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Effect of diabetes, starvation, ethanol and isoniazid on rat liver microsomal 12α-hydroxylase activity involved in bile acid biosynthesis

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12α-Hydroxylation is a step unique for cholic acid* biosynthesis and has been ascribed a role in the regulation of the ratio between cholic acid and chenodeoxycholic acid synthesized from cholesterol [1]. Experimental diabetes has been reported to increase the biosynthesis of cholic acid and decrease or not change the biosynthesis of chenodeoxycholic acid [2–4]. The change observed in bile acid biosynthesis may be due to effects of diabetes on the 12α-hydroxylating cytochrome P-450 [1, 5].

Recently, it has been shown that a species of cytochrome P-450 in rat liver microsomes, cytochrome P-450IIE1, is induced by starvation, diabetes, ethanol and isoniazid [6, 7]. No information is available concerning the possible activity of cytochrome P-450IIE1 towards C_{27}-steroids. In common with cytochrome P-450IIE1, the 12α-hydroxylating cytochrome P-450 is stimulated by starvation [1, 5]. It is not known whether other treatments inducing cytochrome P-450IIE1, e.g. experimental diabetes, have any effects on the 12α-hydroxylating system.

The present communication reports studies of the effect of diabetes, starvation, ethanol and isoniazid treatment on the 12α-hydroxylase system and the possible role of cytochrome P-450IIE1 in 12α-hydroxylation.

Materials and methods

The various ^{14}C- and ^{3}H-labeled substrates were obtained and prepared as described previously [8]. Streptozotocin and *N*-nitrosodimethylamine were from Sigma. Anti-P-450IIE1 IgG and preimmune IgG were generous gifts from Dr M. Ingelman-Sundberg, Stockholm. Other materials were obtained as described previously [9].

Sprague–Dawley male rats (150–200 g) were used. Liver microsomes were prepared from untreated, starved (72 hr), ethanol-treated (10% in drinking water for 3 weeks), isoniazid-treated (0.1% in drinking water for 10 days) and streptozotocin-treated (45 mg/kg body weight i.v. in tail vein) rats and from rats treated with a combination of ethanol and starvation as described previously [10]. An increase in the blood glucose concentration and in the serum cholesterol level, together with an increase in both the total amount as well as in the specific content of cytochrome P-450, confirmed the induction of diabetes in the streptozotocin-treated rats.

Microsomal cytochrome P-450 fractions were prepared from streptozotocin-treated and isoniazid-treated rats according to the procedures described by Ryan *et al.* [11]. The fraction eluted from the octylamine-Sepharose 4B column with the potassium phosphate buffer containing 0.08% Emulgen 911 and 0.33% sodium cholate [11], contained 12α-hydroxylase activity. The cytochrome P-450IIE1 fraction was eluted from the column and subsequently chromatographed on a hydroxylapatite column as described by Ryan *et al.* [11]. The partially purified cytochrome P-450IIE1 fraction from this step was not further purified but chromatographed on hydroxylapatite to remove detergents [10]. The resulting enzyme fraction was called cytochrome P-450IIE1 fraction.

The fractions that contained 12α-hydroxylase activity were purified on a hydroxylapatite column, equilibrated with 10 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 mM EDTA and 0.2% Emulgen 913. The column was washed with the same buffer con-

* The following trivial names and abbreviations used are: cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid.

taining 40 mM and 80 mM potassium phosphate and a fraction of cytochrome P-450 was eluted with 200 mM potassium phosphate in the buffer. This fraction was concentrated and dialyzed against 5 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 0.1 mM EDTA, 0.1% Emulgen 913 and 0.2% sodium cholate. The sample was applied to a Whatman DE-52 column, equilibrated with the same buffer. The column was washed with the equilibrating buffer and a cytochrome P-450 fraction was eluted with equilibrating buffer containing 0.2% Emulgen 913 and 0.5% sodium cholate. The eluted fraction was applied to a hydroxylapatite column, equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA and 0.2% Emulgen 913. The column was washed with the same buffer containing 20, 40 and 80 mM potassium phosphate. The detergent was removed by washing the column with 80 mM phosphate buffer without detergent and the cytochrome P-450 fraction, called cytochrome P-450$_{12\alpha}$ fraction, was eluted with a buffer containing 500 mM phosphate.

The final cytochrome P-450 fractions were dialyzed against 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. Cytochrome P-450 and protein were determined and NADPH-cytochrome P-450 reductase was prepared and assayed as described previously [9].

Incubation procedures and analyses of incubation mixtures were the same as described previously [10] except that incubations with 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol were analyzed by HPLC as described by Andersson and Jörnvall [12]. The mobile phase was 90% methanol for 5β-cholestane-3α,7α-diol and metabolites. Incubations with *N*-nitrosodimethylamine (1 mM or 5 mM) were performed and analyzed as described by Yang *et al.* [13] and Levin *et al.* [14] except that the reactions were terminated by the addition of 50% trichloroacetic acid. Preincubations of microsomal fractions with anti-P-450IIE1 IgG and preimmune IgG, 4 mg of IgG per mg of protein, were performed as described by Thomas *et al.* [6].

Results and discussion

The effect on 12α-hydroxylation of various treatments known to induce cytochrome P-450IIE1 was analyzed in experiments with microsomal fractions. For reasons of comparison, three other hydroxylations in bile acid biosynthesis, the 7α-hydroxylation of cholesterol, the 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol and the 26-hydroxylation of 5β-cholestane-3α,7α-diol, were assayed in the experiments with streptozotocin-treated rats. The demethylation of *N*-nitrosodimethylamine was used as indicator of cytochrome P-450IIE1 activity [14].

Figure 1 summarizes the catalytic properties of microsomal fractions prepared from untreated and streptozotocin-treated rats at different times after the induction of diabetes. The 12α-hydroxylation was up to four times more efficient with microsomes prepared from streptozotocin-treated rats than with microsomes prepared from control rats. The 25-hydroxylation was slightly increased whereas the 7α-hydroxylation and the 26-hydroxylation were decreased by about 50%. These results could indicate that cytochrome P-450IIE1 might be involved in the 12α-hydroxylation as this species of cytochrome P-450 is induced by diabetes [6].

Table 1 shows the effect of various treatments, known to induce cytochrome P-450IIE1. Microsomes from streptozotocin-treated, starved and ethanol-treated plus starved (a treatment reported to have a cumulative induction of cytochrome P-450IIE1 [15]) rats catalyzed 12α-hydroxylation more efficiently than microsomal fractions from untreated rats. Microsomal fractions prepared from isoniazid-treated and ethanol-treated rats catalyzed 12α-hydroxylation somewhat less efficiently than microsomal

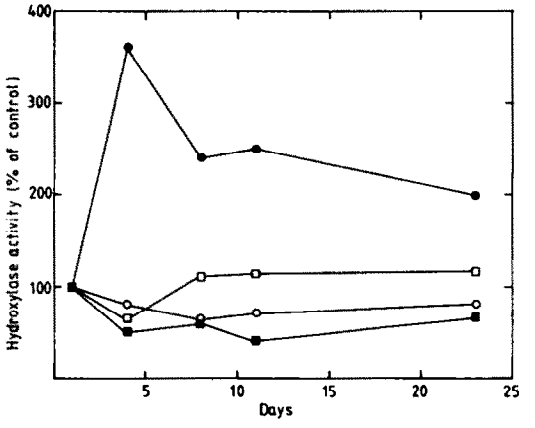

Fig. 1. Effect of induced diabetes on cytochrome P-450 dependent microsomal hydroxylations involved in bile acid biosynthesis. Diabetes was induced by a single injection of streptozotocin prepared in 0.1 M citrate buffer, pH 4.5, (45 mg per mg body weight). Control rats were injected with an equivalent volume of citrate buffer. Microsomes were prepared at different days after the treatment. Labeled steroids (25–100 nmol) were incubated under standard conditions at 37° for 20 min with microsomal fractions (0.2–0.5 nmol of cytochrome P-450) and NADPH (1 μmol) in a total volume of 0.5–0.75 ml of 50 mM Tris–acetate buffer or 150 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The specific hydroxylase activities in microsomes from streptozotocin-treated rats are expressed as percent of the specific activities in microsomes from control rats. The means of experiments with five control and five treated rats were used for calculation of each point. ○—○, 7α-hydroxylation of cholesterol; ●—●, 12α-hydroxylation of 5β-cholestane-3α,7α-diol; □—□, 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol; ■—■, 26-hydroxylation of 5β-cholestane-3α,7α-diol.

fractions from untreated rats. All treatments increased demethylation of *N*-nitrosodimethylamine. It was most efficient with microsomes from ethanol-treated plus starved rats and isoniazid-treated rats. In another set of experiments, antibodies towards cytochrome P-450IIE1 were added to incubations with fractions prepared from starved rats and from rats treated with a combination of ethanol and starvation. Preincubation with anti-P-450IIE1 IgG decreased the demethylation of *N*-nitrosodimethylamine by 50 to 60% whereas the same antibodies did not decrease the 12α-hydroxylation.

From the results described above, it can be concluded that cytochrome P-450IIE1 does not play a role in the 12α-hydroxylation. This conclusion is further supported by the results obtained with purified fractions of cytochrome P-450 from streptozotocin-treated and isoniazid-treated rats, presented in Table 2. Two fractions of cytochrome P-450 were partially purified from streptozotocin-treated and isoniazid-treated rats, respectively. One fraction contained 12α-hydroxylase (cytochrome P-450$_{12\alpha}$ fraction) and the other fraction contained cytochrome P-450IIE1 (cytochrome P-450IIE1 fraction). The cytochrome P-450$_{12\alpha}$ fraction showed one major protein band upon gel electrophoresis whereas the cytochrome P-450IIE1 fraction showed several protein bands. As shown in Table 2, the

Table 1. Hydroxylase activities in liver microsomes prepared from untreated and variously treated rats

Microsomal fractions	Specific content of cytochrome P-450 nmol/mg protein	12α-Hydroxylation of 5β-cholestane-3α,7α-diol pmol/(nmol cytochrome P-450)/min	Demethylation of *N*-nitrosodimethylamine nmol/(nmol cytochrome P-450)/min
Untreated	0.93 ± 0.06	213 ± 2	1.9 ± 0.3
Starved	0.88 ± 0.03	362 ± 27	3.1 ± 0.2
Ethanol-treated	0.80 ± 0.03	159 ± 20	2.9 ± 0.1
Ethanol-treated + starved	1.28 ± 0.06	314 ± 14	7.2 ± 0.2
Streptozotocin-treated	1.49 ± 0.10	656 ± 11	3.1 ± 0.2
Isoniazid-treated	1.10 ± 0.04	163 ± 23	6.3 ± 0.4

5β-Cholestane-3α,7α-diol was incubated under the same conditions as in Fig. 1. Incubations with *N*-nitrosodimethylamine (5 mM) were performed under the same conditions in a total volume of 0.5 ml of 100 mM potassium phosphate buffer, pH 6.8, containing 20% glycerol, 0.1 mM EDTA and 10 mM $MgCl_2$. The results are expressed as mean ± SE (N = 4).

Table 2. Hydroxylase activities in preparations of liver microsomal cytochrome P-450 fractions from streptozotocin-treated and isoniazid-treated rats

Fractions	Specific content of cytochrome P-450 nmol/mg protein	12α-Hydroxylation of 5β-cholestane-3α,7α-diol pmol/(nmol cytochrome P-450)/min	Demethylation of *N*-nitrosodimethylamine nmol/(nmol cytochrome P-450)/min
Streptozotocin-treated			
Microsomes	1.3	677	3.1
Cytochrome $P\text{-}450_{12\alpha}$ fraction	5.0	738	3.8
Cytochrome P-450IIE1 fraction	3.0	314	7.2
Isoniazid-treated			
Microsomes	1.1	195	6.8
Cytochrome $P\text{-}450_{12\alpha}$ fraction	5.0	248	1.9
Cytochrome P-450IIE1 fraction	6.0	<1	6.1

The substrates were incubated under the same conditions as in Table 1. The reconstituted systems contained cytochrome P-450 (0.1–0.5 nmol), NADPH-cytochrome P-450 reductase (2–6 unit), dilauroylglycero-3-phosphorylcholine (25 μg) and NADPH (1 μmol). The results presented are from single experiments. Each preparation was made at least twice and similar results were obtained.

12α-hydroxylase activity was highest in microsomes from streptozotocin-treated rats whereas the demethylase activity was highest in microsomes from isoniazid-treated rats. Fractions that contained the major amount of cytochrome P-450IIE1 showed no or low 12α-hydroxylase activity but catalyzed an efficient demethylation of *N*-nitrosodimethylamine. On the other hand, the cytochrome $P\text{-}450_{12\alpha}$ fractions catalyzed an efficient 12α-hydroxylation whereas the rate of demethylation of *N*-nitrosodimethylamine was as efficient as or lower than with the corresponding microsomes.

In summary, streptozotocin induced diabetes stimulated the 12α-hydroxylation of 5β-cholestane-3α,7α-diol several-fold whereas other hydroxylase activities involved in the biosynthesis of bile acids were not affected to the same extent. The results from experiments with treatments

known to induce cytochrome P-450IIE1, experiments with antibodies towards cytochrome P-450IIE1 and experiments with partially purified fractions of cytochrome P-450, show that cytochrome P-450IIE1 is not involved in the 12α-hydroxylation. The results suggest that 12α-hydroxylation is catalyzed by a species of cytochrome P-450 that is induced by diabetes and starvation in a manner similar to that of cytochrome P-450IIE1. This species of cytochrome P-450 may play a role in the regulation of the ratio between the primary bile acids. The increase in 12α-hydroxylation observed in liver microsomes of diabetic rats could explain the increased pool of cholic acid found in diabetic animals [2–4, 16].

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