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Effects of bezafibrate on dyslipidemia with cholestasis in children with familial intrahepatic cholestasis–1 deficiency manifesting progressive familial intrahepatic cholestasis

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Abstract

No appropriate pharmaceutical therapy has been established for dyslipidemia with cholestasis in progressive familial intrahepatic cholestasis (PFIC)–1. We evaluated the efficacy of bezafibrate in PFIC-1. We monitored the clinical presentation and lipoprotein metabolism of 3 patients, aged 3, 4, and 8 years, with FIC1 deficiency, manifesting PFIC-1, over 12 months of bezafibrate therapy. Pruritus was substantially alleviated in the 3 patients after initiation of bezafibrate. Cholestasis was alleviated in 2 of them. Serum high-density lipoprotein cholesterol and low-density lipoprotein cholesterol increased 1.6- to 2.0-fold and 1.1- to 1.2-fold, respectively; but the values remained low and normal, respectively. Serum lipoprotein X, which was at normal levels before treatment, was elevated to levels above the upper limit of the reference range. High serum triglyceride levels decreased by 15% to 30%, to normal levels, after treatment initiation. The activities of lipoprotein lipase and hepatic triglyceride lipase were increased, but those of high-density lipoprotein regulators remained unchanged. Liver expression of multidrug resistance protein–3, which regulates lipoprotein X synthesis, was enhanced by bezafibrate therapy. Bezafibrate treatment favorably affected pruritus, dyslipidemia, and cholestasis in PFIC-1.

1. Introduction

Progressive familial intrahepatic cholestasis (PFIC), formerly called *Byler syndrome*, is a congenital cholestatic liver disorder with normal γ-glutamyl transpeptidase (GGT) levels [1-3]. This disorder is classified into PFIC-1 and PFIC-2, depending on the mutation. Progressive familial intrahepatic cholestasis–1 is caused by mutations in the *ATP8B1* gene, encoding the FIC1 protein; and PFIC-2 is caused by mutations in the adenosine triphosphate–binding cassette B 11 gene (*ABCB11*) [1-3].

Cholestasis in PFIC usually manifests itself in the first few months of life and is unremitting thereafter, with normal GGT levels. The liver disease typically progresses to cirrhosis before the end of the second decade of life. Markedly short stature, osteoporosis, and severe pruritus due to cholestasis have been noted as serious problems over a long-term follow-up period [1-3].

Unlike other cholestatic liver disorders, serum total cholesterol (TC) levels are normal or low in PFIC patients. However, our previous work provided evidence that PFIC patients have dyslipidemia, characterized by the

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accumulation of triglyceride (TG)-rich low-density lipoprotein (LDL) and depleted high-density lipoprotein (HDL), and a predisposition to cardiovascular disorders involving atherosclerosis [4-11].

To date, no appropriate pharmaceutical therapy has been established for dyslipidemia with cholestasis in PFIC. Bezafibrate, a fibrate, is commonly administered in Japan as an antihyperlipidemic agent. Bezafibrate is known as a ligand of the peroxisome proliferator activator receptor (PPAR) α, a nuclear receptor regulating lipoprotein metabolism. Through this pathway, bezafibrate lowers serum TG levels and raises serum HDL cholesterol (HDL-C) levels [12-15]. PPAR α also enhances hepatic expression of liver multidrug resistance protein–3 (MDR3), which promotes phospholipid secretion into bile [16,17]. Clinically, bezafibrate has recently been used to treat primary biliary cirrhosis; it improved cholestasis and liver function [18-21]. However, the effects of bezafibrate on congenital cholestatic liver disorders have not been studied sufficiently.

We treated 3 PFIC-1 children with bezafibrate for 12 months and compared their clinical presentation and lipoprotein metabolism with those of 2 PFIC-1 patients not receiving bezafibrate therapy. This report describes the beneficial effects of bezafibrate on dyslipidemia and cholestasis in PFIC-1.

2. Subjects and methods

2.1. Subjects

We enrolled 5 patients with PFIC-1 (aged 3-8 years) in this study. Of the 5, 3 patients (patients [Pts]1, 2, and 3) received bezafibrate treatment, whereas the others (Pts 4 and 5) did not, during the study period. For all patients, the diagnosis of PFIC-1 was established based on their clinical course, liver histopathology (light and electron microscopy), and genetic analyses [1-3] (Table 1).

To confirm the abnormality in FIC1 at the protein level, we performed Western blot analyses of frozen liver biopsy samples using a rabbit polyclonal antibody (Sawady Technology, Tokyo, Japan) against amino acid residues 828 to 850 of the human *ATP8B1* sequence (GenBank accession no. AF038007). As a control, the same Western blot analysis was conducted using frozen liver biopsy samples obtained from 3 healthy men aged 31, 36, and 37 years. They were donors for living-related liver transplants for their children with extrahepatic biliary atresia and resulting liver failure. We confirmed that the liver expression of FIC1, encoded by *ATP8B1*, was strikingly decreased in all patients, supporting the diagnosis of PFIC-1 (Fig. 1).

Table 1
Backgrounds of patients with PFIC-1

Patient no. (current age)	M/F	FIC1 gene mutations	Recent liver histology		
			Expansion of portal area	Fibrosis	ILBD degeneration
Pt 1[a] (3 y 11 mo)	F	T380C/ T842C	No	Mild	No
Pt 2[a] (4 y 10 mo)	M	G1235C/ T2021C	No	Mild	No
Pt 3[a] (8 y 2 mo)	F	C1208A/ nt 237(insT)	Yes	Moderate	Yes
Pt 4 (2 y 2 mo)	M	G818A/ C1367T	Yes	Moderate	Yes
Pt 5 (5 y 5 mo)	M	T384C/ A1604T	No	Mild	No

F indicates female; M, male; ILBD, intralobular bile duct.
[a] Three patients (Pts 1, 2, and 3) received bezafibrate for 12 months.

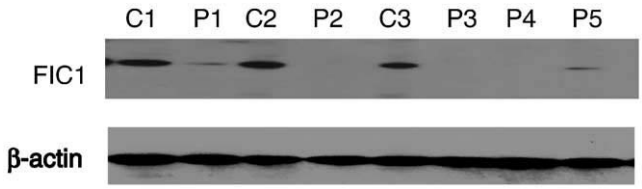

Fig. 1. Hepatic FIC1 protein levels in PFIC-1 children and control subjects. The FIC1 protein levels in the liver were determined by Western blotting, as described in the "Subjects and methods." Liver specimens were obtained from 5 PFIC1 children (Pts 1-5) and 3 healthy controls. C1, C2, and C3 indicate control 1, control 2, and control 3, respectively.

The PFIC-1 children presented with severe pruritus, short stature, low body weight, and mild jaundice. Liver histologies revealed that 3 of the 5 patients had only mild fibrosis (Table 1). They received adequate lipid-soluble vitamins to maintain blood levels within the reference ranges after diagnosis.

Bezafibrate was administered at 5 mg/kg/d (a typical dose for the treatment of dyslipidemia) to Pts 1, 2, and 3. Their liver function and lipoprotein metabolism, as well as their general clinical presentation, were monitored during 12 months of bezafibrate therapy at this dosage [12-15]. Their clinical courses were compared with those of Pts 4 and 5.

Institutional review boards approved this study. Informed consent for treatment with bezafibrate was obtained from the patients and their parents.

2.2. Liver function tests and pruritus scores

Serum levels of aspartate aminotransferase, alanine aminotransferase, total protein, and albumin were determined. As hallmarks of cholestasis, serum levels of GGT, total bile acids (TBAs), and total bilirubin (T-B) were also determined.

Pruritus severity was scored as described in a previous report [22]: 0, none; 1, mild scratching when undistracted; 2, active scratching without abrasion; 3, abrasions; or 4, cutaneous mutilation, with bleeding and scarring.

2.3. Lipids and apolipoproteins

Serum levels of TC, TG, and phospholipids were measured using enzymatic methods with commercial kits. Serum LDL cholesterol (LDL-C) levels were determined

using a homogeneous method with a commercial kit (Cholestest LDL; Daiichi Pure Chemicals, Tokyo, Japan). Serum levels of apolipoprotein (apo) A-I, apo B, and apo C-II were determined using a turbidimetric immunoassay (Apo A-I, Apo B, and Apo C-II Auto N Daiichi; Daiichi Pure Chemicals). Lipoprotein X (LpX) was determined by selective immunoprecipitation as described previously [23]. All measurements were carried out using autoanalyzers (models 7310 and 7170; Hitachi, Tokyo, Japan).

Serum levels of total HDL-C were measured as described previously [4,11]. Briefly, the serum sample was mixed with an equal volume of aqueous 13% polyethylene glycol (PEG 6000; Wako Pure Chemical Industries, Osaka, Japan); and the mixed sample was centrifuged (2000*g*, 15 minutes, room temperature). The cholesterol level was determined in the supernatant as described above.

The chemical composition of LDL was examined according to a method using ultracentrifugation and gel filtration as described previously [4,11]. The chemical compositions of the other lipoproteins (HDL and very low-density lipoproteins) could not be examined because so little was recovered from blood samples, especially before bezafibrate therapy.

2.4. Enzymes related to lipoprotein metabolism

Lecithin-cholesterol acyltransferase (LCAT) activity was determined using an exogenous substrate method with liposomes composed of cholesterol and lecithin (Anasorb LCAT, Daiichi Pure Chemicals). The cholesteryl ester transfer protein (CETP) level was measured using a sandwich enzyme immunoassay.

Activities and protein levels of lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) were determined using postheparin samples. Briefly, a citrated plasma sample was obtained 10 minutes after intravenous injection of heparin (30 IU/kg body weight). The LPL and HTGL activities were measured using nonradioisotopic determination of the amount of released free fatty acid from the substrate triolein, as described previously [24]. The LPL protein level was determined using a sandwich enzyme immunoassay with a commercial kit (LPL-ELISA kit; Dainippon Pharmaceutical, Tokyo, Japan) [25]. The HTGL protein level was determined with a sandwich enzyme immunoassay using monoclonal antibodies [24,25].

2.5. Immunoblot of MDR3 using liver samples

Liver samples were obtained from Pts 1 and 2 by percutaneous liver biopsies before and after 4 months of bezafibrate therapy. Percutaneous liver biopsy was also performed for Pts 4 and 5 before and at 6 to 7 months of this study, and the liver samples were obtained. First, 100 mg of each frozen liver sample were homogenized in 1 mL of lysis buffer containing 10 mmol/L Tris-HCl, 200 mmol/L NaCl, 1 mmol/L EDTA, 5% glycerol, 5 mmol/L 2-mercaptoethanol, 1 mmol/L MgCl2, and 0.5 mmol/L phenylmethylsulfonyl fluoride, a protease inhibitor. After centrifugation (1500*g*, 1 minute), the protein concentration in the supernatant was determined using the Bradford reagent (Sigma Chemical, St Louis, MO) The supernatant was mixed with an equal volume of loading buffer containing 125 mmol/L Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred by electroblotting to nitrocellulose membranes (Millipore, Tokyo, Japan). The membranes were blocked with 10% nonfat dried milk in Tris phosphate-buffered saline. They were then reacted with a commercial antibody for MDR3 (P3II-26; Alexis, Lausen, Switzerland). After washing the membrane, the secondary immunoglobulin G antibody conjugated with peroxidase (The Binding Site, Birmingham, UK) was added to the membranes for 1 hour. The membranes were washed, incubated in enhanced chemiluminescent reagents (Amersham, Buckinghamshire, United Kingdom), and exposed to chemiluminescent film according to the manufacturer's instructions.

3. Results

3.1. Effects on pruritus, cholestasis, and liver function

In Pts 1, 2 and 3, the pruritus scores were highest at baseline and were reduced by bezafibrate therapy. In Pts 1 and 2, the maximum reduction was observed after 4 and 6 months of bezafibrate treatment, respectively. In Pt 3, the slight reduction of the pruritus score was observed after 4 months of treatment; but further improvement was not observed by 12 months (Fig. 2A).

The baseline levels of T-B and TBAs were elevated in these patients, although their GGT levels were normal or low. In Pts 1 and 2, both the T-B and TBA levels decreased after 2 months of treatment but did not change further by 12 months.

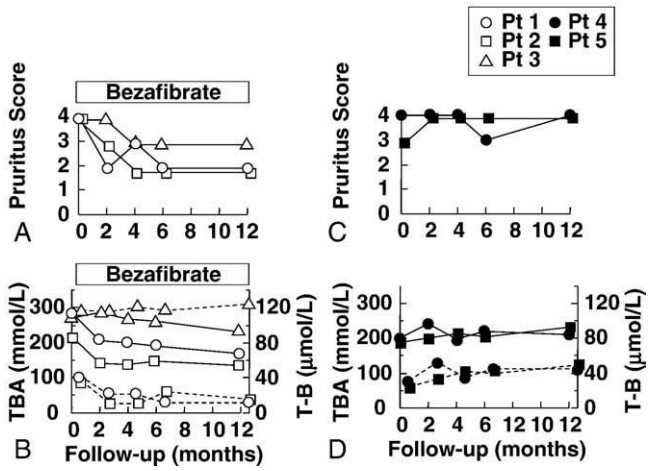

Fig. 2. Effects of bezafibrate on pruritus score, T-B, and TBAs in PFIC-1 children. The PFIC-1 children were followed for 12 months with (A and B) or without (C and D) bezafibrate treatment. TBAs, solid lines; T-B, dotted lines.

In contrast, a slight decrease was observed only in the TBA level in Pt 3 after 12 months of treatment (Fig. 2B).

Increases in the levels of total protein and albumin were also observed in Pts 1 and 2 at 2 months, and the levels increased further over time. To the contrary, no improvement in these liver function tests was seen in Pt 3. Decreases in the aspartate aminotransferase and alanine aminotransferase levels were observed in Pts 1 and 2 after 2 months of therapy; further improvements in liver function were apparent in these patients as therapy continued (data not shown).

None of the possible adverse effects caused by bezafibrate, such as rhabdomyolysis, mucocutaneous syndrome, or any exacerbation of liver dysfunction, was observed in any patient.

Patients 4 and 5, who did not receive bezafibrate therapy, showed no improvement in liver function or pruritus (Fig. 2C-D).

3.2. Effect on lipids, lipoproteins, and apolipoproteins

At baseline, all PFIC-1 children (Pts 1-5) had very low HDL-C levels, slightly high TG levels, and normal LDL-C and LpX levels (Fig. 3A-F). In the patients treated with bezafibrate (Pts 1, 2, and 3), the LDL-C and HDL-C levels increased 1.1- to 1.2-fold and 1.6- to 2.0-fold, respectively, after 2 months of therapy (Fig. 3A). Nevertheless, the posttreatment levels of HDL-C remained below the reference range. In contrast, the TG levels decreased to within the reference range in these patients (Fig. 3B). Although the baseline levels of LpX were normal, the LpX levels increased 2.2- to 4.5-fold, exceeding the upper limit of the reference range, after 12 months of bezafibrate treatment (Fig. 3C).

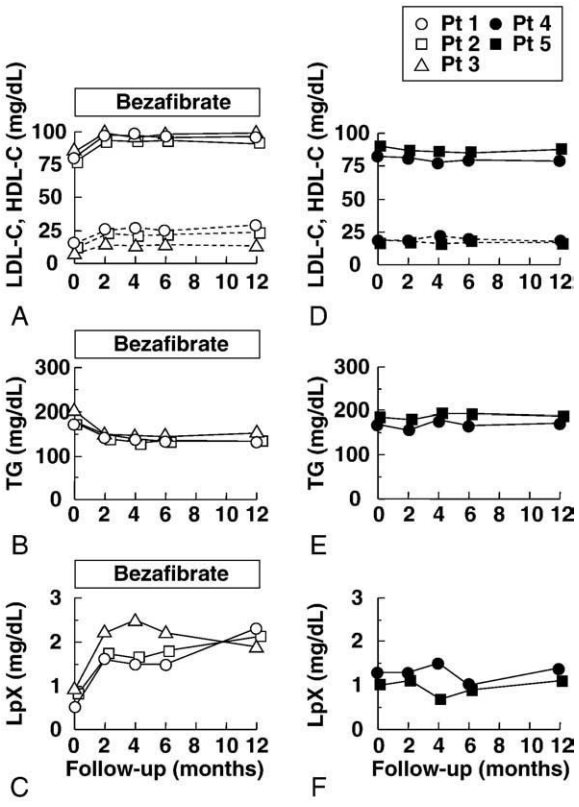

Fig. 3. Effects of bezafibrate on lipids and apolipoproteins in PFIC-1 children. The PFIC-1 children were followed for 12 months with (A, B, and C) or without (D, E, and F) bezafibrate treatment. N indicates reference ranges obtained from 60 age-matched controls consisting of 30 girls and 30 boys with ages ranging from 2 to 8 years. LDL-C, solid lines; HDL-C, dotted lines.

Table 2
Chemical composition of LDL before and after bezafibrate therapy

Patient no.	CE (%)	FC (%)	TG (%)	PL (%)	Protein (%)
Pt 1					
Baseline	22.1	6.7	20.3	26.4	24.5
6 mo	30.2	6.9	15.2	25.2	22.5
Pt 2					
Baseline	19.1	5.9	23.3	25.5	26.2
6 mo	28.9	6.5	16.3	24.4	23.9
Pt 3					
Baseline	12.5	6.2	29.5	26.9	25.9
6 mo	20.3	6.5	23	15.5	24.3
Age-matched control[a]	39.1 ± 1.8	7.2 ± 0.8	8.4 ± 1.5	22.4 ± 0.5	21.3 ± 1.3

Blood samples were obtained at baseline and 6 months after bezafibrate therapy. Data are presented as percentages of the total weight of LDL. CE indicates cholesteryl ester; FC, free cholesterol; PL, phospholipids.

[a] Age-matched control values (mean ± SD) were obtained from 20 children with ages ranging from 2 to 6 years [4].

In the patients treated with bezafibrate, the apolipoprotein levels changed similarly to the HDL-C and TG levels. As for HDL-C, the apo A-I and apo C-II levels at baseline were low in Pts 1, 2, and 3. Both levels increased 1.2- to 1.6-fold but remained below the reference ranges after 2 months of therapy. No further changes were observed thereafter. In contrast, the apo B and apo E levels at baseline were high in all patients. Like the TG levels, the levels of both apo B and apo E decreased by 15% to 30% after bezafibrate treatment and normalized in 2 of the 3 patients (data not shown).

In Pts 4 and 5, who did not receive bezafibrate, the lipid levels showed no significant change (Fig. 3D-F).

Before bezafibrate therapy, the LDL particles from Pts 1, 2, and 3 were rich in TG and proteins but poor in cholesteryl esters, consistent with our previous report [5]. Bezafibrate treatment decreased the TG and protein contents but markedly increased the cholesteryl ester content of the LDL particles (Table 2).

3.3. Effects on LCAT, CETP, LPL, and HTGL activity and protein levels

Before bezafibrate therapy, the LPL activity and protein level were low in Pts 1, 2, and 3. The LPL activity in Pt 3 was disproportionately low, relative to the LPL protein level, reflecting that patient's very low levels of apo C-II, a coenzyme of LPL. The HTGL activity was also reduced in all patients, consistent with its protein level. The LCAT activity and CETP protein level were normal in Pts 1 and 2 but were quite low in Pt 3 (Table 3).

Table 3
Lipoprotein regulators before and after bezafibrate therapy

Patient no.	LPL activity (mmol/[h mL])	LPL protein (ng/mL)	HTGL activity (mmol/[h mL])	HTGL protein (ng/mL)	LCAT activity (nmol/[h mg])	CETP protein (μg/mL)
Pt 1						
Baseline	3.08	61	1.96	189	69.5	1.2
4 mo	4.03	81	2.77	261	70.5	1.3
Pt 2						
Baseline	3.66	70	2.11	202	70.4	1.6
4 mo	4.52	96	2.87	285	71.3	1.4
Pt 3						
Baseline	2.13	56	0.71	85	50.2	0.8
4 mo	2.55	63	1.11	101	49.1	0.8
Normal	7.34-14.10	130-300	8.55-12.21	720-2015	67.3-108.2	1.1-3.5

Blood samples were obtained at baseline and 4 months after bezafibrate therapy.

Bezafibrate therapy increased both the LPL and HTGL activities and the respective protein levels, especially in Pts 1 and 2. On the other hand, the LCAT activity and CETP protein level were not changed, remaining at normal levels in Pts 1 and 2 and at a low level in Pt 3 (Table 3).

3.4. Immunoblot of MDR3 using liver samples

Immunoblot analysis revealed that bezafibrate apparently increased the liver MDR3 expressions in Pts 1 and 2, compared with the baseline level, after 4 months of treatment (Fig. 4). On the other hand, the liver MDR3 expressions in Pts 4 and 5, who did not receive this treatment, were not changed.

4. Discussion

The lipid and lipoprotein profiles changed considerably in all 3 PFIC patients after the initiation of bezafibrate. In 2 of the 3 patients, bezafibrate improved pruritus, cholestasis, and liver dysfunction. High TG levels and low TC levels, in addition to low LDL-C, returned to normal after bezafibrate treatment. The serum levels of HDL-C also increased but remained low.

The improvement of liver dysfunction, along with cholestasis, in our 2 patients may be attributable to the enhancement of MDR3 expression by bezafibrate. The results of recent studies indicate that bezafibrate enhances the expression of MDR3 (ABCB4), a canalicular phospholipid transporter in hepatocytes, via the nuclear receptor PPARα [16,17,20,21]. Phospholipids attenuate the cytotoxicity of hydrophobic bile acids, thereby engendering the development and progression of hepatocholangiopathy [17-19].

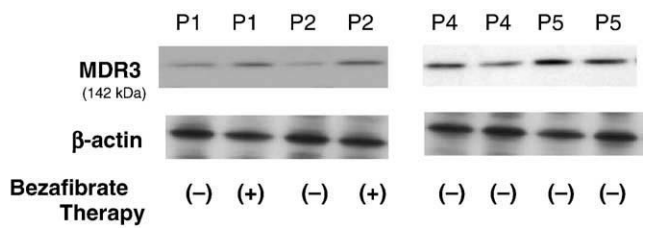

Fig. 4. Hepatic MDR3 levels before and after bezafibrate therapy. In 3 PFIC-1 children (Pts 1, 2, and 3), liver biopsy specimens were obtained immediately before and 2 months after the initiation of bezafibrate therapy. Western blot analysis was carried out as described in "Subjects and methods."

Recently, Shoda et al [17] reported that bezafibrate enhanced the expression of 2 other major canalicular transporters: MRP2 (ABCC2), a transporter of multispecific organic anions involving bilirubin, and BSEP (ABCB11), a transporter of bile acids. Increases in the expression of these transporters may have contributed to the improvement in cholestasis and liver dysfunction in our 2 patients, but we did not directly examine this.

Mutations in the *ATP8B1* gene, encoding FIC1, are responsible for PFIC-1; but the exact role of this protein remains unclear [1,3]. The results of the present study suggest that this protein is associated with many proteins involved in bile acid and bilirubin transport systems, the expressions of which might be stimulated by bezafibrate.

Changes in the lipid and lipoprotein profiles of our patients should be interpreted as a direct effect of bezafibrate on lipoprotein metabolism because such changes were apparent even in Pt 3, whose severe cholestasis was not improved by the treatment.

The enzymes involved in lipoprotein metabolism have not been previously examined in PFIC patients. The results of this study demonstrate low LPL and HTGL activities in PFIC-1 patients and show that both activities were increased after the initiation of bezafibrate therapy. Lipoprotein lipase is an important enzyme for the hydrolysis of TGs in TG-rich lipoproteins, especially chylomicrons and very low-density lipoprotein. Patients with LPL deficiency typically present with prominent hypertriglyceridemia, together with low HDL-C levels [26,27]. Hepatic triglyceride lipase also hydrolyzes TGs in lipoproteins, especially intermediate-density lipoprotein; and HTGL deficiency leads to an accumulation of TG-rich LDL in plasma [28-31]. Based on a comparison of lipid and lipoprotein profiles before and after bezafibrate therapy, it seems likely that the increases in LPL and HTGL activities by bezafibrate therapy contributed

greatly to the increased levels of HDL-C, LDL-C, and TC and the decreased levels of TG in our patients.

The ability of bezafibrate to raise LPL and HTGL activities has been shown in earlier studies [12-15]. The present study confirmed that bezafibrate increased both lipase activities, even in PFIC-1 children. Unlike LPL and HTGL, the HDL regulators CETP and LCAT remained unchanged throughout the course of treatment [6,32-34].

The LDL in our patients was TG-enriched LDL, which displays poor affinity for the LDL receptor; therefore, the LDL in our patients was likely to accumulate in plasma [35,36]. Their high apo B levels before bezafibrate therapy probably reflect such accumulation of LDL because LDL has only 1 apo B per molecule. A decrease in the apo B levels, with a reciprocal increase in the LDL-C levels, after the initiation of this therapy may be explained by an increased affinity of the LDL for the LDL receptor resulting from the increased cholesterol ester content of the LDL at the expense of TG.

After the initiation of bezafibrate therapy, LpX, which was at normal levels before treatment, rose to levels greater than the reference range in all patients. It has been shown that bile acid regurgitation resulting from bile duct damage, together with a decrease in LCAT activity due to liver cell damage, is the main contributor to the formation of LpX [5,7-10,33,34,37,38]. Furthermore, Elferink and colleagues [39] demonstrated that Mdr2 (Abcb4) knockout mice failed to form LpX even during cholestasis; they suggested that the expression of MDR3 regulated the formation of LpX. An increase in MDR3 expression without a change in the LCAT activity was observed in our patients after bezafibrate therapy. In this context, we suggest that increased MDR3 expression by bezafibrate accounted for the increase in the LpX level in the PFIC-1 patients.

This study demonstrated beneficial effects of bezafibrate on cholestasis and dyslipidemia in patients with PFIC-1. The results lead us to consider the possibility that bezafibrate may be a useful therapeutic option for such patients.

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