Recessively inherited L-DOPA-responsive parkinsonism in infancy caused by a point mutation (L205P) in the tyrosine hydroxylase gene


1 University Children’s Hospital, 44791 Bochum, Germany, 2 Department of Biochemistry and Molecular Biology, University of Bergen, 5009 Bergen, Norway, 3 Department of Medical Genetics, University of Bergen, 5021 Bergen, Norway, 4 Hospital for Sick Children, Great Ormond Street, Institute of Child Health, London WC1N 1EH, UK and 5 Institute of Neurology, London WC1N 3BG, UK

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Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), the rate-limiting step in the biosynthesis of dopamine. This report describes a missense point mutation in the human TH (hTH) gene in a girl presenting parkinsonian symptoms in early infancy and a very low level of the dopamine metabolite homovanillic acid in the CSF. DNA sequencing revealed a T614-to-C transition in exon 5 (L205P). Both parents and the patient’s brother are heterozygous for the mutation. Site-directed mutagenesis and expression in different systems revealed that the recombinant mutant enzyme had a low homospecific activity, i.e. ∼1.5% of wt-hTH in E. coli and ∼16% in a cell-free in vitro transcription-translation system. When transiently expressed in human embryonic kidney (A293) cells a very low specific activity (∼0.3% of wt-hTH) and immunoreactive hTH (<2%) was obtained. The expression studies are compatible with the severe clinical phenotype of the L205P homozygous patient carrying this recessively inherited mutation. Treatment with L-DOPA resulted in normalisation of the CSF homovanillic acid concentration and a sustained improvement in parkinsonian symptoms.

INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.1.16.2) catalyzes the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) in a tetrahydrobiopterin-dependent monooxygenase reaction, the rate-limiting step in the biosynthesis of dopamine and noradrenaline/adrenaline. Recently, we described a point mutation in the highly conserved exon 11 of human TH (hTH-Q381 K) in a family of two siblings suffering from a typical form of progressive L-DOPA-responsive dystonia (DRD), representing the first reported mutation in this gene (1,2). The patients were characterized by a clinical onset in the first decade with moderate extrapyramidal symptoms and a dramatic positive therapeutic response to low-dose L-DOPA therapy. The clinical course and diurnal fluctuations suggested a decrease in the synthesis of dopamine (DA) in the nigrostriatal dopaminergic neurons and DA release in the basal ganglia, which has actually been observed by a combined histochemical, immunochemical and enzyme activity study in an autopsied case of DRD (3) demonstrating similar clinical symptoms of dysfunctional dopaminergic neurons.

In the present study we describe a 3 month old girl whose major neurological symptoms are characteristic of parkinsonism, and in addition symptoms of a dysfunction of specific sympathetic neurons in the peripheral nervous system (ptosis) was observed. The biochemical analyses are compatible with a markedly reduced biosynthesis of dopamine in the CNS due to a deficient function of the tyrosine hydroxylase enzyme system, and the symptoms were dramatically responsive to L-DOPA therapy. The parkinsonian symptoms were found to be associated with a missense point mutation (L205P) in the TH gene. When the mutant enzyme was expressed in three different systems a ‘residual activity’ of ∼0.3% was obtained in human embryonic (A293) cells, ∼1.5% in E. coli and ∼16% in a cell-free in vitro transcription-translation system as well as an additional markedly decreased stability when expressed in A293 cells. Thus, all the properties of the recombinant mutant enzyme are compatible with the severe clinical phenotype of the L205P homozygous patient carrying this recessively inherited mutation.

RESULTS

Case report

The patient was the second child of healthy, unrelated Greek parents. The pregnancy was uncomplicated but the mother noted that fetal movements were weaker than those of her first child.

*To whom correspondence should be addressed
She was born at term (birth weight 2.8 kg) by Caesarean section performed because of fetal distress. At the age of 3 months, her main symptoms were brief jerky movements affecting usually the upper limbs but occasionally the lower limbs. An EEG was reported as showing a ‘non-specific generalised dysrhythmia’, CT and MRI scans were normal as was the routine biochemistry. The infant went on to develop generalised rigidity with very little spontaneous movement and continuing involuntary jerky movements. There was no diurnal variability in the symptoms. At the age of 6 months examination revealed an expressionless face, ptosis and drooling. The infant could fix her eyes and follow slowly through 30°. Tongue movements were tremulous. She lay in a frog-like position and had severe head lag and trunkal hypotonia. Tone in the limbs was variable and of cogwheel type. There was a constant tremor most marked in the upper limbs. There were occasional myoclonic jerks. There were no antigravity movements in any limb and deep tendon reflexes were reduced. There were persistent asymmetric tonic neck and Moro reflexes. Ocular instillation of 2.5% (w/v) phenylephrine led to a dramatic improvement in the infant’s ptosis.

Analyses of the cerebrospinal fluid (CSF) revealed a very low level of the dopamine metabolite homovanillic acid, HVA (2.8 SD below the mean for age-matched controls). Reduced dopamine synthesis leading to a low CSF concentration of HVA can occur as a result of disordered pterin synthesis or recycling or as a result of aromatic aminoacid decarboxylase deficiency (AADC) deficiency or, in theory, it could result from isolated deficiency of tyrosine hydroxylase. Disorders of pterin synthesis lead to a reduction in the total blood biotinperin concentration, and ordered recycling is associated with reduced activity of dihydropteridine reductase in blood. Both types of pterin disorder may lead to alterations in the CSF concentrations of neopterin, dihydrobiopterin and tetrahydrobiopterin. Pterin disorders also lead to impaired activity of phenylalanine hydroxylase and hence to hyperphenylalaninemia. Pterin disorders and aromatic aminoacid decarboxylase deficiency lead to reduced synthesis of serotonin and its metabolite, 5-hydroxyindolacetic acid (5-HIAA). AADC deficiency leads to the accumulation of L-DOPA and its metabolites, 3-methoxytyrosine and vanillactic acid and also to accumulation of 5-hydroxytryptophan. As indicated in Table 2, none of these additional abnormalities was present in our patient (all results fell within the normal range for age-matched controls). The results were therefore not suggestive of a pterin disorder or of AADC deficiency but could be explained by isolated tyrosine hydroxylase deficiency.

Treatment with L-DOPA/carbidopa (4:1, w/w) was commenced. A moderate dose of L-DOPA (2 mg/kg body weight given 5 times daily) resulted in normalisation of the CSF HVA concentration (Table 1) and a marked and sustained improvement in the hypokinesia and other parkinsonian symptoms. At the age of 3 years she has mild motor delay (with minimal gait ataxia) and mild speech delay.

### Table 1. Analytes relevant to catecholamine biosynthesis in patient and controls

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentrations</th>
<th>Normal range</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF homovanillic acid (HVA)</td>
<td>18</td>
<td>362–995</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF 5-hydroxyindoleacetic acid (5-HIAA)</td>
<td>173</td>
<td>63–502</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF tetrahydrobiopterin</td>
<td>55</td>
<td>23–55</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF dihydrobiopterin</td>
<td>&lt; 0.4</td>
<td>0.4–13.9</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF neopterin</td>
<td>8</td>
<td>7–65</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF L-DOPA n.d.</td>
<td>&lt;25</td>
<td>n.d.</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF 3-methoxytyrosine n.d.</td>
<td>&lt;80</td>
<td>n.d.</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF 5-hydroxytryptophan n.d.</td>
<td>&lt;20</td>
<td>n.d.</td>
<td>nmol/l</td>
</tr>
<tr>
<td>Whole blood biopterin</td>
<td>3</td>
<td>1.5–4.0</td>
<td>µg/l</td>
</tr>
<tr>
<td>Whole blood dihydropteridine reductase</td>
<td>1.45, 1.84</td>
<td>&gt;0.5</td>
<td>µmol NADH/min/g Hb</td>
</tr>
<tr>
<td>Whole blood serotonin</td>
<td>89</td>
<td>73–280</td>
<td>ng/mol</td>
</tr>
<tr>
<td>Plasma phenylalanine</td>
<td>71</td>
<td>35–100</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Plasma tyrosine</td>
<td>82</td>
<td>30–120</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Urine HVA</td>
<td>1.3</td>
<td>2–15</td>
<td>µmol/l/µmol/creatinine</td>
</tr>
<tr>
<td>Urine vanillactic acid</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>µmol/l/µmol/creatinine</td>
</tr>
<tr>
<td>CSF HVA while on treatment with L-Dopa (1.1 mg/kg x 5 daily)</td>
<td>115</td>
<td>176–851</td>
<td>nmol/l</td>
</tr>
<tr>
<td>CSF HVA while on treatment with L-Dopa (2.0 mg/kg x 5 daily)</td>
<td>459</td>
<td>176–851</td>
<td>nmol/l</td>
</tr>
</tbody>
</table>

The normal controls represent age-matched controls (15, 16 and 17); CSF, cerebrospinal fluid; n.d., not detectable
Mutation in the tyrosine hydroxylase gene

The hTH gene has 13 exons (4). Exons 3, 4, 5, 6, 7, 9, 10, 11 and 12 were PCR amplified from genomic DNA and the primers used for exon 5 are given in Table 2. Sequencing of the 630 bp PCR product revealed a T614-to-C transition causing an amino acid substitution of leucine in codon 205 with proline (Fig. 1) which predicts a change in codon 205 from a leucine to a proline residue in the expressed hTH1 protein.

Expression in E. coli

When the wild-type and mutant form were expressed in E. coli and purified by affinity chromatography on heparin-Sepharose, the recovery of soluble immunoreactive TH protein was 6-fold higher for the wild-type than for the L205P mutant form. Furthermore, whereas the mutant protein revealed two bands of 60 kDa and 57 kDa in almost equal quantities, the wild-type gave only the 60 kDa species (Fig. 2, lanes 5 and 6). The homospecific activity of the L205P mutant enzyme was 1.5 ± 0.2% of the wild-type (100%) corresponding to 259 ± 13 nmol L-DOPA/min/mg TH protein). It remains to be determined whether the two species of L205P mutant enzyme are due to proteolysis or a truncated form as a result of a second initiation of translation (see discussion).

Expression in human embryonic kidney (A293) cells

After transfecting A293 cells with the wild-type and mutant cDNAs, transiently expressed TH was analysed for activity, steady-state level of immunoreactive protein and mRNA. The specific TH activity of the cell lysates after transfection with the mutant cDNAs was 0.3% of immunoreactive protein and mRNA. The specific TH activity of the wild-type (100%, corresponding to 1.09 nmol L-DOPA/min/mg protein) corresponds to 14.9 ± 0.8 % was obtained for the previously reported kinetic variant mutant form Q381K, in good agreement with the cell-free in vitro expression system (2). Western blot analysis revealed immunoreactive bands in the lysates of Q381K and wild-type transfected cells (Fig. 2, lanes 2 and 4), but only barely detectable TH was observed in the L205P cells (< 2% of wt-cells) and the parental A293 cells were negative (Fig. 2, lanes 1 and 3). In contrast, slot blot analyses revealed the same steady-state level of TH mRNA in cells transfected with the wild-type and mutant cDNAs, whereas no TH mRNA was found in the untransfected cells (Fig. 3, slots 1–4). Therefore, we conclude that in spite of a normal TH mRNA level in the L205P transfected cells the very low specific TH activity in these cell lysates is mainly explained by a low cellular level of immunoreactive TH.

In vitro transcription-translation

Selective synthesis of [35S]-labeled TH was obtained both for the wild-type and L205P mutant with a coupled in vitro transcription-
and A293 cells (7,8), whereas the enzyme produced in the coupled in vitro system avoids this problem. Thus, the very low activity of the L205P mutant enzyme in A293 cells is mainly explained by an increased cytosolic degradation of hTH as revealed by the low recovery of immunoreactive enzyme. A reduced stability and catalytic activity of the L205P mutant enzyme was expected since proline is generally considered to be a helix-breaking residue in soluble proteins. Substitution of a leucine residue in an α-helical region by a proline, as in the present mutation, is expected to drastically change the structure and catalytic function of the enzyme (10).

The expression of L205P mutant hTH resulted in a heterogeneous product both in E. coli (Fig. 2) and by the in vitro transcription-translation system (Fig. 3b) on SDS/PAGE. The difference in apparent molecular mass of 2–3 kDa in the in vitro system, also observed for the wild-type enzyme, can be explained by the presence of a second initiation site, i.e. at codon 30 (AUG), which has been reported for another enzyme expressed in the same system (11). Initiation at this site would result in the formation of a truncated gene product in which a 2578 Da peptide is deleted from the N-terminus. This heterogeneity was not observed for the wild-type hTH expressed in A293 cells (Fig. 2, lane 5).

As in the patients with DRD (2,6) all the symptoms of our patient were dramatically responsive to daily L-DOPA therapy. A moderate dose of L-DOPA (2 mg/kg body weight given 5 times daily) resulted in an optimal and sustained clinical response. The CSF homovanillic acid concentration rose from the very low pretreatment level to within the normal range (Table 1).

The two mutations in hTH discovered so far represent quite different clinical and metabolic phenotypes. However, the severity of the disease correlates well with the measured residual TH activity, causing juvenile DRD in the Q381K mutation (2) and early childhood parkinsonism in the L205P mutation (present study). This difference is similar to that observed for mutations in the gene encoding phenylalanine hydroxylase causing persistent hyperphenylalaninemia, but with a marked heterogeneity of metabolic and clinical phenotypes (12). The severe phenotype of our L205P mutation is close to a null mutation, since the child barely survived the perinatal period. Thus, a null mutation in the TH locus has been shown to be lethal at the late embryonic stage in mice (13).

DISCUSSION

In the present report we describe a recessively inherited point mutation in the hTH gene in a girl presenting, in addition to severe parkinsonian symptoms in early infancy, symptoms of a dysfunction of specific sympathetic neurons in peripheral nervous system (ptosis). Thus, in contrast to the typical forms of DRD (6) both dopaminergic and noradrenergic neurons seem to be affected in this patient. The biochemical analyses of the cerebrospinal fluid are compatible with a markedly reduced synthesis of DA in the CNS (very low level of the DA metabolite homovanillic acid) due to a deficient function of the tyrosine hydroxylase enzyme system. The biosynthetic pathway of tetrahydrobiopterin (including the GTP-cyclohydrolyase activity) and the dihydropteridine reductase activity were normal based on a normal or slightly elevated level of tetrahydrobiopterin as well as an increased ratio of reduced/oxidized cofactor in the cerebrospinal fluid (Table 1). The severe symptoms are compatible with the enzymatic phenotype of the L205P mutant form of hTH when expressed in three different systems. The recombinant mutant enzyme revealed a ‘residual activity’ of 0.3 to 16% of the wild-type hTH1 in three complementary expression systems in addition to a decreased stability on transient expression in A293 cells. The differences observed in homospecific activity are of the same order of magnitude as reported by us in the expression studies on mutant forms of the structurally and functionally related enzyme phenylalanine hydroxylase (7,8). The differences between the expression systems can in both cases be explained by a variable degree of proteolysis of mutant enzymes in E. coli (9) and E. coli (9).

MATERIALS AND METHODS

Biochemical investigations

The analytical techniques used for the investigation of suspected inborn errors of neurotransmitter, amine and pterin metabolism have been described previously (14–17).

PCR amplification and DNA sequencing

Exons 3, 4, 6, 7, 9, 10, 11 and 12 of the hTH gene were PCR amplified from genomic DNA. Three hundred ng of each pair of primers was added to 1 µg DNA and 2.5 U Taq polymerase (Perkin Elmer) in a total volume of 100 µl containing 200 mM dNTPs, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂ and 0.001% (w/v) gelatine. The reaction parameters were 94°C for 6 min, then 95°C for 50 s, 69°C for 45 s and 72°C for 3.45 min for 33 cycles, followed by a 13 min extension at 73°C. Exon 5 was amplified using the 5A1 and 5B primers (Table 2).
Site directed mutagenesis

The L205P mutation was introduced into the wild-type hTH cDNA by PCR-based site specific mutagenesis as described (2,18,19). The primers are given in Table 2. The target sequence for the mutagenesis was BstEII/MluI restriction fragment of the hTH cDNA. Positive clones, identified by the loss of a MluI restriction site, were sequenced (Taq DyeDeoxy Terminator Cycling Sequencing Kit 373A automatic DNA sequencer, Applied Biosystems) to verify the introduction of the L205P mutation and to exclude other mutations due to Taq DNA polymerase (Boehringer Mannheim) misincorporation.

Expression of recombinant hTH1 in a cell-free system

Coupled in vitro transcription-translation of wild-type and mutant (L205P) hTH1 were expressed in E. coli using the pET3a-hTH1 expression vector (20) and purified essentially as described (2).

Eukaryotic expression of recombinant hTH1

For transient eukaryotic expression of hTH1 the strong eukaryotic CMV promoter (21) was cloned into the pET3a-hTH1 vector (20). The BamHI/BglII fragment of the pcDNA3 vector (Invitrogen), containing the CMV promoter region, was cloned into the BglII site of the pET3a-hTH1 vector. The human kidney cell line A293 (22) was transfected with wild-type and mutant vector DNA using lipofectamine (GIBCO-BRL) as described by the manufacturer. Cells were harvested after 40 h and kept at −80°C until used. The cells were lysed by sonication in a 20 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM PMSF and centrifuged (15 000 × g, 15 min). The supernatant was used for assay of enzyme activity and immunoblotting.

Immunoisolation, electrophoresis and immunoblotting

hTH expressed in human embryonic kidney (A293) cells were immunoisolated using Dynabeads M-280 (Dynal A.S.) coated sequentially with sheep anti-rabbit IgG and affinity-purified rabbit anti-hTH 1, essentially as described by the manufacturer. SDS/PAGE at 180 V (2 h) in a 10 % (w/v) polyacrylamide gel and immunoblotting was performed using affinity-purified rabbit anti-hTH1 (1.6 µg/ml). The enhanced chemiluminescence (ECL) system from Amersham was used for the immunodetection. The immunoreactivity was quantitated by densiometric scanning of the autoradiograms (Ultrascan XL. laser densiometer from Pharmacia). Different exposure times (10 s to 5 min) and different protein concentrations were used to obtain the most linear concentration range of the densitometric peak integrals.

Extraction of total RNA and assay of specific mRNA

Total RNA was purified from A293 cells and E.coli cells expressing wild-type and mutant hTH using the RNEasy kit (Qiagen). Approximately 8 µg of total RNA was transferred to a Hybond N membrane (Amersham) by a Schleicher & Schüll slot-blot apparatus and crosslinked to the membrane by UV-light. Random priming (Megaprime kit, Amersham) labelled probes were used sequentially after stripping the filters in 0.1% (w/v) SDS/2 mM EDTA for 15 min at 95°C. Slot blot analyses were first performed with the hTH cDNA probe, then stripped and rehybridized with the GAP cDNA probe. Hybridization, washing and stripping of the filter was carried out as described (7). Probes were purified cDNA fragments from the pET3a-hTH1 clone. The GAP cDNA clone (23) was used as an internal control (constitutively expressed protein). For quantitation of hybridizing RNA, the filter was exposed to a B-scanner (Packard Instant Imager) for 1–4 h.

Assay of TH activity

TH activity was assayed at 25°C with 500 µM tetrahydrobiopterin and 35 µM L-tyrosine at pH 7.0 (2). The amount of product was linear with time and amount of enzyme added at the selected assay conditions.

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ABBREVIATIONS

CSF, cerebrospinal fluid; DA, dopamine; HVA, homovanillic acid; L-DOPA, L-dihydroxyphenylalanine; TH, tyrosine hydroxylase.

REFERENCES


