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SPECIES DIFFERENCES IN THE EFFECTS OF BEZAFIBRATE, A HYPOLIPIDEMIC AGENT, ON HEPATIC PEROXISOME-ASSOCIATED ENZYMES

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Abstract—The effects of bezafibrate on hepatic peroxisome-associated enzymes of rats, mice, guinea pigs, hamsters, rabbits, dogs and monkeys were examined. Dogs and monkeys were given bezafibrate orally at 30 mg/kg body wt daily for 2 weeks and at 125 mg/kg body wt daily for 13 weeks, respectively, and other species at 100 mg/kg daily for 2 weeks. In male rats, marked changes were observed in the activities of catalase (1.73-fold), D-amino acid oxidase (DAAO; 0.56-fold), fatty acyl-CoA oxidizing system (FAOS; 12.9-fold) and carnitine acetyltransferase (CAT; 35.8-fold); in female rats, the changes were less than in the males. In mice, there were no apparent sex differences in the responses of hepatic peroxisomal enzymes to bezafibrate and the increases in the activities of catalase, FAOS and CAT were 1.76-, 3.75- and 7.94-fold respectively. In guinea pigs, only slight increases in the activities of FAOS (3.00-fold) and CAT (2.83-fold) were observed. In hamsters, the increases in catalase, FAOS and CAT activities, were 1.23-, 2.19- and 2.77-fold respectively. Although rabbits and dogs showed slight increases in CAT activity, no significant response to the drug was observed in monkeys. Hepatomegaly and the increase of hepatic content of peroxisome proliferation-associated polypeptide (PPA-80), which has been recognized as a peroxisomal bifunctional protein in the fatty acid β-oxidation pathway, were observed only in rats and mice. These results show that there were marked species differences in the effects of bezafibrate on hepatic peroxisomes, and that bezafibrate induced hepatic peroxisome proliferation in rodents, especially rats and mice.

It is well known that various hypolipidemic drugs, such as clofibrate, fenofibrate, nafenopin, Wy-14,643 and tibric acid, induce marked changes in the livers of rats and mice. The initial hepatic responses in rodents are: (1) hepatomegaly, (2) proliferation of the smooth endoplasmic reticulum, and (3) marked proliferation of peroxisomes in association with changes in peroxisome structure and enzyme composition [1–4]. Furthermore, since the finding that certain hypolipidemic peroxisome proliferators induce hepatocellular carcinomas in both mice and rats, the relationships between peroxisome proliferation and hepatocarcinogenicity of these drugs have become extremely important [5–7]. However, it is as yet not established whether there are direct relationships among pharmacological action, peroxisome proliferation and carcinogenicity. It appears, moreover, that there are species differences in hepatic responses to hypolipidemic peroxisome proliferators [8–10]. This has led to doubt as to the relevance of the rodent carcinogenicity findings to humans. There have been a few reports concerning species differences in the hepatic response to hypolipidemic peroxisome proliferators, such as Ly171883 [11], ciprofibrate [12] and gemfibrozyl [13] but there have been no systematic studies of species differences in the response to bezafibrate. We have examined in detail the species differences in the effects of bezafibrate as a hypolipidemic peroxisome proliferator using rats, mice, guinea pigs, hamsters, rabbits, dogs and monkeys.

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MATERIALS AND METHODS

Materials. Bezafibrate was from the Kissei Pharmaceutical Co., Japan. L-Carnitine–HCl was provided by the Earth Pharmaceutical Co., Japan. Acetyl-CoA, NAD, palmitoyl-CoA and bovine serum albumin (BSA, fatty acid free) were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Assay kits for serum triglyceride and cholesterol were obtained from the Daiichi Pure Chemicals Co. Ltd., Japan. Other chemicals, all of reagent grade, were obtained from Wako Pure Chemicals, Japan.

Animals and treatment. Animals used in these experiments were as follows: male and female Wistar rats (10 weeks old), male and female ICR mice (10 weeks old), male Hartley guinea pigs (about 300 g body wt), male Japanese white rabbits (about 3 kg), male beagle dogs (about 10 kg), male golden hamsters (about 80 g), and male and female rhesus monkeys (about 4 kg). These animals were obtained from the Tokyo Laboratory Animals Co. Ltd., Japan. The animals other than dogs and monkeys were treated once a day for 2 weeks with 10 ml/kg body weight of 10 mg/ml of bezafibrate, prepared as a suspension in 0.5% (w/v) methylcellulose. Dogs were treated orally, with the drug in gelatin capsules at 30 mg/kg daily for 2 weeks and monkeys were orally treated

with 125 mg/kg daily (4 ml/kg body wt) for 13 weeks. As described above, hepatocarcinogenesis in the rodent after long-term administration of a hypolipidemic peroxisome proliferator is an important factor in the evaluation of the safety of such drugs for primates, including humans. Thus, in this experiment we have elected to use a dose level of 125 mg/kg and a treatment period of 13 weeks, in order to examine the long-term effect on hepatic peroxisomes and also whether hepatocarcinogenesis would have been produced.

The animals were weighed. Rodents were decapitated whereas rabbits, dogs and monkeys were killed with i.v. barbiturate. The livers were perfused and removed, and then 10% (w/v) homogenates were prepared in 0.25 M sucrose. A part of the homogenate was centrifuged at 3,300 *g* for 10 min and subsequently at 12,500 *g* for 20 min; the resulting pellet was suspended in 0.25 M sucrose at 1 ml/0.1 g original tissue. This light mitochondrial fraction (LM fraction) was used for electrophoretic analysis of hepatic protein composition. Sera obtained from the blood drawn at sacrifice were used for the determination of triglyceride and cholesterol contents.

Assay methods. The activity of the cyanide-insensitive fatty acyl-CoA oxidizing system (FAOS) was determined by measuring the palmitoyl-CoA dependent reduction of NAD at 340 nm [14]. One unit of the activity was defined as the amount of the enzyme that reduced 1 nmol of NAD/min. The activity of carnitine acetyltransferase (CAT) was determined spectrophotometrically by measuring the amount of CoA-SH released during the reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) at 412 nm [15]. One unit of the enzyme activity was defined as the amount of the enzyme that produced 1 nmol of CoA-SH from acetyl-CoA per min. Activities of other peroxisome-associated enzymes such as catalase, urate oxidase and D-amino acid oxidase (DAAO) were determined according to a previous report [16].

Hepatic triglyceride levels were assayed by the Van Handel–Kawade method with a slight modification [17]. Hepatic cholesterol levels were assayed by the method of Kitamura [18]. Serum cholesterol and triglyceride were assayed enzymatically using Autosera CHO-2 and Autosera TG (Daiichi Pure Chemicals Co. Ltd.) respectively. Protein concentration was determined by the method of Lowry *et al.* [19], with BSA as a standard. Statistical evaluations were performed by Student's *t*-test.

RESULTS

There was no significant difference in body weight gain between control and bezafibrate-treated groups of all species throughout these studies (data not shown). Tables 1 and 2 show some biochemical values and the activities of hepatic peroxisome-associated enzymes of control animals respectively. We used male and female animals in the case of rats, mice and monkeys. No significant sex differences in the biochemical values and the peroxisomal enzyme activities of control animals could be found. However, the species differences in lipid levels in liver and serum were marked. In guinea pigs, the lipid levels of the liver and serum were markedly lower than those of other rodents. Except for liver cholesterol contents, the biochemical values for dogs and monkeys resembled each other and, when compared with rodents, the serum cholesterol levels were higher whereas the triglyceride levels were lower. These results show that biochemical values for the control animals, dogs and monkeys were quite similar. In the case of monkeys, after 13 weeks of treatment no production of hepatocarcinogenesis or related changes was observed. As shown in Table 2, there were species differences in the activities of hepatic peroxisomal enzymes. Activity of DAAO was not detectable in mice, rabbits or monkeys, and urate oxidase was not detectable in monkeys. The variations in the activity of catalase were relatively small (30.4 to 113.0 units/g liver), except in the case of rabbits. The activities of FAOS and CAT, which are related to fatty acid metabolism, showed a marked variation depending upon the species. The results of the experiments on the effects of bezafibrate on biochemical value and hepatic peroxisomal enzymes are shown in Tables 3 and 4. After 2 weeks of bezafibrate administration significant hepatomegaly in male rats (45% increase), male mice (44%), and female mice (22%) was observed, whereas no significant changes in relative liver weights were observed in the other species. Although significant decreases in serum triglyceride levels were observed in rats of both sexes (31–41% of control), dogs (40%), rabbits (53%), and male monkeys (37%), serum cholesterol levels decreased only in male rats (71%) and dogs (59%). These results show that bezafibrate had a hypotriglyceridemic action, especially in normolipemic rats, rabbits, monkeys and dogs, and a strong hypocholesterolemic action in rats and dogs at the dose level used in these experiments. Concerning liver lipid levels, a marked decrease was observed in mice and monkeys, whereas in guinea pigs increases of liver cholesterol (47%) and triglyceride (267%) were observed. Table 4 shows the response of hepatic peroxisomal enzymes to bezafibrate after a 2-week administration. In male rats, marked changes were observed in the activities of catalase (1.73-fold), DAAO (0.54-fold), FAOS (12.9-fold) and CAT (35.8-fold). In female rats the changes were less, with no significant increase in catalase activity having been observed, indicating a sex difference in the response of hepatic peroxisome-associated enzymes of rats to bezafibrate. In mice, although a significant sex difference was not seen in the hepatic response, the increases in the activities of catalase, FAOS and CAT were 1.76-, 3.75- and 7.94-fold, respectively, in male mice. In guinea pigs, the increases in FAOS and CAT activities were 3.00- and 2.83-fold respectively. In hamsters, the increases in catalase, FAOS and CAT were 1.23-, 2.19- and 2.77-fold respectively. Although rabbits and dogs showed a slight increase in CAT activity, no significant response to the drug was observed in monkeys. The increase in hepatic content of peroxisome proliferation-associated polypeptide (PPA-80), which has been identified as a bifunctional protein in the peroxisomal β-oxidation system [20], was observed only in rats and mice by analyzing hepatic protein composition using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Table 1. Biochemical values of control animals

Animals	Liver weight (% of body wt)	Liver lipid (mg/g) Cholesterol	Liver lipid (mg/g) Triglyceride	Serum lipid (mg/dl) Cholesterol	Serum lipid (mg/dl) Triglyceride	Liver protein (mg/g)
Rats, Male	3.8 ± 0.2	4.8 ± 0.4	5.8 ± 0.9	60 ± 4	104 ± 32	214 ± 26
Female	3.5 ± 0.1	4.7 ± 0.4	6.9 ± 0.7	68 ± 1	127 ± 22	288 ± 9
Mice, Male	4.8 ± 0.1	5.7 ± 0.4	5.7 ± 0.4	114 ± 21	110 ± 45	207 ± 7
Female	4.9 ± 0.5	5.2 ± 0.6	6.0 ± 1.1	81 ± 15	99 ± 32	224 ± 11
Guinea pigs	6.4 ± 0.8	2.9 ± 0.3	1.3 ± 0.6	40 ± 12	23 ± 5	178 ± 13
Hamsters	4.0 ± 0.3	4.6 ± 0.4	6.1 ± 0.6	164 ± 27	280 ± 21	227 ± 10
Rabbits	2.8 ± 0.1	3.4 ± 0.2	11.7 ± 2.7	33 ± 7	59 ± 22	175 ± 6
Dogs	2.9 ± 0.3	3.8 ± 0.3	9.9 ± 0.8	153 ± 33	30 ± 7	180 ± 5
Rhesus monkeys						
Male	2.1 ± 0.1	14.0 ± 0.8	7.8 ± 1.3	195 ± 20	45 ± 5	247 ± 10
Female	2.3 ± 0.1	14.0 ± 0.8	9.0 ± 3.1	171 ± 6	37 ± 5	257 ± 7

Data are means ± SD, N = 5.

Table 2. Activities of peroxisomal enzymes of control animals

Animals	Activity (units/g liver) Catalase	DAAO	Urate oxidase	FAOS	CAT
Rats, Male	42.9 ± 4.0	1.79 ± 0.25	4.35 ± 1.05	211 ± 54	355 ± 74
Female	30.4 ± 2.3	1.89 ± 0.26	5.01 ± 1.50	247 ± 29	527 ± 24
Mice, Male	58.3 ± 11.9	ND*	4.68 ± 1.62	1886 ± 222	520 ± 150
Female	48.9 ± 9.1	ND	3.81 ± 0.90	1950 ± 129	632 ± 99
Guinea pigs	113.0 ± 13.1	0.62 ± 0.01	1.30 ± 0.10	29 ± 3	816 ± 160
Hamsters	80.2 ± 10.4	1.75 ± 0.12	3.49 ± 0.30	1437 ± 196	2955 ± 575
Rabbits	15.9 ± 2.1	ND	0.94 ± 0.36	152 ± 50	1542 ± 530
Dogs	61.1 ± 4.1	5.43 ± 2.14	2.91 ± 1.03	414 ± 53	1265 ± 203
Rhesus monkeys					
Male	73.0 ± 5.0	ND	ND	30 ± 6	5697 ± 1336
Female	67.7 ± 5.3	ND	ND	35 ± 4	5456 ± 751

Data are means ± SD of five animals.
* Not detectable.

Table 3. Effects of bezafibrate on liver weight, protein content and lipid levels*

Animals	Relative value (%) Liver weight	Liver lipid Chol	Liver lipid TG	Serum lipid Chol	Serum lipid TG	Liver protein
Rats, Male	145†	96	105	71†	31†	135†
Female	109	106	109	99	41†	104
Mice, Male	144†	86†	64†	85	112	102
Female	122‡	87‡	56†	111	72	94
Guinea pigs	88	147‡	267‡	88	174	111
Hamsters	88	99	103	99	86	98
Rabbits	107	90	94	109	53‡	99
Dogs	90	112	97	59†	40†	109
Rhesus monkeys						
Male	105	85‡	69‡	111	73‡	104
Female	106	82	43‡	115	97	93‡

* Data are presented as values relative to control. Abbreviations: Chol, cholesterol; and TG, triglyceride.
†‡ Statistical significance: † $P < 0.01$, and ‡ $P < 0.05$ vs control.
See Table 1 for control values.

Table 4. Effects of bezafibrate on hepatic enzyme activities*

Animals	Activity (units/g liver)				
	Catalase	DAAO	Urate oxidase	FAOS	CAT
Rats, Male	74.3 ± 8.1†	1.00 ± 0.11†	3.09 ± 1.05	2,722 ± 146†	12,705 ± 4,087†
Female	31.9 ± 6.5	1.44 ± 0.20†	5.21 ± 0.53	535 ± 89†	4,096 ± 853†
Mice, Male	102.7 ± 13.8†	ND§	6.15 ± 0.63	7,068 ± 772†	4,127 ± 896†
Female	89.0 ± 9.1	ND	5.36 ± 0.70‡	6,520 ± 1,472†	4,929 ± 1,100†
Guinea pigs	95.0 ± 23.0	0.64 ± 0.13	1.28 ± 0.30	86 ± 30†	2,308 ± 693†
Hamsters	98.9 ± 8.1‡	1.91 ± 0.12	3.25 ± 0.38	3,151 ± 476†	8,184 ± 2,626†
Rabbits	16.1 ± 1.3	ND	0.84 ± 0.21	179 ± 19	3,539 ± 730‡
Dogs	50.9 ± 5.1‡	4.04 ± 0.78	2.29 ± 1.39	484 ± 99	2,098 ± 289†
Rhesus monkeys					
Male	76.2 ± 13.1	ND	ND	43 ± 3	7,195 ± 849
Female	73.4 ± 15.1	ND	ND	41 ± 5	6,757 ± 1,347

* Data are presented as means ± SD, N = 5.
†‡ Statistical significance: † $P < 0.01$, and ‡ $P < 0.05$ vs control.
See Table 2 for control values.
§ Not determined.

DISCUSSION

Several studies have established that certain peroxisome proliferators, when chronically administered in diets, induce hepatocellular tumors in both mice and rats [5–7, 21, 22]. Thus, it is important in the development of new drugs to evaluate the safety of such drugs. Even now, however, the relationships among hepatic peroxisome proliferation, hypolipidemic effect and hepatocarcinogenesis have not been elucidated. These hypolipidemic drugs would be used clinically over a long time for the treatment of hyperlipemic conditions. Thus, the finding of hepatocarcinogenicity of these drugs in rats and mice was remarkably important. This led us to raise the question as to whether these findings in rats and mice could be extrapolated to humans. Concerning hepatocarcinogenesis induced by these drugs, it has been hypothesized that the induction of hepatocellular tumors in hypolipidemic drug-treated animals is related biologically to active products, such as hydrogen peroxide, produced by proliferated peroxisome populations associated with marked enhancement of the activity of oxidases in peroxisomes, such as fatty acyl-CoA oxidase, rather than to direct drug effects [23, 24]. Considering the possible mechanisms of hepatocarcinogenesis by these drugs, studies concerning their effects on the activities of hepatic peroxisome-associated enzymes are a pressing need. Another important problem is species differences. The effects of hypolipidemic drugs on hepatic peroxisomes have been studied in some laboratories using some drugs and animals; those results are summarized by Cohen and Grasso [25]. Our present study shows clearly a species difference in the response of hepatic peroxisomal enzymes to bezafibrate. The administration of bezafibrate for 2 weeks (and 13 weeks in the case of monkeys) caused an increase in the relative liver weight, i.e. hepatomegaly, which is a typical finding after treatment with hypolipidemic peroxisome proliferators, only in male rats and mice. Marked changes in the activities of peroxisome-associated enzymes were found in other species. Although actinomycin D suppresses peroxisome proliferation by clofibrate, hepatomegaly in rats treated with clofibrate has been observed [9]. These results suggest that hepatic peroxisome proliferation by hypolipidemic drugs is not closely related to hepatomegaly, although this may be one of many factors contributing to an increase in hepatic weight. The effect of bezafibrate on FAOS and CAT activities was marked in rodents, such as rats, mice, guinea pigs and hamsters. Among them, the response of male rats was marked (FAOS: 12.9-fold; CAT: 35.8-fold). On the other hand, animals other than rodents showed only slight increases in CAT activity, and no changes in the activities of hepatic peroxisome-associated enzymes were found in the monkey. These results indicate that the effects of bezafibrate on hepatic peroxisomes of rabbits, dogs and monkeys are extremely weak, compared with rodents. In addition, other experiments concerning changes in hepatic hydrogen peroxide content during long-term (29 weeks) treatment of rats with bezafibrate showed that, although FAOS activity increased more than 10-fold during the initial period, the hepatic content of hydrogen peroxide was less than 1.5-fold throughout the experiment (manuscript in preparation).

Considering these experimental results, the effects of hypolipidemic peroxisome proliferators on hepatic peroxisomes may be species dependent, and extrapolaton of the experimental results obtained from rodents to primates, such as the human, may be questionable. Furthermore, data from clinical studies indicate that the human liver does not respond to hypolipidemic agents with peroxisome proliferation [26–28]. Because fixed doses of drugs were used in the present study, the possibility that the effects of the drugs on animals other than rodents would be stronger when higher dose levels were used cannot be excluded. Thus, although definitive statements regarding the effect of bezafibrate on human liver peroxisomes cannot be made, it is possible that the response will not occur in humans since the anti-

cipated clinical dose is 3- to 10-fold less than the dose used in this experiment with dogs and rhesus monkeys [29, 30].

Although there is a marked species difference in induction of peroxisomes, its molecular mechanism has not been established. The following possible mechanisms have been considered: (1) presence of a receptor for drugs, (2) relation to second messenger, and (3) levels of cofactors in the liver. The presence of a receptor for peroxisome proliferators has already been shown [25]. The species specificity in hepatic response to hypolipidemic peroxisome proliferators would be related to the nature of such receptors.

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