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INHIBITION OF FATTY ACID BIOSYNTHESIS BY BEZAFIBRATE IN DIFFERENT RAT CELLS

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Abstract—Bezafibrate is one of the main drugs used in the treatment of human hyperlipemic diseases. Its action on the biosynthesis of fatty acids has been studied and the following conclusions have been drawn: (1) Lipogenesis from glucose is inhibited in hepatocytes and adipocytes isolated from "refed" rats previously treated with bezafibrate. (2) Lipogenesis from glucose is inhibited by bezafibrate in hepatocytes and adipocytes isolated from "refed" rats. (3) Lipogenesis from glucose is also inhibited by bezafibrate in acini isolated from lactating rats. These results show that bezafibrate is an inhibitor of fatty acid synthesis.

Bezafibrate is one of the main drugs used in treating human hyperlipemic diseases [1] and its effects on the lipid metabolism have been widely studied [2, 3]. It decreases the levels of triglycerides and cholesterol in experimental animals [4] and it is effective in lowering elevated levels of VLDL and LDL in patients with hyperlipoproteinemia [5–7]. However, its specific mode of action is not yet completely understood [8].

The effects of this drug on lipid metabolism may be divided into two different kinds: (a) those on the catabolic pathways, and (b) those on the biosynthetic pathways. It is well established that bezafibrate increases the catabolism of lipids via an increase in the activity of the peroxisomal-oxidation system [9]. This supports the hypothesis [10, 11] that the action mechanism of hypolipidemic drugs involves an increase in fatty acid oxidation in hepatic peroxisomes. At the same time, the action of bezafibrate on fatty acid biosynthesis pathways is not completely understood. It has been reported [12] that bezafibrate seems to be an inhibitor of fatty acid biosynthesis *ex vivo*.

In this report we present the effects of bezafibrate on fatty acid biosynthesis. It has been studied in: (1) hepatocytes and adipocytes isolated from "refed" rats; (2) hepatocytes and adipocytes isolated from "refed" rats previously treated with bezafibrate; and (3) acini isolated from lactating rats.

The results support the hypothesis that bezafibrate causes the inhibition of lipogenesis from glucose. This effect, along with that of increasing the fatty acid oxidation, could be important for the hypolipidemic action of bezafibrate.

MATERIALS AND METHODS

Animals. Female Wistar rats, 150–200 g, were divided into three groups: (a) refed rats—rats deprived of food for 48 hr and then allowed free access for the next 48 hr to a carbohydrate-rich diet (Altromin, Germany); (b) refed rats treated with bezafibrate—refed rats treated with bezafibrate during the refeeding periods (400 mg/kg/day by gastric intubation); (c) lactating rats—rats 14–17 days post-partum (peak lactation), kept with eight to ten pups and weighing 280–300 g.

Cell preparations. Isolated adipocytes were prepared by the Rodbell technique [13] and resuspended in a 3% serum albumin phosphate buffer (120 mM NaCl, 4.75 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM Na_2HPO_4, pH 7.4).

Isolated hepatocytes were prepared according to Berry and Friend's method [14] with the modifications described by Cornell *et al.* [15]. Viability was always greater than 90% on the basis of erithrosine exclusion.

To prepare acini, inguinal mammary glands (5–8 g) were removed, chopped and washed as described by Katz *et al.* [16] and Robinson and Williamson [17], and digested in 35 ml of Krebs–Henseleit buffer [18] containing collagenase (1 mg/ml) and 10 mM glucose. Oxygen was supplied by bubbling O_2/CO_2 (19:1) during the 1 hr digestion. The acini were resuspended in the Krebs–Henseleit buffer (10 mg/ml).

Cell incubations. In the final volume of 1 ml each adipocyte incubation included 0.3 ml adipocyte suspension (about 1.5×10^6 cells), 2 mM glucose and 0.125 μCi(U-^{14}C)-glucose (137.5 dpm/nmol glucose) in the 3% albumin phosphate buffer.

Hepatocyte incubation was carried out in 25 ml Ehrlenmeyer glass flasks. Fatty acid synthesis was measured in a final vol. of 2 ml which contained: 0.6 ml hepatocyte suspension (approx. 5×10^6 cells), 15 mM glucose and 1 μCi (U-^{14}C)-glucose (73.33 dpm/nmol glucose) in the 2.5% albumin Krebs–Henseleit buffer. Fatty acid oxidation was measured in a final vol. of 2.5 ml which included 1 ml hepatocyte suspension (approx. 8×10^6 cells), 0.4 mM fatty acid (octanoate or palmitate) and 1 μCi of the labelled compound (1-^{14}C)-octanoate or (1-

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^{14}C)-palmitate) in the 2.5% albumin Krebs–Henseleit buffer.

Silicone-treated 25 ml Ehrlenmeyer flasks were used for acini incubation. The total vol. of 2.0 ml contained 0.5 ml acini suspension, 5 mM glucose and 0.250 μCi (U-^{14}C)-glucose (55 dpm/nmol glucose) in the 3% albumin Krebs–Henseleit buffer.

All incubations were gassed with O_2/CO_2 (19:1) for 20 sec, capped and shaken at 37°. Lipid synthesis assays were left for 60 min and the β-oxidation experiments were left for 20 min.

Correction for background radioactivity in the various assays was carried out by including in the experiments data for flasks where the reaction was stopped at zero time.

Analytical procedures. In order to measure $^{14}CO_2$ production, the reaction was stopped in an ice bath. Following this, 0.2 ml phenylethylamine was injected into the central well of the flasks for hepatocyte and acini incubations, and into the lateral well of the tubes for the adipocyte incubations. Carbon dioxide was collected for 1 hr at 0°.

For the measurement of (U-^{14}C)-glucose incorporation into lipids, esterified lipids and fatty acids were extracted as described by Rodbell [13].

For the fatty acid oxidation studies, reactions were terminated with 0.4 ml of 70% (w/v) perchloric acid. The incorporation of ^{14}C into CO_2 and all acid-soluble products were then determined. The incorporation of ^{14}C into acid-soluble products was measured as described by Mannaerts *et al.* [19].

Chemicals. Bovine serum albumin (Fraction V) was obtained from the Armour Pharmaceutical Company (U.S.A.), and was made fatty-acid free using Chen's method [20]. Collagenase was from the Boehringer Co. (F.R.G.), while (1-^{14}C)-octanoate and (1-^{14}C)-palmitate were purchased from the New England Nuclear (Boston, MA) and (U-^{14}C)-glucose from the Radiochemical Center (Amersham, U.K.). The unlabelled fatty acids were from the Sigma Chemical Co. (Poole, U.K.). All other chemicals were of the highest purity available from commercial sources.

Bezafibrate was a kind gift from the Laboratorios Andreu (Barcelona) and Boehringer (Barcelona, Spain). These bezafibrates are soluble, in the incubation medium with ultrasonic help, at the concentration used.

Calculations. The production of $^{14}CO_2$ from labelled substrates, the incorporation of (U-^{14}C)-glucose into esterified lipids and fatty acids, and the incorporation of ^{14}C into acid-soluble products, were expressed as percentages of controls (in the absence of bezafibrate), as nmol $(10^6 \text{ cells})^{-1}$ or as nmol mg^{-1} dry wt. The values shown in the figures and tables are mean ± SE of at least five different experiments. Statistical analysis was via the Student's *t*-test. $P \leq 0.05$ was taken as significant.

RESULTS

Effect of bezafibrate on lipid utilization in hepatocytes from refed rats treated with bezafibrate

It is well known that the metabolism of fatty acid is, to a considerable degree, dependent on the chain length. Short-chain fatty acids are activated by acyl-CoA synthetase within the mitochondrial matrix [21], whereas the formulation of long-chain acyl-CoA occurs either in the endoplasmic reticulum or in the outer mitochondrial membrane [21, 22]. Moreover, long-chain, in contrast to short-chain, fatty acids can be oxidized by peroxisomes [11], and require esterification with carnitine prior to its transport across the inner mitochondrial membrane.

Fatty acid degradation from labelled compounds can be measured by the formation of both perchloric acid-soluble radioactivity (mainly ketone bodies) and radioactive CO_2.

Table 1 summarizes the effects of bezafibrate on palmitate and octanoate degradation. The $^{14}CO_2$ production from (1-^{14}C)-octanoate, which represents its complete degradation in mitochondria, is not affected by bezafibrate. However, $^{14}CO_2$ production from (1-^{14}C)-palmitate undergoes two-fold increase. Simultaneously, bezafibrate augments the formation of acid-soluble products from (1-^{14}C)-octanoate by a factor of approx. 4. Such products from (1-^{14}C)-palmitate have an almost eight-fold increase.

The overall effect of bezafibrate, measured as CO_2 production and acid-soluble products formation, was twice as large in the case of the degradation of palmitate (approx. a six-fold increase as compared to approx. a three-fold increase for octanoate). These results concur with the increase in activity of the peroxisomal β-oxidation system noted by other authors [9], and support the idea that bezafibrate induces peroxisomal-oxidation of fatty acid and has little or no effect on mitochondrial fatty acid oxidation [9].

Effects of bezafibrate on lipid biosynthesis in different lipogenic cells from refed rats treated with bezafibrate

Lipogenesis from glucose is known to be high in refed rats [23], which we have used in order to facilitate the measurement of fatty acid synthesis from (U-^{14}C)-glucose.

The effects of bezafibrate on lipid biosynthesis in adipocytes and hepatocytes isolated from lipogenic rats previously treated with bezafibrate are summarized in Tables 2 and 3, respectively. As can be seen, bezafibrate, as in the *ex vivo* experiments [12], produces the inhibition of esterified lipids and fatty acid synthesis. This drug inhibited about 67% of the fatty acid synthesis in adipocytes and 93% in hepatocytes. The inhibitory effect on esterified lipid biosynthesis was lower: about 36% in adipocytes and 65% in hepatocytes.

In vitro *effect of bezafibrate on lipid utilization in hepatocytes from refed rats*

There are no appreciable differences in the $^{14}CO_2$ and acid soluble products produced from (1-^{14}C)-octanoate. The CO_2 produced from (1-^{14}C)-palmitate increased slightly, and only the formation of acid-soluble products from this fatty acid increased significantly (results not shown). Similar results have been found by Christiansen [24] for clorfibrate.

In vitro *effects of bezafibrate on lipid biosynthesis in different lipogenic cells from lipogenic rats*

The *in vitro* effects of bezafibrate on lipid biosynthesis studied in hepatocytes and adipocytes isolated

Table 1. Effect of bezafibrate on lipid utilization in hepatocytes from refed rats treated with bezafibrate

Substrate	Treatment	$^{14}CO_2$	Acid-soluble oxidation products (nmol fatty acid/10^6 cells per 20 min)	$^{14}CO_2$ + Acid-soluble oxidation products
(1-^{14}C)-Octanoate	Refed rats	0.80 ± 0.1	14.30 ± 1.4	15.10 ± 1.4
(1-^{14}C)-Octanoate	Refed rats treated with bezafibrate	0.90 ± 0.1	47.30 ± 3.8*	48.20 ± 3.8*
(1-^{14}C)-Palmitate	Refed rats	0.13 ± 0.02	0.55 ± 0.06	0.68 ± 0.6
(1-^{14}C)-Palmitate	Refed rats treated with bezafibrate	0.25 ± 0.02	4.23 ± 0.23†	4.48 ± 0.23*

Hepatocytes from refed rats and refed rats treated with bezafibrate were prepared and incubated for 20 min as described under Materials and Methods. The results are means ± SE, N = 5–6.
* $P < 0.01$.
† $P < 0.001$.

Table 2. Effect of bezafibrate on lipid biosynthesis in adipocytes from refed rats treated with bezafibrate

Incorporation (1-^{14}C)-glucose to:	Control (nmol/mg dry wt per 60 min)	Bezafibrate
$^{14}CO_2$	0.29 ± 0.04 (100%)	0.22 ± 0.02 (75.9%)
Esterified lipids	0.88 ± 0.15 (100%)	0.56 ± 0.09 (63.6%)
Fatty acids	0.60 ± 0.12 (100%)	0.20 ± 0.03 (33.3%)*

Adipocytes from refed rats and refed rats treated with bezafibrate were prepared and incubated for 60 min as described in the text. The results are means ± SE, N = 6–8. The term "esterified lipids" represents the triglicerides.
* $P < 0.05$.

Table 3. Effect of bezafibrate on lipid biosynthesis in hepatocytes from refed rats treated with bezafibrate

Incorporation (U-^{14}C)-glucose to:	Control (nmol/10^6 cells per 60 min)	Bezafibrate
$^{14}CO_2$	40.0 ± 3.4 (100%)	25.1 ± 0.6 (62.8%)*
Esterified lipids	19.9 ± 1.8 (100%)	7.1 ± 1.2 (35.7%)†
Fatty acids	6.1 ± 0.9 (100%)	0.4 ± 0.1 (6.6%)†

Hepatocytes from refed rats and refed rats treated with bezafibrate were prepared and incubated for 60 min as described in Materials and Methods. The results are means ± SE, N = 6–8. The term "esterified lipids" represents the sum of triglicerides plus phospholipids.
* $P < 0.01$.
† $P < 0.001$.

from refed rats and in acini from lactating rats are shown in Fig. 1.

Figure 1a shows that the addition of different concentrations of bezafibrate to the incubation medium of the adipocytes isolated from refed rats results in a marked inhibition of (U-^{14}C)-glucose incorporation into fatty acids, the degree of inhibition being dependent on the concentration of added bezafibrate. A 2 mM concentration of the drug produced an inhibitory effect on fatty acid synthesis of about 50%, and 4 mM bezafibrate produced almost 100% inhibition. It also inhibited the synthesis of esterified lipids, but to a smaller extent (Fig. 1a).

Figure 1b shows the results obtained in hepatocytes where bezafibrate produced similar effects to those described above for adipocytes. The main difference was that a higher concentration of bezafibrate was needed to produce the same effect. The maximum inhibitory effect (100%) was nevertheless reached for a concentration of 4 mM.

Figure 1c shows that bezafibrate effects on lipid biosynthesis in acini are only slightly more marked than the results described for adipocytes. A 2 mM concentration produced a 60% inhibition of fatty acid synthesis, and 100% inhibition was again reached when 4 mM was used.

DISCUSSION

The pharmacological action of bezafibrate, as has been suggested, is accomplished via the induction of fatty acid oxidation in peroxisomes [9].

A decrease in fatty acid synthesis due to bezafibrate has been reported in *ex vivo* experiments [12], though the resulting data could have included bezafibrate effects on fatty acid synthesis as well as fatty acid oxidation. In order to differentiate between the two effects, in particular that on fatty acid synthesis, we studied its effect *in vitro* using different lipogenic tissues.

When isolated cells (hepatocytes and adipocytes) from treated rats were used, we noted that in hepatocytes there was a decrease in the fatty acid biosynthesis pathway (Table 2 and 3) and an increase in fatty acid oxidation (Table 1). Similar to the *ex vivo* experiment, the data might be the result of not only the inhibitory effect but also the increase in fatty acid oxidation. However, the decrease in the fatty acid biosynthetic pathway in adipocytes must be due to the inhibitory effect of bezafibrate. Moreover, in order to make a distinction between the effect of bezafibrate on both pathways, we also studied the effect of this drug when added to the incubation

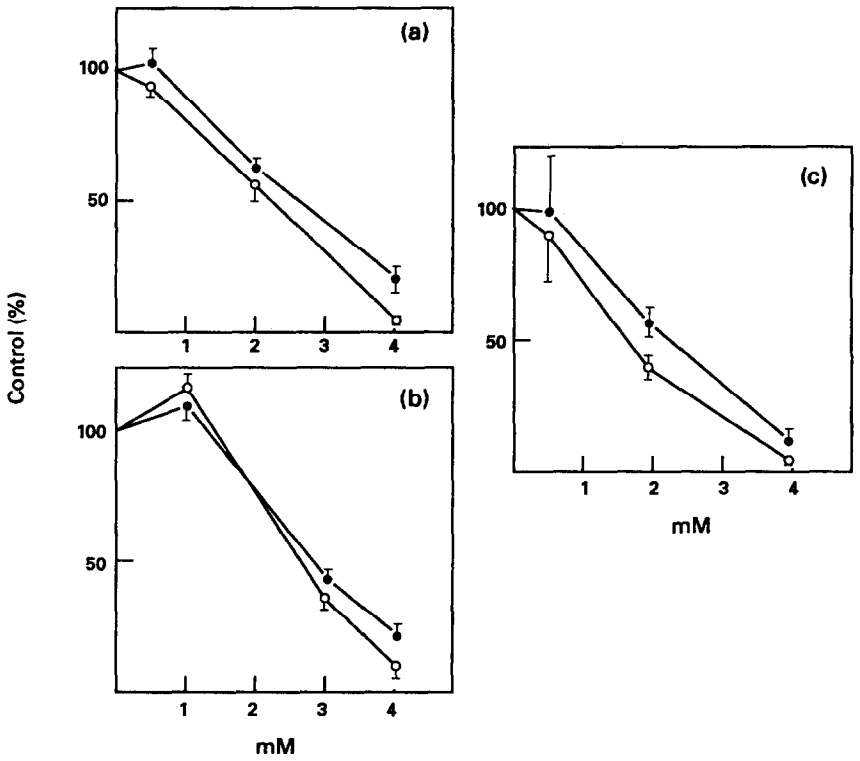

Fig. 1. *In vitro* effect of bezafibrate on synthesis of fatty acids (○) and triglycerides (●), from (U-^{14}C)-glucose: (a) adipocytes; (b) hepatocytes from refed rats, (c) acini from lactating rats, were prepared and incubated for 60 min as described in the text. The results are expressed as a percentage of control (without bezafibrate) and are means ± SE, N = 6–8. The term "esterified lipids" represent the triglicerides in adipocytes and acini, and the sum of triglicerides plus phospholipids in hepatocytes.

medium of different lipogenic cells: adipocytes and hepatocytes from refed rats, and acinis from lactating rats (Fig. 1).

Under these conditions, bezafibrate did not increase the peroxisomal fatty acid oxidation pathway; nor did the utilization of palmitate and octanoate in the presence of bezafibrate produce alterations.

It did, however, produce the inhibition of fatty acid biosynthesis in all lipogenic cells studied: adipocytes (Fig. 1a), hepatocytes (Fig. 1b) and acinis (Fig. 1c). Its effect on hepatocytes was lower than on other cells, contrary to the results obtained from cells isolated from lipogenic rats treated with bezafibrate. This may be a special characteristic of these cells, and could be explained by the fact that, *in vivo*, bezafibrate is mainly accumulated in liver as has been reported by Schmikt *et al.* [25] from pharmacokinetic studies.

This inhibitory effect on fatty acid synthesis is in agreement with the effects ascribed to it in reports, i.e. decrease in the plasma trigliceride levels in healthy subjects [26, 27] as well as in patients [28–31]. It could be effective in all hyperlipidaemia diseases that occur with an increase in VLDL levels. This could be the case for: (a) type IV [7, 32–33] where the increase in blood lipoproteins seems to be caused by an increase in fatty acid biosynthesis from glucides; (b) type III, which accumulates remnants of both chylomicrons and VLDL in the blood stream [34]. This effect arises from the presence of an underlying genetic lesion that affects the primary structure of apo E and diminishes its ability to interact with the receptor [35, 36]. In this lipoproteinemic, Packard *et al.* [37] found an inhibitory effect in the synthesis of VLDL particles, (c) type II, where it lowers plasma triglyceride levels, inducing the fall in VLDL [38–40] and (d) in type V, where the VLDL-lipids had already decreased significantly after 4 weeks [32, 41].

These reports indicate that bezafibrate induced a fall in VLDL independently of hyperlipoproteinaemic phenotype and this could be the result of its inhibitory effect on fatty acid synthesis.

The specific "location" of this inhibitory effect is unknown, but could take place on the acetil CoA carboxylase enzyme reported in kinetic experiments [42].

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