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IDENTIFICATION OF L-PIPECOLATE OXIDASE IN HUMAN LIVER AND ITS DEFICIENCY IN THE ZELLWEGER SYNDROME

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The ability of human liver to oxidize L-pipecolic acid was investigated. Liver from control subjects was found to contain L-pipecolic acid oxidase, an H_2O_2-producing enzyme not previously demonstrated in mammals. In livers from patients with the cerebro-hepato-renal syndrome of Zellweger, a genetic disease characterized by the absence of morphologically distinguishable peroxisomes, L-pipecolic acid oxidase was found to be deficient. These results indicate that L-pipecolic acid oxidase is a peroxisomal enzyme in man and provide an explanation for the fact that elevated levels of L-pipecolic acid are found in body fluids of patients with the Zellweger syndrome.

Lysine catabolism in mammals is known to take a number of diversified routes [1]. It is generally agreed that the major degradative pathway for lysine in mammals, including man [2], proceeds via saccharopine. This pathway involves the sequential action of L-lysine ketoglutarate reductase and saccharopine dehydrogenase [2,3]. The importance of the saccharopine pathway for L-lysine degradation is stressed by the finding of hyperlysinemia in two genetic diseases in man, one with a deficiency of L-lysine ketoglutarate reductase and the other with a deficiency of saccharopine dehydrogenase (for review see [4]). It has long been established that degradation may also proceed via L-pipecolic acid. Studies by Chang [5,6] have shown that in rat brain L-lysine is mainly metabolized via L-pipecolic acid and not via saccharopine, in line with the finding that the activities of lysine ketoglutarate reductase and saccharopine dehydrogenase are negligible in brain [2,3]. Available evidence

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suggests that L-pipecolic acid formation from L-lysine proceeds via Δ^1-piperideine-2-carboxylate followed by subsequent reduction of the latter to L-pipecolic acid [7].

L-pipecolic acid is known to accumulate in body fluids from patients with the cerebro-hepato-renal syndrome of Zellweger, an autosomal recessive disease characterised by the absence of morphologically distinguishable peroxisomes [8,9]. Oral loading tests with DL-pipecolic acid indicated that the accumulation of pipecolic acid might be due to an impairment in pipecolic acid degradation [9]. At this moment, no information is available on the enzymes involved in L-pipecolic acid degradation in mammals. Studies in Pseudomonas species [11,12] (for review see [13]) have led to the identification of a membrane-bound L-pipecolate dehydrogenase. In contrast, Kinzel and Battacharjee [14] isolated an L-pipecolate oxidase from Rhodotorula glutinis. Since peroxisomes contain different oxidases [15] and since the involvement of peroxisomes in L-pipecolic acid degradation in man is indicated by the accumulation of L-pipecolic acid in Zellweger patients, we hypothesized that human liver peroxisomes contain L-pipecolate oxidase activity. In this paper we show that this is, indeed, the case. Furthermore, we show that the activity of the enzyme is deficient in livers from Zellweger patients.

MATERIALS AND METHODS

L-Pipecolic acid oxidase assay

The standard procedure developed for the measurement of L-pipecolic acid oxidase is as follows. An aliquot of human liver homogenate (100-400 µg protein/ml) was added to a medium (final volume 0.25 ml) containing the following standard components: 50 mM sodium pyrophosphate, 1 mM 4-hydroxyphenyl-acetic acid, 0.02% (w/v) Triton X-100, 4 U/ml horseradish peroxidase (Boehringer, grade II), 20 µM FAD, and 10 mM sodium azide (final pH 8.4). After 5 min at 37°C the reaction was started by adding sodium pipecolate (final concentration 15 mM). H_2O_2 formation was monitored by following the horseradish peroxidase catalyzing oxidation of 4-hydroxyphenylacetic acid (non-fluorescent) to 6,6'-dihydroxy-(1,1'-biphenyl)-3,3'-diacetic acid (fluorescent) on a Cobas-Bio Centrifugal Analyzer (excitation wavelength: 318 nm; emission filter: 410-490 nm) (Hoffman-La Roche, Basel, Switzerland) for 60-90 min.

Preparation of human liver homogenates

Pieces of human liver stored at -80°C were thawed on ice in 0.9% (w/v) sodium chloride. The thawed material was subjected to gentle homogenization and sonication (3 cycles of 15 s at 80 W with time intervals of 45 s) at 4°C. Subsequently, homogenates were diluted 1:1 with 0.9% (w/v) sodium chloride plus 0.2% (w/v) Triton X-100. After rigorous vortexing the suspension was centrifuged for 10 min at 10 000 x g_{av} in a Sorvall-RC58 Refrigerated Superspeed Centrifuge. The supernatant was found to contain all L-pipecolic acid oxidase activity and was used in the experiments described in this paper.

Materials

4-Hydroxyphenylacetic acid, L-pipecolic acid, D-pipecolic acid and D-amino-acid oxidase were obtained from Sigma (St. Louis, Mo., USA). Horseradish peroxidase (grade II) was obtained from Boehringer, Mannheim, FRG. All other reagents were of analytical grade.

RESULTS

Initial studies aimed at identifying L-pipecolic acid oxidase activity in human liver were carried out according to Kinzel and Bhattacharjee [14] using diaminobenzidine in the presence of horseradish peroxidase to monitor generation of H_2O_2. The sensitivity of this method was too low to allow measurement of L-pipecolic acid oxidase activity. We therefore adopted the fluorimetric method described by Poosch and Yamazaki [16] for measurement of acyl-CoA oxidase activity. This method involves the use of 4-hydroxyphenylacetate as substrate for peroxidation and allows measurement of low concentrations of H_2O_2 [16]. Using this method we found that addition of L-pipecolic acid to human liver homogenates gives rise to H_2O_2 formation as evidenced by a linear increase in fluorescence for 30-40 min after a small initial lag-phase. Table 1 shows that H_2O_2 production is strictly dependent upon the presence of L-pipecolic acid. Furthermore, FAD and Triton X-100 are required for maximal activity. Table 1 shows that azide had to be present in the assay medium in order to inhibit catalase activity completely (compare [16]). In order to be sure that the activity measured in Table 1 was not due to contamination of commercial L-pipecolic acid with D-pipecolic acid which can be oxidized avidly by the peroxisomal enzyme D-aminoacid oxidase, we added purified D-aminoacid oxidase (370 mU/ml) to stock solutions of sodium L-pipecolate to remove any trace of D-pipecolic acid as described by Rodwell [13]. The enzyme activity measured in control human liver homogenates was found to the same irrespective whether L-pipecolic acid was pretreated with D-aminoacid oxidase or not (not shown).

Fig. 1 shows that L-pipecolic acid oxidase activity was maximal at pH 8.3-8.5.

Table 2 shows that L-pipecolic acid oxidase activity is strongly deficient in livers from Zellweger patients. In accordance with earlier results the activity of the peroxisomal membrane-bound enzyme dihydroxyacetone phosphate

TABLE 1: REQUIREMENTS FOR L-PIPECOLIC ACID OXIDATION IN HUMAN LIVER HOMOGENATES

Ommission from complete medium	Activity (%)
None	100
L-pipecolic acid	0
Triton X-100	25
FAD	30
Sodium azide	1
Peroxidase	0

For experimental details see Materials and Methods. The activity of L-pipecolic acid oxidase in the complete system was 0.43 nmol/min.mg protein.

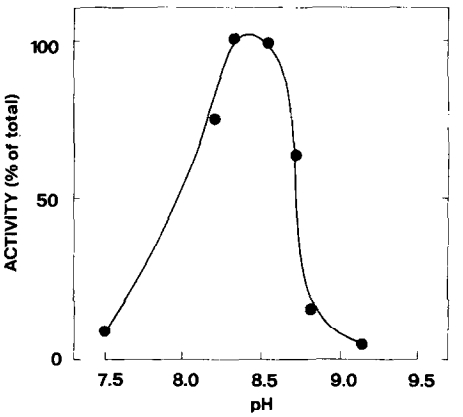

Fig. 1 The activity of L-pipecolic acid oxidase was measured in a human liver homogenate (0.22 mg protein/ml) as a function of pH as described in Materials and Methods.

acyltransferase is deficient in Zellweger patients [17,18], whereas the activity of catalase is normal [19-21].

DISCUSSION

The results described in this paper provide the first evidence that pipecolic acid degradation in man is initiated by L-pipecolate oxidase as in the yeast Rhodotorula glutinis [14] but in contrast to the situation in Pseudomonas species, where pipecolic acid degradation proceeds via a membrane-bound L-pipecolate dehydrogenase [11,12]. The finding that the activity of L-pipecolic acid oxidase is strongly deficient in livers from Zellweger patients (Table 2) not only provides an explanation for the accumulation of L-pipecolic acid in Zellweger patients but also suggests that L-pipecolic acid oxidase is

TABLE 2: ACTIVITY OF L-PIPECOLATE OXIDASE, DIHYDROXYACETONE PHOSPHATE ACYL TRANSFERASE AND CATALASE IN LIVER HOMOGENATES FROM CONTROLS AND ZELLWEGER PATIENTS

Enzyme measured	Controls	Zellweger patients
L-pipecolate oxidase (pmol/min·mg)	413 ± 85 (7)	4.3 ± 2.6 (4)
Dihydroxyacetone phosphate acyltransferase (nmol/2h·mg)	2.63 ± 0.37 (10)	0.20 ± 0.13 (7)
Catalase (μmol O_2/min·mg)	63.1 ± 8.8 (15)	86.4 ± 17.8 (8)

The activity of L-pipecolic acid oxidase was measured as described in Materials and Methods, whereas catalase and dihydroxyacetonephosphate acyltransferase were measured as described in [19]. Values represent mean ± S.D. with the number of livers analyzed within parentheses.

a peroxisomal enzyme in man. Preliminary studies using differential centrifugation of freshly prepared human liver homogenates indicate that the activity of L-pipecolic acid oxidase is highest in the light-mitochondrial fraction containing the bulk of peroxisomes. More detailed studies using density gradient centrifugation in metrizamide [22] are underway to obtain definitive evidence for the peroxisomal localization of L-pipecolic acid oxidase in man. It should be noted that in an independent study reported in abstract form [23] Mihalik and Rhead have recently found that radiolabelled L-pipecolic acid is converted to L-α-aminoadipic acid in a peroxisomal fraction from human liver and that this activity is deficient in the Zellweger syndrome.

Trijbels et al. [24] reported that in rat liver the peroxisomal fraction was most active in the decarboxylation of D,L-[^{14}C] pipecolic acid to [^{14}C]CO_2. A major problem with these studies and with similar studies by Zaar et al. [25] is that both groups used D,L-pipecolic acid as a substrate rather than L-pipecolic acid as used in the present study and that of Mihalik and Rhead [23]. Since D-pipecolic acid is a good substrate for D-aminoacid oxidase [15], which is located in peroxisomes, results obtained with D,L-pipecolic acid as substrate can not provide unequivocal information about the ability of peroxisomes to oxidize L-pipecolic acid.

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