Erythrocyte lipids in triose-phosphate isomerase deficiency

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ABSTRACT   Marked hypoalphalipoproteinemia was found together with relatively low serum cholesterol, triacylglycerol, and LDL levels in a triose-phosphate isomerase (TPI; D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1)-deficient Hungarian family, especially in the two compound-heterozygote brothers. Apart from a slight increase in palmitic and stearic acids together with a slight decrease in oleic and linoleic acids, no other changes were found in the fatty acid composition of the erythrocyte phospholipids. Anisotropy measurements with n-(9-anthroyloxy)stearic and -palmitic acid fluorophores revealed increased motional freedom of the fatty acid chains in the external lipid layers of the intact erythrocytes from all members of the TPI-deficient family as compared with normal age-matched controls. This asymmetric increase in membrane fluidity was found to be significantly higher in the propositus than in his compound-heterozygote brother without any neurological disorders. The change in membrane fluidity may result from as-yet-unresolved aspects of the lipid composition of the plasma membrane. Our findings that the differences between the TPI-deficient individuals and normal controls and the differences between the two compound-heterozygote brothers were all absent in the phospholipid extracts of the same erythrocytes favor the assumption that the increased motional freedom of the fatty acid chains in the external surface of the bilayer is caused by the binding of the mutant TPI molecule to the N-terminal sequence of band 3 protein.

Triose-phosphate isomerase (TPI; D-glyceraldehyde-3-phosphate keto-isomerase, E.C. 5.3.1.1) catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) with high catalytic efficiency (by a factor of 10^{10}). The interconversion favors the formation of DHAP by 20:1. TPI is a highly conserved (1) housekeeping enzyme expressed in all investigated cell types and has an essential role in glycolysis, gluconeogenesis, fatty acid synthesis, and the pentose phosphate shunt (2).

TPI deficiency is a rare autosomal recessive defect. In the absence of consanguinity, clinically affected patients are usually genetic compounds of two defective mutant genes resulting in TPI activity that is <20% of normal. The leading clinical manifestations are (i) chronic nonspherocytic hemolytic anemia and (ii) early death prior to age 6. Infections and cardiac complications had been noted in the minority of the affected cases. Family members with one defective gene and TPI activity around 50% are completely normal.

Based on the energy dependence of mature erythrocytes on glycolysis, the depletion of adenosine triphosphate (ATP) has been proposed to be the cause of the shortened life span of erythrocytes in glycolytic enzymopathies (3). However, low erythrocyte ATP levels are not invariably associated with loss of viability, and circulating ATP levels are not necessarily diminished in patients with glycolytic enzyme defects (4).

Schneider et al. (5) have shown that severely TPI-deficient erythrocytes are capable of normal glycolysis in vitro, even when compared with reticulocyte-rich normal blood, and have raised the possibility of toxic effects of DHAP accumulation in TPI deficiency that may result in lipid abnormality in tissues that—in contrast to mature erythrocytes—are not lacking in α-glycerophosphate dehydrogenase. The role of a possible lipid disorder in the pathomechanism of TPI deficiency was drawn to our attention by the investigation of a unique TPI-deficient Hungarian family with two compound-heterozygote brothers, both having <10% TPI activities in their erythrocytes and chronic hemolytic anemia, one with severe neurologic disorders and the other without any neurologic manifestation of the defect (6–9). This paper presents the results of analyses of the lipid components of the erythrocytes of the members of this Hungarian family with TPI deficiency. Since there is a slow but continuous exchange of lipids between the cell membranes and their environment, the quantitative and qualitative composition of lipoproteins have been investigated as well.

MATERIALS AND METHODS

Patients. The main characteristics of the TPI-deficient Hungarian family are summarized in Table 1. More detailed clinical and biochemical findings have been published earlier (9). There are two striking features in this TPI-deficient family: (i) the neurological disorders developed unusually late (at the age of 11 yr) and remained stable after the age of 13 yr in the propositus (B.J. Jr.), who is now 17 yr old, and (ii) his 24-yr-old brother (A.J.) has severe TPI deficiency and congenital hemolytic anemia. He is, however the only known compound heterozygote who has no neurological manifestation at all.

Analytical Methods. Preparation of Hb-free erythrocyte ghosts [i.e., rightside-out vesicles (ROV)] and inside-out vesicles (IOV) were carried out as described by Sarkadi et al. (10). Analyses of total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, and triacylglycerol were determined by enzymatic methods, while apoproteins AI, AII, and B were determined by immunoturbidimetric assays. Lipid extraction was done as described by Folch et al. (11). Phospholipids and neutral lipids were separated by silicic acid column chromatography. Phospholipid subclasses were separated on precoated silica gel plates (Merck). Fatty acid methyl esters were analyzed according to IUPAC recommendations (12) in a Chromopack-Packard 438A gaschromatograph.

Table 1. Characteristic features in the members of the TPI-deficient family

	Mother (Mrs. B.J.; TPI heterozygote)	Father (B.J.; TPI heterozygote)	Propositus (B.J. Jr.; TPI compound heterozygote)	Brother (A.J.; TPI compound heterozygote)	Brother (T.J.; TPI heterozygote)	Normal range
TPI activity, units/g of Hb	436	644	9.4	6.1	745	1364–1793
DHAP, nmol/ml of RBC	19.8	23.2	904.9	580.9	NT	5.08–13.0
TPI heat instability	Partial	None	Total	Total	Partial	None
Hemolytic anemia	0	0	+	+	0	NA
Neurologic disorders	0	0	+++	0	0	NA

NT, not tested; NA, not applicable.

Fluorescence Anisotropy Measurements. Fluorescence anisotropy measurements were carried out on a computer-controlled and thermostated Hitachi MPF-2A spectrofluorimeter. The lipid-soluble fluophores—3-[*p*-(6-phenyl-1,3,5-hexatrienyl)phenyl]propionic acid (DPH-PA), 2- and 12-(9-anthroyloxy)stearic acid (2-AS and 12-AS), and 16-(9-anthroyloxy)palmitic acid (16-AP)—were from Molecular Probes. Labeling of the washed erythrocytes, ghosts, and IOVs were done according to Dey and Farkas (13). Preparation and labeling of phospholipid vesicles were as described by Dey *et al.* (14). All measurements were carried out as described (14) and corrected for light scattering according to Kuhry *et al.* (15). Measurements were carried out on samples from all members of the TPI-deficient family and 16 healthy age-matched voluntary blood donors. Every data-point on tables and graphs represents the mean of the 10 measurements (means ± SD < 0.05).

RESULTS

All members of the TPI-deficient family have low serum cholesterol, TG, and LDL-Chol levels. Both compound heterozygotes and their mother have significantly lower HDL levels, and both heterozygotes and their father have lower levels of apolipoproteins AI, AII, and B (Table 2). No significant changes were found in the ratio of the main components of very low density lipoprotein (VLDL), LDL, and HDL and in their fatty acid composition. Fatty acid composition of phospholipids from erythrocytes showed a very slight increase in 16:0 and 18:0 fatty acids together with a slight decrease in 18:1 and 18:2 fatty acids, especially in the phosphatidylethanolamine and in the sphingomyelin classes in all members of the family.

The moieties of the series of *n*-anthroyloxy fluorophore probes when used in nonperturbing amounts (i.e., <1.0 μM) locate at a graded series of depths in the outer hemileaflet of the erythrocyte membrane just as in the case of model lipid bilayers (16–18) of isolated biological membranes and living cells (19). 2-AS reflects the motional freedom of the lipids at the level of the second carbon atom (C-2), whereas 12-AS and 16-AP reflect that at the level of C-12 and C-16, respectively. The results of the anisotropy measurements with these anthroyloxy fluorophores revealed significantly increased fluidity in the outer layer of the erythrocyte membrane in all

members of the TPI-deficient family. This increase is most conspicuous at the C-2 level, it is still there at C-12, and disappears in the depths of C-16. This leaflet asymmetry in lipid motional freedom is significantly more expressed in the propositus (B.J. Jr.) with neurologic disorders than in his compound-heterozygote brother without any neurological signs and symptoms (Fig. 1).

DPH-PA labels only the outer leaflet of the plasma membrane (20, 21). The fluorescence anisotropy measurements with DPH-PA revealed a marked increase in the fluidity of the erythrocytes from the mother (Mrs. B.J.) and the two compound heterozygotes (B.J. Jr. and A.J.) as compared with the father and the normal controls (Table 3). This increase was most expressed in the compound heterozygote (A.J.) without any neurological disorder. The fluorescence anisotropy values of the erythrocyte ghosts (ROV) from both groups—that of the family members with TPI deficiency and the normal controls—were decreased as compared with the intact erythrocytes, with the exception of A.J. (the compound heterozygote without neurological disorders). The fluorescence anisotropy values of IOV from both parents and from the controls showed a decrease as compared with that of their ROV, while no difference could be revealed between the motional freedom of the fatty acid chains in ROV and IOV from the two compound heterozygotes.

Because of nonradiative energy transfer to the heme, the fluorescence signal in intact erythrocytes of permeant fluorophores is weighted in favor of the outer hemileaflet of the membrane bilayer, while in erythrocyte ghosts the fluorescence signal is representative of both leaflets (22). In contrast to the normal controls and the father, the difference in anisotropy values between intact erythrocytes and ghosts from the propositus and the mother was much less, and there was no difference at all between the samples obtained from the compound heterozygote without neurological symptoms; these findings plus the complete lack of difference between ROV and IOV from the two compound heterozygotes may point to marked differences in the lipid organization of their erythrocyte membrane. Fluorescence anisotropy measurements of the phospholipids isolated from the lipid extracts from erythrocytes from the TPI-deficient family and the controls showed no difference in anisotropy values (Fig. 2).

DISCUSSION

TPI is involved in fatty acid synthesis and DHAP is a crucial precursor of ether lipids. Lipids form 30–40% of brain tissue

Table 2. Cholesterol, triacylglycerol, and lipoprotein levels in the TPI-deficient family

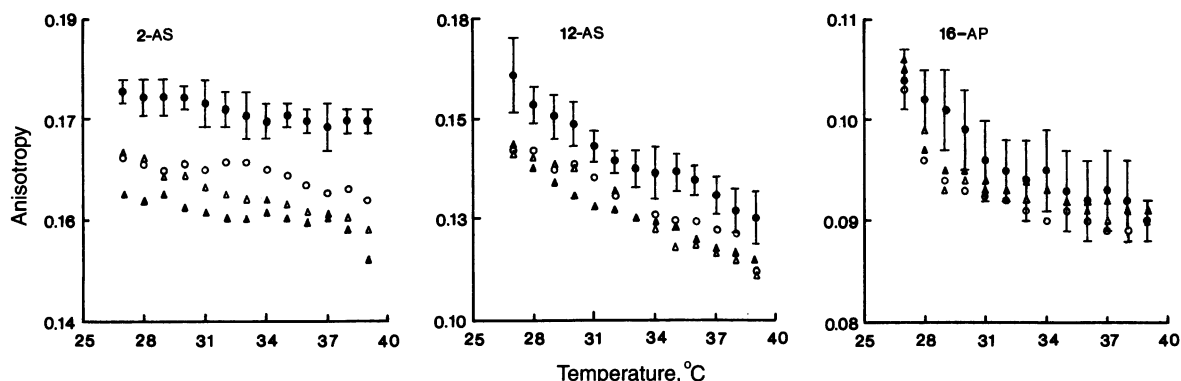|	Mother (Mrs. B.J.)	Father (B.J.)	Propositus (B.J. Jr.)	Brother (A.J.)	Normal range*
Cholesterol, mmol/liter	4.52	2.02	2.40	2.2	4.31 ± 1.08
Triacylglycerol, mmol/liter	1.58	0.37	0.83	0.46	0.81 ± 0.63
LDL-cholesterol, mmol/liter	2.59	0.37	0.84	0.85	2.53 ± 0.62
HDL-cholesterol, mmol/liter	0.59	1.61	0.35	0.31	1.34 ± 0.43
Apoprotein AI, g/liter	1.09	0.75	0.72	0.76	1.33 ± 0.16
Apoprotein AII, g/liter	0.40	0.20	0.19	0.28	0.37 ± 0.09
Apoprotein B, g/liter	0.86	0.41	0.35	0.44	0.92 ± 0.23

*Normal range is expressed as mean ± SD.

FIG. 1.   Fluorescence anisotropy of 2-AS, 12-AS, and 16-AP in the erythrocytes of the TPI-deficient family (means ± SD of control values are represented). ●, Control; ▲, propositus (B.J. Jr.); △, mother (Mrs. B.J.); ○, brother (A.J.).

and ether lipids are essential components of myelin sheaths. Therefore, it seemed justified to look for lipid changes to explain the difference between the two compound-heterozygote brothers with <10% TPI activity, one of which completely lacks the neurological disorders that are cardinal clinical features of TPI deficiency.

The present investigation is limited to erythrocytes, which, in addition to their easy availability, have the advantage of having all lipids in their plasma membrane. Mature erythrocytes are unable to synthesize and metabolize lipids; however, there is a continuous exchange between lipids of the erythrocyte membrane and the plasma. All members of the TPI-deficient family were found to have low-serum cholesterol and triacylglycerol levels. The most conspicuous change was the significantly lower levels of HDL cholesterol and of apolipoproteins AI, AII, and B in the plasma of both compound heterozygotes. No significant changes were found either in the ratio of the main components of VLDL, LDL, and HDL or in their fatty acid composition.

A wide spectrum of conditions are associated with markedly reduced circulating levels of HDL. Although hypoalphalipoproteinemia is considered to have a genetic basis in the vast majority of cases, it has not been established whether the defect is at the synthetic or catabolic level or both (23). Hypoalphalipoproteinemia may manifest either as an isolated entity or in conjunction with other dyslipidemias. Its epidemiology has been studied so far only from the point of view of its linkage to premature coronary heart disease. Low-level HDL disorders resulting from rapid HDL turnover but normal apolipoprotein AI synthetic rates such as Tangier's disease and lecithin-cholesterol acyltransferase deficiency are not strongly associated with high rates of atherosclerosis. These disorders are accompanied, as in this Hungarian family by low levels of LDL. Since the regulation of both synthesis and catabolism of HDLs is very complex, further studies are needed to clarify whether the changes in plasma lipids in this family have direct connection with the TPI defect.

Erythrocyte lipid changes are known to induce chronic hemolytic anemia. The premature breakdown of erythrocytes

has been generally explained by increased rigidity of the cell membrane caused either by increased cholesterol/phospholipid ratio or by increased ratios of saturated to unsaturated fatty acids. None of these changes are present in the erythrocytes from this family. Changes in the levels of 16:0 and 18:0 are insignificant, and 18:1 and 18:2 are slightly decreased. The cholesterol/total phospholipid ratio in the erythrocyte lipids was 1 for Mrs. B.J. (mother), 1.15 for B.J. (father), 1.16 for B.J. Jr. (propositus), 1.20 for A.J. (compound-heterozygote brother), and 0.95 ± 0.08 for normal controls. We have found, however, significant changes in erythrocyte membrane fluidity in the TPI-deficient family. Although the biophysical properties of cell membranes are clearly dependent on their biochemical composition and molecular organization, the specific factors determining the rheological properties of human erythrocytes are not yet completely understood. Fluorescence anisotropy measurements with different permeant and nonpermeant fluorophores have contributed a lot to a better understanding of the ordered states and motional freedom of the bulk and boundary phospholipids in the cell membrane. Shachter *et al.* (22) have shown by using both permeant and impermeant fluorophores greater fluidity in the outer than in the inner leaflet of the human erythrocytes. Fluorescence anisotropy measurements with anthroyloxy fluorophores revealed an even more increased fluidity in the outer layer of the erythrocyte membrane in all members of the TPI-deficient family as compared with normal age-matched controls. This leaflet asymmetry in lipid motional freedom was found to be significantly more expressed in the propositus with neurologic disorders than in his compound-heterozygote brother without any neurological signs and symptoms.

Glyceraldehyde-3-phosphate dehydrogenase, aldolase, and phosphofructokinase are reported to have high-affinity binding sites at the extreme N terminus of the cytoplasmic domain of band 3, the main anion-transport protein of the erythrocyte membrane. It has been suggested that a band 3 tetramer could constitute the nucleus of a glycolytic enzyme complex. This suggestion has been extended to include a glycolytic enzyme complex from TPI to pyruvate kinase bound *in vivo* to the

Table 3.   Anisotropy values of erythrocytes, ghosts (ROV), and IOV with DPH-PA in the TPI-deficient family

	Intact erythrocytes, no.	ROV, no.	IOV, no.
Control	0.302 ± 0.032	0.255 ± 0.004	0.239 ± 0.001
Father (B.J.)	0.289 ± 0.024	0.248 ± 0.005	0.240 ± 0.001
Mother (Mrs. B.J.)	0.250 ± 0.027	0.242 ± 0.002	0.231 ± 0.008
Propositus (B.J. Jr.)	0.260 ± 0.025	0.241 ± 0.001	0.240 ± 0.001
Brother (A.J.)	0.233 ± 0.025	0.235 ± 0.002	0.233 ± 0.004

Determinations of membrane fluidity of intact erythrocytes, ghosts (ROV), and IOV in terms of fluorescence anisotropy measurements with DPH-PA in the TPI-deficient family (mean ± SD of 10 experiments).
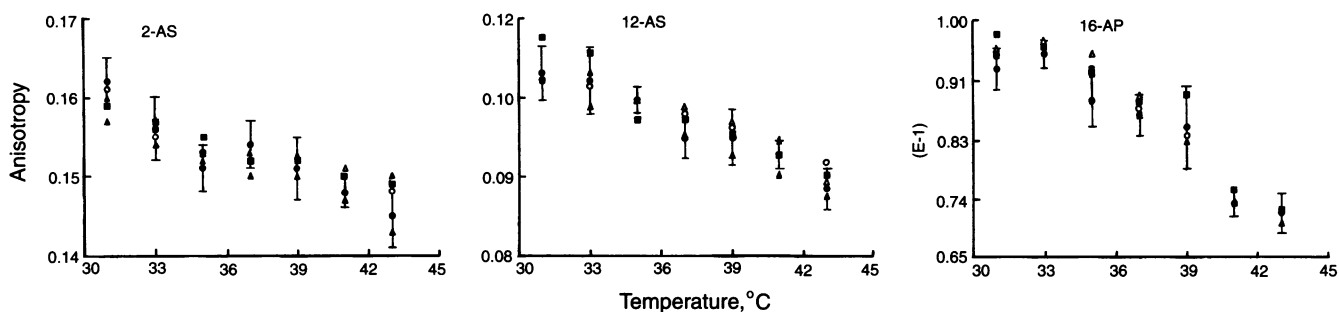
FIG. 2. Fluorescence anisotropy of 2-AS, 12-AS, and 16-AP in the total phospholipids extracted from erythrocytes of the TPI-deficient family (mean ± SD of control values are represented). ●, Control; ▲, propositus (B.J. Jr.); △, mother (Mrs. B.J.); ○, brother (A.J.); ■, father (B.J.).

cytoplasmic domain of band 3 in direct contact with Na^+/K^+-ATPase (24–27). Ovádi (28) has confirmed the direct channeling of substrates among aldolase, TPI, and glyceraldehyde-3-phosphate dehydrogenase in erythrocytes when the protein concentration was held high. The relevance of glycolytic enzyme interactions with band 3 has been repeatedly questioned (29, 30). However, the most recent data of Low *et al*. (31) state that the extreme N terminus of band 3 can bind and inhibit glycolytic enzymes *in vivo* and suggest that the reversible enzyme binding may participate in control of erythrocyte glycolysis. These interactions are markedly influenced by the dynamics and phase behavior of lipids tightly associated with band 3 in the erythrocyte membrane. It is a well-established fact that, with the exception of cholesterol, any molecule penetrating into the lipid bilayer increases the membrane fluidity.

The membrane fluidity changes may originate from different sources in TPI deficiency and may be due to one or more of the following three factors. (*i*) Some as-yet-unresolved aspects of lipid composition of the plasma membrane—i.e., the highly increased amounts of DHAP may change the metabolism of ether lipids. This lipid change may probably be more expressed in cells possessing α-glycerophosphate dehydrogenase. (*ii*) Isoprenes like dolichols are known to increase membrane fluidity. Changes in their concentration and turnover rate have to be studied on cells possessing microsomes. (*iii*) The mutant TPI molecule may cause steric hindrance and induce thereby via the band 3 molecule an increase in membrane fluidity in the external layer of the membrane lipids. We have checked this possibility by measuring the fluorescence anisotropy of the extracted erythrocyte phospholipids. It turned out that the difference in fluorescence anisotropy between both the propositus and his compound-heterozygote brother and the difference between the TPI-deficient family members and the normal controls disappeared completely. This finding may support the suggestion that the interactions of the mutant TPI molecules with membrane constituents are responsible for the observed changes. In view of the still-debated reversible binding of the glycolytic enzymes to band 3, before a final conclusion can be drawn, the influence of the possible changes in ether lipids and isoprenes on the observed alterations in membrane fluidity must be investigated.

Note. A missense mutation within codon 240 [TTC (Phe) → CTC (Leu)] has been revealed (7).

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