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Short Communication

Dihydropyrimidinase deficiency: Confirmation of the enzyme defect in dihydropyrimidinuria

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Dihydropyrimidinase (DHP, EC 3.5.2.2) is the second enzyme in the degradation pathway of uracil and thymine. It catalyses the degradation of both dihydrouracil and dihydrothymine to *N*-carbamyl- β -alanine and *N*-carbamyl- β -aminoisobutyric acid, respectively. So far, four cases of dihydropyrimidinuria (McKusick 222748) have been reported (Duran et al 1991; Henderson et al 1993; Bakkeren et al, personal communication, 1994; Ohba et al 1994). The patients show a variable clinical phenotype comprising seizures or epileptic attacks (3 out of 4 patients) mental retardation (2 patients), growth retardation (1 patient) and dysmorphic features (1 patient). Since these patients excrete large amounts of dihydrouracil and dihydrothymine and moderate amounts of uracil and thymine in their urine, they can easily be detected (Van Gennip et al 1993). On the basis of the characteristic urinary metabolite profile it is assumed that the disease is caused by a deficiency of DHP.

The direct measurement of the activity of DHP in patients has been hampered by the fact that the enzyme is almost exclusively expressed in liver tissue. Here, we provide for the first time direct evidence at the enzyme level for a deficient activity of DHP in liver in a patient with dihydropyrimidinuria.

PATIENT

Patient N.Z., a female born to a consanguineous couple from Lebanon, presented at birth with low anal atresia, clubfoot and hip dysplasia on the right side, hypoplastic end phalanges and nails of fingers and toes, plagiocephaly and some facial dysmorphism. At the age of 3 months no psychomotor development was noticed. Neurological examination revealed severe retardation, convulsions, extrapyramidal dyskinesia and pyramidal signs. Initially, a normal EEG was seen which deteriorated with respect to the background activity and increased irritability.

BAEP was delayed (I–V) consistent with a poor auditory brainstem conduction. MRI revealed retardation of myelination and a severe cerebral cortical atrophy. Brainstem and cerebellar structures appeared to be normal. EMG and morphological examination of muscle and liver tissues revealed no abnormalities.

GC-MS analysis of urinary organic acids showed the presence of large amounts of dihydrouracil and dihydrothymine and of moderate amounts of uracil and thymine, indicating DHP deficiency. A liver biopsy was performed and the liver tissue was analysed for DHP activity. For comparison, 8 control liver samples were investigated using the same procedures.

METHODS

Preparation of tissue homogenates: Homogenates (20%, w/v) of frozen human livers were prepared in a buffer containing 10mmol/L MOPS–NaOH (pH 7.4), 1mmol/L EDTA, 10mmol/L dithiothreitol, 5mmol/L 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (pefabloc) and 10µg/ml leupeptin, with the aid of a Teflon–glass homogenizer. After centrifugation (11 000g at 4°C for 20min) the supernatants were removed and stored in liquid nitrogen until further analysis. Protein concentrations in the supernatants were determined by the copper reduction method using bicinchoninic acid (Smith et al 1985).

Determination of the activity of dihydropyrimidinase: The activity of dihydropyrimidinase was determined in a reaction mixture containing 0.1mol/L Tris-HCl (pH 8.0), 1mmol/L dithiothreitol and 500µmol/L [2-¹⁴C]dihydrouracil. The tube containing the reaction mixture was placed in a scintillation vial which also contained an Eppendorf microtube with 1ml of 2mol/L NaOH. The scintillation vial was sealed with a rubber septum and the supernatants were equilibrated at 37°C in a stirring waterbath for 2min. The reaction was started by the injection of an amount of supernatant corresponding to 0.1–0.2mg of protein into the reaction tube (total volume 100µl). After an appropriate time of incubation (1h) the reaction catalysed by dihydropyrimidinase was terminated by injecting 25 µl of 10% (v/v) perchloric acid through the septum into the reaction tube. A blank value was obtained in the absence of a sample. After termination of the reaction, the scintillation vial was stored at 4°C for 2h to allow the NaOH solution to trap the ¹⁴CO₂. Then the tube containing the reaction mixture was removed from the scintillation vial. The tube containing the NaOH solution was mixed vigorously with 15ml of scintillation liquid and the radioactivity was quantified by scintillation counting. The reaction mixture was centrifuged in a microfuge (11 000g for 5min) to remove the protein. An aliquot of the supernatant was mixed with 5ml of scintillation liquid and the radioactivity was quantified by scintillation counting. The remaining supernatant was stored at –20°C and saved for further analysis by HPLC.

HPLC analysis: The separation of radiolabelled dihydrouracil, radiolabeled uracil and radiolabelled *N*-carbamyl-β-alanine was accomplished by reversed-phase HPLC on a Supelcosil LC-18-S column (250×4.6mm, 5µm particle size). Elution of the column was performed isocratically with 50mmol/L NaH₂PO₄ (pH 4.5) at a flow rate of 1ml/min. The UV detection was performed at 205nm with on-line detection of the radioactivity.

Figure 1 Activity of DHP in 8 control liver samples and in the liver biopsy specimen of the patient with dihydropyrimidinuria (A). DPD activity was measured for comparison (B)

Determination of the activity of dihydropyrimidine dehydrogenase: The activity of dihydropyrimidine dehydrogenase was determined in a reaction mixture containing 35mmol/L potassium phosphate (pH 7.4), 2.5mmol/L MgCl_2 , 1mmol/L dithiothreitol, 2.5mmol/L NADPH and 40 $\mu\text{mol/L}$ $[2\text{-}^{14}\text{C}]$ thymine, essentially as described earlier (Van Kuilenburg et al 1996). Separation of radiolabelled thymine and the reaction products dihydrothymine and β -ureidoisobutyric acid was accomplished by HPLC as described above, with a flow rate of 2ml/min instead of 1ml/min.

RESULTS AND DISCUSSION

Figure 1A shows the activity of DHP in 8 control liver samples and in the liver biopsy specimen of the patient. The activity of DHP in the homogenates of the control livers ranged from 20 to 74nmol/h per mg protein with a mean activity of 55 ± 17 (1SD). In the liver homogenate of the patient the activity of DHP was undetectably low (<0.3nmol/h per mg protein).

For comparison we also measured the activity of dihydropyrimidine dehydrogenase in the liver samples. As can be seen in Figure 1B, the activity of this enzyme in the control livers ranged from 5.0 to 13.8nmol/h per mg protein with a mean activity of 9.3 ± 2.4 (1SD). In the liver homogenate of the patient the activity was 4.2nmol/h per mg protein, which is slightly below the control values. This may have been caused by downregulation of the enzyme *in vivo* by the large concentrations of its products dihydrouracil and dihydrothymine or inhibition of the enzyme by accumulation of these products in the *in vitro* assay. Another possibility may be that the control range is too narrow, because of the small number of controls.

Duran and colleagues (1991) tested the *in vivo* capacity for pyrimidine catabolism in their patient with dihydropyrimidinuria by oral loading tests with dihydrouracil. They found that 91% of the test dose was excreted unchanged in the patient's urine and that the uracil excretion rose slightly. Their results indicated a strongly reduced activity of DHP and also the *in vivo* conversion of dihydrouracil into uracil. We have directly demonstrated the defect at the enzyme level in our patient with dihydropyrimidinuria. Conversion of

dihydrouracil into uracil did not occur in our enzyme assay, probably because of shortage of NADP⁺. Therefore, separation of dihydrouracil and uracil in the HPLC assay is not strictly necessary.

We applied HPLC with on-line detection of radioactivity for the determination of dihydrouracil and its degradation products in the reaction mixture. This method enabled us to measure directly and quantitatively the amount of dihydrouracil and the amount of degradation products formed from dihydrouracil. In this way the enzymatic activity can be determined quantitatively, allowing the detection of total as well as partial enzyme deficiencies.

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