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Effects of benfluorex and fenofibrate treatment on mitochondrial and peroxisomal marker enzymes in rat liver

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Clofibrate (ethyl-*p*-chlorophenoxyisobutyrate) and related compounds are commonly used in the treatment of hyperlipidemia. Besides their beneficial lowering effect on plasma cholesterol and triacylglycerol levels, these drugs may produce some potentially harmful side-effects. Indeed, studies in rodents have demonstrated that clofibrate-like drugs, as well as chemically unrelated hypolipidemic compounds, induce hepatomegaly, hepatic peroxisome proliferation and hepatocarcinoma. These compounds have been labelled peroxisome proliferators and have been proposed as a new class of carcinogens (for a recent review, see ref. [1]). Following the discovery of a β-oxidation pathway for long-chain fatty acids in peroxisomes and of its dramatic stimulation after clofibrate treatment, a causal link has been proposed, but not demonstrated, between the hypolipidemic effect of peroxisome proliferators and the induction of peroxisomal β-oxidation [2]. However, it should be mentioned that these compounds also induce the proliferation of hepatic mitochondria in rats [3], and that peroxisome proliferation in the liver of treated patients is absent or low [4, 5].

Benfluorex (1-(3-trifluoromethylphenyl)-2-[*N*-(2-benoyloxyethyl)amino]propane) is a hypolipidemic agent, chemically unrelated to clofibrate, which does not induce hepatomegaly in experimental animals [6]. It is important to establish whether benfluorex belongs to the family of hypolipidemic peroxisome proliferators or not. In the study presented here this question was approached biochemically, i.e. through measurements of marker enzyme activities specific for peroxisomes and also for mitochondria. This approach rests on the assumption that organelle proliferation should be paralleled by increases in the activity of one or more specific marker enzymes. Benfluorex treatment was compared to treatment with fenofibrate (isopropyl-[4-(*p*-chlorobenzoyl)2-phenoxy-2-methyl]propionate), a powerful clofibrate-like hypolipidemic drug.

Materials and methods

Treatment of animals. Benfluorex (25 or 50 mg/kg) or fenofibrate (25 mg/kg) was given by tube feeding for ten consecutive days to male Wistar rats (starting weight, 100 g). The drugs were suspended (0.25%, w/v) in arabic gum (20%, w/v in H_2O). Control animals received the vehicle only. All animals had free access to food and water.

At 25–50 mg/kg, benfluorex lowers serum triacylglycerols by 40–90% in rats maintained on a high-fat or high-carbohydrate diet, whereas serum cholesterol does not decrease under these conditions [6]. At 25 mg/kg, fenofibrate slightly lowers total serum lipids and cholesterol in the normal rat, but is not, or is less, effective in the hyperlipidemic rat [7].

Liver homogenates and assays. Animals were killed by cervical dislocation. The liver was removed and weighed. A portion was homogenized in 3 vols. of 0.25 M sucrose–3 mM imidazole (pH 7.4)–1 mM EDTA (pH 7.4)–0.1% ethanol. Another portion was freeze-clamped and subsequently homogenized in 0.06 N HCl for glycogen determination [8]. The following parameters were measured on sucrose homogenates: protein [9], total carnitine [10], catalase [11], urate oxidase [12], carnitine acetyl- and palmitoyltransferase [13], glutamate dehydrogenase [14], acyl-CoA oxidase [15, 16], mitochondrial and peroxisomal [17] β-oxidation of long-chain fatty acids. Enzyme activities are reported in the units used by the authors mentioned. Minor changes introduced by the authors have been reported in detail elsewhere [14, 17].

Table 1. Effects of benfluorex and fenofibrate on liver parameters

Liver parameters	Control	Benfluorex (25 mg/kg)	Fenofibrate (25 mg/kg)
Body weight, g	144 ± 3	133 ± 3* (92.4)	150 ± 2 (104.2)
Liver weight, g	6.64 ± 0.16	5.93 ± 0.18* (89.3)	9.73 ± 0.16* (146.5)
Liver weight, g/100 g rat	4.63 ± 0.05	4.44 ± 0.07 (95.9)	6.49 ± 0.09* (140.2)
Protein, mg/100 g rat	922 ± 20	920 ± 26 (99.8)	1470 ± 31* (159.4)
Glycogen, μmole glucose/100 g rat	1637 ± 69	1364 ± 56* (83.3)	1495 ± 42 (91.3)
Carnitine, μmole/100 g rat	1.78 ± 0.14	1.29 ± 0.05* (72.5)	9.79 ± 0.54* (550.0)
Glutamate dehydrogenase, U/100 g rat	1067 ± 20	1067 ± 32 (100.0)	1716 ± 55* (160.8)
Carnitine palmityltransferase, U/100 g rat	13.2 ± 0.5	14.6 ± 1.0 (110.6)	42.3 ± 4.0* (320.5)
Carnitine acetyltransferase, U/100 g rat	2.91 ± 0.19	3.24 ± 0.25 (111.3)	64.3 ± 8.8* (2210.0)
Mitochondrial β-oxidation, U/100 g rat	1.59 ± 0.11	1.68 ± 0.06 (105.7)	4.03 ± 0.20* (253.5)
Catalase, U/100 g rat	293 ± 12	334 ± 10* (114.0)	624 ± 21* (213.0)
Urate oxidase, U/100 g rat	15.2 ± 1.2	17.5 ± 0.5 (115.1)	25.3 ± 1.3* (166.4)
Acyl-CoA oxidase, U/100 g rat	3.59 ± 0.32	3.73 ± 0.20 (103.9)	75.1 ± 3.3* (2091.9)
Peroxisomal β-oxidation, U/100 g rat	1.62 ± 0.07	1.73 ± 0.08 (106.8)	15.5 ± 0.9* (956.8)

Results are mean ± S.E.M. for nine rats in each group. Statistical significance is indicated as: *$P < 0.05$ by Dunnett's *t*-test [18]. Results expressed as percent of control are given in parentheses. Three rats were also treated with benfluorex, 50 mg/kg. Results (not shown) were nearly identical to those obtained at 25 mg/kg.

Reagents. All chemicals used were of analytical grade. Enzymes and coenzymes were from Boehringer Mannheim (F.R.G.).

Results and discussion

Body and liver weight; hepatic protein, glycogen and carnitine content. Table 1 summarizes the results of the study. Compared to controls, benfluorex-treated rats had lower body weights at the end of 10 days of treatment (−7.6%). Liver weight, expressed in g/100 g body weight, was slightly lower after benfluorex (−4%) and, as expected, considerably higher (+40%) after fenofibrate treatment. In order to take these changes in body and liver weight into account, all subsequent results are expressed per 100 g body weight. Hepatic protein content was unaffected by benfluroex, whereas fenofibrate induced a 60% increase. Glycogen and carnitine contents were slightly lowered in benfluorex-treated animals, possibly reflecting the anorexigenic effect. Fenofibrate did not alter hepatic glycogen content but resulted in a more than 5-fold increase in carnitine content. In summary, the drugs under study affected these general hepatic parameters in essentially opposite directions: the effects of benfluorex can possibly be attributed to its moderate and transient anorexigenic effect, whereas, in sharp contrast, fenofibrate displayed hepatotrophic effects, typical of clofibrate-like drugs.

Mitochondrial marker enzymes, mitochondrial β-oxidation. Glutamate dehydrogenase and carnitine palmityltransferase are classical marker enzymes for mitochondria. Carnitine acetyltransferase is present both in mitochondria and peroxisomes. Fatty acid oxidation can be measured under conditions that specifically discriminate for the mitochondrial pathway. This measurement can therefore also be considered as a marker for mitochondria. Benfluorex treatment did not affect the mitochondrial enzyme activities whereas fenofibrate induced increases in all four parameters, as expected for a clofibrate-like compound.

Peroxisomal marker enzymes, peroxisomal β-oxidation. Catalase and urate oxidase are classical marker enzymes for peroxisomes. Acyl-CoA oxidase, the first enzyme of the peroxisomal β-oxidation sequence, is unique to this metabolic pathway and displays all the required properties of a marker enzyme for peroxisomes. In parallel to the situation described for mitochondria, peroxisomal β-oxidation can also be assayed specifically in whole liver homogenates and was therefore included as a marker system for peroxisomes. Benfluorex treatment resulted in a 14% increase in catalase activity, whereas all the other peroxisomal parameters remained unaffected. The effects of fenofibrate confirmed what was expected for a typical peroxisome proliferator: all parameters were increased, especially those reflecting the peroxisomal fatty acid oxidation pathway.

Summary and conclusion

Our results demonstrate that benfluorex at doses that are strongly hypotriglyceridemic does not increase hepatic peroxisomal enzyme activities, whereas fenofibrate at doses that are only slightly hypolipidemic induces a dramatic increase in the activity of these enzymes. Thus, the biochemical approach used in this study reveals that the hypolipidemic drug benfluorex does not belong to the class of hypolipidemic compounds known to induce hepatomegaly, hepatic peroxisome proliferation and hepatocarcinoma in rodents. Morphological studies should confirm the absence of peroxisomal induction.

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Reaction of 4-substituted phenols with benzidine in a peroxidase system

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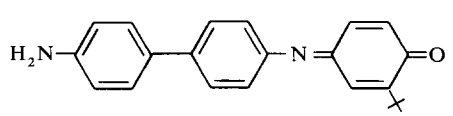
Benzidine and its derivatives are readily oxidized by peroxidases [1, 2]. The oxidation proceeds via a one-electron oxidized intermediate (free radical) which is further oxidized to a quinone-diimine. The oxidation of benzidine by peroxidases generates reactive electrophilic species, which bind to protein and DNA [3] and form glutathione adducts [4]. The peroxidase activity of prostaglandin synthase supports benzidine oxidation [5, 6] and may be involved in benzidine-induced human bladder carcinogenesis.

The reactive intermediate generated in the benzidine/peroxidase/H_2O_2 system may be chemically trapped by the antioxidant 2(3)-*tert*-butyl-*para*-hydroxyanisole (BHA). Thus, when BHA is included in such a system, the yellow color characteristic of the quinone-diimine is not seen; instead, a pink color forms [1]. No such reaction was observed with 2,6-di-*tert*-butyl-*para*-hydroxytoluene (BHT). Phenol reacts similarly to BHA and gives a pink benzidine/phenol adduct [7]. This adduct has been isolated and characterized by NMR and mass spectrometry. The structure of the product is analogous to that of the synthetic dye indoaniline, and results from addition of a benzidine N atom to the phenol ring at the position *para* to the hydroxyl group. Coupling occurred exclusively at the *para* position; no "ortho adduct" was detected. Therefore, the reaction with BHA (in which the *para* position is occupied) demands explanation. In this report, we show that the methoxy group is cleaved from the phenol ring during the trapping reaction.

The published procedure for synthesis of the benzidine/phenol adducts [7] was used. Benzidine, horseradish peroxidase (donor: H_2O_2 oxidoreductase EC 1.11.1.7), and the substituted phenol were mixed in 2 ml acetate buffer, pH 5. The reaction was initiated with H_2O_2. The concentrations of benzidine, phenol, and H_2O_2 were all 250 μM, and peroxidase was 50 μg/ml. After complete development of the color the product was extracted with 5 ml ethyl acetate. TLC was performed on silica gel plates in ether (100%) or $CHCl_3$/ethyl acetate (80 : 20).

The oxidation of benzidine by the horseradish peroxidase/H_2O_2 system yields the yellow quinone-diimine, which slowly decays to the poorly characterized polymeric precipitate "benzidine brown" [8]. In the presence of equimolar phenol, the pink-coloured benzidine/phenol adduct is formed [7]. The behaviour observed with *para*-methoxyphenol was similar to that reported for phenol itself, and the resulting adduct was identical. This was proven by co-chromatography of the *para*-methoxyphenol and phenol adducts in both TLC solvent systems, and identical optical and mass spectra. Thus, the methoxy group must be cleaved from the phenol ring during the reaction. To shed light on this unexpected mechanism, we studied a series of commercially available 4-substituted phenols. Organic-extractable colored adducts were obtained with (in order of decreasing yield) *para*-methoxyphenol, BHA, *para*-fluorophenol, phenol, *para*-chlorophenol, *para*-bromophenol, and *para*-hydroxybenzoic acid. In each case, the adduct was chromatographically identical to the benzidine/phenol adduct. In contrast, *para*-methylphenol (*para*-cresol) and *para*-nitrophenol gave no isolable products and did not prevent "benzidine brown" formation.

BHA (obtained from Sigma) gave one major pink product and numerous other bands (pink to purple colored). BHA is a mixture of 2- and 3-*tert*-butyl substituted isomers, and TLC examination of the commercial material revealed several minor components. Presumably, this accounts for the variety of adducts obtained. The major product was recovered from the TLC plate and submitted for mass spectrometry. The product gave a strong molecular ion (base peak, at m/e = 330, as expected for the structure:



Also present were 331 (^{13}C – parent; relative intensity = 24.8%) and 332 (18.3%). The M + 2 peak is commonly obtained with quinonoid compounds and corresponds to the reduced form of the quinone ring [9].