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SHORT COMMUNICATION

Reduced Cholesterol Accumulation and Improved Deficient Peroxisomal Functions in a Murine Model of Niemann–Pick Type C Disease upon Treatment with Peroxisomal Proliferators

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ABSTRACT. Niemann–Pick type C disease is an inherited disorder characterized by lysosomal accumulation of cholesterol and the mutant gene has recently been identified. The predicted gene product is a transmembrane protein showing homology to proteins involved in the regulation of cholesterol homeostasis, such as 3-hydroxy-3-methylglutaryl-coenzyme A and the sterol regulatory element binding protein cleavage-activating protein. Recent investigations have established a peroxisomal deficiency, which raised the question of whether peroxisomal proliferation could affect this cholesterol-processing error. Mutant mice with Niemann–Pick type C disease were treated with the peroxisomal inducer perfluorooctanoic acid, which increased peroxisomal β-oxidation and catalase activity to the same level as in control mice. Not only the peroxisomal, but also the lysosomal malfunctions were corrected and the cholesterol content was decreased. Clofibrate, another peroxisomal inducer, restored both peroxisomal enzyme activities and ubiquinone content. It appears that in Niemann–Pick type C disease treatment with appropriate peroxisomal inducers restores basic cellular functions, indicating a relationship between peroxisomes and cholesterol homeostasis, and thereby may effectively interfere with the development of the disease. BIOCHEM PHARMACOL **56**;9:1195–1199, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Niemann–Pick; peroxisomes; peroxisomal proliferation; lipid metabolism; cholesterol; dolichol

The majority of cases of human Niemann–Pick disease are represented by the C-type, which is considered to be a lysosomal storage disorder, and characterized by the accumulation of cholesterol, sphingomyelin, glycosphingolipids, and bis-(monoacylglycero)-phosphate [1]. It has been established that the intracellular processing and transport of low-density lipoprotein-derived cholesterol is deficient, resulting in an excessive accumulation of unesterified cholesterol in the lysosomes of several organs [2]. The gene of the major complementation group of NPC[‖] disease (NPC1) has recently been identified, and the predicted gene product is similar to proteins which are involved in the regulation of cholesterol homeostasis. This product has a cholesterol-sensing region resembling that of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and the sterol regulatory element binding protein cleavage-activating protein [3]. A mutant BALB/c mouse strain with NPC is a very good model for this disease, and it has been shown that the affected gene has extensive homology to the human NPC1 gene [4]. It was found that in addition to cholesterol, the metabolism of other mevalonate pathway lipids is also affected [5]. Recent investigations on cellular functions in liver and brain have demonstrated a considerable peroxisomal deficiency in this condition, affecting a number of functions [6]. Significantly, peroxisomal deficiency is a very early sign of the disease in the liver, occurring several weeks before appearance of the clinical symptoms. In this study, peroxisomal inducers were employed to study the importance of peroxisomes in the etiology and development of NPC and to investigate the possibility of correcting peroxisomal deficiencies and suggest future therapeutic interventions.

MATERIALS AND METHODS

The BALB/c npc^{nih} mouse strain was used for all experiments [4]. Induction experiments were performed by supplying standard food containing 0.05% PFOA to the mice for 6 days or 0.6% clofibrate for 3 weeks. The clinical symptoms, mainly ataxia, were noted by visual observation, which did not allow the quantitation of the differences between treated and untreated NPC mice. The experiments performed were approved by the local ethics committee.

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‖ *Abbreviations:* NPC, Niemann–Pick type C disease; C:M:W, chloroform:methanol:water; PMS, succinate-phenazine methosulfate; DCPIP, 2,6-dichlorophenolindophenol; and PFOA, perfluorooctanoic acid.

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Livers were quickly removed and homogenized in 0.25% cold sucrose with an ultraturrax blender.

Total amounts of dolichol, dolichyl-P, and cholesterol were quantified after alkaline hydrolysis performed at 85° for 30 minutes [5]. The samples were extracted with C:M:W (3:2:1) (final concentration). The chloroform phase was evaporated, and the residue was dissolved in C:M:W (1:1:0.3) and subjected to DEAE-Sephadex column chromatography. Neutral lipids were eluted with C:M:W (1:1:0.3) while dolichyl-P was eluted with C:M:W (1:1:0.3) containing 200 mM of ammonium acetate. Neutral lipids were further separated by C_{18} column chromatography by removing cholesterol with methanol and dolichol with n-hexane. Esterified forms of dolichol and cholesterol were extracted with chloroform:methanol (2:1). The lipid residues were applied to a silica column and eluted with chloroform in order to separate neutral lipids from phospholipids. Ubiquinone-9 was extracted with C:M:W (1:1:0.3) at 37° for 1 hour. Ubiquinone-6, dolichol-23, dolichyl-22-P, and ergosterol were used as internal standards.

All neutral lipids were quantified by HPLC using a Hewlett-Packard Hypersil ODS (pore size 3 μm) reversed-phase column [7]. A linear gradient was employed starting with methanol:water (9:1) in pump system A to methanol:2-propanol:n-hexane (2:1:1) in pump system B at a flow rate of 1.5 mL/min. The gradient time was 25 minutes and the program was continued at 100% of solvent B for another 5 to 10 minutes. Quantification was performed by means of a UV detector at 275 nm for ubiquinone and at 210 nm for analysis of the other neutral lipids. The total amount of phospholipids was measured as described previously [8].

NADPH-cytochrome *c* reductase, succinate-PMS-DCPIP reductase, acid phosphatase, urate oxidase, peroxisomal β-oxidation of palmitoyl-CoA, and lauroyl-CoA oxidase and catalase activities were measured as described previously [6, 9]. The protein concentration was determined according to the method of Lowry *et al.* [10].

RESULTS

The total phospholipid content of liver homogenate was similar in the control and NPC mice, whereas the mevalonate pathway lipid pattern was modified (Table I). Cholesterol content increased severalfold, which is the dominating feature of this disease. In the mouse liver, only ca. 2% of cholesterol was esterified and this derivative was not present in increased amounts in the NPC mice. The total amount of dolichol was similar in the control and the diseased state, whereas the esterified form of this lipid was decreased in NPC-affected mice. On the other hand, the dolichyl-P concentration was elevated as much as 270%. Ubiquinone, which has a solanesol side-chain in mice, was decreased significantly. The marker enzyme activities of microsomes and mitochondria, NADPH-cytochrome *c* reductase, and succinate-PMS-DCPIP reductase were unchanged when the control was compared to the diseased mice, in agreement with previous studies which showed that the marker enzymes of endoplasmic reticulum and mitochondria are not modified with respect to enzymatic organization. The increased acid phosphatase activity was the result of the enlargement of the lysosomal compartment. The decreased urate oxidase activity and peroxisomal β-oxidation of palmitoyl-CoA demonstrate that peroxisomal functions are affected by this disease.

TABLE I. Chemical and enzymatic composition of liver homogenates in NPC mice

	Control	NPC
Phospholipid*	0.18 ± 0.03	0.22 ± 0.04
Total cholesterol*	9.7 ± 1.5	108 ± 19¶
% cholesteryl esters	2.3	0.82
Total dolichol†	290 ± 42	276 ± 40
% dolichyl esters	24.5	12.9
Dolichyl-P†	45 ± 6	120 ± 6¶
Ubiquinone†	150 ± 10	98 ± 9¶
NADPH-cyt c reductase‡	3.3 ± 0.5	3.4 ± 0.4
Succinate-PMS-DCPIP reductase‡	56 ± 2.3	52 ± 4.4
Acid phosphatase§	35 ± 2.9	52 ± 2.7
Urate oxidase‡	70 ± 4.5	57 ± 4.6¶
Palmitoyl-CoA oxidation‖	4.6 ± 0.32	2.7 ± 0.29¶

NPC mice studied 3 days after appearance of the symptoms. The control mice were age-matched. The values are the means ± SD (n = 5).

*μg/mg protein.

†ng/mg protein.

‡nmol/(min × mg protein).

§nmol p_i/(min × mg protein).

‖peroxisomal β-oxidation of palmitoyl-CoA, pmol/(min × mg protein).

¶$P < 0.005$.

PFOA is an efficient peroxisomal inducer in mice, causing extensive proliferation of peroxisomes. We included 0.05% PFOA in the diet over a 6-day period in order to investigate the effect of peroxisomal induction in NPC mice exhibiting peroxisomal deficiencies. This treatment in control mice did not influence cholesterol content in the liver to a significant extent but after 6 days of PFOA treatment, the elevated cholesterol level in the diseased mice was observed to be decreased by approximately 25% (Fig. 1A), although it was still considerably higher than the control value. In some experiments, the cholesteryl ester amounts were also estimated. This derivative of cholesterol decreased both in NPC mice and in the control after treatment. The total amount of dolichol was similar in the control and the NPC mice, and upon treatment with the peroxisome proliferator, this amount was decreased significantly in the diseased mice (Fig. 1B). NPC is characterized by a high level of dolichyl-P (Fig. 1C). Upon treatment of mice with PFOA, there was an increase in dolichyl-P concentration in controls, and the high level of this lipid in the diseased mice remained unchanged. Since induction of ubiquinone-9 biosynthesis in the liver requires a longer treatment to obtain an increased amount of this lipid, mice were kept on the clofibrate diet for 3 weeks. This treatment significantly increased the lipid content in both control and NPC mice (Fig. 1D).

The activity of peroxisomal β-oxidation enzymes, such as

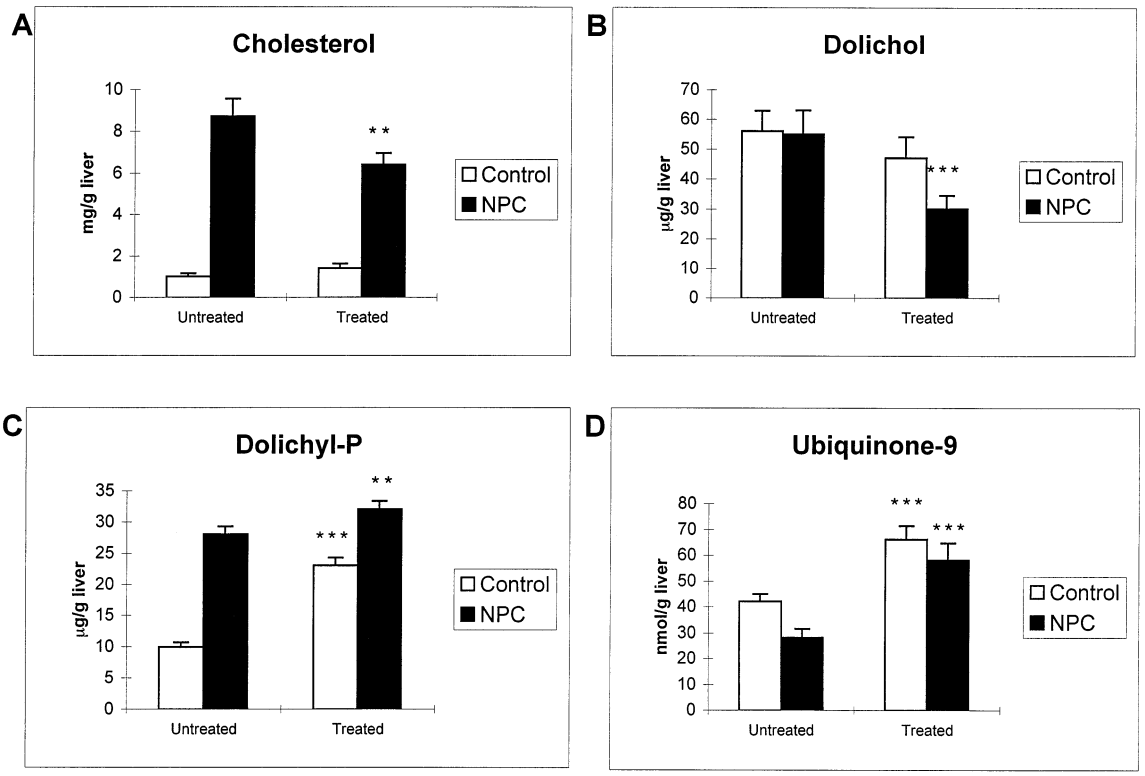

FIG. 1. Mevalonate pathway lipids in the livers of NPC mice after treatment with PFOA or clofibrate. Total cholesterol (A). Total dolichol (B). Dolichyl-P (C). Ubiquinone-9 (D). Control and NPC mice, 3 days after appearance of the symptoms, were either given a diet containing 0.05% PFOA (A, B, and C) or 0.6% clofibrate (D). Six days (PFOA) or 21 days (clofibrate) later, the liver lipids were extracted and analyzed by HPLC. The values are the means ± SD (n = 4). **$P < 0.01$; ***$P < 0.005$.

lauroyl-CoA oxidase, in NPC mice was only half of that found in the control (when analyzed at the initial phase after appearance of clinical symptoms) (Fig. 2A). There was a severalfold induction of oxidase activity both in control and NPC mice treated with PFOA, and after induction the oxidase activity in NPC reached the same level as in control. Catalase activity was decreased in the NPC mice in a similar fashion to the other peroxisomal enzymes (Fig. 2B). The treatment with PFOA did, however, eliminate this deficiency while also inducing the enzyme. The increased acid phosphatase activity in NPC mice (Fig. 2C) is thought to be the consequence of lysosomal enlargement. Interestingly, this nonperoxisomal modification was also normalized upon treatment of mice with the inducer. Peroxisomal proliferators also affect a number of mitochondrial and microsomal enzymes, but this aspect was not investigated.

DISCUSSION

A large number of substances with different chemical structures have been described as inductors of peroxisomes. The induction is mediated by the activation of a member of the nuclear hormone receptor superfamily [11], the peroxisome proliferator-activated receptor α. PFOA and clofibrate are known to be efficient in mice and these were also employed in our study. Since the efficiency and specificity of the various inducers are very different, it is possible that a combined treatment with simultaneous administration of two or several compounds would be optimal to restore all deficient functions in NPC. It is, however, difficult to perform large experiments with diseased animals of approximately the same age, since the recessively inherited disease results in relatively few diseased animals even from a large colony.

NPC is classified as a lysosomal storage disease dominated by the inhibited transport of cholesterol from lysosomes, mainly affecting the endocytic portion of this lipid [2]. The recent finding concerning general peroxisomal deficiency was unexpected and the question arises as to what extent it is related to the process of the disease. Since the use of peroxisomal inducers restores the original functional capacity of this organelle, the importance of peroxisomes in the etiology and development of this disease is further emphasized. Interestingly, induction of peroxisomes also decreases the total amount of cellular cholesterol in NPC mice, probably due to increased cholesterol transport. The inhibited transport of cholesterol is one of the cardinal symptoms of this disease, also apparent from studies of the recently identified NPC1 gene, whose protein product showed homology to proteins involved in cholesterol homeostasis [3]. It is not quite clear to what extent peroxi-

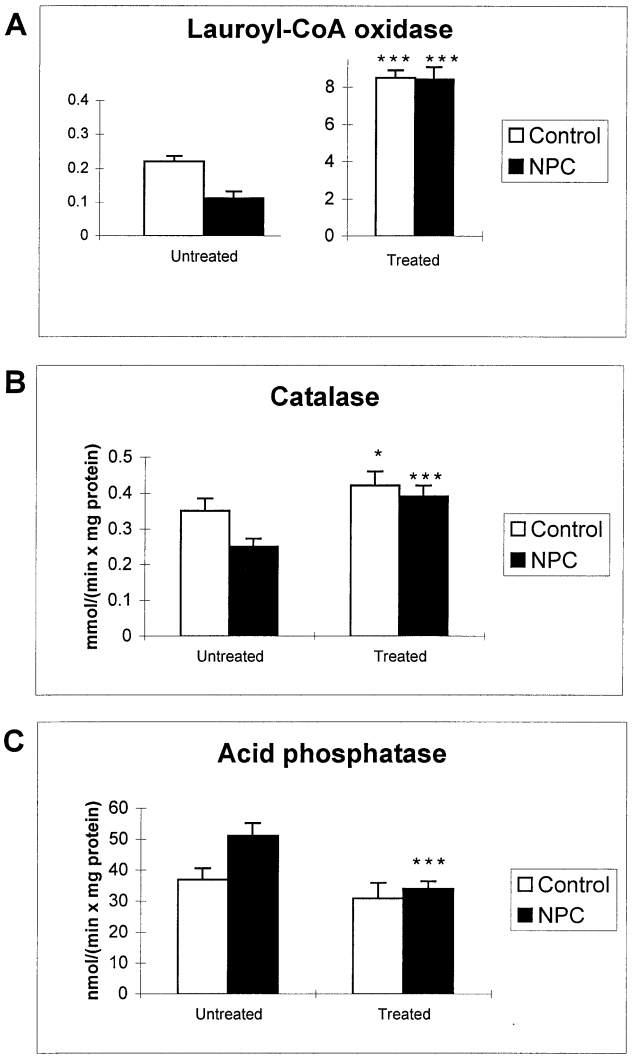

FIG. 2. Peroxisomal and lysosomal enzyme activities in the livers of NPC mice after treatment with PFOA. Lauroyl-CoA oxidase (A). Catalase (B). Acid phosphatase (C). Treatment was performed as in Fig. 1. The values are the means ± SD (n = 4). *$P < 0.05$; ***$P < 0.005$

somes are involved in cholesterol transport, but there are several indications that these are indeed implicated. Sterol carrier protein 2, which is mainly localized in peroxisomes, is suggested to be a carrier in cholesterol transport and its amount is greatly decreased in NPC [12]. Investigations performed on fibroblasts from peroxisome-deficient patients have shown a decreased low-density lipoprotein cholesterol uptake [13]. In our studies, the decreased acid phosphatase activity after the treatment also indicates that the peroxisomal proliferation partly normalizes the size of the lysosomal compartment. We treated the diseased mice after appearance of the symptoms and were able to eliminate peroxisomal deficiencies and reduce cholesterol accumulation. An interesting possibility for further study would be to start administration of the inducers at an earlier phase of life, long before the disease becomes apparent. Since the disease can be diagnosed shortly after birth by the polymerase chain reaction as well as by filipin staining of liver biopsies, we may in future start peroxisomal induction at an earlier phase of life. By correcting peroxisomal malfunctions, it may be possible to postpone the onset of the disease symptoms. In humans, peroxisomal proliferation in response to peroxisomal inducers is limited, and therefore the possibility of using such drugs for treatment of NPC is not clear. However, since these inducers display hypolipidemic effects in humans, a human clinical trial may be of value.

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