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THE EFFECT OF BECLOBRIC ACID AND CLOFIBRIC ACID ON PEROXISOMAL β-OXIDATION AND PEROXISOME PROLIFERATION IN PRIMARY CULTURES OF RAT, MONKEY AND HUMAN HEPATOCYTES

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Abstract—The peroxisome-proliferating effects of clofibric acid and beclobric acid were studied in primary cultures of hepatocytes derived from rat, monkey (*Macaca fascicularis*) and human liver. Determination of peroxisomal fatty acid β-oxidation and morphometrical analysis of the peroxisomal compartment were performed after incubation of 1-day-old hepatocyte cultures for 3 days with either compound. In rat liver cell cultures both compounds gave a 10-fold increase in peroxisomal β-oxidation, a 3-fold increase in the relative number of peroxisomes and a 1.5-fold increase in the mean size of peroxisomes. Beclobric acid gave its maximal effect at a concentration of 10 μM, which is at least one order of magnitude lower than the maximum-effect concentration of clofibric acid. At concentrations >300 μM beclobric acid was cytotoxic. No stimulation of peroxisomal fatty acid β-oxidation was found in either monkey or human hepatocyte cultures. Morphometrical analysis also showed no increase in the peroxisomal compartment in cultures derived from these species, as indicated by the lack of increase in both relative number and size of peroxisomes. In all three species tested beclobric acid was equally cytotoxic for hepatocytes *in vitro*. These results are of relevance for the interpretation of the peroxisome-proliferating effects of clofibrate and similar compounds in rats. Since peroxisome proliferation may be correlated to increased hepatic tumour incidences in the rat, the absence of peroxisome proliferation in primates suggests the absence of tumourogenic activity by hypolipidemic compounds in these species.

A number of hypolipidemic drugs such as clofibrate induce hepatic peroxisome proliferation in rodents [1, 2] and there is evidence that some of these compounds increase liver tumour incidences in rats and mice [3]. The same features have been observed after treating rats and mice with phthalate esters [4] and there are indications that in rats peroxisome proliferation can also be achieved by a high-fat diet [5]. These liver changes are accompanied by alterations in a number of enzymatic activities such as increases in peroxisomal fatty acid β-oxidation [6], in cytochrome P450-dependent fatty acid ω- and (ω-1)-hydroxylation [7] and in mitochondrial 2,4-dienolyl-CoA reductase [8]. In species other than the rat or the mouse, i.e. hamsters [9, 10], guinea-pigs [11], dogs or monkeys [10, 12, 13] the effects of these types of compounds are much less pronounced or even absent. Although compounds like clofibrate do have a distinct hypolipidemic effect in man, it has remained unclear whether peroxisome proliferation and hepatocarcinogenesis also occur in man [13–16].

Peroxisome proliferation can also be induced in primary cultures of rat hepatocytes [17]. In this *in vitro* system the induction of peroxisomal changes by compounds like clofibrate requires the presence of hydrocortisone in the culture medium [18]. The use of hepatocyte cultures enables a further clarification of the mechanisms of action of these compounds by studying their effects under well defined external conditions. Employing liver cell cultures derived from various species thus provides the possibility to study the mechanisms underlying the reported species differences in susceptibility.

Beclobrate (2-[4-[(4-chlorophenyl)methyl]phenoxy]-2-methyl-ethylbutyrate) is a new and potent hypolipidemic drug in man [19]. In comparison with the clofibrate it has been shown to be up to 40 times more potent in normolipidemic rats [20].

In the present study we compared the effects of the free carbonic acids of beclobrate and clofibrate on the activity of peroxisomal β-oxidation as well as their potency to induce peroxisome proliferation in primary hepatocyte cultures derived from the rat, the monkey (*Macaca fascicularis*) and man.

MATERIALS AND METHODS

Chemicals. Beclobric acid (2-[4-[(4-chlorophenyl)methyl]phenoxy]-2-methylbutaric acid) was kindly provided by Siegfried Ltd (Zofingen, Switzerland). Clofibric acid (ethyl-2-(4-chlorophenoxy)-2-methylpropionic acid) was commercially obtained from Janssen Chimica (Beerse, Belgium). Media used for isolation and culture of hepatocytes were

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obtained from Gibco Europe (Breda, The Netherlands). Dimethylformamide (DMF¶) was supplied by British Drug Houses (Poole, U.K.). All other chemicals were of analytical grade.

Isolation and primary culture of hepatocytes. Male Wistar rats (180–230 g body weight) were bred in our laboratory and had free access to water and food (standard laboratory food, Hope Farms, Woerden, The Netherlands). Rat hepatocytes were isolated using the two-step perfusion technique as originally described by Seglen [21] and modified by Paine *et al.* [22]. For the isolation of monkey hepatocytes a similar procedure was followed as for rat hepatocytes, with the following modifications. Monkeys (*Macaca fascicularis (=cynomolgus)*) served as kidney donors for kidney cell preparation. These kidney cells were used in the production of poliomyelitis vaccine. Livers of five of these animals (three males aged 2.5, one female aged 2.5, one female aged 12) were used for our experiments. Anesthetized animals were cannulated through the portal vein immediately after nephrectomy. After opening the vena cava near the diaphragm, the liver was perfused with 500 mL Ca^{2+}–Mg^{2+}-free Hanks Balanced Salt Solution (HBSS), containing 0.25 mM EGTA and 10 mM HEPES, pH 7.4, followed by 1 L of the same perfusate without EGTA. The liver was then excised, placed on ice and transported to the laboratory (*ca.* 15 min). An additional perfusion took place with 1 L bicarbonate-buffered (pH 7.4) HBSS, 37°, gassed with 95% O_2–5% CO_2, followed by a 30 min recirculating perfusion with the same buffer containing 0.05% collagenase and 2.5 mM $CaCl_2$. The liver was gently torn apart and cells were dispersed in HBSS containing 2.5% bovine serum albumin (BSA). Centrifugation, resuspension and incubation was the same as with hepatocytes derived from the other species. Cell yield was *ca.* 8×10^8 cells/liver, viability was between 85 and 95% as determined by trypan blue exclusion.

Post-mortem human liver tissues were obtained from three kidney donors, two males aged 40 and 42 and one female aged 43. Isolation of human hepatocytes was as previously described by Rijntjes *et al.* [23].

Hepatocytes were cultured in Waymouth MB 572/1 medium containing 3% newborn calf serum, insulin (10^{-6} M), hydrocortisone (10^{-5} M) and gentamycin (50 mg/L). Cells were incubated at a density of 85×10^3 viable cells/cm^2. The medium was changed after 4 hr. The cultures were preincubated for 24 hr, after which the medium was replaced by a medium containing various concentrations of beclobric acid or clofibric acid dissolved in dimethylformamide (DMF) (final conc. 0.25%). The culture media were renewed after 48 and 72 hr. Cells were cultured in Sterilin plastic dishes (diameter *ca.*60 mm). For electron microscopy the cells were cultured in Lux Permanox plastic dishes (diameter *ca.* 60 mm).

The appearance of monolayers derived from all three species was very similar: cells attached and spread at a similar rate and responded to the presence of beclobric acid at higher concentrations in the same way. In parallel experiments it was shown that monkey hepatocytes in primary culture did respond to known inducers of cytochrome P450 isoenzymes: addition of phenobarbital, β-naphthoflavone and clofibric acid caused enhancements of ethoxyresorufin and pentoxyresorufin O-dealkylation activities (Mennes *et al.*, unpublished results) that are very similar to those in rat hepatocyte culture [24]. Furthermore, monkey hepatocyte primary cultures also responded to EGF with an increase in replicative DNA synthesis (Bieri and Blaauboer, unpublished results). For human hepatocytes such data are not yet available.

Analytical methods. Cells were harvested after 96 hr by removing the medium, washing the monolayers twice with saline and scraping the cells in a sucrose–EDTA–Tris buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris–HCl, pH 7.4, 4°). The cells were then ultrasonified for 10 sec in an MSE 100 Watt Ultrasonic Disintegrator equipped with a titanium rod with tip diameter 3.5 mm, amplitude 6 μm, frequency 20 kHz. Cell homogenates were stored at −70°. Peroxisomal β-oxidation activity was determined according to Mitchell *et al.* [18].

Electron microscopy. Cell monolayers growing in Lux Permanox dishes were rinsed twice at 4° with phosphate buffer I (Sörensen, 175 mM, pH 7.4) and then fixed at 4° for 2 hr in 3% glutaraldehyde in phosphate buffer II (Sörensen, 50 mM, pH 7.4). After three rinses in phosphate buffer I, peroxisomes were cytochemically stained for catalase by incubating at 37° in 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 in glycine–NaOH buffer (0.1 M, pH 10.5) for 1 hr [25–27]. The cells were then washed three times in phosphate buffer I and postfixed at room temperature for 45 min in 1% osmiumtetroxide in phosphate buffer II, dehydrated in graded ethanols and flat-embedded in the dishes in polarbed (Polaron, Watford, U.K.). The cells were thin sectioned (70–90 nm) parallel with the dish's surface on a Reichert OMU-3 ultramicrotome with a diamond knife. Sections were stained with a saturated uranyl acetate solution in 70% ethanol and with Reynolds lead citrate [28]. Electron micrographs were made at a magnification of 1100× and 3800× using a Zeiss EM10 electron microscope. Electron micrographs taken at 3800× were enlarged photographically 5×. The surface area of each peroxisomal profile was measured using a digitizer tablet (Minimop, Kontron Bildanalyse, Eching, F.R.G.). Statistical analysis of frequency histograms of the relative size of peroxisomes was performed using the Kolmogorov–Smirnov two-sample test [29]. The number of peroxisomes was determined by counting the organelles on micrographs of grid openings (300 mesh) that were completely covered with cells.

RESULTS

In rat hepatocyte monolayer preparations studied

¶ Abbreviations: BSA, bovine serum albumin; DMF, dimethylformamide; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid; HBSS, Hanks Balanced Salt Solution; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N′*-(2-ethane sulfonic acid).

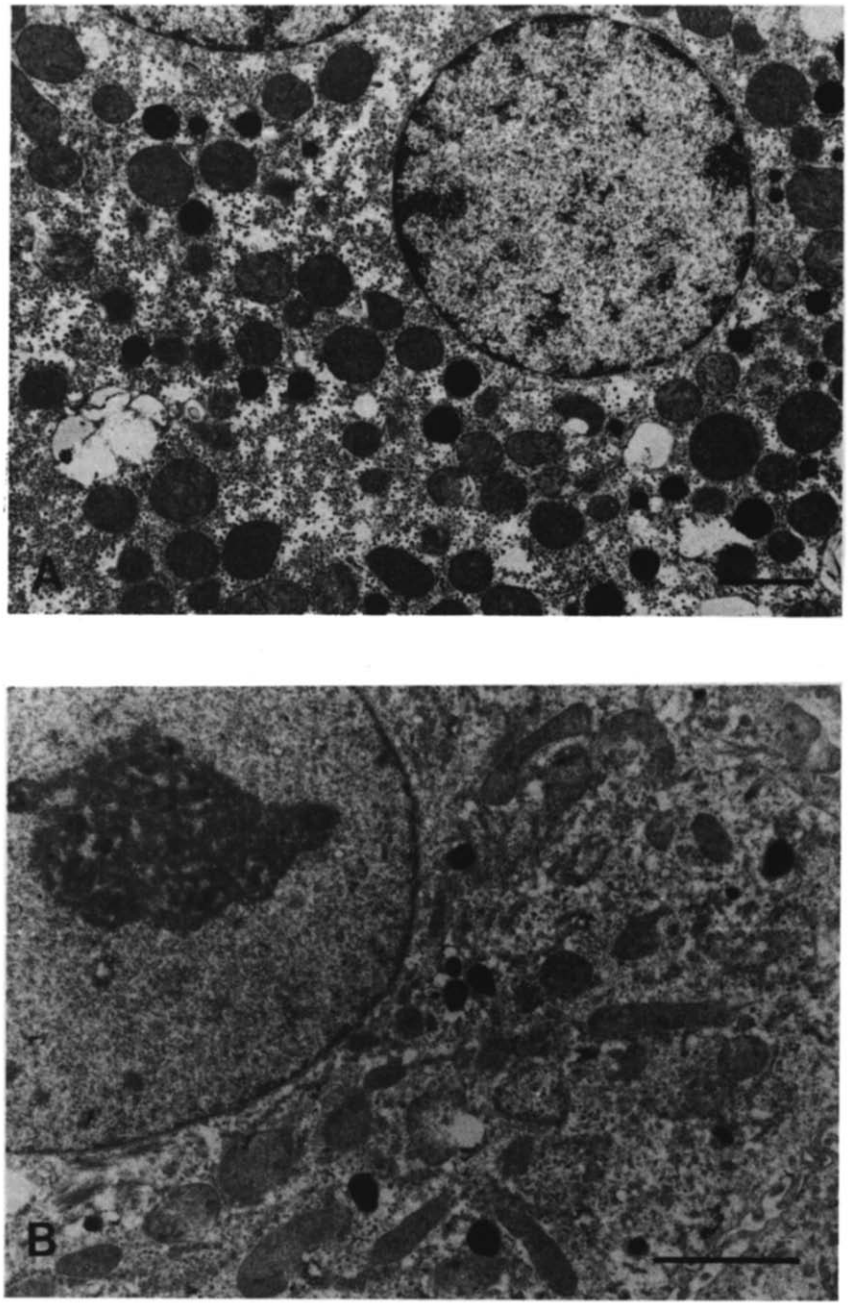

Fig. 1. Electron micrograph of monkey (A) and human (B) hepatocytes in primary culture after incubation for 3 days with 100 μM beclobric acid. The cells are stained for catalase activity. Bar = 2 μm.

in the electron microscope peroxisomes can be recognized by the presence of a crystalline-like structure called the peroxisome nucleoid. However, not all organelles that could be peroxisomes showed such a sub-organelle structure and in monkey hepatocytes the peroxisomal nucleoid is hardly recognizable at all. In human hepatocyte peroxisomes the nucleoid appears to be absent [27]. Therefore, it was necessary to apply a staining method specific for catalase, producing electron-dense structures. All organelles staining in this procedure are nominated peroxisomes. Figure 1 shows electron micrographs from monkey (A) and human (B) hepatocyte cultures stained for catalase.

Light-microscopical examination of the cultures showed that 1000 μM clofibric acid did not give obvious changes in the appearance of monolayers. With beclobric acid the cells were killed within 24 hr after addition of 1000 μM of the compound, while 300 μM caused changes in the monolayer's density and cell appearance: the treated cells were less cubical than control cells. These effects were observed in hepatocyte cultures derived from all three species examined.

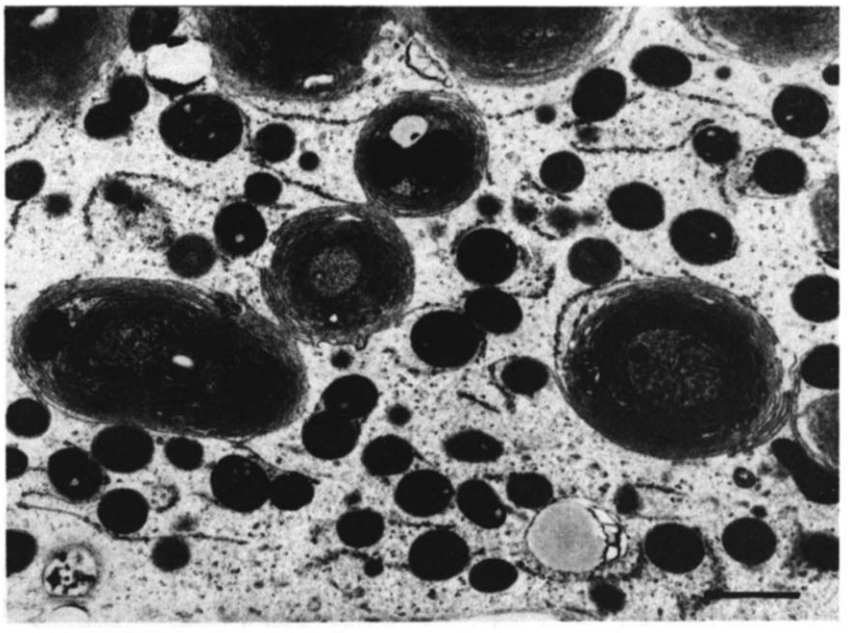

Fig. 2. Electron micrograph of rat hepatocytes in primary culture after incubation for 3 days with 300 μM beclobric acid. Concentric membranous bodies are shown. Bar = 1 μm.

In hepatocyte cultures incubated with 300 μM beclobric acid changes in the cells could be observed electron micrographically. Figure 2 shows an example of these multiple organellar changes consisting of concentric membranous bodies, in many cases showing inclusions of mitochondria. Here too, no differences were observed between rat, monkey and human hepatocyte cultures.

Determination of cyanide-insensitive (peroxisomal) β-oxidation in hepatocyte cultures derived from the rat showed a concentration-related increase in this parameter with both clofibric acid and beclobric acid (Table 1). This increase occurred at a clofibric acid concentration of 30 μM, whereas even 1 μM becloric acid gave a significantly elevated activity for this reaction. Maximal activities were similar for both compounds, but with beclobric acid this maximum was reached at 10 μM, while with clofibric acid this was ≥300 μM. Table 1 shows that in monkey and human hepatocyte primary cultures no increased activity of peroxisomal β-oxidation was found after incubation with both compounds.

In rat hepatocyte primary culture addition of both clofibric acid and beclobric acid resulted in an increased number of peroxisomes (Table 2). For both compounds similar minimal-effect concentrations were found as with the biochemical determination of β-oxidation activity: a significant increase in the number of peroxisomes was observed at concentrations of 3 and 300 μM beclobric acid and clofibric acid, respectively. Thus, beclobric acid was about two orders of magnitude more active than clofibric acid. Table 2 also shows the highest concentration not resulting in cytotoxicity did not give rise to an increased number of peroxisomes in monkey and human hepatocytes.

Morphometric analysis of electron micrographs of rat hepatocyte cultures demonstrated an effect of both compounds on the distribution of peroxisomes over size classes. Figure 3 shows this shift in peroxisome size for 300 μM clofibric acid and 100 μM beclobric acid. Chi-square tests showed a non-normal distribution of data in the treated rate hepatocyte cultures, which is apparently caused not only by an increase in the mean size of peroxisomes but also by the appearance of small peroxisomes. The Kolmogorov–Smirnov two-sample test showed that even at 3 μM beclobric acid and at 10 μM clofibric acid a significant increase in the size of peroxisomes was found. In contrast, in monkey and human hepatocyte cultures no changes in the size of peroxisomes could be observed. Table 3 summarizes the results of this test.

DISCUSSION

In rat hepatocyte cultures peroxisomal β-oxidation of fatty acids is stimulated in the presence of beclobric acid at concentrations one order of magnitude lower than with clofibric acid. This is in agreement with the *in vivo* data [20] in which beclobrate was shown to be a very effective hypolipidemic agent. Data presented by Kocarek and Feller [30] also showed that beclobric acid is a more potent peroxisome proliferator in rat hepatocyte cultures than clofibric acid. However, our results with hepatocyte primary cultures derived from *Macaca* monkey and man show that in this *in vitro* system with these

Table 1. Effect of clofibric acid and beclobric acid on peroxisomal β-oxidation activity in hepatocyte cultures derived from rat, monkey and human liver

	Clofibric acid			Beclobric acid		
Conc. (μM)	Rat	Monkey	Human	Rat	Monkey	Human
0	0.34 ± 0.13	0.71 ± 0.07	0.43 ± 0.15	0.34 ± 0.13	0.71 ± 0.07	0.43 ± 0.15
1	ND	ND	ND	0.71 ± 0.14*	ND	ND
3	ND	ND	ND	2.12 ± 0.11‡	ND	ND
10	0.66 ± 0.18	0.73 ± 0.11	0.46 ± 0.05	3.95 ± 0.69†	0.85 ± 0.07	0.41 ± 0.08
30	0.77 ± 0.06†	0.82 ± 0.03	0.34 ± 0.05	3.48 ± 1.17*	0.74 ± 0.01	0.37 ± 0.11
100	1.58 ± 0.35†	0.69 ± 0.02	0.37 ± 0.09	3.01 ± 0.24‡	0.86 ± 0.17	0.50 ± 0.26
300	3.12 ± 0.67†	0.82 ± 0.06	0.49 ± 0.06	2.80 ± 2.05	0.78 ± 0.07	0.62 ± 0.08
1000	ND	0.76 ± 0.16	0.41 ± 0.12	Cytotoxicity	Cytotoxicity	Cytotoxicity

Hepatocytes were preincubated for 24 hr, followed by 72 hr treatment.
Peroxisomal β-oxidation is expressed as nmol NAD^+/min/mg protein.
ND, not determined.
Statistics: Student's *t*-test vs control. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$. Data are means ± SD, N = 3 incubations, derived from one individual. Experiments were repeated, three times for rat and human hepatocytes and five times for monkey hepatocytes; two-way analysis of variance showed that although differences existed in the absolute β-oxidation activity in hepatocyte cultures derived from different individuals, there was a significant concentration-dependent effect of clofibric acid and beclobric acid in rat hepatocytes but not in monkey and human hepatocytes.

Table 2. Relative number of peroxisomes in primary cultures of hepatocytes derived from rat, monkey and human liver after *in vitro* treatment with clofibric acid and beclobric acid

	Clofibric acid			Beclobric acid		
Conc. (μM)	Rat	Monkey	Human	Rat	Monkey	Human
0	55.5 ± 12.9	24.1 ± 5.5	59.0 ± 14.7	55.5 ± 12.9	24.1 ± 5.5	59.0 ± 14.7
1	ND	ND	ND	58.9 ± 18.4	ND	ND
3	ND	ND	ND	70.8 ± 12.8*	ND	ND
10	62.6 ± 16.2	ND	ND	80.4 ± 17.0†	ND	ND
100	66.6 ± 13.4	ND	ND	94.8 ± 17.8‡	ND	ND
300	152.0 ± 27.1‡	ND	ND	167.1 ± 24.4‡	22.7 ± 3.9	50.0 ± 10.1
1000	123.9 ± 36.5‡	24.1 ± 2.9	70.5 ± 12.7	Cytotoxicity	Cytotoxicity	Cytotoxicity

Measurements were carried out in cultures treated for 72 hr after a preincubation for 24 hr. Data are means ± SD, N = 8 determinations/conc., i.e. eight grid openings completely covered with cells were counted on a 300 mesh grid. Data are derived from one individual, experiments were repeated three times; two-way analysis of variance showed that although differences existed in the absolute numbers of peroxisomes per grid opening in hepatocyte cultures derived from different individuals, there was a significant concentration-dependent effect of clofibric acid and beclobric acid in rat hepatocytes but not in monkey and human hepatocytes.
ND, not determined.
Statistics: Student's *t*-test vs 0 μM, * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

species proliferation of peroxisomes and increases in peroxisomal β-oxidation do not occur. The absence of peroxisome proliferative activity of clofibric acid and beclobric acid was observed in cell cultures which responded to known inducers of cytochrome P450 isoenzymes (e.g. clofibric acid) and to EGF. This indicates that under these culture conditions, hepatocyte monolayers are able to express several responses related to the effects of hypolipidemic compounds. These results therefore show that the *in vivo* hypolipidemic action of these compounds does not correlate with their peroxisome proliferating activity in different mammalian species. In rodents as well as in man fibrates are known to produce a lowering of blood VLDL levels [19, 20]. Hanefeld *et al.* [15] examined human liver biopsies taken before and during clofibrate treatment and observed that, although there was a slight but significant increase in the numerical density of peroxisomes, there was no significant increase in the volume density of the organelle. These authors therefore concluded that no specific peroxisomal changes occurred in human liver and that mitochondrial changes were more prominent. De La Iglesia *et al.* [16] reported the absence of peroxisome proliferation in man after prolonged treatment with gemfibrozil. Thus, no evidence exists for gross changes in the size and the biochemical activity of the peroxisomal compartment after exposure to this type of compound in man. This is further substantiated by the agreement between the biochemical determination of cyanide-insensitive β-oxidation of fatty acids and the morphometrical

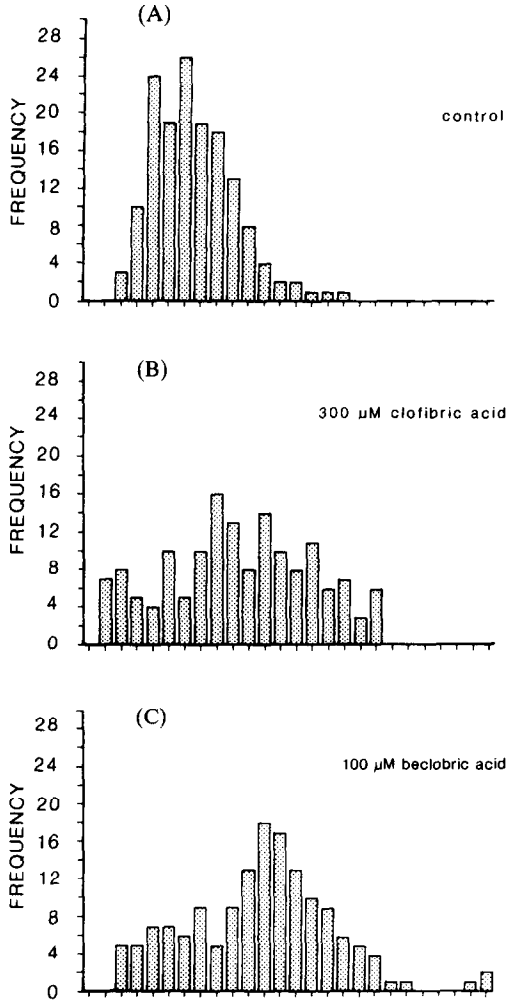

Fig. 3. Distribution over size classes of peroxisomes in rat hepatocyte primary cultures: (A) untreated cells; (B) cells treated with clofibric acid (300 μM); (C) hepatocytes treated with beclobric acid (100 μM). Peroxisome size is given as the surface area of the organelle on electron micrographs as determined on a digitizer tablet, in mm^2 ($100\ mm^2 \approx 0.277\ \mu m^2$). The Kolmogorov–Smirnov two-sample test showed that the distribution in (B) and (C) are significantly different from the distribution in (A) (see Table 3).

Table 3. Effect of clofibric acid and beclobric acid on the size of peroxisomes in hepatocyte primary cultures derived from rat, monkey and human hepatocytes

	Clofibric acid			Beclobric acid		
Conc. (μM)	N	Mean	K.S.	N	Mean	K.S.
Rat hepatocytes						
0	151	24.3		151	24.3	
1		ND		151	24.8	
3		ND		151	26.7	*
10	151	35.5	†	151	28.3	†
100	151	28.3	†	151	31.1	†
300	151	35.5	†	151	41.0	†
1000	151	27.8	†	Cytotoxicity		
Monkey hepatocytes						
0	100	20.9		100	20.9	
100		ND		100	19.7	
1000	100	21.7		Cytotoxicity		
Human hepatocytes						
0	150	17.1		150	17.1	
100		ND		150	18.3	
1000	150	15.0	*	Cytotoxicity		

Measurements were carried out in cultures treated for 72 hr with the compounds as indicated in the table after a preincubation for 24 hr.

Peroxisome size is given as the surface area of the organelle on electron micrographs as determined on a digitizer tablet, in mm^2 ($100\ mm^2 \approx 0.277\ \mu m^2$). Data are means ± SD, derived from one individual. Experiments were repeated three times; two-way analysis of variance showed that although differences existed in the absolute peroxisome sizes in hepatocyte cultures derived from different individuals, there was a significant concentration-dependent effect of clofibric acid and beclobric acid in rat hepatocytes but not in monkey and human hepatocytes.

Statistics: Kolmogorov–Smirnov (K.S.) two-sample test vs 0 μM. * $P < 0.05$; † $P < 0.01$.

indices of peroxisome number and size, as determined in our *in vitro* experiments with hepatocyte cultures derived from all three species. This raises the question whether the ability to produce peroxisome proliferation of a compound is a good reflection of its hypolipidemic action.

It has been suggested that peroxisome proliferation in rat hepatocytes is associated with the production of long-chain dicarboxylic acids by a cytochrome P450-dependent reaction in the endoplasmic reticulum [31]. Indeed, many hypolipidemic compounds are strong inducers of cytochrome P450IVA1, the isoenzyme responsible for long-chain fatty acid ω- and (ω-1)-hydroxylation. These dicarboxylic acids cannot be metabolized in the mitochondria of rat hepatocytes. The peroxisomal β-oxidation can produce medium- and short-chain compounds and these are substrates for mitochondrial lipid metabolism. However, as far as we are aware nothing is known of the regulation of these processes in non-rodent liver.

Since these fibrates do have hepatocarcinogenic properties in rodents and since the ability to produce peroxisome proliferation and liver tumours is also shared by other compounds, i.e. phthalic esters, the question arises whether assessment of these phenomena in one species is of relevance for the evaluation of the toxicological risk in another species. Results obtained with a number of hypolipidemic agents suggests that no causality exists between the ability to produce peroxisome proliferation and tumourogenic capability [32]. Reddy and coworkers [2, 3, 33] have postulated that the tumourogenic activity of hypolipidemic compounds may be the result of an imbalance between the production of hydrogen peroxide in peroxisomes, formed during β-oxidation and the detoxification of this form of active oxygen. Peroxisomal catalase as well as cytosolic GSH peroxidase play a role in these detoxification processes [34]. The hypolipidemic compound nafenopin was found to be

an inhibitor of GSH peroxidase in rat liver, while the peroxisomal catalase activity was elevated [35]. These findings support the assumption that peroxisome proliferating compounds do cause an excessive hydrogen peroxide production in rats. However, no data are available concerning the balance between peroxisomal peroxide production and detoxification in different species, including man.

In conclusion, clofibric acid and beclobric acid show marked differences in their ability to produce peroxisome proliferation and increased fatty acid β-oxidation in rat hepatocyte primary cultures as compared with primary hepatocyte cultures derived from *Macaca* monkey and man. In rat liver cell culture, beclobric acid has a higher efficacy to produce this effect, being active at concentrations at least one order of magnitude lower than clofibric acid. A good correlation was found between the effects on cyanide-insensitive β-oxidation and the morphometrically determined enlargement of the peroxisomal compartment. Both effects showed a dose-related increase in rat hepatocytes. However, none of the effects was found in monkey and human liver cell cultures.

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