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EFFECT OF HYPOLIPIDEMIC COMPOUNDS ON LAURIC ACID HYDROXYLATION AND PHASE II ENZYMES

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Abstract—Treatment of male Fischer 344 rats with various hypolipidemic drugs of different peroxisome proliferating potency (1-benzylimidazole, acetylsalicylic acid, clofibrate, tiadenol) led to an induction of liver lauric acid hydroxylase, whereas probucol, which is not a peroxisome proliferator, did not induce this enzyme.

Activity of bilirubin UDP-glucuronosyltransferase was increased by all the compounds tested. The highest increase was observed after treatment with acetylsalicylic acid (2.3-fold).

High correlation ($r = 0.953$) was observed between the activities of lauric acid hydroxylase and the corresponding activities of cytosolic epoxide hydrolase reported previously.

The amount of microsomal expoxide hydrolase was not changed by any of the compounds.

Whereas clofibrate and tiadenol decreased glutathione *S*-transferase activity with 1-chloro-2,4-dinitrobenzene as substrate, 1-benzylimidazole and probucol increased this activity. With 4-hydroxynonenal as a substrate qualitatively the same results were obtained with the exception that probucol did not affect the enzyme activity. When glutathione *S*-transferase activity was measured with *cis*-stilbene oxide as substrate only the more than five-fold increase after treatment with 1-benzylimidazole was significantly different from control values.

Activity of dihydrodiol dehydrogenase was increased after treatment of rats with 1-benzylimidazole (1.5-fold), whereas application of tiadenol led to a decrease of enzyme activity.

Feeding of male guinea pigs with clofibrate did not change the activity of peroxisomal β-oxidation, cytosolic epoxide hydrolase or lauric acid hydroxylase. However, treatment with tiadenol caused an increase of these activities.

Hypolipidemic agents are used in clinical therapy to reduce elevated serum lipids. However, most of them have been shown to give rise to peroxisome proliferation in rodents, and all peroxisome proliferators tested so far in long-term studies have been found to induce hepatocellular carcinomas in rats and mice [1]. As they have no mutagenic activity [2] and do not bind covalently to DNA [3, 4] hypolipidemic peroxisome proliferators are considered a novel class of chemical carcinogens [5].

In addition to proliferation of peroxisomes, administration of these compounds to rats or mice led also to liver enlargement and proliferation of endoplasmic reticulum [6]. Concurrently, several enzyme activities are affected, most of them being associated with lipid metabolism (e.g. palmitoyl CoA oxidase, carnitine acyltransferase, palmitoyl CoA hydrolase [6, 7]. Furthermore, activities of drug metabolizing enzymes are changed [8, 9]. We have previously shown that a concomitant induction of rat liver cytosolic epoxide hydrolase with peroxisomal β-oxidation occurred after treatment of rats with hypolipidemic compounds of differing peroxisome proliferating potency and action on serum lipids [10]. In order to examine more comprehensively the effect of these compounds on drug metabolizing enzymes, we have now extended our previous study to lauric acid hydroxylase, UDP-glucuronosyl-transferase, glutathione *S*-transferases and dihydrodiol dehydrogenase.

It has been shown that clofibrate and other peroxisome proliferators induce a special form of cytochrome P-450 [11, 12]. The induction of this cytochrome P-450 isoenzyme is reflected by an increase of lauric acid hydroxylation [13]. This enzyme also hydroxylates arachidonic acid [12, 14].

UDP-glucuronosyltransferases are a family of membrane-bound enzymes, which catalyze the conjugation of endogenous as well as exogenous compounds with glucuronic acid [15]. Clofibrate specifically induced bilirubin glucuronidation, whereas activities of 3-methylcholanthrene and phenobarbitone inducible isoenzymes were not increased [9].

Another enzyme family involved in the conjugation of electrophilic xenobiotics are the glutathione *S*-transferases. Treatment of rats with clofibrate and some other peroxisome proliferators lowered their specific activity when 1-chloro-2,4-dinitrobenzene was used as substrate [16, 17].

Rat liver contains one form of dihydrodiol dehydrogenase, which was shown to be identical with 3α-hydroxysteroid dehydrogenase [18]. The enzyme is able to efficiently reduce the mutagenicity of metabolically activated benzo(*a*)pyrene [19] as well as that of the ultimate carcinogen *anti*-benz(*a*)anthracene 8,9-dihydrodiol-10,11-oxide [20].

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In order to prove the linkage between peroxisome proliferation, induction of cytosolic epoxide hydrolase and lauric acid hydroxylation respectively, we extended the study to the guinea pig, which is regarded to be non-responsive to the action of hypolipidemic compounds as neither clofibrate [21] nor diethylhexylphthalate [22] caused a proliferation of peroxisomes in this species.

MATERIALS AND METHODS

Chemicals. 1-benzylimidazole and tiadenol were purchased from Aldrich (Steinheim, F.R.G.) and clofibrate from Serva Feinbiochemica (Heidelberg, F.R.G.). Acetylsalicylic acid and 1-chloro-2,4-dinitrobenzene were obtained from Sigma (Deisenhofen, F.R.G.). Probucol was provided by Laboratoires Fournier (Fontaine les Dijon, France) and 4-hydroxynonenal by Dr H. Esterbauer (University of Graz, Austria).

UDP-glucuronic acid, lauric and 12-hydroxylauric acids and NADPH were obtained from Boehringer (Mannheim, F.R.G.).

Cis-stilbene oxide (0.48 GBq/mmol) and benzene dihydrodiol were synthesized as described in Refs 23 and 24, respectively.

Animal experiments. Male Fischer 344 rats (150–160 g) and male Hartley guinea pigs (340–410 g) were obtained from Charles River Wiga (Sulzfeld, F.R.G.). Animals were kept at constant temperature, under a constant light–dark cycle with free access to water and a defined diet (Altromin).

For the induction studies, animals were fed a pelleted diet containing the hypolipidemic compound prepared as described in [25]. As the tested compounds differ markedly in their hypolipidemic potency the doses used in human therapy were taken as a guideline. For the experiments with rats the lowest doses used were two-fold, on a body weight basis, the therapeutic dose used in man. Doses of 1-benzylimidazole and acetylsalicylic acid, which are not used as hypolipidemic drugs, were in the same order of magnitude as described in Ref. 2. Rats received the diet for 7 days and guinea pigs for 4 weeks.

Liver homogenate was prepared in ice-cold 10 mM Tris–HCl buffer, pH 7.4 containing 0.25 M sucrose. The 25% (w/v) homogenate was centrifuged for 10 min at 600 *g* and for 15 min at 10,000 *g*. The resultant supernatant was centrifuged for 60 min at 100,000 *g* to give the cytosolic supernatant. The pellet was resuspended in the same buffer to give the microsomal fraction. Subcellular fractions were stored for 6 months at −20° and were identical to those which were used to determine the enzyme activities reported in Ref. 10.

Enzyme assays. Activities of lauric acid hydroxylase and bilirubin UDP-glucuronosyltransferase were determined in the microsomal fraction as described previously [26, 27]. Microsomal epoxide hydrolase was quantified using an immunoassay [28]. Peroxisomal β-oxidation was measured in the 600 *g* supernatant [29].

Dihydrodiol dehydrogenase [30] and cytosolic epoxide hydrolase activities [31] were evaluated in the cytosol. Activity of glutathione *S*-transferase was determined using 1-chloro-2,4-dinitrobenzene [32], 4-hydroxynonenal [33] and *cis*-stilbene oxide [34] as substrates and cytosol as enzyme source.

Enzyme assays were performed under conditions of substrate saturation and linearity.

RESULTS

In the rats, with the exception of probucol and acetylsalicylic acid, all compounds tested caused a dose-dependent increase of the liver/body weight ratio [10].

The hypolipidemic compounds affected lauric acid hydroxylation in different ways (Table 1). At the highest dose tested, probucol decreased lauric acid hydroxylase to about 50% of the control activity. In contrast, the other compounds caused a dose-dependent increase in enzyme activity. Tiadenol was the most potent drug leading to a 15-fold induction at the highest dose tested.

Activity of UDP-glucuronosyltransferase was significantly increased by the investigated hypolipidemic drugs (Table 1). The strongest effect was observed upon feeding of tiadenol and acetylsalicylic acid resulting in a two-fold and 2.3-fold induction respectively. However, based on the dose, tiadenol was a 10 times more potent inducer.

As the enzyme activities shown in Table 1 and those reported previously [10] were estimated from the same samples, a correlation analysis could be performed. A high correlation (Table 2) was observed between the activities of lauric acid hydroxylase (Table 1) and the activities of peroxisomal β-oxidation ([10]; $r = 0.993$) and cytosolic epoxide hydrolase ([10]; $r = 0.953$) respectively. Correlation between UDP-glucuronosyltransferase activity and activity of lauric acid hydroxylase, peroxisomal β-oxidation or cytosolic epoxide hydrolase was significantly lower.

We have furthermore quantified the amount of microsomal epoxide hydrolase using an enzyme immunoassay. Treatment of rats with the hypolipidemic compounds did not significantly change the content of microsomal epoxide hydrolase in rat liver microsomes compared with control animals (Table 1).

In contrast to the effect on the other enzyme activities treatment of rats with tiadenol reduced liver glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene to 30% of control (Table 3). A significant decrease was also observed upon clofibrate feeding, whereas probucol and 1-benzylimidazole caused a 1.5-fold and 2.4-fold increase of glutathione *S*-transferase activity towards this substrate.

Whereas treatment of rats with probucol did not affect glutathione *S*-transferase towards 4-hydroxynonenal, the changes by the other hypolipidemic compounds were similar to those observed with 1-chloro-2,4-dinitrobenzene as substrate.

When *cis*-stilbene oxide was used to measure glutathione *S*-transferase activity only the more than five-fold increase after treatment with 1-benzylimidazole was significantly different from control values.

Activity of dihydrodiol dehydrogenase determined

Table 1. Effect of hypolipidemic compounds on specific activity of lauric acid hydroxylase and bilirubin UDP-glucuronosyltransferase and the content of microsomal epoxide hydrolase in Fischer-344 rat liver

Compound	Drug included in the diet at % (w/w)	Mean dose intake (mg/kg/day)	Lauric acid hydroxylase†	Bilirubin UDP-glucuronosyl-transferase	Microsomal epoxide hydrolase‡
Control	—	—	0.65 ± 0.13	0.97 ± 0.29	21.6 ± 4.2
Probucol	0.025	40	0.48 ± 0.03	1.28 ± 0.09	21.1 ± 1.3
	0.125	102	0.35 ± 0.09**	1.44 ± 0.03*	21.9 ± 3.1
1-Benzylimidazole	0.1	64	1.34 ± 0.06***	1.56 ± 0.03*	26.8 ± 0.9
Acetylsalicylic acid	0.2	190	1.39 ± 0.24**	1.42 ± 0.11*	24.8 ± 3.40
	1.0	630	6.17 ± 2.24***	2.26 ± 0.33**	24.6 ± 4.7
Clofibrate	0.05	56	3.18 ± 0.40***	1.32 ± 0.08	27.1 ± 2.4
	0.25	205	8.30 ± 0.26***	1.77 ± 0.09**	19.0 ± 1.3
Tiadenol	0.04	37	3.75 ± 0.28***	1.48 ± 0.23*	23.0 ± 3.7
	0.1	99	9.08 ± 1.61***	1.93 ± 0.09***	21.3 ± 4.1
	0.5	548	9.66 ± 0.37***	2.00 ± 0.34**	28.0 ± 4.8

Results represent mean ± standard deviation for three rats (control: five rats) treated and processed individually.
† Specific activity is given in nmol/min × mg protein.
‡ Results are expressed as μg epoxide hydrolase/mg total microsomal protein.
P values for results significantly different (Student's *t*-test) from control data at *P < 0.05, **P < 0.01, ***P < 0.001.

Table 2. Correlation of enzyme activities in rat liver after treatment with hypolipidemic drugs

Enzymes			Linear regression	
Cytosolic epoxide hydrolase	vs	Lauric acid hydroxylase	$y = 0.59 + 0.02x$	$r = 0.953$
Peroxisomal β-oxidation	vs	Lauric acid hydroxylase	$y = 1.08 + 0.10x$	$r = 0.953$
Peroxisomal β-oxidation	vs	Cytosolic epoxide hydrolase	$y = 25.66 + 5.10x$	$r = 0.972$
UDP-glucuronosyl transferase	vs	Lauric acid hydroxylase	$y = -8.31 + 7.78x$	$r = 0.795$
UDP-glucuronosyl transferase	vs	Peroxisomal β-oxidation	$y = -65.50 + 59.49x$	$r = 0.668$
UDP-glucuronosyl transferase	vs	Cytosolic epoxide hydrolase	$y = -356.00 + 333.0x$	$r = 0.713$

Enzyme activities given in Table 1 and corresponding data for peroxisomal β-oxidation and cytosolic epoxide hydrolase published in Ref. 10 were used for correlation analysis.

Table 3. Effect of hypolipidemic compounds on glutathion S-transferase activities in Fischer 344 rat liver

Compound	Drug included in the diet at % (w/w)	Substrate		
		1-Chloro-2,4-dinitrobenzene†	4-Hydroxynonenal†	Cis-stilbene oxide†
Control	—	395 ± 57	636 ± 48	31.9 ± 8.2
Probucol	0.125	623 ± 82***	623 ± 13	47.3 ± 18.2
1-Benzylimidazole	0.1	962 ± 46***	1047 ± 13***	170.6 ± 39.7***
Clofibrate	0.25	267 ± 35**	373 ± 18***	44.4 ± 13.7
Tiadenol	0.5	120 ± 9***	201 ± 37***	41.5 ± 11.9

Results represent mean ± standard deviation for three or four rats (control: nine rats, for 4 hydroxynonenal: five rats) treated and processed individually.

† Specific activity is given in nmol/min × mg protein.

P values for results significantly different (Student's *t*-test) from control data at $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 4. Effect of hypolipidemic compounds on specific activity of dihydrodiol dehydrogenase in Fischer 344 rat liver

Compound	Drug included in the diet at % (w/w)	Dihydrodiol dehydrogenase†
Control	—	50.6 ± 7.3
Probucol	0.125	47.0 ± 5.5
1-Benzylimidazole	0.1	79.0 ± 13.2**
Clofibrate	0.25	49.7 ± 0.6
Tiadenol	0.5	23.0 ± 1.7***

Results represent mean ± standard deviation for three rats (control: five rats) treated and processed individually.

† Specific activity is given in nmol/min × mg protein. Benzene dihydrodiol was used as substrate and $NADP^+$ as co-factor.

P values for results significantly different (Student's *t*-test) from control data at $^{**}P < 0.01$, $^{***}P < 0.001$.

with benzene dihydrodiol as substrate was not changed when animals were fed a diet containing probucol or clofibrate (Table 4). Activity was decreased after tiadenol treatment to 45% of the control value. 1-Benzylimidazole led to 1.5-fold increase in enzyme activity (Table 4). When tested *in vitro* these compounds did not affect dihydrodiol dehydrogenase activity (data not shown).

We have furthermore investigated the effect of the fibric acid derivate clofibrate and of tiadenol, which is structurally dissimilar [10], on some enzyme activities in the guinea pig liver. The drugs were included in the diet corresponding to the highest concentrations used for the treatment of rats. With guinea pigs no hepatomegaly was observed (data not shown). Treatment with clofibrate did not change any of the enzyme activities presented in Table 5.

However, tiadenol caused a 2.2-fold increase of peroxisomal β-oxidation. Activity of cytosolic epoxide hydrolase was 1.7-fold and that of lauric acid hydroxylase three-fold higher than the control values. The effect of tiadenol was confirmed by an experiment in which guinea pigs were fed a diet containing 1% of this drug. Increases of enzyme

Table 5. Effect of clofibrate and tiadenol on peroxisomal β-oxidation, cytosolic epoxide hydrolase and lauric acid hydroxylase in Hartley guinea pig liver

Compound	Drug included in the diet at % (w/w)	Mean dose intake (mg/kg/day)	Peroxisomal β-oxidation†	Cytosolic epoxide hydrolase†	Lauric acid hydroxylase†
Control	—	—	2.3 ± 0.4	0.294 ± 0.007	0.32 ± 0.06
Clofibrate	0.25	199	1.9 ± 0.1	0.297 ± 0.018	0.34 ± 0.01
Tiadenol	0.5	325	5.2 ± 0.3**	0.517 ± 0.014***	0.96 ± 0.09***

Results represent mean ± standard deviation for three animals treated and processed individually.
† Specific activity is given in nmol/min × mg protein.
P values for results significantly different (Student's *t*-test) from control data at $^{**}P < 0.01$, $^{***}P < 0.001$.

activities were comparable to those obtained with 0.5% tiadenol (data not shown).

DISCUSSION

Hypolipidemic properties and peroxisome proliferating potencies of the compounds tested in this study have been described in Refs 2, 10 and 35 respectively.

The activity of bilirubin UDP-glucuronosyltransferase was enhanced only by few compounds, most of them being structurally related to clofibrate or other arylcarboxylic acids [9, 27]. Results presented in Table 1 show that this is not a prerequisite as treatment of rats with tiadenol, 1-benzylimidazole or probucol caused a dose dependent significant increase in this enzyme activity.

As with clofibrate some of the other compounds or their metabolites may be glucuronidated. However, it is unlikely that substrate induction occurs as conjugation of clofibric acid is probably not catalyzed by the isoenzyme which conjugates bilirubin [9]. An explanation of the effect of hypolipidemic compounds is complicated by the fact that some of them did also increase glucuronidation of group I and group II substrates (1-benzylimidazole [35]) whereas others did not (clofibrate [9]).

Lauric acid is a suitable marker substrate for cytochrome P-452 [11]. Data given in Table 1 represent total 11- and 12-hydroxylation of lauric acid. As shown for clofibrate and some other peroxisome proliferators [13], those compounds preferentially induced 12-hydroxylation whereas 11-hydroxylation activity was only moderately changed. Induction of lauric acid hydroxylation reflected peroxisome proliferating potency of the compounds tested.

At present it is unclear whether the excellent correlation (Table 2) between induction of lauric acid hydroxylase (Table 1) and cytosolic epoxide hydrolase as well as peroxisomal β-oxidation [10] is due to a causal co-induction by hypolipidemic drugs. Possibly the observed effects are mediated by a common cytosolic receptor protein [1]. But the changes in the enzyme activities may also be related to altered metabolic changes in lipid metabolism caused by the action of hypolipidemic compounds considering that all three enzymes take fatty acids or their derivatives as substrates [2, 5, 14, 36, 37].

The assumption of a common linkage between induction of peroxisomal β-oxidation, cytosolic epoxide hydrolase and lauric acid hydroxylase is supported by the experiments with guinea pigs as tiadenol produced a concomitant increase in these enzyme activities (Table 5). Furthermore, this species cannot be longer regarded to be insensitive to the action of hypolipidemic compounds.

We have previously observed a five-fold increase in rat liver microsomal epoxide hydrolase activity after treatment of animals with 1-benzylimidazole [10]. This seems not to be due to an induction but most probably to an activation, as the amount of microsomal epoxide hydrolase in rat liver was not significantly elevated (Table 1). Another possibility is the induction of a microsomal epoxide hydrolase with different immunological properties. In contrast to our results, in mouse liver both activity and content of mouse liver microsomal and cytosolic epoxide hydrolase were increased after treatment of animals with 2-ethylhexanoic acid [38]. When the effect of 2,4-di- and 2,4,5-trichlorophenoxyacetic acid on mouse liver cytosolic epoxide hydralase was tested, changes in the amount of enzyme did not parallel changes in activity [39].

Confirming previous observations [16, 17] clofibrate and tiadenol decreased hepatic glutathione *S*-transferase activity towards 1-chloro-2,4-dinitrobenzene (Table 3) while 1-benzylimidazole in contrast increased this activity. This was not due to an activation as the compound inhibited the enzyme slightly when tested *in vitro* at a concentration of 1 mM (data not shown). Therefore reduction of glutathione *S*-transferase activity by peroxisome proliferators is not a general rule. This is underlined by the fact that the peroxisome proliferator 2,4,5-trichlorophenoxyacetic acid increased mouse liver glutathione *S*-transferase activity [39].

With 4-hydroxynonenal, a product of lipid peroxidation [40] glutathione transferase 8-8 showed a severalfold higher specific activity compared with 1-chloro-2,4-dinitrobenzene [40]. The effect of hypolipidemic agents on the activity of glutathione *S*-transferases against this specific substrate has not been studied yet. Our results indicate that the effects on acidic glutathione *S*-transferases did generally not differ from those on other forms (Table 3).

Epoxide metabolism of glutathione *S*-transferases when measured with *cis*-stilbene oxide as substrate was nearly exclusively studied in mouse liver. The activity was slightly elevated after treatment of mice with clofibrate [41] or tridiphane [42]. However, the

results cannot be generalized as it was shown that the effect of clofibrate and diethylhexylphthalate depends on the mouse strain [43]. In Fischer rats neither clofibrate nor tiadenol did significantly change glutathione *S*-transferase activity with *cis*-stilbene oxide as substrate, which contrasts the findings with the other substrates (Table 3). The high increase of glutathione *S*-transferase activity after treatment with 1-benzylimidazole is not due to enzyme activation as this compound did not affect glutathione *S*-transferase activity *in vitro* (data not shown).

These observations and the fact that cytosolic epoxide hydrolase is induced by the investigated compounds [10] may indicate an elevated liver epoxide metabolism.

Although a series of compounds were tested as inducers of dihydrodiol dehydrogenase, up to now only thyroxine is known to cause a moderate increase in enzyme activity [44]. We have therefore investigated the effect of hypolipidemic compounds on dihydrodiol dehydrogenase.

As probucol and clofibrate did not affect the activity of dihydrodiol dehydrogenase (Table 4), the changes observed after treatment with tiadenol and 1-benzylimidazole, which were neither due to inhibition nor activation as outlined by *in vitro* experiments (data not shown), did not depend on their hypolipidemic or peroxisome proliferating activity.

In conclusion, our results present evidence for a dose dependent and concomitant induction of peroxisomal β-oxidation, cytosolic epoxide hydrolase and lauric acid hydroxylase by hypolipidemic compounds with peroxisome proliferating activity in rat and, to a certain extent, in guinea pig. Based on this finding one may assume a common but yet unknown regulatory principle for these enzymes. On the other hand, activity of UDP-glucuronosyl-transferase and dihydrodiol dehydrogenase are not or randomly influenced by these compounds, suggesting individual regulatory responses.

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