Research Letters

Proliferation of peroxisomes without simultaneous induction of the peroxisomal fatty acid β-oxidation

E. Baumgart[1], A. Völkl[1], J. Pill[2] and H.D. Fahimi[1]

[1]*Department of Anatomy and Cell Biology II, University of Heidelberg, Heidelberg* and [2]*Medical Research Department, Boehringer Mannheim, Mannheim, FRG*

Received 23 February 1990

Marked proliferation of rat hepatic peroxisomes is observed after treatment with a new potent hypolipidemic drug BM 15766, as well as after bezafibrate. Whereas the relative specific activity of the peroxisomal fatty acid β-oxidation system is not affected by BM 15766 it is significantly increased after bezafibrate. This is also confirmed by immunoblot analysis of individual β-oxidation enzymes in highly purified peroxisome fractions. These observations suggest that peroxisome proliferation and the induction of the fatty acid β-oxidation are regulated separately.

Peroxisome proliferation; Lipid β-oxidation; Hypolipidemic drug

1. INTRODUCTION

The fatty acid β-oxidation system in rat liver peroxisomes (PO) is induced by a variety of hypolipidemic drugs which simultaneously give rise to PO proliferation [1]. In most previous studies, the PO proliferation has been closely associated with marked induction of the β-oxidation of long-chain fatty acids [2]. Indeed, the distinct elevation of an 80 kDa protein, which is most probably identical with the bifunctional protein (hydratase-dehydrogenase) has been used as a specific marker for the proliferation of PO [2]. Since the carcinogenicity of PO proliferators observed in rodents has been attributed to the increased activity of the fatty acid β-oxidation system and the possible oxidative damage of DNA [3], the search for hypolipidemic drugs which do not induce the PO β-oxidation system has been intensified. In this study we present evidence that a new potent hypolipidemic drugs, BM 15766 [4], induces marked proliferation of liver PO *without* significant elevation of the β-oxidation system in highly purified PO fractions. These findings suggest that PO proliferation and the induction of the β-oxidation enzymes are regulated separately.

2. MATERIALS AND METHODS

2.1. *Animals*

Male Sprague-Dawley rats, weighing 200–250 g, kept on a standard laboratory diet and water ad libitum, were used. For biochemical and morphological studies groups of 5 animals each were administered by gastric intubation for 14 days either 50 or 75 mg/kg per day benzafibrate (2-{4-[2-(4-chlorobenzamide)ethyl]phenoxy}-2-methylpropionic acid) or 75 mg/kg per day BM 15766 (4-{2-[4-(4-chlorocinnamyl)piperazin-1-yl]ethyl}benzoic acid), suspended in methylcellulose. The control group received the corresponding amount of methylcellulose. The triglycerides and total sterol content of the serum were determined [5] in blood samples obtained from the retrooccular plexus after 7 and 14 days of treatment.

2.2. *Morphology*

Animals were anesthetized with chloralhydrate and the livers were perfused via the portal vein with a fixative containing 0.25% glutaraldehyde, 2% sucrose in 0.1 M Pipes buffer, pH 7.4. For the cytochemical visualization of PO, 80 μm tissue sections were incubated in alkaline 3,3′-diaminobenzidine [6], post-fixed with reduced OsO_4 and embedded in Epon.

2.3. *Isolation of peroxisomes (PO)*

Rat livers were perfused for 5 min with physiological saline through the portal vein. After homogenization an enriched PO fraction (D-fraction) was isolated as described before [7]. Highly purified PO fractions (>98%) were obtained by Metrizamide-gradient centrifugation [7].

2.4. *Enzyme activity determinations*

Carnitine acetyltransferase, catalase and the cyanide insensitive fatty acid β-oxidation activity were measured as described [7,8]. Protein was determined by Bradford [9] or Lowry et al. [10] procedures.

2.5. *Immunoblotting*

Antibodies against PO proteins were raised in rabbits and their specificity was assessed by immunoblotting as described before [11]. Equal amounts of protein [9] from highly purified PO fractions were subjected to SDS-PAGE (12.5% gel, 9 × 5 × 0.1 cm), using a microslab electrophoresis apparatus. After electrotransfer onto nitrocellulose, the blots were immunostained as described previously

Correspondence address: H.D. Fahimi, Institut für Anatomie und Zellbiologie (II), Im Neuenheimer Feld 307, D-6900 Heidelberg, FRG

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

[11]. The immunoblots were subjected to quantitative analysis using a television-based image analyzer [11]. The same gels used for immunoblotting were stained with silver after electrotransfer to assess the overall alterations of PO proteins [12].

3. RESULTS

Whereas no differences in body weight gains were observed, the relative liver weights were increased to 114% with BM 15766 and 145% with 50 mg/kg per day bezafibrate.

3.1. *Serum lipids*

Treatment either with BM 15766 or bezafibrate reduced the serum triglyceride and sterol levels (Table I).

3.2. *Morphology*

Marked proliferation of PO was observed in 1 μm liver sections, stained for the cytochemical localization of catalase, after both bezafibrate and BM 15766. Whereas PO proliferation was more pronounced in pericentral hepatocytes after BM 15766, it was uniformly distributed in the liver lobule in bezafibrate-treated rats (Fig. 1). By electron microscopy the proliferation of PO in all treated groups was confirmed. Moreover, clustering of enlarged angular PO with proliferation of SER was observed in pericentral hepatocytes of BM 15766-treated animals (Fig. 1c).

3.3. *Enzyme activities*

Whereas carnitine acetyltransferase activity in total liver homogenates of bezafibrate-treated rats was increased by a factor of 10.5 (controls: 9.2 ± 0.1 mU/mg protein, [10]), it showed only a minor elevation (1.4-fold) after BM 15766. The catalase activity (U/g liver) in D-fractions was increased significantly both in BM 15766- and 50 mg/kg per day bezafibrate-treated animals (Table II). Its relative specific activity (RSA) (RSA is defined as the specific enzyme activity (U/mg protein) in the enriched PO-fraction divided by that in the total homogenate), however, was slightly elevated in the BM 15766-treated group but was not significantly changed after 50 mg/kg per day bezafibrate, and was reduced by 40% in the group treated with 75 mg/kg per day bezafibrate. The cyanide insensitive fatty acid β-oxidation activities in D-fractions were increased in all experimental groups, with bezafibrate exerting a much stronger effect than BM 15766 (Table II). The corresponding RSA values, however, were significantly increased (2.2-fold) only in bezafibrate-treated animals (50 mg/kg per day), with BM 15766 showing no significant change. Whereas the ratio of activities of fatty acid β-oxidation to catalase was not significantly altered after BM 15766 it was markedly increased in bezafibrate-treated animals (Table II).

3.4. *SDS-gels and immunoblots*

Whereas the polypeptide pattern of silver-stained SDS-gels from highly purified PO fractions of control and BM 15766-treated animals was almost identical, preparations from bezafibrate-treated animals exhibited marked changes in protein composition of PO (Fig. 2). Thus, a marked increase in all β-oxidation enzyme bands and a decrease in the catalase band was noted (Fig. 2). This impression was confirmed in immunoblots and their quantitative densitometric analyses (Figs 3–5). As shown in Fig. 3, acyl-CoA oxidase was substantially increased in PO of bezafibrate-treated animals, although at different rates with respect to the individual subunits A-C. On the other hand, only subunit C of BM 15766-treated rats showed an increase with a concomitant reduction of subunits A and B. Bezafibrate treatment further increased the bifunctional protein drastically (Fig. 4), whereas the control and BM 15766 bands showed essentially the same intensity. Whereas the PO thiolase (Fig. 5) was only slightly changed with BM 15766, it was markedly increased after bezafibrate. The catalase protein was reduced more intensely after bezafibrate than BM 15766 treatment (Fig. 6).

Table I

Effects on serum lipids after 7 and 14 days of treatment with BM 15766 and bezafibrate (75 mg/kg) in comparison to control values

	Triglycerides (mg/dl)		Total sterols (mg/dl)	
	7 days	14 days	7 days	14 days
Control	146.2 ± 12.67 (100%)	155.1 ± 11.22 (100%)	80.14 ± 2.61 (100%)	82.43 ± 3.83 (100%)
BM 15766	80.9 ± 7.59 (55.3%)	79.2 ± 4.84 (51%)	33.87 ± 1.09 (42.3%)	24.44 ± 1.11 (29.6%)
Bezafibrate	59.1 ± 6.46 (40.4%)	73.4 ± 9.95 (47.3%)	56.43 ± 2.77 (70.4%)	53.71 ± 4.02 (65.2%)

$n = 5$; for all treated groups $P < 0.005$

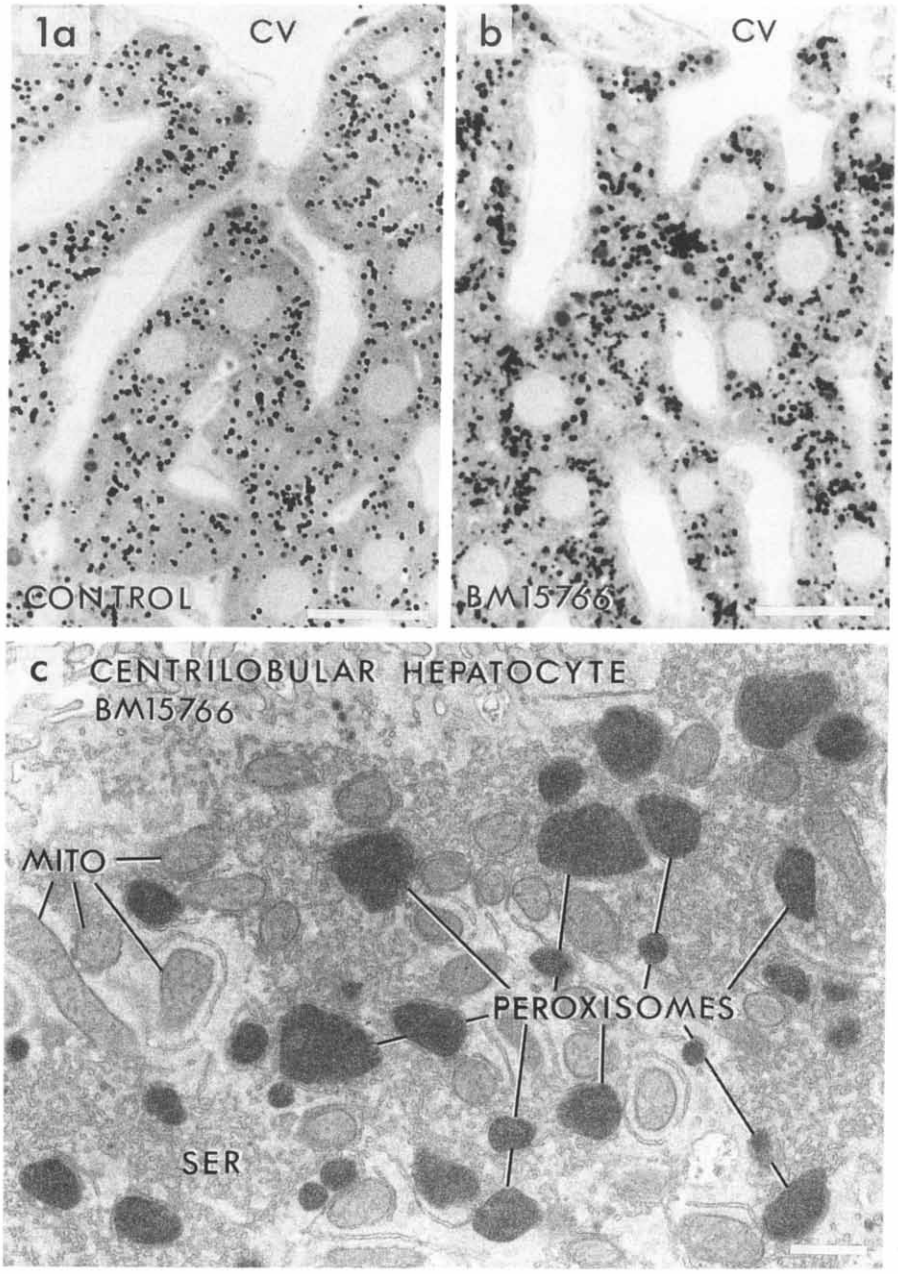

Fig. 1. Sections of rat liver stained for visualization of PO. (a) Light micrograph of a control rat with normal distribution of PO around a central vein (CV). Bar = 25 μm. (b) Liver of a rat treated with BM 15766 showing marked pericentral proliferation of PO. Bar = 25 μm. (c) Electron micrograph of a centrilobular hepatocyte from BM 15766-treated animals. Note marked proliferation of PO and smooth endoplasmic reticulum (SER). Bar = 800 nm.

4. DISCUSSION

Treatment with both bezafibrate and BM 15766 reduced significantly the serum lipids (Table I) and induced marked proliferation of PO. The proliferation of peroxisomes was also adduced by the increased activities of the PO marker enzyme catalase and the cyanide insensitive fatty acid β-oxidation system in enriched PO fractions (Table II). The corresponding RSA values, however, indicate an increase of the β-oxidation system only after bezafibrate but not with BM 15766. This is also clearly demonstrated by the ratios of the fatty acid β-oxidation activity to catalase, which was markedly elevated only after bezafibrate. These results were confirmed by SDS-PAGE and immunoblot analysis of highly purified PO fractions. All together they demonstrate that BM 15766 induces PO proliferation with only minor effects on the polypeptide composition of the organelle. This finding implies that PO proliferation is not necessarily accompanied by

Table II

Effects on peroxisomal enzymes in enriched peroxisomal fractions (D-fractions) after 14 days of treatment with BM 15766 and bezafibrate (50 mg/kg)

	Control	BM 15766	Bezafibrate
Catalase (U/g liver)	1.91 ± 0.13	2.80 ± 0.10**	3.06 ± 0.62**
β-oxidation (U/g liver)	18.41 ± 1.39	25.39 ± 1.04**	56.09 ± 8.50**
Catalase (RSA)	3.68 ± 0.29	5.29 ± 0.38*	3.07 ± 0.33
β-oxidation (RSA)	2.76 ± 0.16	3.05 ± 0.17	5.98 ± 0.38**
β-ox/Cat (RSA)	0.75	0.58	1.95**

$n = 5$; * $P < 0.05$; ** $P < 0.005$

simultaneous induction of the β-oxidation enzymes and that these two phenomena may be regulated independently. Whereas the main pharmacologic effect of BM 15766 is exerted by the competitive inhibition of the 7-DHC-Δ7-reductase in cholesterol biosynthesis [4], the exact mode of action of bezafibrate and related substances is still subject of intensive research [13]. An important enzyme system influencing the substrate flow to the PO β-oxidation is the cytochrome P450 IVA1-system. Cytochrome P450 IVA1 catalyzes the ω-hydroxylation of fatty acids and its activity is strongly increased during treatment with hypolipidemic agents [14]. The ω-hydroxyl fatty acids are further oxidized in the cytosol to dicarboxylic acids, which can only be degraded in PO and may lead to the drastic increase of

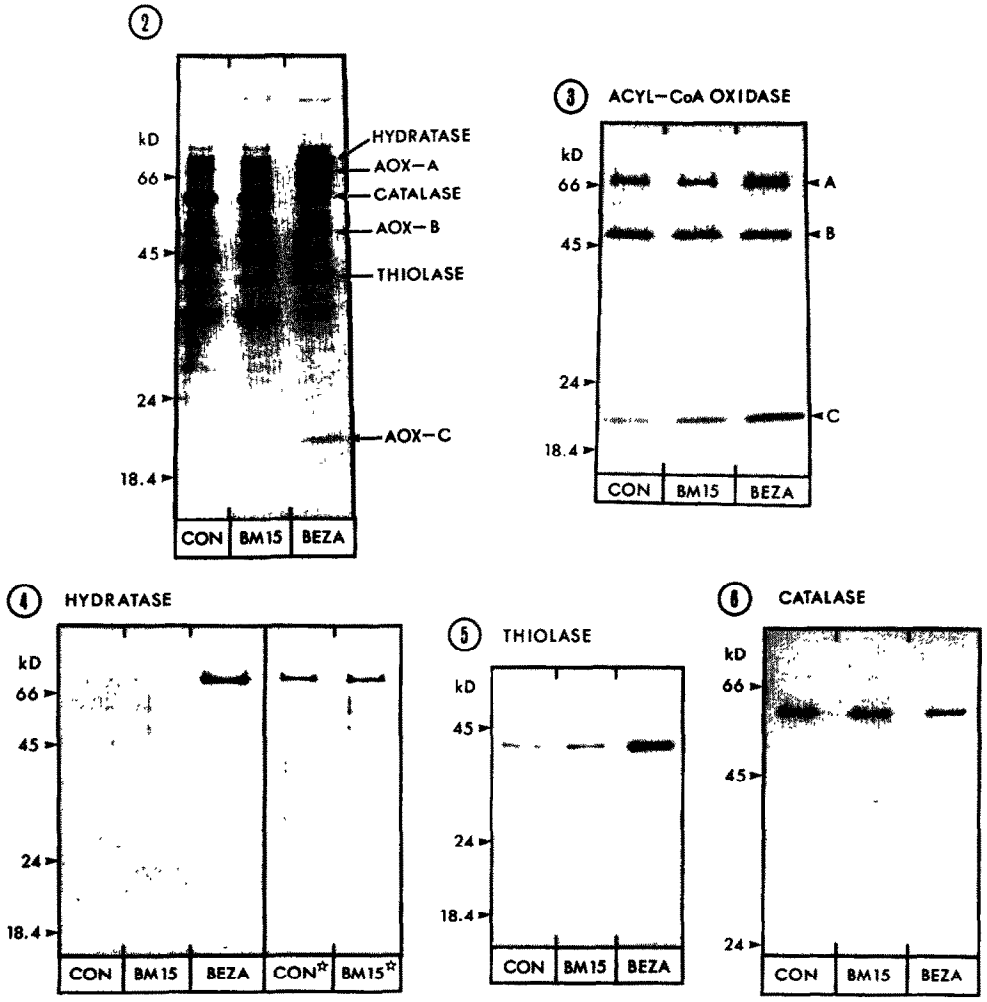

Fig. 2. SDS-PAGE of highly purified PO fractions (3 μg protein) obtained from controls, and BM 15766 as well as bezafibrate-treated rat livers. After the electrotransfer of polypeptides, the gel was stained with silver [12]. Standard molecular weight markers are indicated on the left and PO β-oxidation enzymes, as well as catalase on the right. Note the marked increase of β-oxidation polypeptides and the reduction of catalase after bezafibrate treatment.

Figs 3–6. Immunoblots of highly purified PO-fractions from control and treated animals. The densitometric values relative to control are shown in parentheses. Fig. 3: Acyl-CoA oxidase (3 μg protein). Three subunits with molecular masses of 72, 52 and 20.5 kDa (A, B and C respectively) are identified, showing increased intensities (A: 211%; B: 120%; C: 265%) in bezafibrate-treated animals. Note also the slightly more intense subunit C (175%) with concomitant decrease of subunits A (76%) and B (86%) after BM 15766 treatment. Fig. 4: Bifunctional protein (hydratase-dehydrogenase) (1.5 μg of protein at left and 4 μg protein at right – stars): Bezafibrate induced drastically this protein (20-fold), while BM 15766 showed little effect. Fig. 5: Thiolase (1.5 μg protein). Note the marked increase with bezafibrate (650%) and the minor effect of BM 15766 (130%). Fig. 6: Catalase (3 μg protein). Both treated groups show reduction with bezafibrate being more pronounced than BM 15766.

the PO β-oxidation system during fibrate treatment in rodents [14,15]. The induction of cytochrome P450 IVA1-system after treatment with hypolipidemic drugs correlates with the hepatomegaly and the increase in carnitine acetyl-transferase activity [15]. It is of interest that BM 15766 does not give rise to marked hepatomegaly or elevation of the carnitine acetyl-transferase activity, a finding which is consistent with the lack of induction of the PO β-oxidation enzymes in highly purified PO-fractions. It has been proposed that induction of PO proliferation is mediated by amphipathic carboxylic acid derivatives and that the carboxylic function is necessary for the proliferation of PO, whereas changes in the 'hydrophobic backbone' of the compounds may only vary the efficacy of PO proliferation [16]. Since BM 15766 is an amphipathic compound with a carboxylic group it seems to fulfill the requirements necessary for the induction of PO proliferation. In contrast to other PO proliferators, however, BM 15766 does not induce the PO β-oxidation enzymes. This suggests that additional factors such as interaction with other enzyme systems of liver lipid metabolism, changes in substrate flow to fatty acid or glycerolipid synthesis, and overload of the liver mitochondrial β-oxidation system with fatty acid derivatives may be necessary to induce the PO β-oxidation system. Since recent studies in our laboratory indicate that BM 15766 treatment induces an elevation of the 70 kDa integral PO membrane protein [17] without marked alterations of the β-oxidation enzymes and other PO matrix proteins, this drug could provide a suitable tool to elucidate the exact mechanism of PO proliferation and its possible relationship to hepatic carcinogenesis.

REFERENCES

[1] Lazarow, P.B. (1977) Science 197, 580–581.
[2] Reddy, J.K. and Lalwani, N.D. (1983) CRC Crit. Rev. Toxicol. 12, 1–58.
[3] Fahl, W.E., Lalwani, N.D., Watanabe, T., Goel, S.K. and Reddy, J.K. (1984) Proc. Natl. Acad. Sci. USA 81, 7827–7830.
[4] Aufenanger, J., Pill, J., Schmidt, F.H. and Stegmeier, K. (1986) Biochem. Pharmacol. 35, 911–916.
[5] Wahlefeld, A.W. (1974) in: Methoden der Enzymatischen Analyse (Bergmeyer, H.U. ed.) vol. 2, pp. 1878–1882, Verlag Chemie, Weinheim.
[6] Fahimi, H.D. (1969) J. Cell Biol. 43, 275–288.
[7] Völkl, A. and Fahimi, H.D. (1985) Eur. J. Biochem. 149, 257–265.
[8] Baumgart, E., Stegmeier, K., Schmidt, F.H. and Fahimi, H.D. (1987) Lab. Invest. 56, 554–564.
[9] Bradford, M. (1976) Analyt. Biochem. 72, 248–254.
[10] Lowry, O.H., Rosebrough, N.J., Farr, L.A. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–278.
[11] Beier, K., Völkl, A., Hashimoto, T. and Fahimi, H.D. (1988) Eur. J. Cell Biol. 46, 383–393.
[12] Gooderham, K. (1984) in: Methods in Mol. Biol. (Walker, J.M. ed.) vol. 1, pp. 113–118, Humana, Clifton, NJ.
[13] Lock, E.A., Mitchell, A.M. and Elcombe, C.R. (1989) Annu. Rev. Pharmacol. Toxicol. 29, 145–163.
[14] Sharma, R., Lake, B.G., Makowski, R., Bradshaw, T., Earnshaw, D., Dale, J.W. and Gibson, G.G. (1989) Eur. J. Biochem. 184, 69–78.
[15] Bains, S.K., Gardiner, S.M., Mannweiler, K., Gillett, D. and Gibson, G.G. (1985) Biochem. Pharmacol. 34, 3221–3229.
[16] Hertz, R., Arnon, J. and Bar-Tana, J. (1985) Biochim. Biophys. Acta 836, 192–200.
[17] Baumgart, E., Völkl, A., Hashimoto, T. and Fahimi, H.D. (1989) J. Cell Biol. 108, 2221–2231.