



Effects of Lorenzo's Oil on Peroxisomes in Healthy Mice

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We investigated peroxisomal alterations in mice treated with different doses of Lorenzo's Oil (a therapy for X-linked adrenoleukodystrophy patients) for up to 100 days. Hepatic erucic acid levels were already significantly increased 2.2-fold and 2.6-fold in mice treated with 10% and 20% Lorenzo's Oil for 21 days, respectively. No lipidosis was found in liver, myocardium and kidney of any of the treated mice. While hepatic catalase, lauroyl-CoA oxidase and glycolate oxidase, and renal catalase activities were not induced by either diet, myocardial catalase activity was increased in most groups. This suggests that the mechanism of the effect of Lorenzo's Oil in X-linked adrenoleukodystrophy patients may not be a direct effect on the peroxisomes.

Keywords: Adrenoleukodystrophy (X-linked); glycerol trierucate; glycerol trioleate; catalase; peroxisomal oxidases

Introduction

Lorenzo's Oil, a 4:1 mixture of glycerol trioleate and glycerol trierucate is prescribed as a diet supplement to patients with X-linked adrenoleu-

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kodystrophy. These patients suffer from progressive demyelination of the central nervous system, adrenal insufficiency, and accumulation of very-long-chain fatty acids. Peroxisomal β -oxidation of very-long-chain fatty acids is deficient. X-linked adrenoleukodystrophy is caused by a defect of a gene coding for an ATP-binding transporter (1). This is believed to affect the import and function of very-long-chain acyl-CoA synthetase in peroxisomes and not the function of peroxisomal acyl-CoA oxidases or of other peroxisomal enzymes.

The most widely used therapy consists of a diet free of very-long-chain fatty acids and supplemented with Lorenzo's Oil. It reduces very-long-chain fatty acid levels but amelioration of clinical manifestations is seldomly observed (2).

Large-scale trials of this dietary therapy are already being conducted (3–5) while no experimental studies on the effects of Lorenzo's Oil have been reported. In literature, data on the effects of oleic acid or erucic acid containing diets sometimes are conflicting and do not allow to make a prediction on the effect of Lorenzo's Oil on peroxisomal metabolism. Our aim was to investigate light microscopic characteristics of peroxisomes and the activity of several peroxisomal enzymes in mice fed diets supplemented with Lorenzo's Oil.

Materials and Methods

Adult male NMRI mice (35–40g) were fed a powdered chow (A04, UAR, Epinay, France), supplemented with 2 and 10% (w/w) Lorenzo's Oil (Scientific Hospital Supplies, Liverpool, U.K.) during 3, 21 and 100 days. All animals had free access to food and water. The chow contained 3% (w/w) lipids. Matched control mice only received the standard chow.

The concentrations of Lorenzo's Oil approximated the daily doses administered to children with X-linked adrenoleukodystrophy. We considered the body surface area as reference point to convert doses from children to mice (6): a dose of 3 g/kg/day (maximum dose found in literature (3)) given to a child weighing 20 kg corresponds to a daily intake of 25 g/kg body weight in the mouse. The daily intake of Lorenzo's Oil by the mice on a diet with 2% or 10% (w/w) Lorenzo's Oil was 6.3 and 31.5 g/kg body weight, respectively. An additional experiment using a 20% Lorenzo's Oil diet for 3 and 21 days was set up to study the effects of a "high-fat" diet on the peroxisomes. These animals consumed 63 g Lorenzo's Oil/kg body weight/day.

Animals were killed by cervical dislocation after ether anaesthesia. For histological analysis, cryosections of fixed material (from the middle lobe of the liver, the myocardial left ventricle wall and the kidney cortex) were incubated with diaminobenzidine at pH 10.5 (catalase staining of peroxisomes) and evaluated by means of a comparative light microscope.

The organs were kept at -80°C before enzyme assays were performed.

All measurements were performed within the linear range for enzyme activity as previously described (7). Catalase (EC 1.11.1.6) activity was measured at 0°C in homogenates of liver, myocardium and kidney cortex using the titanium oxysulphate method. One U is the amount of catalase which breaks down 90% of the substrate (1.5 mM H₂O₂) in a volume of 50 mL at 0°C in 1 min.; maximal reaction time is 10 min. Hepatic peroxisomal fatty acyl-CoA oxidase (EC 1.3.99.3; the rate limiting enzyme for peroxisomal β -oxidation of fatty acids) and L- α -hydroxyacid oxidase (EC 1.1.3.15; an enzyme not involved in β -oxidation of fatty acids) were assayed by a fluorometric method.

For the assay of erucic and oleic acid in liver, total lipids were extracted from 0.5 mL of liver homogenates with chloroform/methanol (2:1) using the method of Folch et al. (8). Ten μ g of nonadecanoic acid (Sigma, St Louis, USA) was added as internal standard. Fatty acids were transesterified by incubating 12 h at 80°C with methanol/H₂SO₄. Fatty acid methyl esters were separated on a DBI capillary column (J&W Scientific, Rancho Cordova, USA) on a gas chromatograph (Varian, Walnut Creek, USA). Quantification of erucic and oleic acids was done with the standard of nonadecanoic acid.

Each experimental group consisted of five animals. All results are presented as the mean \pm SEM. For statistical analysis the Kruskal-Wallis test was used. Significance was obtained when $p < 0.05$.

This study was approved by the Ethical Committee of the Vrije Universiteit Brussel and was conform to the ethical guidelines of the Declaration of Helsinki.

Results

Control mice showed no time-dependent differences in weight data, activities of lauroyl-CoA oxidase, glycolate oxidase or catalase, nor in peroxisomal morphology and therefore, were pooled into one control group.

No differences in appetite between groups of animals were observed. Mean body weight as well as mean weight of liver, heart and kidneys were not significantly changed in Lorenzo's Oil-treated animals. No correlations between the weight data and the duration of feeding or dietary concentration of Lorenzo's Oil were found.

The liver of untreated mice contained 0.63 ± 0.02 μ g erucic acid/mg protein and 27.28 ± 1.03 μ g oleic acid/mg protein. A diet containing 2% Lorenzo's Oil provoked an 1.3-fold increase in hepatic erucic acid concentration when administered for 100 days. In mice fed a diet with 10% Lorenzo's Oil for 21 and 100 days, the concentration of erucic acid in the liver was significantly increased 2.2-fold and 2.6-fold, respectively. Mice treated with the 20% Lorenzo's Oil-supplemented diet for 21 days revealed a 2.6-fold increase in hepatic erucic acid level. The ratio erucic acid/oleic acid did not differ between mice treated with different Lorenzo's Oil-containing diets.

TABLE 1. Enzyme activities in mice treated with different dietary concentrations of Lorenzo's Oil for up to 100 days

Concentration and duration of Lorenzo's Oil treatment	Hepatic lauroyl-CoA oxidase activity (nmol H ₂ O ₂ /min.mg protein)	Hepatic glycolate oxidase activity (nmol H ₂ O ₂ /min.mg protein)	Hepatic catalase activity (mU/mg protein)	Myocardial catalase activity (mU/mg protein)	Renal catalase activity (mU/mg protein)
0% (controls)	2.95 ± 0.48	1.31 ± 0.23	507 ± 63	1.20 ± 0.08	33 ± 2
2% during 3 days	3.82 ± 0.28	2.21 ± 0.45	428 ± 27	1.17 ± 0.07	31 ± 2
2% during 21 days	2.60 ± 0.10	0.80 ± 0.26	478 ± 40	1.55 ± 0.03 ^a	37 ± 3
2% during 100 days	4.19 ± 0.46	0.60 ± 0.16	505 ± 47	1.53 ± 0.06 ^a	40 ± 5
10% during 3 days	3.54 ± 0.62	1.92 ± 0.40	360 ± 27 ^a	2.03 ± 0.04 ^a	36 ± 2
10% during 21 days	5.25 ± 1.06	2.08 ± 0.71	558 ± 64	2.12 ± 0.08 ^a	33 ± 3
10% during 100 days	3.28 ± 0.27	0.96 ± 0.24	517 ± 16	2.26 ± 0.10 ^a	36 ± 2
20% during 3 days	3.67 ± 0.34	1.28 ± 0.22	368 ± 32 ^a	1.93 ± 0.09 ^a	33 ± 2
20% during 21 days	4.33 ± 0.43	2.14 ± 0.47	601 ± 17	2.27 ± 0.12 ^a	39 ± 3

Each experimental group consisted of 5 animals.

Data are significantly different from control data ($p < 0.05$).

Mean hepatic peroxisomal lauroyl-CoA oxidase and glycolate oxidase activities were unchanged in either group of treated mice (Table 1). No time-dependent nor dose-dependent changes in fatty acyl-CoA oxidase and glycolate oxidase activities were observed.

The diet with 2% Lorenzo's Oil did not affect hepatic catalase activity (Table 1). Diets containing 10% and 20% Lorenzo's Oil only provoked a significant change (i.e., decrease) in hepatic catalase activity after 3 days of treatment. When all individual enzymatic data were pooled, no significant linear correlation between the activity of catalase (a H₂O₂-scavenging enzyme) on the one hand and the activity of glycolate oxidase and/or lauroyl-CoA oxidase (two H₂O₂-producing enzymes) on the other hand was found.

Myocardial catalase activity was significantly increased in all treated groups except in mice fed 2% Lorenzo's Oil for 3 days (Table 1). Renal catalase activity was unaffected by any dose of Lorenzo's Oil tested (Table 1).

Light microscopic evaluation revealed no changes in peroxisomal number, size, distribution or catalase staining in hepatocytes, myocardiocytes and epithelial cells of the proximal renal tubules of mice fed the Lorenzo's Oil-supplemented diets. No accumulation of lipid droplets was observed in the liver, myocardium and kidney of any of the treated mice.

Discussion

The increased concentrations of erucic acid measured in liver homogenates of mice fed Lorenzo's Oil-containing diets for longer periods indi-

cate that the diet supplement is absorbed by the intestinal tract of the mice. Thus the failure to induce changes in hepatic and renal peroxisomal enzyme activities can not be ascribed to intestinal malabsorption of Lorenzo's Oil.

Our data indicate that Lorenzo's Oil diet has no effect on the activity of hepatic peroxisomal fatty acyl-CoA oxidase (the H_2O_2 -producing and rate limiting enzymatic step in peroxisomal fatty acid β -oxidation) in mice. In literature only reports dealing with high-fat diets containing important amounts of oleic acid or erucic acid are present. Hepatic fatty acyl-CoA oxidation was unchanged in rodents fed high-fat diets containing varying amounts (up to 73.8%) of oleic acid (9). High-rapeseed oil containing 44% erucic acid had a negative modulating effect on peroxisomal β -oxidation enzyme system in rats (10) while 6% erucic acid added to a 20% soybean oil diet had no effect (11). Subcutaneous (s.c.) injections of erucic acid decreased the cyanide-insensitive palmitoyl-CoA dependent dehydrogenase activity by 35% in rats (10). The absence of peroxisomal β -oxidation induction in mice fed 20% Lorenzo's Oil indicates that neither oleic acid nor erucic acid is responsible for the often observed induction provoked by the more complex natural oils when supplemented in high doses.

Lorenzo's Oil supplemented diets did not alter hepatic glycolate oxidase activity. Only one other hepatic oxidase not involved in fatty acid oxidation, was previously studied in diet experiments: urate oxidase was unchanged in rats fed high caloric concentrations (30%) of partially hydrogenated marine oil (12). The pure oil contained 15.1% oleic acid and 14.5% erucic acid (11).

Diets with Lorenzo's Oil supplementation did not induce hepatic catalase activity. This is in accordance to the lack of induction of lauroyl-CoA oxidase and glycolate oxidase and also suggests the absence of induction of other H_2O_2 -producing metabolic pathways in the liver of treated mice. The normal peroxisomal morphology is consistent with these findings. A high-fat diet based on olive oil (which contains 65.6% oleic acid) decreased catalase activity with $\pm 23\%$ while a high-fat diet based on partially hydrogenated peanut oil (which contains 63.6% oleic acid) had no effect (13). The effect of erucic acid on hepatic catalase activity has not been investigated.

Lorenzo's Oil treatment induces myocardial catalase activity. This was also observed in rats fed 20% partially hydrogenated fish oil + 5% soybean oil (14). Since partially hydrogenated fish oil contains about 15% erucic acid (11), the final dietary concentration approximated 3% which holds the middle between the erucic acid concentration in 10% and 20% Lorenzo's Oil diets. Catalase induction may be linked to a simultaneous increase in peroxisomal β -oxidation: the diet with 20% partially hydrogenated fish oil + 5% soybean oil also induced the cyanide-insensitive palmitoyl-CoA dependent NAD^+ -reduction (14). A diet with 18.6% olive

oil (contained 78.7% oleic acid) did not change antimycin- and rotenone-insensitive oxidation rates of [$1\text{-}^{14}\text{C}$] palmitate in the heart of rats (15). We therefore assume that the induction of myocardial catalase activity in mice treated with Lorenzo's Oil is due to the presence of erucic acid in the diet.

The increases in myocardial catalase activity were not reflected in an enhanced staining intensity of the individual peroxisomes. Apparently, the intensity of the oxidized diaminobenzidine in sections cytochemically stained for catalase is little susceptible to the observed changes in activity of this enzyme.

The well-known myocardial lipidosis provoked by erucic acid-containing diets (16) was absent in mice treated with Lorenzo's Oil. This may be explained by the transient nature of this phenomenon: the rapid increase in catalase activity (already after 3 days with the highest 2 Lorenzo's oil concentrations) indicates a fast adaptation of the myocardial metabolism.

We found no renal peroxisomal changes. No data on effects of oleic acid and erucic acid containing diets on renal peroxisomes are available.

Our data demonstrate that in the liver of normal mice, Lorenzo's Oil does not induce lauroyl-CoA oxidase and catalase. This contrasts with the effects of peroxisome proliferators and high doses of certain complex vegetable and animal oils (7,17). This difference together with the absence of visual lipid accumulation in the liver of mice fed the Lorenzo's Oil-supplemented diets may indirectly suggest that the hepatic peroxisomal β -oxidation capacity in treated mice was not overloaded by the excess of erucic and oleic acids.

X-linked adrenoleukodystrophy patients have a defective capacity to β -oxidize very-long-chain fatty acids due to a deficiency of very-long-chain fatty acyl-CoA synthetase (18). However peroxisomal β -oxidation of erucic acid is not impaired in skin fibroblasts from these patients (19). Therefore, no accumulation of both monoenoic fatty acids present in Lorenzo's Oil is expected in liver of X-linked adrenoleukodystrophy patients and an overload of the peroxisomal β -oxidation capacity is unlikely.

The mechanism of the effect of Lorenzo's Oil in serum of X-linked adrenoleukodystrophy patients (i.e., the normalization of the level of very-long-chain fatty acids) may not be a direct effect on the peroxisomes. The long monoenoic fatty acids of the oil are believed to compete with and thus reduce the elongation of saturated fatty acids to very-long-chain fatty acids in the endoplasmic reticulum (20,21).

At present there is no evidence of clinically relevant benefit of Lorenzo's Oil-treatment in symptomatic X-linked adrenoleukodystrophy patients; a preventative effect of this treatment is currently under investigation (22,23). Side-effects of dietary Lorenzo's Oil are lower platelet counts (24) and bleeding diathesis due to decreased platelet membrane anisotropy (25).

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