Vol. 123, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 28, 1984 Pages 1054-1061

ACTIVITY OF PEROXISOMAL ENZYMES AND INTRACELLULAR DISTRIBUTION OF CATALASE IN ZELLWEGER SYNDROME

R.J.A. Wanders*, M. Kos*, B. Roest*, A.J.Meijer***, G. Schrakamp**, H.S.A. Heymans*, W.H.H. Tegelaers*, H. van den Bosch**, R.B.H. Schutgens* and J.M. Tager***

* Department of Pediatrics, University Hospital Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands

** Laboratory of Biochemistry, University of Utrecht Padualaan 8, 3584 CH Utrecht, The Netherlands

*** Laboratory of Biochemistry, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands

Received August 2, 1984

The activity of peroxisomal enzymes was studied in human liver and cultured human skin fibroblasts in relation to the finding (Goldfischer, S. et al.(1973) Science 182, 62-64) that morphologically distinct peroxisomes are not detectable in patients with the cerebro-hepato-renal (Zellweger) syndrome. In homogenates of liver from the patients, dihydroxyacetone phosphate acyltransferase, a membrane-bound peroxisomal enzyme, is deficient (Schutgens, R.B.H., et al. (1984) Biochem. Biophys. Res. Commun. 120, 179-184). In contrast, there is no deficiency of the soluble peroxisomal matrix enzymes catalase, L-α-hydroxyacid oxidase and D-aminoacid oxidase. Catalase is also not deficient in homogenates of cultered skin fibroblasts from the patients. The results of digitonin titration experiments showed that in control fibroblasts at least 70% of the catalase activity is present in subcellular particles distinct from mitochondria or lysosomes. In contrast, all of the catalase activity in fibroblasts from Zellweger patients is found in the same compartment as the cytosolic marker enzyme lactate dehydrogenase.

The cerebro-hepato-renal (Zellweger) syndrome is a rare familial disease characterized by a variety of clinical and biochemical aberrations (for a review see ref. 1). In 1973 Goldfischer et al. (2) reported that peroxisomes were absent in liver and kidney from patients with Zellweger syndrome. Furthermore, the mitochondria were abnormal in appearance (2) and were reported to be defective in oxidative phosphorylation (2,3).

Although the role of peroxisomes in mammalian metabolism has long remained an enigma, recent studies have shown that essential steps in ether phospholipid biosynthesis (4,5), bile acid synthesis (6), very long chain fatty oxidation (7) and dicarboxylic acid oxidation (8) take place in the peroxisomes. Hajra and coworkers (4,5) showed that two of the enzymes involved in ether phospholipid

0006-291X/84 $1.50

biosynthesis, dihydroxyacetone phosphate acyltransferase and alkyldihydroxyacetone phosphate synthase, are localized in the peroxisomal membrane. We have recently shown that dihydroxyacetone phosphate acyltransferase is deficient in patients with Zellweger syndrome (9) and that plasmalogens, the main end products of ether phospholipid biosynthesis in mammals, are virtually absent in tissues from the patients (10,11).

We have now extended this investigation to peroxisomal matrix enzymes in liver and cultured skin fibroblasts from patients with Zellweger syndrome. The results described in this paper show that peroxisomal matrix enzymes are not deficient in the patients. Furthermore at least one of these enzymes, catalase, has an aberrant intracellular distribution.

MATERIALS AND METHODS

Enzyme activity measurements

D-aminoacid : O_2 oxidoreductase (EC 1.4.3.3) was assayed by incubation of an aliquot of human liver homogenate (in 250 mM sucrose, 10 mM Tris-HCl, pH 7.5) at 37°C for 2h in a reaction medium containing 50 mM sodium pyrophosphate, 25 µM FAD, 50 mM D-alanine and 0.1% (w/v) Triton X-100. The final pH was 8.5. The 2-oxoacid formed (pyruvate) was measured as its 2,4-dinitrophenylhydrazone at 450 nm according to Nakano et al. (12).

L-α-hydroxyacid : O_2 oxidoreductase (EC 1.1.3.15) was assayed by incubating an aliquot of human liver homogenate for 30 min at 37°C in a reaction medium containing 50 mM sodium pyrophosphate, 0.5 mM EDTA, 50 mM sodium glycolate and 0.1% (w/v) Triton X-100. The final pH was 8.5. After termination of the reactions with trichloroacetic acid, the glyoxylate formed was measured as its 2,4-dinitrophenylhydrazone adduct at 450 nm (12).

Catalase (H_2O_2 : H_2O_2 oxidoreductase, EC 1.11.1.6) was measured in human liver homogenates and skin fibroblast homogenates by registering the production of O_2 polarographically at 20°C in a medium containing 50 mM potassium phosphate, 10 mM sodium perborate and 0.025% (w/v) sodium cholate; the final pH was 7.4. The activity of *dihydroxyacetone phosphate acyltransferase* (EC 2.3.1.42) was measured exactly as described before (9).

Latency measurements

Fibroblasts were cultured according to standard procedures and harvested after confluency in a medium containing 250 mM sucrose plus 10 mM HEPES (pH 7.4). Intact fibroblasts from control subjects and Zellweger patients were incubated in isotonic media containing different concentrations of digitonin and the activity of lactate dehydrogenase, catalase, β-hexosaminidase and glutamate dehydrogenase was measured as follows.

Lactate dehydrogenase was assayed by recording the decrease in absorbance at 340 nm in a medium (2 ml) containing fibroblasts (about 5 µg protein/ml), 300 mM sucrose, 5 mM HEPES, 0.2 mM NADH and 0.5 mM pyruvate; the final pH was 7.4. The activity of *catalase* was measured at 20°C by following the production of O_2 polarographically in a medium containing fibroblasts (about 40 µg protein/ml), 300 mM sucrose and 10 mM sodium perborate; the final pH was 7.4. For measurements of *β-hexosaminidase* activity fibroblasts (about 100 µg protein/ml) were incubated in a medium containing 300 mM sucrose, 100 mM sodium acetate and 2 mM 4-methylumbelliferyl-deoxy-acetyl-β-D-glucosaminide; the final pH was 5.4. After terminating the reaction with glycine/ NaOH (pH 10.0), the 4-methylumbelliferone generated was measured fluorimetrically. For measurements

of *glutamate dehydrogenase* activity fibroblasts (about 100 µg protein/ml) were incubated in a medium containing 300 mM sucrose, 10 mM HEPES, 2.5 mM EDTA, 5 mM 2-oxoglutarate, 0.5 mM NADH, 50 mM ammonium acetate and 1 mM ADP; the final pH was 7.4. After stopping the reaction with perchloric acid and subsequent neutralization, the glutamate produced was measured enzymically (13) in the protein free supernatant. Protein was measured by the method of Lowry (14).

RESULTS

Acyl-CoA: dihydroxyacetone phosphate acyltransferase is deficient in liver, brain and cultured skin fibroblasts from patients with Zellweger syndrome (9). In order to establish whether the absence of peroxisomes in Zellweger patients is correlated with a generalized deficiency of peroxisomal enzymes, we measured the activity of D-amino acid oxidase, L-α-hydroxyacid and catalase in homogenates of post mortem liver from patients and controls. The results are shown in Table 1. In contrast to dihydroxyacetone phosphate acyltransferase, D-amino acid oxidase, L-α-hydroxyacid oxidase and catalase are not deficient in liver from Zellweger patients.

In normal human liver at least 60% of the catalase is found in the peroxisomes (15). Consequently the question arose of the intracellular localization of these enzymes in patients with Zellweger syndrome. Since this problem could not be studied in post mortem livers subjected to freezing and thawing, cultured skin fibroblasts were used.

TABLE 1 ACTIVITY OF PEROXISOMAL MATRIX ENZYMES IN LIVER HOMOGENATES FROM CONTROL SUBJECTS AND PATIENTS WITH ZELLWEGER SYNDROME

ENZYME	ACTIVITY IN	
	CONTROLS	ZELLWEGER
D-aminoacid oxidase (nmol/min · mg protein)	0.73 ± 0.16 (11)	0.62 ± 0.14 (4)
L-α-hydroxyacid oxidase (nmol/min · mg protein)	1.61 ± 0.41 (11)	0.83 ± 0.15 (4)
Catalase (μmol O_2/min · mg protein)	58.9 ± 8.3 (11)	82.3 ± 19.7 (4)
Acyl-CoA: dihydroxyacetone phosphate acyltransferase (nmol/2h · mg protein)	2.47 ± 0.30 (7)	0.23 ± 0.15 (4)

The activities of D-aminoacid oxidase, L-α-hydroxyacid oxidase, catalase and acyl-CoA: dihydroxyacetone phosphate acyltransferase were measured in human liver homogenates as described in Materials and Methods. Results are expressed as mean ± S.E.M. with the number of subjects in parentheses.

TABLE II ACTIVITIES OF CATALASE AND ACYL-CoA: DIHYDROXYACETONE PHOSPHATE ACYLTRANSFERASE IN FIBROBLASTS FROM PATIENTS WITH ZELLWEGER SYNDROME AND CONTROLS

ENZYME	ACTIVITY IN	
	CONTROLS	ZELLWEGER
Catalase (μmol O_2/min · mg protein)	12.59 ± 1.57 (15)	26.72 ± 2.17 (17)
Acyl-CoA: dihydroxyacetone phosphate acyltransferase (nmol/2h · mg protein)	9.80 ± 0.40 (27)	0.66 ± 0.17 (9)

Catalase and acyl-CoA: dihydroxyacetone phosphate acyltransferase were assayed in fibroblast homogenates as described in Materials and Methods. Results are expressed as mean ± S.E.M. with the number of cell lines in parenthesis.

As shown in Table 2 the activities of dihydroxyacetone phosphate acyltransferase and catalase show the same pattern in cultured skin fibroblasts as in liver; whereas dihydroxyacetone phosphate acyltransferase is deficient in fibroblasts from Zellweger patients, catalase is clearly not. In control fibroblasts catalase has been shown to be particle bound (16,17). In order to establish the subcellular localization of catalase in fibroblasts from Zellweger patients and controls, use was made of the phenomenon of latency (i.e. lack of activity of an enzyme due to the presence of an impermeable membrane that prevents accessibility to the enzyme of its substrate(s)). A titration was carried out with digitonin. This compound, by forming a complex with cholesterol, makes membranes permeable. Only a small amount of digitonin is required to render the plasma membrane permeable since the cholesterol content of this membrane is particularly high (18). The cholesterol content of intracellular membranes is lower than that of the plasma membrane (18) so that greater amounts of digitonin are required to abolish the latency of an enzyme in an intracellular organelle.

The results of a typical experiment are shown in Fig. 1. As markers for different intracellular compartments lactate dehydrogenase (cytosol), β-hexosaminidase (lysosomes) and glutamate dehydrogenase (mitochondria) were used. The amount of digitonin required to release the latency was least with lactate dehydrogenase, greater with β-hexosaminidase and greater still with glutamate dehydrogenase. This is in accordance with the data of Colbeau et al. (18) on the

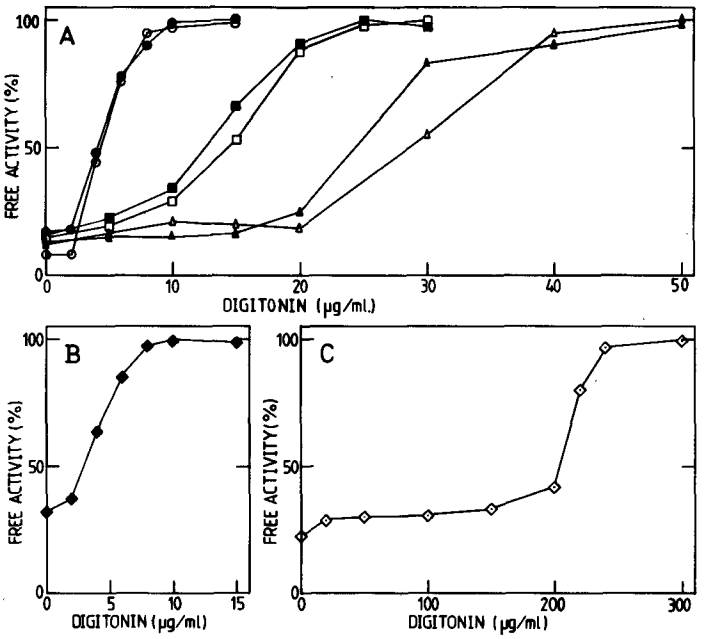

Fig. 1 Titration of the amount of digitonin required to release the latency of catalase and marker enzymes in fibroblasts from control persons and patients with Zellweger syndrome.
Confluent fibroblasts were incubated in an isotonic medium containing the concentrations of digitonin indicated and the free activity of lactate dehydrogenase (○,●), glutamate dehydrogenase (△,▲), β-hexosaminidase (□,■) (Fig. 1A) and catalase (◇,◆) (Fig. 1B,C) was measured as described in Materials and Methods.
Open symbols represent control fibroblasts, whereas closed symbols represent Zellweger fibroblasts. The values for the free activity of each enzyme are expressed as a percentage of the total activity of that enzyme, measured in the presence of 0.1% (w/v) Triton X-100.
Simular results were obtained in 3 other experiments (see text).
Note that different scales are used in the abscissa in A, B and C.

cholesterol content of various intracellular membranes in rat-liver. Furthermore, the amounts of digitonin required to release the latency of lactate dehydrogenase, β-hexosaminidase and glutamate dehydrogenase, respectively, were exactly the same in cultured skin fibroblasts from Zellweger patients as in those from control persons (Fig. 1A).

In fibroblasts from a control subject, about 80% of the catalase activity was latent. Upon incubation with 20 µg/ml digitonin, the lowest concentration used, the latency decreased to 70%. In order to abolish the latency of catalase completely a concentration of digitonin of 240 ± 20 µg/ml (n = 4) was required (Fig. 1C). In contrast, upon titration with digitonin of fibroblasts from Zellweger patients the latency of catalase was abolished completely at a digi-

tonin concentration of 8 ± 1 µg/ml (n = 4) (Fig. 1B), i.e. about the same concentration as that required to release the latency of lactate dehydrogenase (9 ± 1 µg/ml digitonin (n = 4)).

DISCUSSION

The results presented in this paper show that in liver homogenates from Zellweger patients several peroxisomal enzymes are present at near normal amounts (Table 1), even though peroxisomes are absent (2). An important distinction between acyl-CoA: dihydroxyacetone phosphate acyltransferase, which was shown to be severely deficient in Zellweger patients (9), and the three peroxisomal enzymes we have found to be present in near normal amounts in the patients is that acyl-CoA: dihydroxyacetone phosphate transferase is a membrane-bound enzyme (4,5), whereas the other enzymes are soluble peroxisomal proteins (19).

The data of Fig. 1 suggest that, in contrast to the situation in control fibroblasts, catalase is a cytosolic enzyme in fibroblasts from Zellweger patients. Obviously no studies such as that of Fig. 1 could be carried out on frozen liver material. The normal catalase activity in liver from Zellweger patients as reported in this paper, and the results of histochemical studies showing that catalase-positive particles can not be detected in liver from Zellweger patients (2,20), provide presumptive evidence that catalase (and, presumably, other soluble peroxisomal matrix enzymes) are localized in the cytosol in the liver of Zellweger patients.

In man a single locus, on chromosome 11, codes for catalase (21). Our results show that in cultured human skin fibroblasts catalase is predominantly particle-bound. Furthermore, the fact that higher concentrations of digitonin are required to release the latency of catalase than those required to release the latency of β-hexosaminidase and glutamate dehydrogenase indicate that the catalase-containing particles are distinct from lysosomes and mitochondria and presumably represent peroxisomes.

The results of this study suggest that functional peroxisomes are not required for the formation of at least some active, soluble peroxisomal matrix

enzymes. Catalase is a haem-containing, tetrameric protein, consisting of 4 identical subunits (22). It has been suggested (23) that oligomerization of catalase and addition of haem occur after uptake of the apomonomer into the peroxisomes in rat-liver (23). The results of our studies suggest either that formation of the active enzyme can occur not only in the peroxisomes but also in the cytosol, or that the localization of these steps differs in rat and human material. Studies are in progress to differentiate between these possibilities.

ACKNOWLEDGEMENTS

The authors are grateful to Piet Borst for his valuable suggestions, Ronald Oude Elferink and Andre Schram for their helpful advice, Anneke Strijland for her help in some preliminary experiments, Ellen Meijboom for culturing the fibroblasts used in the experiments described in this study, Gerrit-Jan Romeyn and Petra van de Bergh for expert technical assistance and Dr. M.F. Niermeyer for providing some of the cell lines used in this study. This work was supported financially by the "Princess Beatrix Fund", the Hague, The Netherlands.

REFERENCES

1. Kelley, R.I. (1983) Am. J. Med. Gen. 16, 503-517.
2. Goldfischer, S., Moore, C.L., Johnson, A.B., Spiro, A.J., Valsamis, M.P., Wisniewska, H.K., Ritsch, R.H., Norton, W.T., Rapin, I. and Gartner, L.M. (1973) Science 182, 62-64.
3. Trijbels, J.M.F., Berden, J.A., Monnens, L.A.H., Willems, J.L., Janssen, A.J.M., Schutgens, R.B.H. and van den Broek-van Essen, M. (1983) Pediatr. Res. 17, 514-517.
4. Hajra, A.K. and Jones, C.L. (1979) J. Biol. Chem. 254, 10896-10900.
5. Hajra, A.K. and Bishop, J.E. (1982) Ann. N. Y. Acad. Sci. 386, 170-182.
6. Kase, F., Björkhem, I. and Pedersen, J.I. (1983) J. Lipid. Res. 24, 1560-1567.
7. Osmundsen, H. (1982) Ann. N. Y. Acad. Sci. 386, 13-29.
8. Mortensen, P.B., Kølvraa, S., Gregersen, N. and Rasmussen, K. (1982) Biochim. Biophys. Acta 713, 393-397.
9. Schutgens, R.B.H., Romeyn, G.J., Wanders, R.J.A., van den Bosch, H., Schrakamp, G. and Heymans, H.S.A. (1984) Biochem. Biophys. Res. Comm. 120, 179-184.
10. Heymans, H.S.A., Schutgens, R.B.H., Tan, R., van den Bosch, H. and Borst, P. (1983) Nature 306, 69-70.
11. Heymans, H.S.A., van den Bosch, H., Schutgens, R.B.H., Tegelaers, W.H.H., Walther, J.U., Müller-Höcker, J. and Borst, P. (1984) Eur. J. Pediatr. 142, 10-15.
12. Nakano, M., Ushijima, Y., Saga, M., Tsutsumi, Y. and Asami, H. (1968) Biochim. Biophys. Acta 167, 9-22.
13. Bergmeyer, H.U. (ed.) (1979) Methoden der Enzymatischen Analyse, 2 nd. Edn., Verlag Chemie, Weinheim.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Noguchi, T. and Takada, Y. (1979) Arch. Biochem. Biophys. 196, 645-647.
16. Böck, P. (1973) Z. Zellforsch. 144, 539-547.
17. Mellman, W.J., Schimke, R.T. and Hayflick, L. (1972) Exp. Cell. Res. 73, 399-409.

18. Colbeau, A., Nachbaur, J. and Vignais, P.M. (1971) Biochem. Biophys. Acta 249, 462-492.
19. De Duve, C. and Baudhuin, P. (1966) Physiol. Rev. 46, 323-357.
20. Mooy, W.J., Dingemans, K.P., van den Bergh-Weermans, M.A., Jöbsis, A.C., Heymans, H.S.A. & Barth, P.G. (1983) Ultrastruct. Pathol. 5, 135-144.
21. Wieacker, P., Mueller, C.R., Mayerova, A., Grzeschik, K.H. and Ropers, H.H. (1980) Ann. Genet. 23, 73-77.
22. Aebi, H., Wyss, S.R., Scherz, B. and Skvaril, F. (1974) Eur. J. Biochem. 48, 137-145.
23. Lazarow, P.B. and de Duve, C. (1973) J. Cell. Biol. 59, 491-524.