Stimulation of fecal fat excretion and the disposal of protoporphyrin in a murine model for erythropoietic protoporphyria


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ERYTHROPOIETIC PROTOPORPHYRIA (EPP) is characterized by genetically decreased activity of ferrochelatase (Fch) (2). Fch is the enzyme that catalyzes the final reaction in the heme biosynthetic pathway: the insertion of iron into protoporphrin (PP) to form heme. Reduced Fch activity in humans results in accumulation of photoactive PP in erythrocytes, blood, and the liver (2, 6, 33). The symptomatology of EPP involves immediate and extreme photosensitivity. Exposure to light with a wavelength between 400 and 410 nm leads to generation of reactive oxygen radicals from photoreactive PP in erythrocytes, blood, and the liver (2, 6, 33). Photosensitivity usually appears during infancy or childhood. In a subset of patients with EPP, liver disease may occur, which might develop into progressive liver failure and is probably related to hepatic accumulation of PP (23, 27).

PP is a hydrophobic compound that, under physiological conditions, is disposed from the body via secretion in bile and subsequently excretion via the feces (33). Apparently, this pathway has insufficient capacity to accommodate the increased production of PP in Fch-deficient conditions. Treatment options for EPP are disappointingly limited. To interrupt the enterohepatic circulation of PP, the administration of cholestyramine is suggested. However, it is only used in severe liver damage (14, 21). Other treatments are β-carotene (19), parenteral heme administration (either as such or in the form of blood transfusion) (7, 8, 31, 34, 37), cysteine (20), ursodeoxycholic acid (1), or chenodeoxycholic acid (32, 36). However, these treatments have been investigated only in a limited number of patients, and the outcomes have been inconsistent.

A mouse model for EPP became available in 1991. Fch-deficient (fch/fch) mice have a point mutation in the gene encoding for Fch (35). Similar to human EPP, the phenotype of these mice is characterized by high concentrations of PP in blood and the liver. Fch/fch mice have photosensitivity and develop progressive liver failure. Fch/fch mice have proven to be a valuable model for human EPP (5). This animal model allows us to evaluate the consequences of EPP-related liver disease and possible novel treatment options (5, 24).

We hypothesized that the hydrophobic character of PP offers the possibility to enhance its disposal from the body by stimulation of fecal fat excretion. According to our hypothesis, hydrophobic compounds associate with nonabsorbed fats in the intestinal lumen and are subsequently disposed via the feces. The hydrophobic nature of PP may suggest that it would readily partition in an oil phase. McDonagh et al. (22) demonstrated in an in vitro partitioning model showing that partitioning of PP between an oil and aqueous phase is pH dependent. We performed similar in vitro experiments, based on PP quantitation by optical density rather than on visual interpretation, as in the short report of McDonagh et al. Our quantitative data showed that at pH 7.4, 68% of PP was partitioned in the intestinal oil phase and, at pH 7.8, 84% of PP was recovered from the aqueous buffer, implying a pH-dependent partitioning between the dietary oil phase and the aqueous buffer. Our data implied that a small change in pH had a major effect on the PP partitioning between the oil and aqueous phase. Since the pH in the intestinal lumen is not well known, we hypothesized that generation of an unabsorbed intestinal dietary oil phase would be able to enhance the disposal of PP.

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To a certain extent, PP has similar physicochemical characteristics as unconjugated bilirubin. The validity of the hypothesis for treatment of the accumulation of hydrophobic compounds was recently demonstrated for unconjugated bilirubin. Gunn rats have a genetic deficiency of the enzyme UDP-glucuronosyltransferase, leading to an accumulation of unconjugated bilirubin. In hyperbilirubinemic Gunn rats, fecal fat excretion was stimulated by orlistat treatment (13, 29). Orlistat treatment increased fecal fat and fecal bilirubin excretion and decreased plasma bilirubin concentrations in Gunn rats (12, 13, 29). Sucrose polyester is a noncaloric fat, since it is neither digested nor absorbed. Rather, sucrose polyester is recovered unchanged in the stools (3, 11, 15). Sucrose polyesters are a mixture of sucrose molecules esterified with six to eight long-chain fatty acids. The composition of these molecules creates a steric hindrance to endogenous lipases (gastric and pancreatic), which is responsible for their “nonabsorbable fat” character.Sucrose polyester did not increase fat excretion beyond that of the excreted sucrose polyester: the absorption of triacylglycerides did not decrease in the presence of sucrose polyester.

Recently, we (25) demonstrated that sucrose polyesters strongly induce fecal excretion of fat, in the form of the ingested sucrose polyester, and of the hydrophobic brominated polyesters did not decrease in the presence of sucrose polyester.

MATERIALS AND METHODS

Mice. Fch/fch mice (Balb/c background) were generously supplied by X. Montagutelli (Institut Pasteur, Paris, France) for establishing a breeding program at the Central Animal Laboratory, Faculty of Medical Science (University of Groningen, Groningen, The Netherlands). Mice were individually housed in a temperature- and light-controlled environment and were protected from direct light and its phototoxicity by means of a yellow filter. Mice were decently cared for according to local guidelines. The local Ethics Committee for Animal Experiments approved the study and the involved experimental procedures. Diet and water were supplied ad libitum.

Diets. All diets were produced by Hope Farms (Woerden, The Netherlands). Sucrose polyester was a generous gift from Dr. J. Westrate (Unilever, Vlaardingen, The Netherlands). The semisynthetic, purified high-fat diet (code 4141.07) contained 35 energy % fat and 16.2 wt % long-chain fatty acids in the form of triacylglycerol. The fatty acid composition was determined by gas chromatography (in mol %): C8-C12, 4.4; C16:0, 28.5; C18:0 3.9; C18:1n-9, 33.2; C18:2n-6, 29.3; and C18:3n-3, 0.2). It was used as a control diet except in the experiments in which it was compared with the semisynthetic low-fat diet (code 4063.02). The low-fat diet was a semisynthetic, purified diet containing 13 energy % fat and 5.2 wt % long-chain fatty acids [fatty acid composition (in mol %): C8-C12, 6.9; C14-0, 0.7; C16:0, 30.0; C18:0, 3.7; C18:1n-9, 29.9; and C18:2, 28.8). The composition of the experimental orlistat diets was identical to control diets except for supplementation with orlistat (200 or 800 mg/kg diet; code 4141.13). For the sucrose polyester diet (also custom synthesized), 25% of the fat content of the high-fat diet (i.e., 4 wt %) was replaced by sucrose polyesters with predominantly unsaturated acyl chains resulting in a 16 wt % fat diet, of which 12 wt % was absorbable fat. No fat-soluble vitamins were supplemented except for those supplied in the diets.

**Dietary treatment groups.** The Chow fed to the mice was standard laboratory chow containing 6 wt% fat and 14 energy % fat. After the collection of baseline blood samples, adult *fch/fch* mice were randomly assigned to a semisynthetic high-fat or low-fat diet group for 8 wk. Other groups of adult *fch/fch* mice were fed the high-fat diet for 4 wk, after which they were randomly assigned for another 4 wk to continue with the same high-fat diet, to the high-fat diet supplemented with the nonabsorbable fat sucrose polyester, or to the high-fat diet supplemented with orlistat (200 or 800 mg/kg diet).

**Experimental procedures.** In each of the experimental designs described above, blood samples (75 µl) were collected by tail bleeding under isoflurane anaesthesia for measuring PP concentrations in red blood cells (RBCs) every 2 wk. Blood samples were immediately transferred into EDTA-containing tubes for separation of RBCs and plasma. At regular intervals, fecal samples were collected for 3–120 h and stored at −20°C for fat and porphyrin determinations. Body weights were assessed every 2 wk.

At the end of the experimental period, mice were anesthetized with isoflurane and an intraperitoneal injection with Hypnorm (1 ml/kg fentanyl/fluanisone) and diazepam (10 mg/kg). Under light-protected conditions, their gallbladders were cannulated as described previously (38), and bile was collected for 30 min. Bile flow was determined gravimetrically, assuming a density of 1 g/ml. Large blood samples (0.8–1.0 ml) were collected by cardiac puncture in EDTA-containing tubes for separation of erythrocytes and plasma by centrifugation at 4,000 rpm for 10 min. Erythrocytes were washed with PBS and stored for porphyrin analysis at −20°C until analysis. Livers were excised and weighed; samples for porphyrin analysis were stored until measurements at −20°C.

**Statistical analyses.** All values are expressed as means ± SD. Differences between the treatments were analyzed with Student’s t-test (two treatments) or with one-way ANOVA with post hoc comparison by Bonferroni. The level of significance was set at *P* values of <0.05.

**RESULTS**

**Effects of high- or low-fat diet on fecal fat excretion and PP concentrations in RBCs and the liver.** First, we determined the fecal fat excretion of *fch/fch* mice that were fed a semisynthetic low-fat or high-fat diet. In a previous study (13), we demonstrated in Gunn rats that fecal excretion of fat depends strongly on dietary fat content. In *fch/fch* mice, the high-fat diet significantly increased fecal fat excretion compared with the low-fat diet (Fig. 1A). The high-fat diet tended to decrease PP concentrations in RBCs, from 27.4 ± 12.2 µmol/l prior to the high-fat diet to 13.3 ± 4.8 µmol/l after 8 wk of the high-fat diet, but statistical significance was not reached (*P* = 0.1; Fig. 1B). The PP concentration in RBCs on the low-fat diet varied considerably, without showing a significant alteration over the 8 wk of treatment and without a significant difference compared with *fch/fch* mice fed the high-fat diet. Liver function was severely affected by PP accumulation in *fch/fch* mice, but hepatic PP concentrations were similar.
after fch/fch mice were fed the high-fat or low-fat diet for 8 wk (Fig. 1C). Fecal PP excretion initially decreased in both groups after the start of the experimental (semisynthetic) diets (Fig. 1D). After treatment with high-fat and low-fat diet, the effect appeared transient since fecal PP excretion returned to pretreatment levels after 6 wk.

Effects of sucrose polyester diet on fecal fat excretion and PP concentrations in RBCs and the liver. Dietary administration of nonabsorbable fat, sucrose polyester, significantly increased fecal fat excretion compared with the high-fat diet (Fig. 2A). After 2 wk of experimental diet, RBC PP concentrations were significantly higher in fch/fch mice fed the diet supplemented with sucrose polyesters (P < 0.05). At 4 wk, however, RBC PP concentrations were similar in fch/fch mice fed the control high-fat diet (7.2 ± 0.6 μmol/l, not significant; Fig. 2B). Hepatic PP concentrations did not differ between mice treated with the high-fat diet and with the high-fat diet supplemented with sucrose polyesters (Fig. 2C). Dietary administration of sucrose polyesters significantly decreased fecal PP excretion in fch/fch mice compared with the control diet (Fig. 2D). At the end of the experiment, fecal PP excretion increased again.

Effects of different concentrations of orlistat on fecal fat excretion and PP concentrations in RBCs and the liver. Orlistat treatment significantly increased fecal fat excretion in a dose-dependent fashion (Fig. 3A). During the runin period of 4 wk, RBC PP concentrations decreased from 21.3 ± 5.0 to 10.3 ± 2.2 μmol/l (data not shown). Feeding fch/fch mice with one of the two orlistat diets did not further affect RBC PP concentrations. At the end of the 8-wk treatment period, RBC PP concentrations in the high-fat diet-treated mice were virtually identical to those in mice treated with orlistat (Fig. 3B). After 8 wk of treatment, hepatic PP concentrations were not significantly different among the three groups (Fig. 3C). In fch/fch mice fed the 200 mg/kg orlistat diet, fecal PP excretion transiently increased after 2 wk of treatment. After 2 and 4 wk of treatment with the 800 mg/kg orlistat diet, fecal PP excretion profoundly decreased (Fig. 3D).

Effects of high-fat, low-fat, olestra, and orlistat diet on fecal PP and fecal fat excretion. Dietary administration of sucrose polyester and different concentrations of orlistat to the high-fat diet significantly increased fecal fat excretion compared with the control high-fat diet. In previous studies, we demonstrated a positive correlation between fecal fat and fecal BDE-47 excretion in Wistar rats fed with a sucrose polyester diet (25) and an increase in fecal fat and fecal bilirubin excretion in Gunn rats fed with orlistat at different concentrations (12, 13, 29). Fecal PP excretion did not correlate positively with fecal fat excretion during high-fat or low-fat diet (r = −0.224, P > 0.05; Fig. 4). After treatment for 4 wk with the control orlistat diet, the effect appeared transient since fecal PP excretion returned to pretreatment levels after 6 wk.
sucrose polyester diet, fecal fat excretion was negatively correlated with fecal PP excretion ($r = -0.77, P < 0.01$). There was no significant linear relation ($r = 0.24, P = 0.52$) between fecal fat and fecal PP excretion in fch/fch mice treated with the 200 mg/kg orlistat diet and no positive correlation between fecal fat and fecal PP excretion at the end of treatment with the 800 mg/kg orlistat diet ($r = -0.87, P < 0.001$).

**DISCUSSION**

We tested the hypothesis that stimulation of fecal fat excretion by a high-fat diet, by nonabsorbable sucrose polyesters, or by orlistat treatment enhances the disposal and decreases the accumulation of PP in fch/fch mice, a well-established model for EPP. However, the results clearly demonstrate that increasing fecal fat excretion did not increase the rate of fecal excretion or decrease the hepatic accumulation of PP in fch/fch mice. The present results are in contrast with earlier studies (13, 25) in which stimulation of fecal fat excretion enhanced the disposal of other hydrophobic (endogenous or exogenous) compounds.

Feeding fch/fch mice with the high-fat diet increased fecal fat excretion. The high-fat diet decreased PP concentrations in RBCs. At the end of the 8-wk experimental period, however, hepatic PP concentrations in mice on the high-fat diet were very similar to those on the low-fat diet. Apparently, PP concentrations in RBCs do not closely correlate with hepatic PP accumulation. Theoretically, the extent of hepatic PP accumulation could be extremely overwhelming, and thus a possible limited therapeutic effect of the high-fat diet will not be discernable as for the imposed treatments. However, fecal PP excretion was not increased in the high-fat group and did not correlate with fecal fat excretion.

We (13, 25) previously demonstrated that the stimulation of fecal fat excretion by the high-fat diet in rats is relatively mild compared with treatment with sucrose polyesters or orlistat. Meijer et al. (25) showed that sucrose polyesters strongly enhanced fecal excretion of fat and of the hydrophobic brominated organohalogen BDE-47 in Wistar rats. We reasoned that the presence of sucrose polyesters would guarantee the permanent presence of a lipophilic phase in the intestinal lumen, which could hypothetically allow for the association with hydrophobic compounds like PP. Sucrose polyesters have been demonstrated to enhance the disposal of various persistent organic pollutants in humans (10, 28). As expected, fecal fat excretion was profoundly higher in sucrose polyester-treated fch/fch mice compared with high-fat diet-treated fch/fch mice. Nevertheless, sucrose polyester treatment did not enhance fecal PP excretion and did not affect PP concentrations in RBCs and hepatic PP concentrations.

**Fig. 2.** Effects of the control high-fat diet and control high-fat diet supplemented with sucrose polyester on fecal fat excretion (A), RBC PP concentration (B), hepatic PP concentration (C), and fecal PP excretion (D) in fch/fch mice fed a control high-fat diet for 4 wk followed by another 4 wk with the same diet or followed by the control high-fat diet supplemented with sucrose polyester for 4 wk. Data are means ± SD; n = 5 mice/group. *P < 0.05; **P < 0.01. Control high-fat diet supplemented with sucrose polyesters significantly increased the excretion of fat (sucrose polyester) in the feces compared with treatment with the control high-fat diet. Assuming a mean molecular weight of fecal fatty acid of 280, fecal fat loss in the sucrose polyester group amounted to ~80 mg/day (not taking into account the contribution of sucrose). After 8 wk of treatment, erythrocyte PP concentrations were lower in fch/fch mice fed the control high-fat diet compared with fch/fch mice fed the control high-fat diet supplemented with sucrose polyester. Hepatic concentrations of PP in fch/fch mice 4 wk after being switched to the diet showed no significant difference between the 2 groups. Fecal PP concentrations in fch/fch mice decreased significantly after dietary administration of sucrose polyesters to the control high-fat diet compared with feeding of the control high-fat diet alone.

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the liver compared with high-fat controls, and, finally, fecal PP excretion was not correlated positively with fecal fat excretion during sucrose polyester treatment of *fch/fch* mice. It is unlikely that the amount of fecal fat was (still) too low for enhancing the disposal of hydrophobic compounds. This reasoning was supported by the orlistat results. Orlistat treatment effectively increased fecal fat excretion during the high-fat diet, even more profoundly than sucrose polyester treatment. However, orlistat did not enhance fecal PP excretion in *fch/fch* mice. Again, fecal PP excretion was not related positively to fecal fat excretion in orlistat-treated *fch/fch* mice. These observations demonstrate unequivocally that orlistat does increase fecal fat excretion, as demonstrated previously, but that orlistat does not enhance fecal excretion of PP.

Feeding *fch/fch* mice with the orlistat-containing high-fat diet or the sucrose polyester diet did not have a decreasing effect on RBC PP concentration. Our present results are in accordance with those from Meerman et al. (24), who showed that PP concentrations in RBCs of *fch/fch* mice on a regular (low-fat) chow diet were approximately three times higher than presently observed after 8-wk treatment with a high-fat diet (with or without sucrose polyesters or orlistat). The physiological importance of this decrease, however, may not be relevant.
since hepatic PP levels did not alter due to treatment with a high-fat diet, sucrose polyester diet, or orlistat diet. Treatment with the 800 mg/kg orlistat diet even decreased fecal PP excretion in *fchl/fch* mice. In humans, only a few therapeutic studies have been performed in patients with EPP. From these studies, it has become apparent that the PP concentration in the feces of EPP patients varies considerably (between 213 and 3,378 nmol/g feces) (16, 26, 40). Similarly, reported RBC PP concentrations vary considerably in these studies, among 30 μmol/l (26), 0.3 and 1.1 μmol/l (30), 0.4 μmol/l (16), and 2.1 and 2.9 μmol/l (40). In the present study, fecal and RBC PP concentrations were in the range of 358 nmol/g and between 0.3 and 30 μmol/l, respectively, indicating much higher PP concentrations in *fchl/fch* mice compared with RBCs and fecal PP concentrations in humans.

Clearly, our data do not support our hypothesis that stimulation of fecal fat excretion will enhance fecal PP excretion. At present, we can only speculate about the apparent discrepancy between the in vitro data on PP affinity for dietary lipids (22), the positive effects of enhanced fecal fat excretion on the disposal of accumulated bilirubin (12, 13, 29), and of the hydrophobic brominated organohalogen BDE-47 in rats (25) and the present negative results on PP disposal in *fchl/fch* mice. One explanation may involve the pH in the colonic lumen. The physiological nature of the lipophilic phase of unabsorbed dietary fat in the intestinal lumen is rather unexplored, and we cannot exclude that the pH is too high for an efficient interaction between PP and (unabsorbed) fat. Another possible explanation for the present negative results may be related to the *fchl/fch* mouse model itself. As shown by Meerman et al. (24), *fchl/fch* mice have profound liver disease, related most likely to PP accumulation. The phenotype of *fchl/fch* mice may be too severe to observe positive effects of enhanced fecal fat excretion. We cannot exclude that results would be different in a much milder animal model for EPP, like the exon 10-deleted heterozygous mice (18).

In conclusion, treatment of *fchl/fch* mice with a high-fat diet, a nonabsorbable dietary fat, or with the lipase inhibitor orlistat increases fecal excretion of fat but did not increase fecal excretion of PP or decrease the hepatic concentration of PP. The present data indicate that, in contrast to unconjugated hyperbilirubinemia, accumulation of PP is not amenable to stimulation of fecal fat excretion.

REFERENCES


