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Hepatic peroxisomes in isolated hyperpipecolic acidaemia: evidence supporting its classification as a single peroxisomal enzyme deficiency

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Abstract Hyperpipecolic acidaemia is still regarded as a peroxisomal assembly deficiency. The enzyme responsible for the accumulation of pipecolic acid is located in the peroxisomes in man. We studied the appearance and alterations of peroxisomes in liver biopsy material from three unrelated children suffering from isolated hyperpipecolic acidaemia, in which only the metabolism of pipecolic acid is disturbed, using light and electron microscopy after cytochemical staining for visualisation of peroxisomes. Morphometric results showed the presence of normal-sized to small peroxisomes, an increase in number and abnormally shaped organelles, suggesting enhancement of metabolic efficiency. In one case enlarged organelles were observed. Skin fibroblasts were studied in all patients: their peroxisomes appeared to be normal. The obvious presence of peroxisomes in isolated HPA indicates that this disorder should be classified as a single peroxisomal enzyme deficiency.

Key words Peroxisomes · Pipecolic acid · Hyperpipecolic acidaemia · Genetic disorders · Morphometry

Introduction

Peroxisomal disorders are genetic disorders linked to peroxisomal dysfunction. They are subdivided into groups, depending upon the deficiency or presence of peroxisomes and loss of multiple peroxisomal functions or a single function. The first group contains peroxisomal biogenesis deficiencies, in which peroxisomes are markedly deficient or absent, resulting in Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy and hyperpipecolic acidaemia (HPA) [15, 30].

HPA (McKusick 23940) was first described in four cases [3, 9, 27], whose serum contained the unusual compound pipecolic acid; subsequently elevated levels of very long chain fatty acids (VLCFA) were found in cultured skin fibroblasts. There was a strong resemblance in clinical and biochemical presentation between these HPA patients and peroxisomal deficiency disorder patients.

In 1988 Mihalik and Rhead [18] have reported that in man the conversion of L-pipecolic acid, a minor intermediate in L-lysine catabolism, to alpha aminoadipic acid takes place in peroxisomes; the oxidation is catalysed by L-pipecolate oxidase within the peroxisomes in human beings [29]. Confirmation has come from purification and characterisation of peroxisomal L-pipecolic acid oxidase from monkey liver [19]. This explains the accumulation of pipecolic acid in classic Zellweger patients.

Wanders et al. [28] studied cultured fibroblasts from the patient described by Thomas et al. [27] and found a multiple loss of peroxisomal functions due to a deficiency of peroxisomes. They cited studies by Moser et al. revealing the same results in cultured fibroblasts from the patients described by Gatfield et al. [9] and Burton et al. [3]. However, in both HPA patients of Burton et al. [3], Challa et al. [4] reported the presence of morphologically normal liver peroxisomes, but the report did not include micrographs. Roels et al. [24] mentioned the presence of catalase containing organelles in the liver of two patients with increased pipecolic acid without VLCFA accumulation. The presence of peroxisomes in HPA liver suggests that classification together with Zellweger syndrome as a peroxisomal biogenesis defect may be unjustified. HPA (isolated) should be assigned to a separate disease category (single enzyme defect). Cox and Dancis [5] suggested that the previously reported cases of HPA with associated

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neuropathy and hepatomegaly were probably unrecognised examples of Zellweger syndrome, but underline the need for more research into isolated HPA.

It seems, then, that the term HPA was assigned to patients on the basis of the observed accumulation of pipecolic acid prior to the discovery of multiple peroxisomal defects. We should ask, however, whether the term HPA, in particular isolated HPA, should be reserved for patients with elevated pipecolic acid only [20].

HPA associated with Joubert syndrome has been observed in three siblings [21]. Liver biopsy revealed the presence of peroxisomes, but with abnormal morphological features.

We have studied the presence of hepatic peroxisomes, and morphometric alterations in their size, number, surface and volume densities and shape, in liver biopsies from three patients with isolated HPA.

Patient histories

The first patient was a 3375 g male born to healthy parents. The pregnancy and birth were normal. At birth the baby was found to have cheilopalatoschisis bilaterally, for which surgical intervention was initiated when he was 6 months old. By the age of 5 months he presented psychomotor retardation, mild facial dysmorphia, normal weight (60th percentile) and length (60th percentile), and a head circumference at the 97th percentile. At 12 months a peroxisomal disorder was suspected, because of mildly increased plasma values of pipecolic acid and phytanic acid associated with normal plasma VLCFA (Table 1). Also plasma lysine and urine lysine values were normal. The pipecolic acid level in cerebrospinal fluid (CSF) was elevated when it was estimated at 15 months and at 23 months of age. At this time the liver biopsy was taken. Liver functions were normal. Funduscopic examination was normal, and MRI of the brain showed enlarged ventricles without other abnormalities.

Peroxisomal functions in cultured skin fibroblasts (Table 2) were normal for de novo plasmalogen synthesis, DHAPAT activity, phytanic acid alpha oxidation, VLCFA, pristanic acid and C26:0 beta oxidation, immunoblot analysis (anti-acyl-CoA oxidase type 1; anti-L- and anti-D-bifunctional protein; anti-thiolase I) and immunofluorescence with anti-catalase and anti-ALDP (normal presence of peroxisomes). Investigations on fibroblasts concerning L-pipecolic acid oxidase (complementation analysis; antibodies) are impossible at present.

The isolated deficiency of L-pipecolate oxidase has not yet been investigated by techniques of molecular genetics: the cDNA/gene coding for human L-pipecolate oxidase has not yet been cloned.

The second patient was a girl, aged 3.5 months at the time of the biopsy. She was born after an uneventful pregnancy, with a birth weight of 2755 g. Her twin sister was healthy. She presented

Table 1 Biochemical parameters in plasma, urine and cerebrospinal fluid (*CSF*) in the three hyperpipecolic acidaemia (*HPA*) patients versus normal values

Patient no., sex	Age at time of biopsy	Plasma pipecolic acid (μmol/l)	Urine pipecolic acid (μmol/mmol creatinine)	CSF pipecolic acid (μmol/l)	Plasma phytanic acid (μmol/l)
1, boy	23 months	5.08 (age 12 months)	–	9.37 (at age 15 months) 0.35 (at age 23 months)	27.62
2, girl	3.5 months	6.49	69 (at age 1 month) (normal<6 months: 0.55–24)	Normal	Normal
3, boy	8 years 4 months	41.5	6.2 (at age 8 years) 6.6 (at age 9 years)	0.94	Normal
Normal value		<5	0.01–1.5	<0.12	<8.6

Table 2 Peroxisomal functions in cultured fibroblasts of the three HPA patients

Parameter measured	Patient 1	Patient 2	Patient 3	Control values[a]
1. DHAPAT activity (nmol/2 h per mg protein)	6.6	9.1	8.0	10.9±2.5 (75)[a]
2. VLCFA (very long chain fatty acid) levels				
C26:0 (nmol/mg protein)	0.28	0.17	0.35	0.25 (0.18–0.38)[b]
C26:0/C22:0	0.04	0.03	0.05	0.04 (0.03–0.07)
3. Peroxisomal fatty acid oxidation (pmol/h per mg protein)				
C26:0	1932	1306	1174	1937±440 (40)
Pristanic acid	1127	880	862	1126±267 (40)
4. Phytanic acid α-oxidation (pmol/h per mg protein)	62	61	37	68±19 (40)
5. Catalase immunofluorescence	Normal	Normal	Normal	Normal
6. Immunoblot analysis				
Acyl-CoA oxidase	Normal	Normal	Normal	Normal
L-Bifunctional protein	Normal	Normal	Normal	Normal
Peroxisomal thiolase	Normal	Normal	Normal	Normal

[a] Each value is mean ±SD, with number of observations in parentheses
[b] Values represent the mean with the 5–95% interval between parentheses

muscular hypotonia with normal deep tendon reflexes, and enlarged fontanelles associated with a normal head circumference (30th percentile) and absence of dysmorphic features. An electroretinogram showed a decreased scotopic b-wave. Pipecolic acid levels were elevated in urine at 1 month of age and in plasma, and normal in CSF (Table 1). Plasma VLCFA, phytanic acid, pristanic acid, lysine and liver function values were normal, as were urinary bile acid excretion and lysine values. MRI of the brain was normal.
As in patient 1, studies on cultured fibroblasts revealed normal peroxisomes (Table 2).

The third patient, a boy, was normal until he was 4 years old, when he presented delayed mental development and aggressive behaviour. By 8 years he showed normal weight, height, head circumference, muscle tone, hearing and vision. Pipecolic acid concentrations were elevated in plasma, urine and CSF (Table 1). At 8 years and 4 months a liver biopsy was taken. Liver functions were normal, as were other peroxisomal functions. VLCFA concentration in plasma and plasma and urine lysine values were normal. MRI of the brain was normal at 12 years of age.

Plasma and urine proline were extremely high, which is compatible with hyperprolinaemia type II. It remains to be established whether the anomalies of proline and pipecolic acid metabolism are independent phenomena.

Cultured fibroblast analysis showed no peroxisomal disturbances (Table 2).

Methods

Biopsy material was processed for light and electron microscopy. In order to visualise peroxisomes, cytochemical localisation of catalase activity (a peroxisomal marker enzyme) was performed by staining with diaminobenzidine (DAB) and hydrogen peroxide at alkaline pH [25]. Therefore, liver specimens were fixed for 18–24 h in 4% formaldehyde in 0.12 M sodium cacodylate buffer (pH 7.4) containing 1% calcium chloride (w/v) at room temperature. Cryostat or chopper sections were incubated in DAB solution (diaminobenzidine · 4 HCl in Theorell buffer; pH 10.5) and hydrogen peroxide for 3 h at room temperature with shaking. Visualisation of mitochondria was inhibited by KCN added to the catalase medium. Sections were postosmicated with 1% OsO_4 (1–1.5 h) for light microscopy and embedded in plastic. For electron microscopy sections were postosmicated overnight at 4°C with 4% OsO_4 and embedded in plastic.

Immunocytochemistry with antibodies against the peroxisomal enzymes and proteins alanine/glyoxylate aminotransferase (AGT), acyl-CoA oxidase (AOX) and bifunctional enzyme (PH) was done at the light microscopic level, and against the peroxisomal membrane protein (PMP43 kDa) at electron microscopic level in patient 3 as described by Espeel and Van Limbergen [7]. Therefore, specimens were postfixed with 0.5% glutaraldehyde for 1 h at 4°C after the formaldehyde-calcium fixation. Dehydration then followed over a graded ethanol series, followed by two impregnation steps with Unicryl (Biocell, Cardiff, UK), and the specimens were then left in Unicryl overnight at 4°C. Thereafter specimens underwent polymerisation for 5 days. Semithin sections were mounted on A.P.S.-coated glass slides, incubated with antibody overnight at 4°C, and subsequently incubated with protein A–colloidal gold for 1 h; then stabilisation of the antigen-antibody-protein A colloidal gold complex was done, followed by silver enhancement. Ultrathin sections were collected on Formvar-coated nickel grids. The procedure for immunostaining was essentially the same as for the semithin sections, but no silver enhancement was done.

Polyclonal antibodies against AGT were raised in rabbits against the purified enzyme from human liver [10]; polyclonal antibodies against acyl-CoA oxidase and bifunctional protein were raised in rabbits against the isolated enzyme from rat liver (IgG fractions obtained by ammonium sulfate precipitation); polyclonal antibodies against PMP43 kDa were raised in rabbits against the membrane from human liver peroxisomes [26].

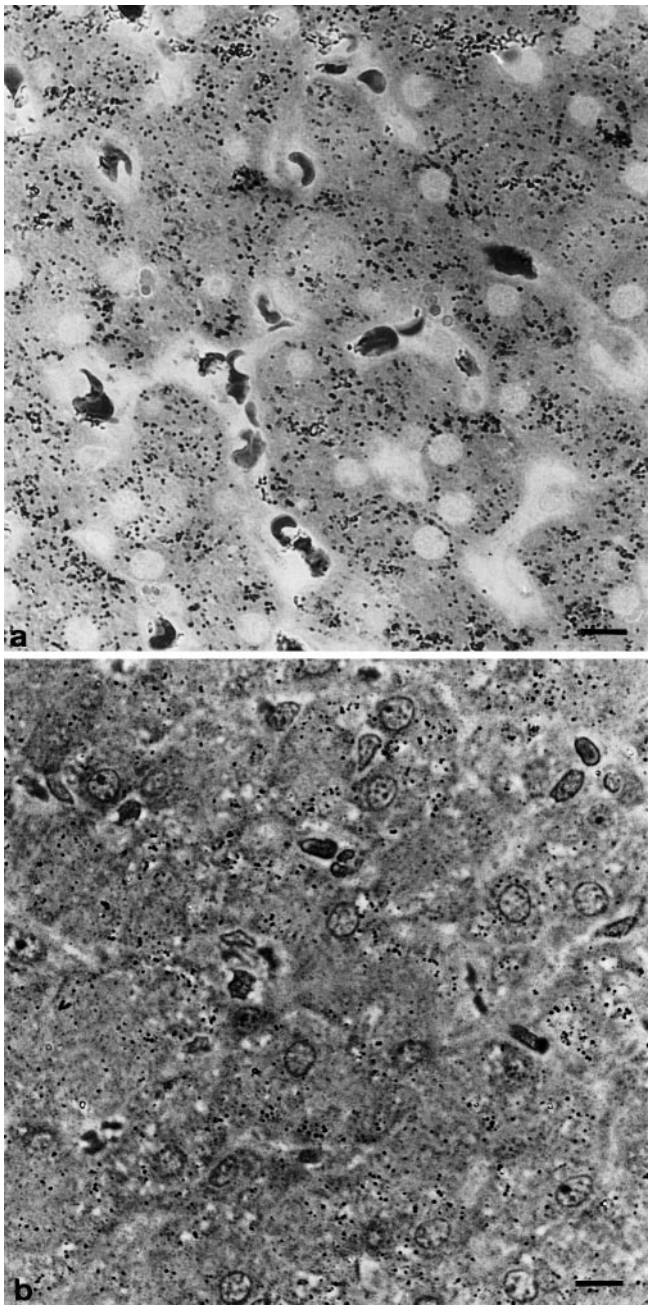

Fig. 1a, b Light microscopical visualisation of peroxisomes. The distribution of the organelles (*dark granules*) in the parenchymal cells is normal. ×625, *scale bar* 10 µm. **a** Localisation of catalase activity after DAB staining in a 2 µm section of the liver of patient 3 (*bright field*). **b** Localisation of immunoreactivity of peroxisomal bifunctional enzyme in the liver of the same patient. Protein A–colloidal gold-silver enhancement in 2 µm acrylate embedded section of liver biopsy (phase contrast)

Morphometry of peroxisomes was performed according to Kerckaert et al. [12], on randomly taken electron micrographs of ultrathin sections after DAB staining. Microscope magnification was calibrated for each series of photographs. The results in tissue of the patient were compared to control values. Before concluding that an altered value of a new patient is significant, the normal range of control values should be taken into account (95% confidence limits, indicating a *P*-value of 0.05).

The Lojda method for acid phosphatase activity (AcPP; visualisation of macrophages), Oil-Red-O and Sudan Black B staining for lipids were used as described in Roels et al. [25]. Sections stained with Perl's (demonstration of iron storage), periodic acid–Schiff (visualisation of glycogen) and trichrome (presence of connective tissue) were examined. The presence of trilamellar structures in lysosomes, a typical feature in many peroxisomal defects, was investigated by electron microscopy, and birefringent inclusions in macrophages by polarising light [11, 25].

Results

Light microscopy

After DAB staining for catalase activity peroxisomes are obviously present in the liver parenchymal cells of all three patients (Fig. 1a). In patient 1 the organelles seemed to be rather small, while in the other two patients the peroxisomal size looked normal (visual evaluation). They were abundant in patient 1 (subjective observation).

Some enlarged macrophages were seen after AcPP staining in patients 1 and 2, and many PAS-positive macrophages in patient 3. No polarising inclusions were observed. The presence of diffuse or granular iron particles was noted in about half of parenchymal cells of patient 2. In none of the patients was steatosis detected; glycogen was present in the parenchymal cells, as it is in most liver biopsies. No fibrosis had developed.

Immunocytochemical localisation of peroxisomal proteins in semithin sections of liver tissue of patient 3 revealed a normal distribution, i.e. in the peroxisomal matrix (Fig. 1b).

Electron microscopy and morphometry

In all patients peroxisomes were clearly present in parenchymal cells (Fig. 2).

In patient 1 they were seen as a population of many, mostly small, organelles; frequently peroxisomes with abnormal forms (elongated or oval) were observed. Morphometric measurements (Table 3) in this patient showed a moderate reduction in size, the corrected mean d-circle value being decreased (just below the 95% confidence limit). The diminution of the form-ellipse value (axial ratio) indicated the presence of elongated or abnormally shaped peroxisomes. Their number (N_v) was nearly doubled (193% of the normal value); volume and surface densities were within the confidence limits.

In patient 2 morphometric analysis (Table 3) revealed normal parameters, yet a subpopulation of enlarged peroxisomes was present (above the 95th percentile value with regard to control values). The mean peroxisomal d-circle is within the confidence limits. The corrected mean d-circle was normal, but high (yet within the confidence limits). The axial ratio was slightly decreased: again some irregu-

Table 3 Morphometric results of peroxisomes in liver biopsies from the three HPA patients versus control values. (*Number* number of measured organelles, *Size* (*d-circle*) diameter of the circle with the same area as the measured profile, *Corrected mean* correction for section thickness, *above 95th percentile* mean of values of the 5% largest organelles, *Form ellipse* the ratio between the shortest and the longest diameter (expresses the roundness of an organelle), N_v numerical density: number of peroxisomes per unit of cellular volume, V_v volume density: total peroxisomal volume expressed as a fraction of cellular volume, S_v surface density: total peroxisomal surface area expressed as a fraction of cellular volume)

	Patient 1	Patient 2	Patient 3	Controls		
				Adults (7)	Infant 6 weeks	Infant 4 months
				(95% confidence limits)		
Number	107	142	105	989	117	135
d-Circle (μm)						
Mean	0.450	0.559	0.502	0.525 (0.433–0.617)	0.445	0.518
SEM (±)	0.012	0.017	0.014	0.018		
Corrected mean	0.549	0.700	0.618	0.643 (0.569–0.717)	0.555	0.640
Max.	0.700	1.040	0.870	0.940	0.848	1.027
Above 95th percentile	0.668	0.967	0.824	0.768	0.769	0.848
SEM (±)	0.010	0.021	0.018	0.015		
Form ellipse (axial ratio)						
Mean	0.736	0.766	0.750	0.861	0.808	0.884
Max.	0.940	0.970	0.944	0.995		
Min.	0.390	0.400	0.311	0.410	0.436	0.569
Volume parameters						
N_v	0.193	0.089	0.167	0.100 (0.044–0.156)	0.110	0.128
SEM (±)				0.009		
V_v (%)	1.284	1.602	1.577	1.047 (0.499–1.595)	0.712	1.183
SEM (±)				0.100		
S_v	0.152	0.143	0.163	0.110 (0.054–0.166)	0.085	0.131
SEM (±)				0.010		

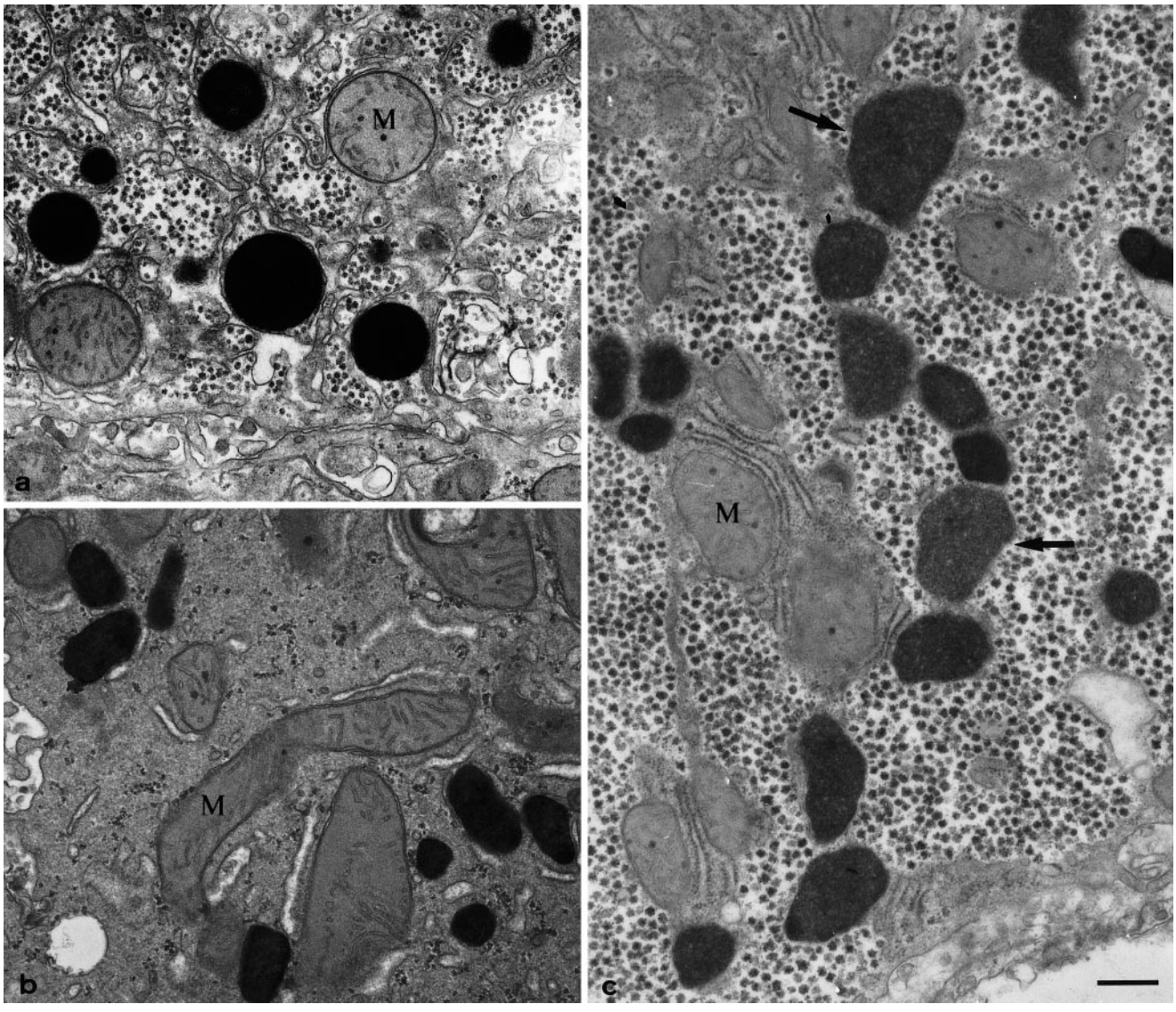

Fig. 2a–c Electron microscopical visualisation of peroxisomes after DAB-staining of catalase activity in liver (*M* mitochondria). ×18,000, *scale bar* 0.5 µm. **a** Control liver of 22-month-old girl. Size and shape of organelles are normal. **b** Liver of patient 3, male, 8 years and 4 months of age. Peroxisomes with normal mean size compared with control liver. Abnormally shaped organelles (*oval, elongated*) are confirmed by low axial ratio at morphometry. **c** Cluster of mostly large (*arrows*) peroxisomes in the liver of patient 2, female, 3.5 months of age. Unusual forms (*elongated, blocked, tailed*). Heterogeneity of catalase activity between individual organelles is striking but has been reported in many human livers [23]

larly or oval shaped peroxisomes were observed. In some cells clusters of peroxisomes were seen. The numerical density N_v was normal, as was the surface density S_v; the volume density V_v was high (just above the confidence limit).

In the third patient morphometric analysis (Table 3) showed a significant augmentation of the number of peroxisomes with 67% compared with the control value. The mean peroxisomal size was normal. The axial ratio (form-ellipse) was low: abnormally shaped peroxisomes (oval) were seen. The values of the volume and surface densities were high, but within the confidence limits.

No trilamellar inclusions, pathognomonic for severe peroxisomal disorders, were found, even in patient 3 at the age of more than 8 years, although their appearance is age dependent [11, 25].

Immunocytochemical localisation of the 43 kDa-PMP showed a normal reaction at the peroxisomal membrane.

Discussion

Microscopic investigation of liver biopsy material from our three patients with isolated HPA revealed the presence of peroxisomes in the parenchymal cells; morphometry showed some alterations. Poll-The et al. [21] reported that the peroxisomal number in some hepatocytes seemed to be decreased or was normal (visual evaluation); in some cells oval and elongated forms were seen; morphometry revealed slightly smaller peroxisomes than

in a control liver. Elongation as a proliferation-related change in shape, increases in number or the appearance of clusters, and increases in surface and/or volume densities, often accompanied by a decrease in size, as proliferation signs, are considered to be expressions of adaptation to a disturbed hepatic metabolism [6, 14]. These morphometric features were also found in our patients.

In isolated HPA the deficient activity of one peroxisomal enzyme, L-pipecolic acid oxidase, results in a block in the degradation of pipecolic acid. In an other peroxisomal single enzyme deficiency disorder, i.e. primary hyperoxaluria type 1 [12, 13], in which augmentation of metabolic efficiency was also assumed, similar peroxisomal alterations (decrease in size, increased number, abnormally shaped organelles) were observed.

Throughout the studies made by several investigators, discrepancies in the presence and functions of peroxisomes between liver and cultured skin fibroblasts were detected (see also "Introduction"). Challa et al. [4] described normal hepatic peroxisomes in HPA liver of the two patients originally reported by Burton et al. [3]. Wanders et al. [28] found cytoplasmic catalase and no organelles in cultured fibroblasts of Thomas et al.'s HPA patient [27], but Wiemer et al. [31] found punctate fluorescence in the same cell line. Because of such discrepancies, Leroy et al. [16] debated whether HPA should be classified as a peroxisomal biogenesis disorder.

In other peroxisomal disorders too, discrepancies have been described between peroxisomes in liver and cultured fibroblasts. In some patients normal peroxisomal functions were detected in fibroblasts together with clear abnormalities in blood and urine biochemistry and in liver tissue. Mandel et al. [17] described a patient with a clinical picture of peroxisomal disorder with elevated serum VLCFA and bile acids, but VLCFA were normal in fibroblasts; cytochemistry and immunochemistry of liver biopsy revealed the absence of peroxisomes in most hepatocytes (mosaic distribution). Mosaicism of peroxisomal occurrence in liver tissue was described in three more patients by Espeel et al. [8]; in one of these fibroblasts were again normal. Beard et al. [2] reported peroxisomes in fibroblasts from a patient with infantile Refsum disease without any such finding in the liver. Absence of peroxisomes in liver together with normal plasmalogen synthesis and DHAPAT activity in fibroblasts was described in Baumgartner et al. [1]. Roels [22] listed many examples of such discrepancies. All this illustrates that alterations may be tissue specific and that studies in cultured fibroblasts should be combined with examination of liver. In our patients investigations on fibroblasts revealed 'normal' peroxisomes in agreement with the liver data. This supports our opinion that isolated HPA is not a Zellweger variant.

Classification of HPA as a peroxisomal biogenesis defect similar to the cerebro-hepato-renal syndrome of Zellweger may be justified in two of the original cases, in which pipecolic acid disturbance was detected before other peroxisomal biochemical parameters were found to be disarranged. In contrast, in isolated HPA with only one abnormal metabolite, as in the three patients we studied, peroxisomes were clearly present with some morphological alterations, which indicates a compensatory mechanism. We propose classifying isolated HPA as a single peroxisomal enzyme deficiency disease, as Poggi et al. [20] have done.

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Note added in proof

We recently examined the liver of a girl aged 15 years demonstrating a novel case of isolated hyperpipecolic acidaemia. She presented with a progressive neurologic disorder (spastic paraparesis and cognitive decline). The pipecolic acid level was elevated; however, other peroxisomal parameters (including VLCFA) were normal. Peroxisomes were well stained by catalase and AGT localization; they were visibly increased in number and showed signs of recent proliferation. In collaboration with Dr. B. Cohen and A. Divincenzo (Dept. of Neurology, Cleveland Clinic).

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