protein thiols) [25,26]. Increased plasma and urine of D-lactate of the propositus suggests that the propositus sustains a particularly high flux of MG formation. The small but significant increases in plasma D-lactate of both parents may reflect the moderate increases in DHAP in these subjects, with respect to controls. The estimates of DHAP and MG-related variables in the propositus, compound heterozygote and normal controls has been summarised in Table 2.

A further independent measure of increased MG exposure comes from the levels of MG-derived AGEs. When the AGE is formed irreversibly with a chemical stability much greater than the lifespan of RBCs, as applies for CEL and MOLD, the AGE levels are expected to reflect exposure to MG in the 6–8 weeks prior to sampling. When the AGE is formed reversibly and has chemical stability less than the RBC lifespan, as applies for MGH1 (with a half-life of ca. 12 days under physiological conditions), the AGE level reflected MG exposure in this shorter period (2–4 weeks) [15]. The increased levels of Hb MG-H1, CEL and MOLD reflect exposure to increased levels of MG in the medium term in all family members with the highest increases and severity of MG levels in the propositus and compound heterozygote.

Table 2
Metabolites in blood plasma and red blood cells of the triosephosphate isomerase deficient propositus, compound heterozygote and normal healthy controls

Analyte	Compartment (units)	Propositus	Compound heterozygote	Normal controls
TPI activity†	RBCs (U/g Hb)	9.4	6.1	1364–1793
DHAP†	RBCs (nmol/ml RBCs)	905	581	5.1–13.0
DHAP → MG	Proportion of DHAP (%)	3.6	2.3	0.5
MG	RBCs (pmol/ml RBCs)	724	625	455 ± 157
	Plasma (pmol/ml)	180	198	139 ± 98
	RBCs (nmol/ml RBCs)	31.4	24.7	8.8 ± 3.8
D-Lactate	Plasma (nmol/ml)	44.8	35.3	12.5 ± 5.4
	Urine (μmol/24 h)	119	49	28 ± 14

† Data taken from Ref. [22]; otherwise from this work. The proportion of DHAP metabolised to methylglyoxal was deduced from previous measurements with RBC incubations in culture with and without added dihydroxyacetone, which is rapidly converted to DHAP by triokinase. The flux of methylglyoxal formation was estimated by the increase in D-lactate concentration; D-lactate is not metabolised in RBCs [34].

The estimation of FL adducts in hemoglobin by LC-MS/MS gave high estimates for FL. Most fructosamine-modified Hb has a β-chain terminal *N*-fructosyl-valine residues that represents ca. 60% of total glycated hemoglobin HbA1; FL residues are mainly on the α-chains and represent ca. 40% of glycated hemoglobin HbA1 [27]. Assay of HbA1 by mass spectrometric techniques gives estimates approximately twice that determined by affinity and ion exchange separation techniques because intact glycated subunits of Hb retained on chromatography columns undergo subunit exchange such that the HbA1 eluted has greater than one fructosamine adduct per Hb tetramer [27]. The concentration of FL was decreased in the propositus and compound heterozygote in the absence of evidence of hypoglycemia in these subjects. This may be a feature of oxidative stress in the normoglycemic status of TPI deficiency where increased oxidative degradation of FL occurs. The marker of oxidative damage to protein, dityrosine [28], was increased significantly in the propositus—consistent with the oxidative stress in TPI deficiency. MetSO was not increased similarly but this may be due to the countering effects of MetSO reductase activity repairing the oxidative damage [29].

Cellular proteins containing MG-H1 and CEL residues are degraded and the free AGEs are released into the circulation for urinary excretion; AGEs in plasma proteins may be removed by degradation of plasma protein by renal tubular epithelial cells after leakage into the glomerular filtrate. The plasma concentration of MG-H1, however, was increased in the plasma of the propositus and compound heterozygote. Increased glycation of proteins by MG is associated with enzyme inactivation, protein denaturation and cross-linking [11,13,13,30]. This may contribute to the TPI deficiency syndrome.

3-Nitrotyrosine was determined as a marker of protein nitrosation mediated by reactions of peroxynitrite and nitryl chloride [31]. This marker was below the limit of detection in hemoglobin (< 0.05% Hb) but it was found free in plasma at concentrations similar to those reported [32]. The urinary excretion of 3-nitrotyrosine was increased from control levels only in the propositus. Increased protein nitrosation has been linked to cholinergic dysfunction occurring in neurodegenerative diseases where formation of 3-nitrotyrosine was associated with inhibition of acetylcholine synthesis and choline acetyltransferase [33]. Increased activity of acetylcholine esterase was found in an earlier study in the red cell membrane preparations of the neurologically affected Hungarian patient with TPI deficiency as compared to his neurologically symptom-free brother and normal controls [22]. The neuromuscular disorders seen in TPI deficiency syndrome may be mediated by increased tyrosine nitration.

The two brothers studied have germ-line identical TPI, suffer from severe TPI deficiency, but only one developed neurological disorders. The neurologically intact compound heterozygote had a lower MG concentration in RBCs, a lower plasma D-lactate concentration and lower urinary D-lactate than the propositus. This suggests that the exposure to high fluxes of MG was lower in the compound heterozygote than in the propositus. Increased MG accumulation and glycation in the propositus may contribute directly to the pathogenesis of the neurodegeneration.

Acknowledgements

We thank the Wellcome Trust (UK) for support for our LC-MS/MS-related protein biomarker research at the University of Essex.

References

[1] A.S. Schneider, Triosephosphate isomerase deficiency: historical perspectives and molecular aspects, Bailliere's Clin. Haematol. 13 (2000) 119–140.
[2] I.O. Daar, P.J. Artymiuk, D.C. Phillips, L.E. Maquat, Human triosephosphate isomerase deficiency: a single amino acid substitution results in a thermolabile enzyme, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 7903–7907.
[3] M.L. Chang, P.J. Artymiuk, X. Wu, S. Hollan, A. Lammi, L.E. Maquat, Human triosephosphate isomerase deficiency resulting from mutations of the Phe-240, Am. J. Hum. Genet. 52 (1993) 1260–1269.
[4] C. Valentin, M. Cohen-Solal, L. Maquat, M. Horanyi, M. Inselt-Kovacs, S. Hollan, Identical germ-line mutations in the triosephosphate isomerase alleles of two brothers are associated with distinct clinical phenotypes, Life Sci. 323 (2000) 245–250.
[5] S. Hollan, H. Fujii, A. Hirono, K. Hirono, H. Karro, S. Miwa, V. Harsanyi, E. Gyodi, M. Inselt-Kovacs, Hereditary triosephosphate isomerase (TPI) deficiency: two severely affected brothers one with and one without neurological problems, Hum. Genet. 92 (1993) 486–490.
[6] A.S. Schneider, M. Cohen-Solal, Hematologically important mutations: triosephosphate isomerase, Blood Cells Mol. Diseases 22 (1996) 82–84.
[7] W.N. Valentine, A.S. Schneider, M.A. Baughan, Hereditary hemolytic anemia with triosephosphate isomerase deficiency: studies in kindreds with coexistent sickle cell trait and erythrocyte glucose-6-phosphate dehydrogenase deficiency, Am. J. Med. 41 (1966) 27–41.
[8] A.S. Schneider, I. Dunn, K.H. Ibsen, I.M. Weinstein, Triosephosphate isomerase deficiency. B. Inherited triosephosphate isomerase deficiency, in: E. Beutler (Ed.), Erythrocyte Carbohydrate Metabolism and Preliminary Studies of the Erythrocyte Enzyme, 1968, pp. 273–279, New York.
[9] E. Karg, I. Nemeth, M. Horanyi, S. Pinter, L. Vecsei, S. Hollan, Diminished blood levels of reduced glutathione and α-tocopherol in two triosephosphate-deficient brothers, Blood Cells Mol. Diseases 26 (2-29-2000) 91–100.
[10] S.A. Phillips, P.J. Thornalley, The formation of methylglyoxal from triose phosphates. Investigation using a specific assay for methylglyoxal, Eur. J. Biochem. 212 (1993) 101–105.
[11] P.J. Thornalley, The glyoxalase system in health and disease, Mol. Aspects Med. 14 (1993) 287–371.
[12] E.A. Abordo, H.S. Minhas, P.J. Thornalley, Accumulation of α-oxoaldehydes during oxidative stress. A role in cytotoxicity, Biochem. Pharmacol. 58 (1999) 641–648.
[13] P.J. Thornalley, Clinical significance of glycation, Clin. Lab. 45 (1999) 263–273.
[14] M. Shinohara, P.J. Thornalley, I. Giardino, P.J. Beisswenger, S.R. Thorpe, J. Onorato, M. Brownlee, Overexpression of glyoxalase I in bovine endothelial cells inhibits intracellular advanced glycation endproduct formation and prevents hyperglycaemia-induced increases in macromolecular endocytosis, J. Clin. Invest. 101 (1998) 1142–1147.
[15] N. Ahmed, O.K. Argirov, H.S. Minhas, C.A. Cordeiro, P.J. Thornalley, Assay of advanced glycation endproducts (AGEs): surveying AGEs by chromatographic assay with derivatisation by aminoquinolyl-*N*-hydroxysuccimidyl-carbamate and application to N_{ε}-carboxymethyl-lysine- and N_{ε}-(1-carboxyethyl)lysine-modified albumin, Biochem. J. 364 (2002) 1–14.
[16] A.C. McLellan, P.J. Thornalley, Synthesis and chromatography of 1,2-diamino-4,5-dimethoxybenzene, 6,7-dimethoxy-2-methylquinoxaline and 6,7-dimethoxy-2,3-dimethylquinoxaline for use in a liquid chromatographic fluorimetric assay of methylglyoxal, Anal. Chim. Acta 263 (1992) 137–142.
[17] A. Lapp, M.S. Dunn, DL-Methionine sulphoxide, Biochem. Prep., (1955) 80–85.
[18] T.G. Huggins, M.C. Wells-Knecht, N.A. Detories, J.W. Baynes, S.R. Thorpe, Formation of *o*-tyrosine and dityrosine in proteins during radiolytic and metalcatalysed oxidation, J. Biol. Chem. 268 (1993) 12341–12347.
[19] M. Sokolovsky, J.F. Riordan, B.L. Vallee, Tetranitromethane. A reagent for the nitration of tyrosyl residies in proteins, Biochemistry 5 (1966) 3582–3589.
[20] M. Meldal, W. Kindtler, Synthesis of a proposed antigenic hexapeptide from Escherichia-coli k88 protein fimbriae, Acta Chem. Scand., Ser. B, Org. Chem. Biochem. 40 (1986) 235–241.
[21] P.A. Finot, J. Mauron, Le blocage de la lysine par la reaction de Maillard: I. Synthese de *N*-(desoxy-1-D-fructosyl-1)-et *N*-(desoxy-1-D-lactulosyl-1)-L-lysine, Helv. Chim. Acta 52 (1969) 1488–1495.
[22] S. Hollan, M. Magocsi, G. Fodor, M. Horanyi, Search for the pathogenesis of the differing phenotype in two heterozygote Hungarian brothers with the same genotypic triosephosphate isomerase deficiency, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10362–10366.
[23] A.C. McLellan, P.J. Thornalley, Sample storage conditions for the assay of glyoxalase activities in whole blood samples, Ann. Clin. Biochem. 29 (1992) 222–223.
[24] A.C. McLellan, S.A. Phillips, P.J. Thornalley, Fluorimetric assay of D-lactate, Anal. Biochem. 206 (1992) 12–16.
[25] A.C. McLellan, S.A. Phillips, P.J. Thornalley, The assay of methylglyoxal in biological systems by derivatization with 1,2-diamino-4,5-dimethoxybenzene, Anal. Biochem. 206 (1992) 17–23.
[26] T.W.C. Lo, M.E. Westwood, A.C. McLellan, T. Selwood, P.J. Thornalley, Binding and modification of proteins by methylglyoxal under physiological conditions. A kinetic and mechanistic study with N_{α}-acetylarginine, N_{α}-acetylcysteine, N_{α}-acetyllysine, and bovine serum albumin, J. Biol. Chem. 269 (1994) 32299–32305.
[27] X. Zhang, K.F. Medzihradszhy, J. Cunningham, P.D.K. Lee, C.L. Rognerud, C.-N. Ou, P. Harmatz, H.E. Witkowska, Characterization of glycated hemoglobin in diabetic patients: usefulness of electrospray mass spectrometry in monitoring the extent and distribution of glycation, J. Chromatogr., B, Biomed. Sci. Appl. 759 (2001) 1–15.
[28] S. Bhattacharjee, S. Pennathur, J. Byun, J. Crowley, D. Mueller, J. Gischler, R.S. Hotchkiss, J.W. Heinecke, NADPH oxidase of neutrophils elevates *o*,*o*′-dityrosine crosslinks in proteins and urine during inflammation, Arch. Biochem. Biophys. 395 (2001) 69–77.
[29] H. Weissbach, F. Etienne, T. Heinemann, S.H. Hoshi, W.T. Lowther, B. Matthews, G. St John, C. Nathan, N. Brot, Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function, Arch. Biochem. Biophys. 397 (2002) 172–178.
[30] T.P. Degenhardt, S.R. Thorpe, J.W. Baynes, Chemical modification of proteins by methylglyoxal, Cell. Mol. Biol. 44 (1998) 1139–1145.
[31] J.P. Eiserich, M. Hristova, C.E. Cross, A.D. Jones, B.A. Freeman, B. Halliwell, A. van der Vliet, Formation of nitric oxide-derived inflam-

matory oxidants by myeloperoxidase in neutrophils, Nature 391 (1998) 393–397.

[32] J.P. Gaut, J. Byun, H.D. Tran, J.W. Heinecke, Artifact-free quantitation of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry, Anal. Biochem. 300 (2002) 252–259.

[33] Y.M. Gaudry-Talarmain, N. Moulian, F.A. Meunier, B. Blanchard, D. Angaut-Petit, L. Faille, C. Ducrocq, Nitric oxide and peroxynitrite affect differently acetylcholine release, choline acetyltransferase activity, synthesis, and compartmentation of newly formed acetylcholine in *Torpedo marmorata* synaptosomes, Nitric Oxide-Biol. Chem. 1 (2002) 330–345.

[34] S.A. Phillips, P.J. Thornalley, Formation of methylglyoxal and D-lactate in human red blood cells in vitro, Biochem. Soc. Trans. 21 (1993) 163.