Alkyl dihydroxyacetone phosphate synthase in human fibroblasts and its deficiency in Zellweger syndrome

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Abstract The cerebro-hepato-renal (Zellweger) syndrome is an autosomal recessive disorder biochemically characterized by the absence of morphologically distinguishable peroxisomes. Key enzymes involved in the biosynthesis of ether phospholipids, i.e., dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase, are located in mammalian (micro)peroxisomes. We have previously shown a strikingly reduced activity of dihydroxyacetone phosphate acyltransferase in liver, brain, and cultured skin fibroblasts from Zellweger patients (Schutgens et al. 1984. *Biochim. Biophys. Res. Commun.* **120:** 179–184). We have now extended these investigations by studying alkyl dihydroxyacetone phosphate synthase in cultured human skin fibroblasts. Enzymatic activity was determined by measuring the formation of radioactive alkyl dihydroxyacetone phosphate from palmitoyl dihydroxyacetone phosphate and [1-^{14}C]hexadecanol as substrates. The enzyme was optimally active at pH 8.5 and was stimulated (about 2–3-fold) by the presence of 0.05% (v/v) Triton X-100. The apparent K_M values for the enzyme in control fibroblasts amounted to 35 μM for palmitoyl dihydroxyacetone phosphate and 90 μM for hexadecanol. The reaction became inhibited at higher concentrations of both Triton X-100 and palmitoyl dihydroxyacetone phosphate. Control skin fibroblasts showed alkyl dihydroxyacetone phosphate synthase activity of 69 ± 28 pmol · min^{-1} · mg^{-1} (n = 7), while fibroblasts from patients had an activity of only 6.3 ± 1.7 pmol · min^{-1} · mg^{-1} (n = 7). Alkyl dihydroxyacetone phosphate synthase was also found to be deficient in tissue homogenates of Zellweger patients. The specific activity of this enzyme in liver, kidney, and brain homogenates from Zellweger patients was less than 15% of that in the corresponding tissues from controls. These findings together with the reported deficiency of dihydroxyacetone phosphate acyltransferase provide an explanation for both the virtual absence of plasmalogens in tissues from Zellweger patients as well as the impaired de novo biosynthesis of plasmalogens in fibroblasts from these patients. — **Schrakamp, G., C. F. P. Roosenboom, R. B. H. Schutgens, R. J. A. Wanders, H. S. A. Heymans, J. M. Tager, and H. van den Bosch.** Alkyl dihydroxyacetone phosphate synthase in human fibroblasts and its deficiency in Zellweger syndrome. *J. Lipid Res.* 1985. **26:** 867–873.

Supplementary key words cerebro-hepato-renal (Zellweger) syndrome • inborn errors • glycerol ether lipid biosynthesis • enzyme deficiency • peroxisomes • peroxisomal dysfunctions

The cerebro-hepato-renal (Zellweger) syndrome (ZS) is an autosomal recessive disorder, clinically characterized by hypotonia, craniofacial dysmorphia, disturbances in liver function, and severe psychomotor retardation (1–4). Goldfischer et al. (1) reported in 1973 a total absence of morphologically distinct peroxisomes in hepatocytes and renal tubule cells of ZS patients. In the course of the last decade it has become clear that peroxisomes are involved in important metabolic functions both in the plant and animal kingdoms (5). In recent years several disturbances in ZS patients have been described that might be caused by peroxisomal dysfunctions. Kase, Björkhem, and Pedersen (6) found that conversion of trihydroxycoprostanoic acid to cholic acid is catalyzed by a peroxisomal enzyme. Peroxisomes also contain a fatty acid β-oxidation system (7) that seems specially equipped for the oxidation of very long chain fatty acids (8, 9). Hence, the accumulation of trihydroxycoprostanoic acid (3) and of very long chain fatty acids (8, 10) in ZS patients might well be ascribed to the absence of peroxisomes in these patients.

Hajra, Burke, and Jones (11) and Hajra and Bishop (12) showed that acyl-CoA:DHAP acyltransferase (EC 2.3.1.42) and alkyl DHAP synthase are located in peroxisomes of rodent liver and in microperoxisomes of rodent brain. The peroxisomal localization of the former enzyme was recently confirmed by others (13, 14). These membrane-bound enzymes catalyze the initial steps in the biosynthesis of glycerol ether lipids (12, 15–19). Indeed, we recently found plasmalogens, the major end products of ether phospholipid synthesis, to be virtually absent in tissues of

Abbreviations: DHAP, dihydroxyacetone phosphate; ZS, Zellweger syndrome.

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ZS patients (20, 21). Subsequently, we reported that acyl-CoA:DHAP acyltransferase activity is strikingly reduced in tissues and cultured skin fibroblasts from these patients (22). This result was essentially confirmed by Datta, Wilson, and Hajra (23), although these authors claimed that DHAP acyltransferase was completely absent in leukocytes of one and fibroblasts of two ZS patients.

In this study we report on the second peroxisomal membrane-bound enzyme involved in ether lipid biosynthesis, i.e., alkyl DHAP synthase, the enzyme that actually introduces the glycerol ether bond by replacing the long acyl group in acyl DHAP by a long chain alcohol. The properties of this enzyme in cultured human skin fibroblasts were investigated and a comparison of the enzymatic activities in fibroblasts and some tissues of control subjects and ZS patients was made.

MATERIALS AND METHODS

Materials

[1-^{14}C]Hexadecanol (55 mCi/mmol) was obtained from IRE-Nederland, Soesterberg, The Netherlands. Glycolic acid, oxalylchloride, n-nitroso toluol-4-sulfomethylamide, phenylhydrazine, silica gel 60 HR, and solvents were from Merck, Darmstadt, FRG. Palmitoyl chloride was from Fluka AG, Buchs, Switzerland. Diethyleneglycolmonoethylether was from BDH Chemicals, Poole, England. DEAE-cellulose circles (∅ 2.3 cm) were from Whatmann, Maidstone, England.

Enzyme sources

Fibroblasts were cultured in F_{10} medium containing initially 15% fetal calf serum and subsequently 15% newborn calf serum. Cells were harvested according to standard procedures (21) and stored at −70°C. Control fibroblasts were obtained from skin biopsies of healthy volunteers. Detailed clinical and biochemical findings concerning the Zellweger patients are presented elsewhere (21). Washed fibroblasts were resuspended in 5 mM Tris-HCl (pH 7.5) and 50 mM NaCl at a protein concentration of about 2.5 mg/ml. Tissue homogenates were prepared in a Dawnson homogenizer in the same buffer as described above. Tissue homogenates and fibroblast suspensions were processed further by sonicating three times for 15 sec at 80 W with 45-sec intervals for cooling, followed by three cycles of freezing in liquid nitrogen and thawing.

Preparation of substrates

Palmitoyl DHAP was synthesized as described by Hajra, Saraswathi, and Das (24), using the intermediate 1-O-palmitoyl-3-diazohydroxyacetone (25). This intermediate was decomposed with H_3PO_4 and the product was purified by silicic acid chromatography. [1-^{14}C]Hexadecanol was purified by anion exchange chromatography in ethanol to remove traces of fatty acid. Both substrates were stored under nitrogen at −20°C.

Enzyme assay

The alkyl-DHAP synthase assay was based on the DEAE-filter paper assay described by Brown and Snyder (19). The standard alkyl DHAP synthase assay contained 100 mM Tris-HCl (pH 8.5), 50 mM NaF, 0.05% (v/v) Triton X-100, 240 μM palmitoyl-DHAP, 100 μM [1-^{14}C]hexadecanol (100,000 dpm/nmol), and 100 μg of homogenate protein in a total volume of 100 μl. Palmitoyl-DHAP and hexadecanol were added as sonicated suspensions in a buffer containing 5 mM Tris-HCl (pH 8.0). After incubation at 37°C for 20 min the reaction was stopped by an extraction according to Bligh and Dyer (26). The water layer contained 2 M KCl and 0.2 M H_3PO_4. Next, 0.1 ml of the chloroform layer was spotted on a DEAE-cellulose disk. The filters were dried and washed three times for 15 min in absolute ethanol at 0°C. The filters were dried and placed in scintillation vials containing 10 ml of emulsifier fluid (Packard, Belgium) and the radioactivity was measured.

In initial experiments the chloroform layer resulting from the Bligh and Dyer (26) extraction after incubation was used for product identification. Alkaline hydrolysis was done according to Davis and Hajra (16). Protein contents of homogenates were measured according to the method of Lowry et al. (27).

RESULTS

Product identification

Although human skin fibroblasts appear to be capable of de novo synthesis of alkoxy lipids (28, 29), the presence of alkyl DHAP synthase activity in fibroblasts has not been documented. In order to identify the chloroform-soluble radioactive product formed in the synthase assays, the chloroform layer was subjected to thin-layer chromatography (silica 60 HR) with chloroform–methanol–acetone–acetic acid–water 80:40:80:40:20 (v/v) as development solvent. Two radioactive spots appeared, one large spot at the front (excess of [1-^{14}C]hexadecanol) and one small radioactive spot which coincided with reference palmitoyl DHAP. After reaction with phenylhydrazine (19), this radioactivity had the same R_f value as the phenylhydrazone of palmitoyl DHAP, indicating the presence of a carbonyl group. In contrast to palmitoyl DHAP, the ^{14}C-labeled product was stable to alkaline hydrolysis (16). These properties identify the radioactive product as alkyl DHAP.

Properties of alkyl DHAP synthase

In order to make an accurate comparison of the alkyl DHAP synthase activity in fibroblasts from control subjects and Zellweger patients, the optimal assay conditions were investigated. It can be seen from **Fig. 1** that enzyme activity is linear with time for 20 min and linear up to 100 μg homogenate protein under the standard assay conditions described in the Materials and Methods section. Alkyl DHAP synthase from control human fibroblasts showed a pH optimum of 8.5 (**Fig. 2**). The shape of the pH-dependency curve, with a small shoulder around pH 6.5, was confirmed in three independent experiments using different fibroblast homogenates. Although the meaning of this shoulder is not clear at present, it is noteworthy that similar pH/activity profiles were reported for alkyl DHAP synthase from Ehrlich ascites cells (16), and from rabbit harderian gland (15). The synthase activity in fibroblasts from ZS patients was very low at all pH values investigated (Fig. 2). This result indicates not only a strikingly reduced alkyl DHAP synthase activity at pH 8.5 in ZS fibroblasts, but, in addition, demonstrates that this is not caused by a change in the pH optimum of the synthase in those cells. The synthase activity in control fibroblasts was stimulated by Triton X-100 up to concentrations of 0.05% (v/v), while higher concentrations became inhibitory (**Fig. 3**).

Fig. 4A shows the dependence of reaction velocity on hexadecanol concentration. The apparent K_M for the enzyme in control fibroblasts as deduced from a double-reciprocal plot amounted to 90 μM. A V_{max} of 0.13 nmol · min^{-1} · mg^{-1} was calculated. The activity in fibroblast homogenates from ZS patients proved too low to allow an accurate measurement of the apparent K_M and V_{max} values. The substrate concentration dependency curve for palmitoyl DHAP is shown in Fig. 4B. This curve clearly shows that alkyl DHAP formation is completely dependent on the addition of palmitoyl DHAP to the incubation mixtures. Up to 120 μM substrate, the reaction rate increased with substrate concentration. Higher substrate concentrations appeared to become inhibitory, probably due to the detergent nature of acyl DHAP. This observation is in agreement with results reported for the enzyme from ascites cells (16). From the data in the ascending part of the substrate concentration curve, an apparent K_M of 35 μM for palmitoyl DHAP was calculated for the enzyme in control fibroblasts. Again, the activity of the enzyme in fibroblasts from a ZS patient was too low to calculate a reliable K_M value. There was, however, also a tendency for the enzyme activity in ZS fibroblasts to become inhibited at higher palmitoyl DHAP concentrations.

Comparison of alkyl DHAP synthase activities in patients and controls

Table 1 compares the alkyl DHAP synthase activities of fibroblasts from controls and ZS patients. It is obvious from this table that the synthase activity in ZS fibroblasts is only about 10% of that of controls. The specific enzymatic activity in ZS fibroblasts ranged from 3 to 8 pmol · min^{-1} · mg protein^{-1} and its maximum value measured was fivefold lower than the minimal value found in control fibroblasts. When alkyl DHAP synthase activities in some control and Zellweger tissues were measured, the results in **Table 2** were obtained. The product formed in experiments with control tissues was identified as [^{14}C]alkyl DHAP by the same methods as described for fibroblast enzyme. Comparable values for the specific activities were found in the different tissues from controls

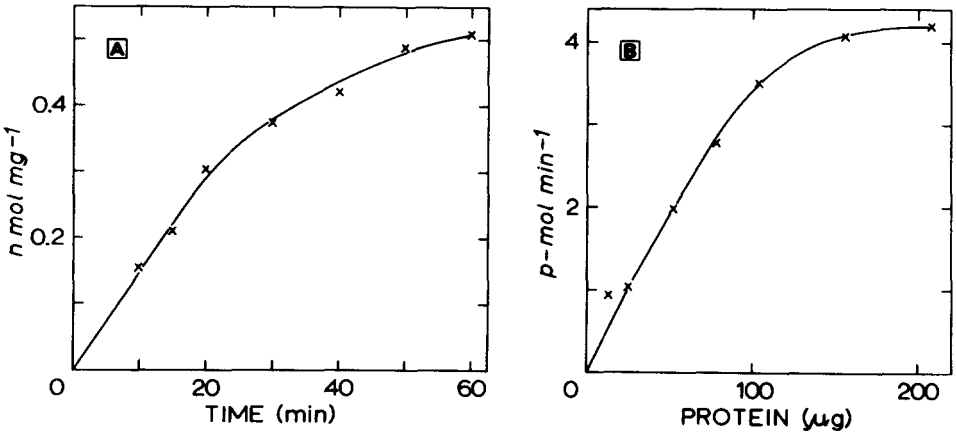

Fig. 1. Alkyl DHAP synthase activity in human skin fibroblasts as a function of time and protein concentration. The assay mixtures contained 120 μM palmitoyl DHAP, 100 μM hexadecanol, 100 mM Tris/HCl (pH 8.5), 50 mM NaF, 0.05% Triton X-100, and were incubated at 37°C. The amount of homogenate protein used for the time curve (A) was 100 μg and the time of incubation in the concentration curve (B) was 20 min. Essentially the same results were obtained with different homogenates, although the absolute values differed. (Compare Table 1.)

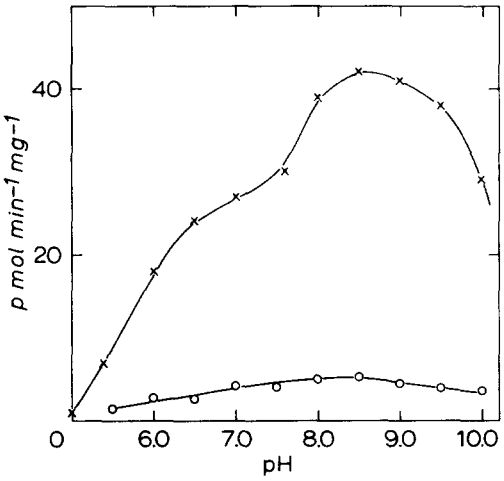

Fig. 2. Effect of pH on the alkyl DHAP synthase activity in human skin fibroblasts. The assays were performed under standard conditions (for details see Materials and Methods section) in buffers containing 100 mM Tris/maleate at the indicated pH values. Activity was measured in homogenates of control fibroblasts (x–x) and fibroblasts of Zellweger patients (O–O). The shape of these curves was confirmed in three independent experiments.

but these were about fourfold lower than those found in control fibroblasts. Although this difference could be intrinsic, it could also be caused by partial loss of enzymatic activity as a result of postmortem changes in tissues or by the assay conditions that were not optimized separately for the enzyme in human tissues. Despite the few tissues available for investigation, it is clear from Table 2 that tissues of Zellweger patients are deficient in alkyl DHAP synthase activity.

DISCUSSION

Peroxisomes appear to have several important metabolic functions (5). Recent studies have shown the presence in mammalian peroxisomes of enzymes involved in the β-oxidation of (very) long chain fatty acids (7–10), the biosynthesis of bile salts (6), and the biosynthesis of glycerol ether lipids (11, 12). Our finding that human skin fibroblasts contain a DHAP acyl-transferase activity with properties similar to those of the peroxisomal enzyme in rat and guinea pig liver has led us to suggest that (micro) peroxisomes are present in human fibroblasts (22). Using histochemical techniques, Novikoff et al. (30) demonstrated the presence of catalase-positive particles in rat fibroblasts.

The observation that [1-^{14}C]hexadecanol is incorporated into the alkenyl moiety of plasmalogens in human skin fibroblasts (28, 29), indicating that de novo glycerol ether bond formation occurs in these cells, suggested the presence of another peroxisomal enzyme, i.e., alkyl DHAP synthase. The results reported in this study provide the first unequivocal evidence for the occurrence of this enzyme in human skin fibroblasts. The human fibroblast enzyme shares several properties with that from Ehrlich ascites cells (15, 16). Both enzymes are optimally active at pH 8.5, become inhibited at higher concentrations of the substrate palmitoyl DHAP, but not of the other substrate, hexadecanol, and exhibit apparent K_M values for both substrates that differ by less than a factor 2.

As demonstrated for the first time by Goldfischer et al. (1), the cerebro-hepato-renal (Zellweger) syndrome is characterized by the absence of morphologically distinguishable peroxisomes. Our finding that DHAP acyltransferase, a membrane-bound peroxisomal enzyme (11, 12), was deficient in cultured skin fibroblasts from nine patients with ZS (22) is thus in accordance with the observation of Goldfischer et al. (1). Subsequently, Datta et al. (23, 31) reported the absence of this enzyme in fibroblasts from two ZS patients. With regard to alkyl DHAP synthase, these authors claimed in their preliminary report that the activity of this enzyme in fibroblasts from the two ZS patients was comparable to that of normal control fibroblasts. Datta et al. (31) subsequently reported that the activity of this enzyme in fibroblasts from three ZS patients was 15–45% of the mean value in fibroblasts from three control subjects. In contrast, our results (Table 1 and Figs. 2 and 4) clearly indicate that alkyl DHAP synthase activity is strikingly reduced (less than 10% of the mean control value) in fibroblasts from seven ZS patients. The reason for these discrepancies is not clear.

In theory, the low enzymatic activity in ZS fibroblasts as determined in the standard assay developed for control fibroblasts (Table 1) could be caused by deviating properties of the enzyme in ZS fibroblasts. Due to its low activ-

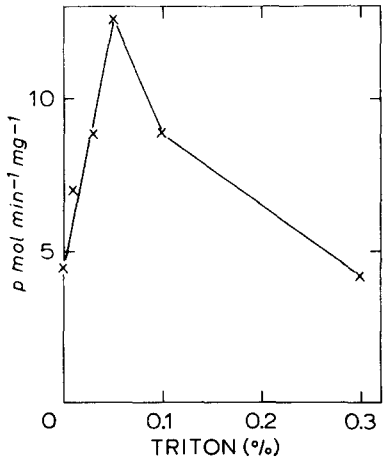

Fig. 3. Influence of the detergent Triton X-100 on the alkyl DHAP synthase activity in human skin fibroblasts. The assays were performed under standard conditions (for details see Materials and Methods section) with variable Triton X-100 concentrations as indicated.

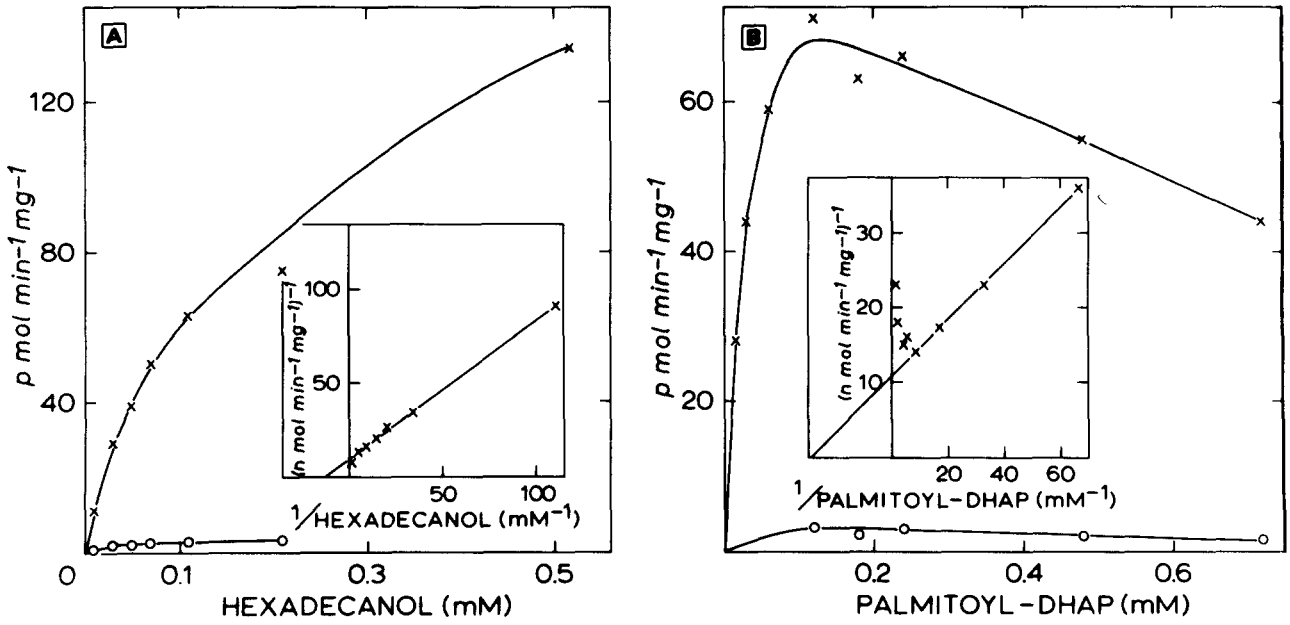

Fig. 4. Alkyl DHAP synthase activity in human skin fibroblasts as a function of substrate concentrations. Standard incubation mixtures were used except that the concentrations of hexadecanol (A) and palmitoyl DHAP (B) were varied as indicated. x-x, Control fibroblasts; O-O, ZS fibroblasts.

ity, the kinetic constants for the enzyme in ZS fibroblasts could not be reliably determined. It is clear, however, that no indications were obtained for the presence of an alkyl DHAP synthase activity in ZS fibroblasts with a radically different pH optimum (Fig. 2). In addition, the conclusion of a deficiency in alkyl DHAP synthase in Zellweger patients is substantiated by the data on this enzyme in liver, kidney, and brain from controls and patients (Table 2).

Not all peroxisomal enzymes appear to be deficient in ZS patients. Wanders et al. (32) have found that catalase, D-aminoacid oxidase, and L-α-hydroxy acid oxidase exhibit activities in liver and fibroblasts from ZS patients that are fully comparable to control values. Digitonin titration of fibroblasts showed that catalase, a soluble peroxisomal matrix enzyme, is located in the cytoplasm of ZS fibroblasts. Apparently, catalase protein is stable in the absence of functional peroxisomes. By using immunological techniques, Tager et al. (submitted for publication) have recently established that three peroxisomal β-oxidation enzyme proteins were markedly deficient in liver of ZS patients. Thus, in contrast to the situation with catalase, functional peroxisomes appear to be required for the stability of these peroxisomal β-oxidation enzymes. The question arises whether a similar situation exists with regard to the membrane-associated enzymes DHAP acyltransferase and alkyl DHAP synthase. If this were so, the residual activity in ZS patients of DHAP acyltransferase (22, 31) and of alkyl DHAP synthase (this paper, 31) could be due to traces of enzyme proteins still present. An alternative possibility is that the enzyme proteins are not deficient but that the reduction in activity is due to lack of association of the proteins with peroxisomal membranes or to some defect in proper processing.

TABLE 1. Alkyl DHAP synthase activity in cultured skin fibroblasts

Fibroblasts	Number of Cell Lines	Alkyl DHAP Synthase Activity	
		Mean ± S.D.	Range
		pmol · min⁻¹ · mg⁻¹	
Controls	7	69 ± 28	40–130
Zellweger	7	6.3 ± 1.7	3–8

TABLE 2. Alkyl DHAP synthase activity in tissues

Tissue	Alkyl DHAP Synthase	
	Controls	Zellweger Patients
	pmol · min⁻¹ · mg⁻¹	
Liver	18	2
	15	3
Kidney	20	
	14	3
Brain	11	n.d.

Enzymatic activity was determined in standard assay mixtures using 100 μg of homogenate protein from the indicated tissues. Values represent the mean of duplicate incubations for each tissue from different controls or patients. Abbreviations: n.d., not detectable.

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