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SHORT NOTE
Triosephosphate isomerase activity-deficient mice show haemolytic anaemia in homozygous condition

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Summary

A triosephosphate isomerase (TPI) mutant, *Tpi1*$^{a\text{-}m6Neu}$, with approximately 57% residual enzyme activity in blood compared with wild-type was detected among offspring of triethylenemelamine-treated male mice. Homozygous mutants with about 13% residual enzyme activity were recovered in progeny of *inter se* matings of heterozygotes. The loss of TPI activity was evident both in blood and in other tissue extracts. Values for haematocrit, haemoglobin, number of red blood cells (RBC), mean corpuscular volume of RBC, mean corpuscular haemoglobin concentration and spleen weight show significant differences between wild-type animals and homozygous mutants. Sequence analysis revealed a substitution (c.A149G) in the *Tpi1* gene. This mutation results in an Asp to Gly substitution at codon 49 in exon 2 at a highly conserved position located in the functional domain of the TPI protein which is responsible for the correct dimerization of the subunits. As a potential animal model, *Tpi1*$^{a\text{-}m6Neu}$ represents the only available TPI-deficient homozygous viable mouse mutation.

1. Introduction

During the screening of offspring from triethylenemelamine-treated male mice for activity variants of ten different erythrocyte enzymes, a female was found with low triosephosphate isomerase (TPI) activity. In this paper, we describe the genetical, physiological and molecular characterization of the mutation.

2. Materials and methods

(i) *Mutation induction and genetical characterization*

Male (101/ElxC3H/El)F_1 hybrid mice, 12 weeks old, were treated i.p. with 2 mg/kg body weight triethylenemelamine and then immediately caged with untreated test-stock females (Ehling, 1978). F_1 offspring of this experiment were screened for activity variants of ten different enzymes (Charles & Pretsch, 1987). Preparation of blood samples, determination and calculation of the specific enzyme activity, as well as the genetic confirmation and characterization, are described elsewhere (Charles & Pretsch, 1987; Merkle & Pretsch, 1989). All mice used were obtained from colonies maintained in Neuherberg. The mutant line is available as cryopreserved sperm from the European Mouse Mutant Archive (EMMA ID 2461).

(ii) *Physiological characterization of the TPI mutation*

Ten-week-old animals of both sexes were used. Heterozygous mutant offspring were selected and backcrossed at least nine generations to the inbred C3H/El wild-type strain in order to transfer the mutant gene to a defined inbred genetic background. Heterozygotes originating from such backcrosses were mated *inter se* to recover homozygous mutants. Examination of haematological and other physiological parameters was performed as described previously (Merkle & Pretsch, 1989).

Heat stability of erythrocyte TPI was determined by incubating erythrocyte lysate at 50 °C. At 5 min time intervals, aliquots were taken and chilled

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Table 1. *Physiological characterization of the TPI-deficient mouse mutation*

Tpi1 genotype[a]	a/a	a/a-m6Neu	a-m6Neu/a-m6Neu
Haematocrit (%)	47·9 ± 1·0	47·4 ± 1·7	46·3 ± 1·6*
Haemoglobin (g/dl)	17·2 ± 0·6	16·9 ± 0·9	15·7 ± 1·0**
RBC ($\times 10^{12}$/l)	918 ± 48	920 ± 45	845 ± 40*
MCH (pg/RBC)	19 ± 1	18 ± 1	19 ± 1
MCV (fl)	52 ± 3	52 ± 2	55 ± 3*
MCHC (g Hb/dl)	36 ± 2	36 ± 2	34 ± 2*
Body weight (g)	23·1 ± 2·1	23·0 ± 0·8	23·4 ± 1·6
Spleen somatic index[b]	0·41 ± 0·11	0·47 ± 0·15	0·59 ± 0·16**
TPI activity (% of wild-type) in			
blood	100 ± 3	57 ± 4**	13 ± 5**
lung	100 ± 12	57 ± 16**	31 ± 8**
spleen	100 ± 18	68 ± 8**	22 ± 9**
heart	100 ± 12	71 ± 13**	37 ± 10**
liver	100 ± 11	75 ± 4**	48 ± 7**
kidney	100 ± 10	76 ± 10**	54 ± 9**
brain	100 ± 10	77 ± 14**	45 ± 11**

[a] a: wild-type allele; a-m6Neu: TPI-deficient allele.
[b] Spleen weight × 100/body weight.
Data are given as means ± S.D. for ten animals. Tested offspring are descendants from intercrosses of heterozygous mutants. MCH, mean cell haemoglobin; MCV, mean cell volume; MCHC, MCH concentration; Hb, haemoglobin.
* Significant difference ($P < 0{\cdot}05$; *t*-test) between wild-types and mutants.
** Significant difference ($P < 0{\cdot}01$; *t*-test) between wild-types and mutants.

immediately with ice-cold buffer. After sedimentation of precipitated haemoglobin, residual TPI activity was assayed.

(iii) *PCR amplification and DNA sequencing*

For the molecular characterization of the mutation, RNA was extracted from kidneys of C3H/El control animals and homozygous mutants using the RNeasy® Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers for two overlapping PCR fragments were designed to cover the entire coding region of *Tpi1*. The primers were selected using Oligo Primer Analysis software (http://ihg.gsf.de/ihg/ExonPrimer.html). Primer sequences are available upon request. All PCR amplifications were carried out according to the manufacturer's specifications (AccessQuick™ RT-PCR System; Promega, Madison, WI). PCR products were sequenced commercially (SequiServe, Vaterstetten, Germany).

3. Results

(i) *Mutation induction, original mutant and genetic characterization*

In a mutagenicity experiment, 10 195 offspring derived from triethylenemelamine-treated spermatogonial cell stages were screened for mutations affecting the activity of ten different erythrocyte enzymes. Three mutants with altered TPI activity were detected. One of these was a female with decreased TPI activity (TPI9770; allele designation: *Tpi1*$^{a\text{-}m6Neu}$).

In backcrosses of heterozygous mice having roughly 57% TPI residual activity in blood with wild-type C3H/El animals, a ratio of approximately 1 : 1 (189 : 184) was seen between wild-type and heterozygous offspring. In matings between two heterozygotes, homozygous mutants with approximately 13% of wild-type TPI activity were obtained. There was no significant deviation from the 1 : 2 : 1 (46 : 108 : 56) ratio for wild-type, heterozygous and homozygous mutants. Mean litter sizes of backcrosses, heterozygous and homozygous *inter se* crosses did not differ significantly ($7{\cdot}4 \pm 1{\cdot}5$, $n = 38$; $7{\cdot}2 \pm 3{\cdot}3$, $n = 29$; $7{\cdot}3 \pm 1{\cdot}6$, $n = 11$; *t*-test).

(ii) *Physiological characterization and TPI activity in different tissues*

Routine haematological tests were performed to determine the possible effect of the TPI deficiency on erythrocyte metabolism and to exclude the possibility that the reduced TPI activity in blood results indirectly from altered erythrocyte dynamics. In homozygous mutants significant deviations from the wild-type values were observed for haematocrit, haemoglobin, number of red blood cells (RBC), mean corpuscular volume of RBC, mean corpuscular haemoglobin concentration and spleen weight (Table 1).

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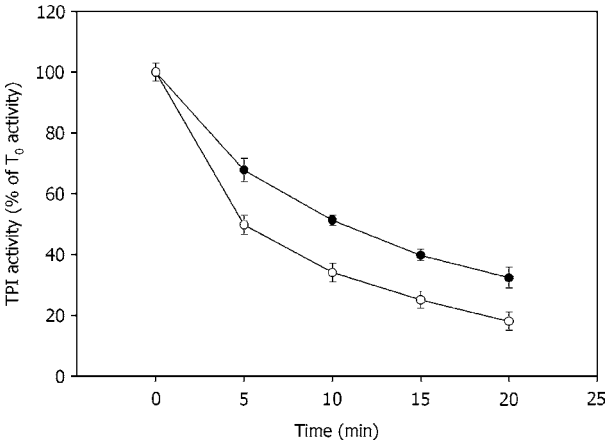

Fig. 1. Percentage residual activity of erythrocyte TPI of wild-type (closed circles) and heterozygous TPI mutants (open circles) after incubation at 50 °C. Values are given as the means for ten animals. Bars represent ±S.D.

The activity of TPI has been determined in blood, lung, spleen, heart, liver, kidney and brain of wild-type, heterozygous and homozygous animals (Table 1). A highly significant TPI activity decrease can be recognized in all tissues of hetero- and homozygous mutants.

Studies comparing the erythrocytic heat stability at 50 °C revealed a significantly lower stability in heterozygous mutants compared with wild-types (Fig. 1). TPI heat lability in homozygous mutants is so strong that the activity is reduced to zero after only a few minutes of incubation.

(iii) *Molecular characterization*

PCR primers based on the published mouse TPI RNA sequence (GenBank/EMBL accession number NM009415) were used to amplify two overlapping fragments, thereby covering the entire coding region of the mouse *Tpi1* transcript. The sequence of the mutant *Tpi1*$^{\text{a-m6Neu}}$ has been compared with that of the wild-type C3H/El (which is identical with the literature). An exchange of A to G at position 149 (counting the first base in the ATG start codon as 1) of the *Tpi1* gene, which leads to an Asp to Gly substitution at codon 49 in exon 2, co-segregated with the mutant phenotype.

4. Discussion

TPI (EC 5.3.1.1) is the glycolytic enzyme that catalyses the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. TPI plays an important role in several metabolic pathways (glycolysis, gluconeogenesis and triglyceride synthesis) and is essential for efficient energy production. It is a dimer of identical subunits, each of which is made up of 249 amino acid residues. The enzyme is only active as a dimer (Waley, 1973). TPI is a housekeeping enzyme expressed in all tissues and encoded by a single gene in human and mice. Its amino acid sequence is highly conserved among all known TPI proteins (Schneider, 2000).

Bulfield *et al.* (1987) described a mouse mutant, *Tpi-1*$^{\text{b}}$, for which homozygotes expressed 42% TPI residual activity in erythrocytes. The authors demonstrated that the mutant *Tpi-1*$^{\text{b}}$ was associated with increased heat instability. An unusual feature of this mutation was that, although homozygous mutants had low TPI activity in erythrocytes, they did not have altered activity in liver, kidney or brain. A possible explanation for this difference in TPI activity between erythrocytes and other organs is that it is due to the increased heat instability, since a lack of synthesis in erythrocytes would result in a depletion of enzyme activity, whereas active enzyme synthesis and turnover in liver, kidney or brain would maintain near-normal enzyme activity. This feature can also be observed in the currently described mutant (Table 1), but is more dramatic than in the *Tpi-1*$^{\text{b}}$ mutant.

Previously, we identified four heterozygous TPI mutants with approximately 50% activity in blood compared with wild-type (Charles & Pretsch, 1987). Breeding experiments displayed an autosomal, dominant mode of inheritance for the mutations. All mutations were found to be homozygous lethal at an early post-implantation stage of embryonic development, probably due to a total lack of TPI activity and consequently to the inability to utilize glucose as a source of metabolic energy (Merkle & Pretsch, 1989). Zingg *et al.* (1995) demonstrated that the observed 50% reduction in enzymatic activity in the four independently induced mouse mutants is due in three cases to an A:T to T:A transversion and in one case to an A:T to C:G transversion. Each of the sequence alterations has a potential impact on the structure of the TPI protein that is consistent with the existence of a null allele.

In contrast with these homozygous lethal mutations, the mutant *Tpi1*$^{\text{a-m6Neu}}$ represents the only available homozygous viable TPI-deficient mouse mutation. Genetical experiments revealed a semidominant mode of TPI-deficiency inheritance with complete penetrance and full fertility of homozygous animals. The observed reduction in enzymatic activity in the mutant is due to an A:T to G:C transition resulting in an Asp to Gly substitution at codon 49. This amino acid is located in exon 2, an exon in which the amino acid sequence is highly conserved in mammals (*Homo sapiens*, *Macaca mulatta*, *Pan troglodytes*, *Rattus norvegicus*, *Oryctolagus cuniculus* and *Mus musculus*). Relevant to an inference about the clinical significance of a mutation at this site is the observation that this residue is 100% conserved in mammals, but only 33% conserved in lower species

(C. Halfman, personal communication, 2002). 49Asp was found to directly participate in the dimer interface and is therefore in contact with residues in the other subunit (Schneider, 2000). Mutation sites in or interacting with the dimer interface would be expected to exhibit molecular instability manifested as thermolability. In fact, studies comparing the heat stability of erythrocytes at 50 °C showed a lower stability of TPI activity in heterozygous mutants compared with wild-types.

TPI deficiency in human is a rare autosomal recessive multisystemic disorder characterized by a decreased enzyme activity of 2–45% of normal in RBC of homozygotes or compound heterozygotes (Orosz *et al*., 2006). Heterozygotes are clinically normal. The clinical syndrome in homozygotes or compound heterozygotes is marked by profoundly decreased enzyme activity in all tissues that have been studied and is characterized by lifelong haemolytic anaemia and severe progressive neuromuscular degeneration, most often beginning about the seventh month of life. An increased tendency to infection is almost always noted, infectious episodes often being linked with increased anaemia and episodic hypotonia. Nearly all cases result in death before the age of 5 (Schneider, 2000). No effective therapy is available for TPI deficiency.

Two factors appear to be relevant to the TPI deficiency as a unique glycolytic enzymopathy coupled with neurodegenerative disorder. The presence of the mutant protein can result in the formation of toxic protein aggregates and/or the impairment of energy metabolism (Orosz *et al*., 2006). It has been documented that in other neurodegenerative diseases, unfolded or misfolded proteins form aberrant protein–protein interactions that lead to the formation of toxic protein aggregates causing neuronal dysfunction. The accumulation of unfolded or misfolded proteins can impair energy metabolism by mechanisms that are not fully understood. Neural dysfunction resulting from misfolded proteins and impaired energetics may both significantly account for chronic neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease or Huntington's disease. The major hurdle to elucidate the pathomechanism of TPI deficiency in human is the lack of brain tissues available for experimental purposes (Orosz *et al*., 2006).

Due to the mutation of a dimer interface residue in the described mouse mutant, we assume that the altered enzyme is a molecular unstable protein causing the reduced enzyme activity. Additionally, the physiological characterization of homozygous mutants demonstrates features for the presence of haemolytic anaemia. Therefore, this line could be a potential model animal for a whole field of neurodegenerative disorders and the neurological analysis of TPI mutant mice could be promising for the clarification of these diseases.

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