Prevention of peroxisomal proliferation by carnitine palmitoyltransferase inhibitors in cultured rat hepatocytes and *in vivo*

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1. The induction of peroxisomal β -oxidation activities by bezafibrate in cultured rat hepatocytes and in the rat *in vivo* was prevented by inhibitors of carnitine acyltransferase, e.g. 2-bromopalmitate, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate or 2-tetradecylglycidic acid. 2. The prevention of peroxisomal proliferation by carnitine palmitoyltransferase inhibitors could not be accounted for by inhibition of mitochondrial β -oxidation, since 2-bromo-octanoate, acting as an inhibitor of β -oxidation, did not prevent the induction of peroxisomal activities in cultured rat hepatocytes. 3. The putative role of the acylcarnitine derivative of bezafibrate was analysed by studying the formation of bezafibroylcarnitine with bezafibroyl-CoA as substrate. However, no bezafibroylcarnitine formation was demonstrated in the presence of rat liver preparations capable of catalysing transfer to carnitine of medium- or long-chain fatty acids. 4. The prevention of peroxisomal proliferation by carnitine acyltransferase inhibitors may help in dissecting the causal relationship between the multiple effects mediated by peroxisomal proliferators.

INTRODUCTION

Peroxisomal proliferation and the induction of peroxisomal activities in cultured rat hepatocytes have been reported to be essentially similar to that observed *in vivo*, with respect to the morphometric parameters and the specific activities induced (Gray *et al.*, 1983; Bieri *et al.*, 1984; Furukawa *et al.*, 1984; Hertz *et al.*, 1985). The induction of peroxisomal proliferation in culture by peroxisomal proliferators added to the culture medium has indicated that a structural principle shared by all peroxisomal proliferators may be directly responsible for triggering the induction of peroxisomal activities (Hertz *et al.*, 1985). Indeed, most presently available peroxisomal proliferators consist of xenobiotics having a carboxylic function carried on an amphipathic backbone, or alternatively a functional group capable of being metabolized to a carboxylic acid. Clofibrate (Hess *et al.*, 1965), clofibrate analogues (Fahimi *et al.*, 1982), phthalate and adipate plasticizers (Moody & Reddy, 1978), acetylsalicylic acid (Ishii & Suga, 1979) and others seem all to fall into this category. The induction of peroxisomal activities in culture by long-chain fatty acids added to the culture medium (Hertz *et al.*, 1985) and the peroxisomal proliferation induced *in vivo* by fat-rich diets (Neat *et al.*, 1980) seem to corroborate this general rule. Thus the free carboxylic function or a derivative thereof may be assumed to be involved in initiating the induction of peroxisomal proliferation, and the nature of the hydrophobic backbone may determine the efficacy.

The present paper evaluates the presumed role of the carboxylic function of xenobiotic carboxylic compounds in initiating peroxisomal proliferation in culture and *in vivo*, by analysing the induction exerted by bezafibrate {2-[4-(chlorobenzamidoethyl)phenoxy]-2-methyl

propionic acid} (Fahimi *et al.*, 1982) as a model compound in the presence of specific inhibitors of fatty acid metabolism.

EXPERIMENTAL

Methods

Cultured rat hepatocytes were prepared as described by Berry & Friend (1969) with modifications as previously reported (Hertz *et al.*, 1985). The viability of cells before culture was 60–95%, depending on the collagenase preparations used. Some 1.0×10^6 – 1.4×10^6 cells/plate were plated on collagen-precoated 35 mm-diam. culture dishes in 1 ml of Ham F-10 medium containing 15% (w/v) fetal-calf serum, 50 μ g of streptomycin sulphate/ml and 50 μ g of penicillin G/ml. At 90 min after plating, the culture medium was changed to Dulbecco's modified Eagle's medium containing 15% fetal-calf serum, 1 munit of insulin/ml and 10 μ g of cortisol/ml. Bezafibrate or fatty acid were added for the time periods as indicated and the medium was changed daily.

Peroxisomal enoyl-CoA hydratase, peroxisomal palmitoyl-CoA oxidation (cyanide-insensitive) and overall palmitate β -oxidation in culture were determined in replicate as previously described (Hertz *et al.*, 1985). [1,3- 3 H]Glycerol incorporation into cellular or medium triacylglycerols in culture was determined in replicate as described by Mayorek & Bar-Tana (1983).

Peroxisomal palmitoyl-CoA oxidation and enoyl-CoA hydratase in rat liver homogenates were determined by homogenizing the liver in 4 vol. of 0.25 M-sucrose/0.1% ethanol, followed by centrifuging the homogenate at 700 g for 10 min and measuring the activities in the super-

nant. Heat-labile peroxisomal enoyl-CoA hydratase was assayed spectrophotometrically as described by Lalwani *et al.* (1981). Cyanide-insensitive palmitoyl-CoA oxidation was determined as described by Mannaerts *et al.* (1979).

Carnitine acyltransferase in rat liver homogenate and subcellular fractions was assayed in the direction of acylcarnitine formation as described by Bremer (1981). The reaction mixture contained 75 mM-KCl, 50 mM-mannitol, 25 mM-Hepes, pH 7.3, 2 mM-KCN, 0.2 mM-EGTA, 1 mg of fatty acid-free albumin/ml and 0.5 mM-L-[methyl- ^3H]carnitine (5 $\mu\text{Ci}/\mu\text{mol}$) in a final volume of 0.5 ml. The reaction was started by adding 200–600 μg of protein of the enzyme fraction and was carried out at 30 °C for 5 min. With palmitoyl-CoA as substrate, the reaction was terminated by adding 1.5 ml of 0.8 M-HCl, followed by extracting the palmitoyl-carnitine product into butanol (Bremer, 1981). With either octanoyl-CoA or bezafibroyl-CoA as substrate, the reaction was terminated by adding 1.0 ml of 2-methylpropan-1-ol, followed by extracting the acylcarnitine product into the 2-methylpropan-1-ol phase as described by Solberg (1972). Rat liver mitochondria, peroxisomes and endoplasmic reticulum for carnitine acyltransferase assays were prepared by differential centrifugation as described by de Duve *et al.* (1955). Mitochondrial membranes were prepared as described by McGarry *et al.* (1978).

Protein was determined by the method of Lowry *et al.* (1951).

Materials

Bezafibroyl-CoA was synthesized by reaction of an excess of bezafibroylimidazole with CoA. Bezafibroylimidazole was synthesized as described by Kawaguchi *et al.* (1981) for the preparation of CoA thioesters of fatty acids. For the preparation of bezafibroyl-CoA, 10 μmol of CoA was treated with 25 μmol of bezafibroylimidazole in 0.2 ml of tetrahydrofuran/water (2:1, v/v). The mixture was incubated for 15 min at room temperature until CoA had completely reacted (Ellman, 1958). After evaporation of tetrahydrofuran under N_2 , the aqueous mixture was suspended in 1 ml of water, acidified by HCl to pH 1.0–2.0, and the precipitated bezafibroyl-CoA was extracted into 1 ml of added butan-1-ol. The butanol phase was washed with water (pH 1.0–2.0), evaporated to dryness, and the residue was suspended in 2 ml of 0.15 M-acetate buffer (pH 4.0) and was extracted with 2 $\times$ 5 ml of dichloromethane. The aqueous solution of bezafibroyl-CoA was aerated with N_2 to remove traces of dichloromethane and kept at –18 °C. The overall yield amounted to 55%. The A_{260}/A_{232} ratio of the purified product was 0.83. The concentration of bezafibroyl-CoA was determined by its free thiol content (Ellman, 1958) after its hydrolysis in 0.7 M-KOH at 60 °C for 15 min.

[1- ^{14}C]Palmitoyl-CoA was synthesized enzymically as described by Kornberg & Pricer (1953). The product was purified as described by Seubert (1960).

Ham F-10 medium, Dulbecco's modified Eagle medium and fetal-calf serum were from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Tissue-culture dishes (35 mm diam.) were from Falcon (Oxnard, CA, U.S.A.) and were precoated with collagen as described by Michalopoulos & Pitot (1975). Insulin was from Nordisk (Insulin Laboratory, Copenhagen, Denmark) and cortisol succinate was from Upjohn (Tuurs,

Belgium) Bovine serum albumin (essentially fatty acid-free), egg albumin, collagenase and octanoyl-CoA were from Sigma. 2-Bromopalmitate was from Fluka. Bezafibrate, POCA and TDGA were kindly given by Boehringer (Mannheim, Germany), Byk Gulden (Konstanz, Germany) and McNeil Pharmaceuticals (Springhouse, PA, U.S.A.) respectively. [1- ^{14}C]Palmitic acid, L-[methyl- ^3H]carnitine and [1,3- ^3H]glycerol were from Amersham International (Amersham, Bucks., U.K.).

RESULTS AND DISCUSSION

The induction of peroxisomal activities in cultured rat hepatocytes by bezafibrate or long-chain fatty acids added to the culture medium has implicated the carboxyl group of either the xenobiotic or the natural fatty acids in the induction of peroxisomes (Hertz *et al.*, 1985; Ikeda *et al.*, 1985), and has motivated this search for a putative carboxylate metabolite responsible for initiating peroxisomal proliferation. The possible requirement for enzymic thioesterification of the proliferator to its corresponding acyl-CoA derivative (Bronfman *et al.*, 1986), followed by conjugation of the thioester with glycerol 3-phosphate, carnitine, amino acid or similar acceptors, was evaluated in the presence of added 2-bromopalmitate, acting as a general inhibitor of fatty acid thioesterification (Pande *et al.*, 1971), esterification into lipids (Evans & Denton, 1977) and mitochondrial β -oxidation (Chase & Tubbs, 1972). As shown in Fig. 1, the induction of peroxisomal activities by bezafibrate in culture was prevented in the presence of added 2-bromopalmitate within the concentration range 10–30 μM . The 2-bromopalmitate effect could not be ascribed to elimination of cellular bezafibrate owing to inhibition of its transport, since bezafibrate was still effective in decreasing triacylglycerol secretion into the medium, with a concomitant accumulation of cellular acylglycerols (results not shown).

In the search for the site of action of 2-bromopalmitate, its effect on carboxylate thioesterification and glycerol esterification into acylglycerols was verified in a concentration range and under conditions where the induction of proxisomal activities was prevented. As shown in Table 1, the esterification of exogenous glycerol into triacylglycerols in culture remained essentially unchanged in the presence of 5–20 μM of 2-bromopalmitate, whereas the apparent ED_{50} for the prevention of peroxisomal proliferation by 2-bromopalmitate was 10 μM (Fig. 1). Hence the 2-bromopalmitate effect had to be ascribed to a site of action characterized by a higher affinity for 2-bromopalmitate than that of the long-chain fatty acyl-CoA synthase or glycerol esterification. The high sensitivity of mitochondrial β -oxidation to inhibition by 2-bromopalmitate in either mitochondrial preparations (Chase & Tubbs, 1972) or cultured rat hepatocytes (Mahadevan & Sauer, 1971) (Table 1) seemed to implicate the mitochondrial metabolism of fatty acids in the 2-bromopalmitate effect. Indeed, the induction of peroxisomal activities by bezafibrate in culture could be eliminated completely in the presence of added oxirane inhibitors of mitochondrial carnitine acyltransferase, e.g. POCA (Bartlett *et al.*, 1981) (Fig. 2a) or TDGA (Tutwiler & Dellerigne, 1979) (Fig. 2b) within the concentration range of 0–100 μM , and under conditions where mitochondrial β -oxidation was effectively inhibited (e.g. 80% inhibition by

Fig. 1. Effect of 2-bromopalmitate on the induction of peroxisomal activities by bezafibrate in culture

Cultured rat hepatocytes were incubated as described under 'Methods' in the absence ($\diamond$) or presence of 0.05 mM-bezafibrate ($\square$), 0.1 mM-bezafibrate ($\bullet$), 0.2 mM-bezafibrate ($\circ$), 0.4 mM-bezafibrate ($\triangle$) and 2-bromopalmitate as indicated. After incubation for 48 h, the heat-labile enoyl-CoA hydratase (a) and the cyanide-insensitive palmitoyl-CoA oxidation (b) activities were assayed as described under 'Methods'. Results are means $\pm$ S.D. for three individual culture dishes.

Table 1. Effect of 2-bromopalmitate on glycerol esterification and palmitate β -oxidation in cultured rat hepatocytes

Cultured rat hepatocytes were preincubated for 1 h in the presence of 2-bromopalmitate as indicated. The incorporation of glycerol into cellular triacylglycerols was then determined for the time periods as indicated in the presence of 1.0 mM-[1,3- 3 H]glycerol (4.3 μ Ci/ μ mol) and the specified concentrations of 2-bromopalmitate. Palmitate β -oxidation to water-soluble products was determined by washing the preincubated cultures in modified Hanks buffered saline and further incubating the cultures for 30 min in 1 ml of the same buffer supplemented with 0.03% of fatty acid-free albumin, 0.1 mM-[1- 14 C]palmitate (1.2 μ Ci/ μ mol) and 2-bromopalmitate as indicated. Results are means $\pm$ S.D. for three individual culture dishes.

Concn. of 2-bromopalmitate added to culture medium	Glycerol incorporation into cellular triacylglycerols		Palmitate oxidation (nmol/30 min per plate)
	(nmol/30 min per plate)	(nmol/60 min per plate)	
—	2.0 $\pm$ 0.1	5.4 $\pm$ 0.1	7.5 $\pm$ 0.2
5 μ M	2.6 $\pm$ 0.2	5.2 $\pm$ 0.5	3.7 $\pm$ 0.1
10 μ M	2.5 $\pm$ 0.1	4.2 $\pm$ 0.4	3.7 $\pm$ 0.1
20 μ M	2.3 $\pm$ 0.1	4.3 $\pm$ 0.5	3.7 $\pm$ 0.1

20 μ M-POCA). The prevention of peroxisomal proliferation by carnitine palmitoyltransferase inhibitors in culture was also observed in the presence of peroxisomal proliferators unrelated to fibrates. Thus peroxisomal proliferation induced in culture by β β -methyl-substituted hexadecanedioic acid (Bar-Tana *et al.*, 1985) was prevented in the presence of 2-bromopalmitate or POCA added to the culture medium (results not shown). The oxiranes employed were poor inhibitors of microsomal long-chain fatty acyl-CoA synthase (apparent K_i 100 μ M).

The prevention of peroxisomal proliferation by either 2-bromopalmitate or POCA was also exemplified in the rat *in vivo*. Thus the induction of either peroxisomal crotonyl-CoA hydratase or palmitoyl-CoA oxidation by bezafibrate *in vivo* was blunted by treating rats orally

with either 2-bromopalmitate or POCA (Table 2). The prevention of peroxisomal proliferation by inhibitors of carnitine acyltransferase *in vivo* was observed in starved, but not in fed, rats (results not shown). This sensitivity of POCA in the starved state was similar to that reported previously for other effects mediated by POCA *in vivo*, e.g. inhibition of carnitine acyltransferase I in mitochondria isolated from rats treated by POCA, the hypoglycaemic effect and the hypoketonaemic effect (Wolf & Engel, 1985).

Prevention of peroxisomal proliferation by inhibitors of mitochondrial carnitine acyltransferase could not be ascribed to a requirement for a functional mitochondrial β -oxidative pathway. Thus inhibition of mitochondrial thiolase by added 2-bromo-octanoate (Raake & Lowenstein, 1979) with a concomitant overall inhibition of

Fig. 2. Prevention of bezafibrate-dependent peroxisomal proliferation by POCA and TDGA in culture

Cultured rat hepatocytes were incubated as described under 'Methods' in the absence ($\diamond$) or presence of 0.2 mM-bezafibrate ($\circ$), 0.4 mM-bezafibrate ($\triangle$) and either POCA (a) or TDGA (b) as indicated. After incubation for 48 h in culture, the indicated peroxisomal activities were assayed as described under 'Methods'. Results are means $\pm$ s.d. for three individual culture dishes.

Table 2. Prevention of bezafibrate-dependent peroxisomal proliferation by 2-bromopalmitate and POCA in the rat *in vivo*

Rats were starved for 4 consecutive days. From day 2 of starvation the animals were randomized into groups of five rats each and were given daily by gavage a 2 ml solution of 1% methylcellulose 400 containing bezafibrate (1.5 mg/100 g body wt.) and 2-bromopalmitate (0.6 mg/100 g body wt.) as indicated in (a), or bezafibrate (1.2 mg/100 g body wt.) and POCA (0.23 mg/100 g body wt.) as indicated in (b). The animals were killed by decapitation 18 h after the last administration, and liver peroxisomal enoyl-CoA hydratase and cyanide-insensitive palmitoyl-CoA oxidation activities were determined as described under 'Methods'. Results are means $\pm$ s.d. ($n = 5$); *significantly different from the respective bezafibrate group ($P < 0.02$ by Mann-Whitney U-test).

	Treatment mode	Peroxisomal enoyl-CoA hydratase (units/mg of protein)	Peroxisomal palmitoyl-CoA oxidation (munits/mg of protein)
(a)	Non-treated	1.0 $\pm$ 0.2	6.7 $\pm$ 0.8
	2-Bromopalmitate	3.0 $\pm$ 0.7	10.9 $\pm$ 2.5
	Bezafibrate	10.2 $\pm$ 2.5	23.0 $\pm$ 3.6
	Bezafibrate + 2-bromopalmitate	6.1 $\pm$ 2.3*	14.3 $\pm$ 2.5*
(b)	Non-treated	0.9 $\pm$ 0.2	
	POCA	3.2 $\pm$ 0.5	
	Bezafibrate	9.5 $\pm$ 1.4	
	Bezafibrate + POCA	4.0 $\pm$ 0.7*	

mitochondrial β -oxidation could not prevent the induction of peroxisomal proliferation by bezafibrate in culture (Table 3). Moreover, as 2-bromo-octanoate inhibits microsomal diacylglycerol acyltransferase (Mayorek & Bar-Tana, 1985), but does not prevent the induction of peroxisomal activities by bezafibrate, xenobiotic 'mixed lipids' (Caldwell & Marsh, 1983; Fears & Richards, 1981; Fears *et al.*, 1978) are probably not involved in initiating the induction of peroxisomal proliferation.

The prevention of peroxisomal proliferation by specific inhibitors of carnitine acyltransferase, e.g. POCA and TDGA, but not by an inhibitor of mitochondrial

β -oxidation, e.g. 2-bromo-octanoate, could imply a requirement for the bezafibroylcarnitine ester in initiating the bezafibrate-dependent induction of peroxisomal activities. The putative role played by the acylcarnitine derivative of bezafibrate was analysed by studying the formation of bezafibroylcarnitine from synthetic bezafibroyl-CoA as substrate, under experimental conditions where an activity approx. 0.2% of that observed with either medium-chain (octanoyl-CoA) or long-chain (palmitoyl-CoA) fatty acids could still be detected. However, no carnitine transferase activity was observed in the presence of mitochondrial, submitochondrial, microsomal or peroxisomal preparations capable of

Table 3. Induction of peroxisomal activities by bezafibrate in the presence of 2-bromo-octanoate in culture

Cultured rat hepatocytes were incubated for 48 h in the presence of bezafibrate and 2-bromo-octanoate as indicated. Overall β -oxidation was determined after 24 h in culture as described in Table 1 and in the presence of 2-bromo-octanoate and bezafibrate as indicated. Peroxisomal enoyl-CoA hydratase was determined at 48 h as described under 'Methods'. Results are means $\pm$ S.D. for three individual culture dishes.

Additions to culture medium	Overall β -oxidation (nmol/30 min per plate)	Peroxisomal enoyl-CoA hydratase (unit/mg of protein)
–	1.67 $\pm$ 0.10	0.19 $\pm$ 0.02
Bezafibrate (0.4 mM)	0.85 $\pm$ 0.06	0.33 $\pm$ 0.02
2-Bromo-octanoate (0.05 mM)	0.52 $\pm$ 0.04	0.18 $\pm$ 0.02
Bezafibrate (0.4 mM) + 2-bromo-octanoate (0.05 mM)	0.16 $\pm$ 0.02	0.34 $\pm$ 0.03

catalysing transfer to carnitine of medium- or long-chain fatty acids. Similarly, no bezafibroylcarnitine formation was observed with the respective carnitine transferase preparations derived from livers of bezafibrate-treated rats. Hence the involvement of the carnitine acyltransferase activity in the induction of peroxisomal proliferation could implicate the possible sequestration of mitochondrial CoA (Ball *et al.*, 1979; Berge *et al.*, 1983) or some possible novel function of the transferase protein.

Amphipathic carboxylic compounds which serve as inhibitors of carnitine acyltransferase present an interesting case where the same compound may either act as a peroxisomal proliferator, in view of its amphipathic carboxylic nature, or alternatively may prevent peroxisomal proliferation as a result of inhibiting the carnitine acyltransferase reaction. The resultant capacity of these compounds as peroxisomal proliferators may thus depend on their respective efficacies as potential inducers of peroxisomal proliferation and as carnitine acyltransferase inhibitors. These efficacies may vary as a function of the experimental conditions employed. Indeed, POCA could not induce peroxisomal proliferation in cultured rat hepatocytes (Fig. 2a), but did induce liver peroxisomal proliferation *in vivo* (Van Hoof *et al.*, 1981) (Table 2). Its lower efficacy *in vivo* as compared with fibrates (Bone *et al.*, 1982) may perhaps reflect the extent of inhibition of the carnitine acyltransferase activity *in vivo*.

The prevention of peroxisomal proliferation by carnitine acyltransferase inhibitors in culture or *in vivo* may be exploited for dissecting the causal relationship between the multiple effects exerted by peroxisomal proliferators in various biological systems. Thus the hypertrophic-hyperplastic response to peroxisomal proliferators (Reddy *et al.*, 1982), the hypotriglyceridaemic effect of peroxisomal proliferators (Reddy & Krishnanthe, 1975) or the enhanced conversion of pre-adipocytes into mature fat-loaded adipocytes in culture in the presence of added peroxisomal proliferators (Brandes *et al.*, 1986, 1987) may now be analysed under conditions of prevention of peroxisomal proliferation, thus making possible the evaluation of the apparent mutual linkage between the concerned phenomena.

This research was supported by the Herta Schnap Foundation for Cancer Research and the Joint Research Fund of the Hebrew University and Hadassah.

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Received 18 November 1986/16 February 1987; accepted 19 February 1987