Induction of peroxisomal β -oxidation by a microbial catabolite of cholic acid in rat liver and cultured rat hepatocytes

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The capability of (4*R*)-4-(2,3,4,6,6*a* β ,7,8,9,9*a* α ,9*b* β -decahydro-6*a* β -methyl-3-oxo-1*H*-cyclopenta[*f*]quinolin-7*\beta*-yl)valeric acid (DCQVA), a catabolite of cholic acid produced by enterobacteria, to induce peroxisome proliferation *in vivo* and *in vitro* was studied. Rats given 0.3 % DCQVA in the diet for 2 weeks showed marked increases in peroxisomal β -oxidation, mitochondrial 2,4-dienoyl-CoA reductase and microsomal laurate ω -oxidation activities in the liver compared with control rats given the diet

without DCQVA. Cultured rat hepatocytes treated with DCQVA for 72 h also exhibited greatly enhanced β -oxidation activity. The increased activity was concentration-dependent and the effective concentrations were comparable with those of clofibric acid that produced the same degree of induction in the assay. The results demonstrate that DCQVA is a potent peroxisome proliferator that occurs naturally in rat intestine.

INTRODUCTION

A number of structurally unrelated compounds have been shown to induce hepatic peroxisome proliferation in rodents [1,2]. Several hypolipidaemic agents such as clofibrate, certain phthalate ester plasticizers and fatty acid analogues that are metabolically inert are known to be potent proliferators [1,2]. Similar effects are evoked by high-fat diets [3,4] or diabetes [5], which cause intracellular accumulation of fatty acids. Induction of peroxisome proliferation is associated with the induction of several fatty acid-metabolizing enzymes, notably those involved in peroxisomal β -oxidation and in microsomal ω -oxidation [6], as well as mitochondrial 2,4-dienoyl-CoA reductase (EC 1.3.1.34) [7–10], which plays an essential role in the β -oxidation of unsaturated fatty acids [11]. Thus it has been hypothesized that a physiological role of peroxisome proliferation is in regulating fatty acid homeostasis and that the intracellular accumulation of fatty acids is the stimulus for triggering peroxisome proliferation [1].

Peroxisomes also play an important role in the formation of bile acids, such as cholic acid and chenodeoxycholic acid, from cholesterol [12]. In addition, peroxisomes, as well as microsomes, are capable of synthesizing cholesterol from acetyl-CoA [13,14]. It may therefore be expected that cholesterol, bile acids or their derivatives might cause peroxisome proliferation and might thereby contribute to the regulation of cholesterol/bile acid homeostasis. However, whether any of these compounds induces peroxisome proliferation is not known. Bile acids produced in the liver are conjugated with glycine or taurine and excreted into the bile [15]. In the intestine, a major fraction of the bile acids is taken up by the enterocytes and returned to the liver via the enterohepatic circulation, while a portion of unabsorbed bile acids undergoes direct degradation by the intestinal flora [16]. Degradation of the ring structure of cholic acid yields δ -oxohexahydroindane carboxylic acid derivatives, compounds still containing intact C and D rings, which can then be further converted to nitrogen-containing metabolites, probably via formation of the corresponding δ -oxoamides and subsequent cyclization to the enaminolactams [16,17]. (4*R*)-4-

(2,3,4,6,6*a* β ,7,8,9,9*a* α ,9*b* β -Decahydro-6*a* β -methyl-3-oxo-1*H*-cyclopenta[*f*]quinolin-7*\beta*-yl)valeric acid (DCQVA) is one of these nitrogenous metabolites produced by *Corynebacterium equi* (Figure 1) [18]. Since preliminary experiments with a number of microbial metabolites of cholic acid showed that DCQVA produced hepatomegaly in rats, we have focused on DCQVA in this study to determine whether it is a peroxisome proliferator.

MATERIALS AND METHODS

Materials

DCQVA was a gift from Professor Shohei Hayakawa (Hiroshima University, Hiroshima, Japan), and had been prepared as described previously [18]. The methyl ester of DCQVA was analysed by gas chromatography/mass spectrometry and found to be pure. Sorbic acid and crotonic anhydride were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan); clofibric acid, NADPH, NAD and CoA from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and [1-¹⁴C]lauric acid from Amersham International p.l.c. (Amersham, Bucks., U.K.). Crotonyl-CoA was synthesized from crotonic anhydride [19]. All other biochemicals and chemicals used were reagent grade.

Animals and treatment

Male F344 rats (5 weeks old; 105–120 g body weight) were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and fed either a powdered CRF-1 diet containing 6 % fat, 23 % protein and 53 % carbohydrate (Oriental Yeast Inc., Tokyo, Japan) or this diet containing 0.3 % (w/w) DCQVA for 2 weeks.

Subcellular fractionation of the liver

Immediately after the rats were killed, the livers were excised. A part of the liver was taken and frozen at -70°C until enzyme activities in whole-liver homogenate (Table 1) were determined. The other part of the liver was homogenized in a glass-Teflon homogenizer with cold 0.25 M sucrose containing 10 mM Tris/HCl (pH 7.4) and 1 mM EDTA, and fractionated into nuclear, heavy mitochondrial, light mitochondrial, microsomal and cyto-

Abbreviations used: DCQVA, (4*R*)-4-(2,3,4,6,6*a* β ,7,8,9,9*a* α ,9*b* β -decahydro-6*a* β -methyl-3-oxo-1*H*-cyclopenta[*f*]quinolin-7*\beta*-yl)valeric acid; THCA, trihydroxycholestanoic acid.

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Figure 1 Chemical structure of DCQVA

solic fractions by the method of de Duve et al. [20]. Each fraction was redispersed and homogenized again in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.1 % (w/v) Triton X-100 prior to enzyme assay. For the assay of laurate ω -oxidation, microsomes were suspended in 0.25 M sucrose. The frozen liver was homogenized in 9 vol. of 0.05 M phosphate buffer (pH 7.4) containing 0.1 % (w/v) Triton X-100 prior to the assay, and the supernatant after centrifugation at 600 *g* for 10 min was used.

Enzyme assays

The activities of 2,4-dienoyl-CoA reductase, crotonase [trans-2-enoyl-CoA hydratase (EC 4.2.1.17), determined using crotonyl-CoA as substrate], the subcellular marker enzymes glutamate dehydrogenase (EC 1.4.1.2; mitochondrial matrix), catalase (EC 1.11.1.6; peroxisomal matrix), urate oxidase (EC 1.7.3.3; peroxisomal core) and NADPH:cytochrome *c* reductase (EC 1.6.2.4; endoplasmic reticulum), and peroxisomal β -oxidation (CN-insensitive palmitoyl-CoA oxidation) were determined spectrophotometrically as described previously [21]. Heat-stable (mitochondrial) crotonase activity was determined in the homogenates after treatment at 57 °C for 5 min [22]; heat-stable activity was subtracted from whole crotonase activity in the intact homogenates to obtain heat-labile (peroxisomal) crotonase activity [22]. Laurate hydroxylation (total of ω - and ω -1-hydroxylation) was assayed with [1-¹⁴C]lauric acid as reported by Ortiz de Montellano and Reich [23]. Protein was determined by the method of Lowry et al. [24].

Hepatocytes and culture

Hepatocytes were prepared from rat liver by a previously described collagenase perfusion method [25]. The cells were plated on 60-mm collagen-coated dishes (Iwaki Glass Co., Tokyo, Japan) at a density of 2.4×10^6 cells in 4 ml of L-15 medium containing 5 % fetal-calf serum, 300 μ M ethanolamine and 100 μ g/ml kanamycin. After 4 h the medium was exchanged with fresh medium containing either 0.25 % dimethyl sulphoxide or DCQVA in 0.25 % dimethyl sulphoxide. The medium was renewed every 24 h. After 72 h, hepatocytes were washed once with cold PBS, scraped with a rubber policeman into 1.5 ml of PBS and centrifuged at 200 *g* for 5 min. The cell pellet was suspended in 0.2 ml of 0.05 M potassium phosphate (pH 7.4) containing 0.1 % Triton X-100 and centrifuged at 15000 *g* for 5 min. Enzyme activities were determined in the supernatant.

RESULTS

Administration of DCQVA in the diet for 2 weeks caused remarkable increases in CN-insensitive palmitoyl-CoA oxidation (19-fold) and heat-labile crotonase (46-fold) activities in liver

Table 1 Effect of DCQVA on the activities of some liver enzymes

Rats were fed on a diet containing 0.3 % DCQVA for 2 weeks. The values represent means $\pm$ S.D. of five rats. The specific activity was measured in homogenates (nmol/min per mg of wet liver)^a and microsomes (nmol/min per mg of protein)^b, as indicated below. Statistically significant effects were assayed using unpaired Student's *t* test and are indicated at the *P* < 0.001 level (*)

	Activity	
	Control rats	DCQVA-treated rats
Crotonase ^a		
Heat-stable	1400 $\pm$ 120	1450 $\pm$ 90
Heat-labile	216 $\pm$ 50	9870 $\pm$ 580*
Dienoyl-CoA reductase ^a	2.80 $\pm$ 0.42	15.23 $\pm$ 0.82*
Glutamate dehydrogenase ^a	2.51 $\pm$ 0.28	2.51 $\pm$ 0.31
Peroxisomal β -oxidation ^a	1.27 $\pm$ 0.18	24.37 $\pm$ 1.98*
Urate oxidase ^a	0.406 $\pm$ 0.090	0.388 $\pm$ 0.042
Laurate hydroxylation ^b	1.88 $\pm$ 0.19	22.0 $\pm$ 0.6*

homogenates (Table 1). In contrast, the activities of heat-stable crotonase and urate oxidase were unchanged. The activity of 2,4-dienoyl-CoA reductase in the liver homogenate increased by 5.4-fold. Laurate hydroxylation in the microsomal fraction also increased markedly (12-fold). These changes were accompanied by an increase (65 %) in liver weight (12.6 ± 0.5 g in treated rats compared with 7.62 ± 0.84 g in control rats; *n* = 5).

Subcellular fractionation of the liver homogenate showed a marked increase in peroxisomal β -oxidation caused by DCQVA treatment (Figure 2a). Heat-stable crotonase was localized in the M (heavy mitochondrial) fraction and heat-labile crotonase in the L (light mitochondrial) fraction; a pronounced induction of heat-labile crotonase only was observed (Figures 2c and 2d). 2,4-Dienoyl-CoA reductase was distributed almost totally in the N (nuclear), M and L fractions (Figure 2b). A comparison of this distribution pattern with those of marker enzymes suggests that 2,4-dienoyl-CoA reductase was localized mainly in mitochondria but was also present in peroxisomes. DCQVA treatment caused increases in the activity of this enzyme in both the M and L fractions. No appreciable changes were seen in the patterns for glutamate dehydrogenase (Figure 2f) or NADPH:cytochrome *c* reductase (Figure 2g).

The induction of peroxisomal β -oxidation by DCQVA was also observed in cultured rat hepatocytes (Figure 3). DCQVA induced peroxisomal β -oxidation in a concentration-dependent manner over the same concentration range as clofibrate, a commonly used peroxisome proliferator, and was significantly more potent at ≤ 100 μ M.

DISCUSSION

In the present study DCQVA, a catabolite of cholic acid produced by enterobacteria, when administered in the diet to rats was shown to induce peroxisomal β -oxidation, heat-labile (peroxisomal) crotonase, microsomal ω -oxidation and mitochondrial 2,4-dienoyl-CoA reductase activities in rat liver. In contrast the activity of urate oxidase, a known peroxisomal enzyme, was unchanged. Thus DCQVA apparently has characteristic features shared by various peroxisome proliferators [1,6–10]. This is a first observation of a naturally occurring metabolite of cholic acid that is a peroxisome proliferator.

The possibility exists that induction of hepatic peroxisome-associated enzymes by the administration of DCQVA results from an indirect effect of DCQVA on the liver cells. That is,

Figure 2 Subcellular distribution of peroxisomal β -oxidation, 2,4-dienoyl-CoA reductase, crotonase and some marker enzymes in the control and DCQVA-fed rat livers

The livers from control rats ($n = 5$) or rats ($n = 5$) fed with 0.3% DCQVA for 2 weeks were homogenized and fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and cytosolic (S) fractions, and enzyme activities were determined as described in the Materials and methods section. (a) Peroxisomal β -oxidation activity with sorboyl-CoA; (b) 2,4-dienoyl-CoA reductase; (c) crotonase; (d) crotonase after heat treatment; (e) catalase; (f) glutamate dehydrogenase; (g) NADPH:cytochrome *c* reductase.

Figure 3 Induction of peroxisomal β -oxidation by DCQVA and clofibric acid in cultured rat hepatocytes

Cultured rat hepatocytes were prepared as described in the text and incubated for 72 h with DCQVA (●) or clofibric acid (○). The culture medium was replaced every 24 h. Results are means $\pm$ SD for three dishes. Specific activities of control dishes at 4 h after plating was 14.7 ± 2.2 nmol/min per mg of protein, decreasing to 5.63 ± 0.54 nmol/min per mg of protein after a 72 h culture.

DCQVA administered in the diet might simply perturb the enterohepatic circulation of bile acids by changing their rate of reabsorption or their metabolic pattern in the intestine, thereby raising the levels of endogenous proliferator(s), i.e. other metabolite(s) of bile acids, which may be responsible for the effect. The experiments with cultured rat hepatocytes in which DCQVA added to the medium induced peroxisome β -oxidation at comparable concentrations to those of clofibric acid demonstrates that DCQVA can directly produce the effect itself. Since induction of peroxisome β -oxidation *in vivo* evoked by the administration of 0.3% DCQVA in the diet was similar to the reported value with 0.25% clofibric acid [26], the correspondence in *in vitro* and *in vivo* potencies strongly indicates that DCQVA

was transported to the liver and acted as a direct peroxisome proliferator.

The level of DCQVA under physiological conditions, although it has not been measured, is presumably low, since a major fraction of bile acids secreted from the liver is efficiently reabsorbed via the enterohepatic circulation and only a minor fraction could be degraded by enterobacteria [27]. However, if this enterohepatic circulation was disrupted for some reason, the increased level of cholic acid in the intestine would raise the level of DCQVA. It has been well documented that decreased amounts of bile acids returning to the liver from the intestine induce cholesterol 7α -hydroxylase, the rate-limiting enzyme of bile acid biosynthesis from cholesterol, in the liver [15,27]. Thus the production of cholic acid from cholesterol would be enhanced in this situation. Possibly, an increase in peroxisomal fatty acid oxidation induced by enhanced levels of DCQVA could increase the supply of acetyl units for the biosynthesis of cholesterol and consequently bile acids. Indeed, acetyl units resulting from peroxisomal fatty acid oxidation were shown to be preferentially incorporated into cholic acid [28]. Furthermore, the presence of a cholesterol biosynthetic pathway in peroxisomes [13,14] suggests that there may be channelling between the two pathways of cholesterol synthesis and fatty acid oxidation. Thus, under these conditions where the level of bile acids returning to the liver regulates bile acid synthesis from cholesterol, DCQVA may contribute to this regulation and thereby maintain cholesterol/bile acid homeostasis. However, since in species other than the mouse or rat the effects of xenobiotic peroxisome proliferators are much less pronounced or even absent [1], it should be noted that the regulation of cholesterol/bile acid metabolism by DCQVA is likely to be restricted to rodents and cannot be extrapolated to other mammalian species.

The biosynthetic pathway of bile acid production from cholesterol constitutes a series of modifications of sterol ring structures, catalysed by several microsomal and cytosolic enzymes, and subsequent oxidative cleavage of the sterol side chain [12,15,27]. The latter step takes place in peroxisomes, and

produces chenodeoxycholic acid and cholic acid from di- and trihydroxycholestanic acid (THCA) respectively [12,15]. This sequential oxidation is thought to follow a pathway similar to that for the β -oxidation of fatty acids in this organelle. Genetic evidence indicates that a single peroxisomal thiolase [29] and a bifunctional enzyme [30] act on both fatty acid oxidation and bile acid formation. In contrast, the peroxisomal first step of bile acid formation is catalysed by THCA-CoA oxidase, an enzyme distinctly different from fatty acyl-CoA oxidase [31]. Although the present results indicate that fatty acyl-CoA oxidase was induced by DCQVA, the response of THCA-CoA oxidase to DCQVA is not known; however, it is possible that DCQVA, acting like a partially hydrogenated fish oil diet [32], may induce THCA-CoA oxidase. In contrast, conflicting evidence has been obtained that treatment of rats with clofibrate did not induce THCA-CoA oxidase [33], although the same treatment did increase bile acid formation *in vivo* [34]. Thus these results may suggest that, although the latter peroxisomal steps initiated by THCA-CoA oxidase are essential for bile acid formation [12], the levels of the peroxisomal enzymes in untreated liver may be sufficient for bile acid synthesis, and that cholesterol 7 α -hydroxylase is indeed the rate-limiting enzyme of bile acid synthesis, as previously reported [15,27].

In this study DCQVA, a naturally occurring catabolite of cholic acid, has been shown to modulate fatty acid metabolism by induction of peroxisome-associated enzymes in rat liver. The present results may suggest a close relationship between fatty acid, cholesterol and cholic acid metabolism in rat liver, in which peroxisomes play a central role.

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