

SIMILARITIES AND DIFFERENCES IN THE REGULATION OF HEPATIC CYTOCHROME P-450 ENZYMES BY DIABETES AND FASTING IN MALE RATS*

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Abstract—The effects of streptozotocin-induced diabetes and fasting on hepatic cytochrome P-450 enzymes in sexually mature male rats were studied by immunochemical techniques and enzyme assays. The level of cytochrome P-450ac (an acetone/ethanol inducible form), 65 pmol/mg microsomal protein in control rats, increased 4- to 5-fold in diabetic rats and 3- and 5-fold in fasting rats. In contrast, P-450 UT-A (a male specific form) decreased drastically from 295 pmol/mg in the control group to about 10% of this value in diabetic rats and to 50% in fasting rats. P-450 PCN-E (a 16 α -cyanopregnenolone/dexamethasone inducible form), on the other hand, decreased from 151 pmol/mg to 38% in diabetic rats and increased 2-fold in fasting rats. These changes were also reflected in catalytic activities using *N*-nitrosodimethylamine, benzphetamine, and erythromycin as substrates. Slight changes in cytochromes P-450 UT-F, P-450 UT-I and P-450 PB-C were also observed under these conditions, but the biological significance is not known. These results suggest that different mechanisms exist for the regulation of the expression of cytochrome P-450 enzymes in diabetic and fasting rats.

Cytochrome P-450 (P-450_{||}), a family of microsomal hemoproteins, catalyzes the oxidative metabolism of a wide array of substrates [1–4]. Up to now, more than ten different forms of P-450 have been purified from rat liver microsomes, and their properties have been characterized. These P-450 enzymes, with different primary structures, are known to have different but overlapping substrate specificities and to be regulated differently. Because alteration of the composition of P-450 enzymes can affect the metabolism of drugs and steroid hormones as well as the bioactivation of toxic compounds, the regulation of P-450 enzymes is a subject of considerable importance.

Diabetes and fasting are known to affect the metabolism of drugs, hormones and carcinogens. It has been demonstrated that fasting and diabetes decrease the metabolism of hexobarbital, codeine, chlorpromazine, aminopyrine, and other drugs as well as testosterone 16 α -hydroxylase activity in male rats [5–10]. On the other hand, fasting and diabetes also cause an increase in microsomal aniline

hydroxylase and ethoxycoumarin deethylase activities [6, 8, 9, 11–13]. Recent studies from one of our laboratories demonstrated that both fasting and diabetes induce the activity of NDMA demethylase [14–16], an enzyme responsible for the bioactivation of NDMA [17, 19], and that this increase is due to the induction of P-450ac [19, 20], a P-450 species probably identical to the P-450_j purified from isoniazide-induced rat liver microsomes [21, 22]. Both fasting and diabetes share the common feature of causing an elevation of the mRNA levels of this enzyme [19, 20].

Because of this similarity in the regulation of P-450ac by fasting and diabetes, it would be interesting to know whether such a similarity also exists with regard to the regulation of other P-450 enzymes. To characterize the possible changes of different P-450 enzymes, the conditions of diabetes and fasting were studied simultaneously to elucidate the mechanisms by which these conditions affect the regulation of P-450 enzymes.

MATERIALS AND METHODS

Materials. Streptozotocin, NADPH, cytochrome c, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and erythromycin were purchased from the Sigma Chemical Co. (St. Louis, MO). *N*-Nitrosodimethylamine was from the Aldrich Chemical Co. (Milwaukee, WI). "Chemstrip ug" for the urinary glucose test was obtained from Boehringer Mannheim (Indianapolis, IN). Benzphetamine-HCl and *p*-nitroanisole were from the Upjohn Co. (Kalamazoo, MI) and the Eastman Organic Co. (Rochester, NY) respectively. The nitrocellulose sheet was from Schleicher & Schuell (Keene, NH).

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|| Abbreviations: P-450, cytochrome P-450; and NDMA, *N*-nitrosodimethylamine. The nomenclature of the rat hepatic P-450 enzymes is the same as used previously [1–3]. Apparently equivalent forms and the names of the genes are as follows: P-450ac = P-450_j encoded by P-450 IIE1 gene; P-450 UT-A = P-450_h = P-450 2c; P-450 PCN-E = P-450 PB-2a; P-450 UT-F = P-450_a = P-450 3 encoded by P-450 IIA1 gene; P-450 PB-C = P-450 PB-1 encoded by P-450 IIC6 gene; and P-450 UT-I = P-450_i = P-450 2d [3].

Table 1. Effects of diabetes and fasting on body weight, liver weight, and microsomal enzymes of rats

Treatment group	Change in body weight (g)	Liver weight (g)	P-450 content (pmol/mg)	Reductase activity (nmol cytochrome <i>c</i> reduced/min/mg)
C	+88	15	610	286
D1	-105	8	654	318
D2	-95	9	640	344
F1	-15	9	752	266
F2	-36	7	834	262

Rats were treated as described in Materials and Methods. C, D1, D2, F1, and F2 represent the following treatment groups: control, moderate diabetes (rats received a third injection of streptozotocin), severe diabetes (diabetes developed after two injections), fasting 39 hr, and fasting 87 hr respectively. Livers from two rats were pooled as one sample. P-450 content and cytochrome *c* reductase activity were the averages of two samples for groups C, F1, and F2 or the averages of three determinations of one sample for groups D1 and D2.

The sources of other reagents have been described previously [23, 24].

Animals. Nine-week-old male Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY) and maintained in metabolic cages in air-conditioned quarters with 12-hr light-dark cycles. The rats (body weights around 295 g) were made diabetic by two daily s.c. injections of freshly prepared streptozotocin (in 0.05 M citrate buffer, pH 4.5) at a dose of 65 mg/kg. Control rats received an equal amount of the buffer. Detection of urinary glucose with "Chemstrip" and analyses of food consumption and body weight changes were used to determine the onset of diabetes. For the rats that did not develop marked diabetes (urinary glucose <5%), a third injection of streptozotocin (100 mg/kg) was given on day 7. For fasting experiments, one group of rats was fasted 39 hr and the other group was fasted 87 hr. All rats were killed between 9:00 and 10:00 a.m. on the same day, i.e. on day 14 after the first streptozotocin injection.

Microsomes and enzyme assays. Livers from two rats of the same group were pooled as one sample for microsomal preparation by differential centrifugation [23]. Previous methods were used for the determination of protein concentration and P-450 content, as well as activities of NADPH-cytochrome *c* reductase, NDMA demethylase, benzphetamine demethylase, and *p*-nitroanisole demethylase [14, 23]. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were used for the NADPH generating system [23]. The method for erythromycin demethylase assay was similar to that for NDMA demethylase assay except that 0.4 mM erythromycin was used as the substrate. All assays were run in duplicate, and the differences between the duplicates were less than 10% of the means.

Antibodies and immunoblot analysis. P-450ac was purified from acetone-induced rat liver microsomes [1]. Anti-P-450ac IgG was prepared from rabbit, and its properties have been described previously [24]. In preliminary experiments, this anti-P-450ac IgG preparation was subjected to immuno-adsorption by immobilized hepatic microsomal proteins (from 3-methylcholanthrene-treated rats) according to Thomas *et al.* [25]. However, this adsorption step

did not affect the results on the quantitation of P-450ac in different types of microsomes and, therefore, was not used in this study. The presently used anti-P-450ac IgG preparation did not cross-react with P-450s PB-B, PB-C, UT-I, and UT-F in immunoblot experiments; but it did cross-react with P-450s UT-A and PCN-E, showing intensities approximately 5–10% that of an equimolar P-450ac. This cross-reactivity may affect the quantitation of this P-450 form to a slight degree. Nevertheless, it should not affect the conclusions of this study. Purified P-450s UT-A, UT-I, UT-F, PCN-E, and PB-C and their corresponding antibodies were prepared as described [2]. Gel-electrophoresis and immunoblot analyses were performed using previous procedures [24]. For quantitation of individual P-450 forms, samples from all groups were electrophoresed on the same gel. For each sample, three different concentrations of protein within a linear range, determined in preliminary experiments, were analyzed. A standard curve was constructed for each P-450 enzyme by analyzing the immuno-stained bands of purified P-450 standard of microsomes of known levels of the specific P-450 forms on the same gel. Densitometry of each band on the nitrocellulose sheet was carried out using a Shimadzu dual-wavelength TLC scanner, model CS-930 (Shimadzu Scientific Instruments, Inc., Columbia, MD). Results were expressed as picomoles of P-450 enzyme per milligram of microsomal protein.

RESULTS

All rats in the treated groups were found to have obvious loss of body weight and to have lower liver weights than the control rats (Table 1). Total P-450 content increased from 610 to 752 and 834 pmol/mg microsomal protein in 39-hr and 87-hr fasting groups, respectively; no obvious increases were observed in the diabetic groups. NADPH-cytochrome *c* reductase activities in treated groups were in the same range as that of control rats with the possible exception of the severely diabetic group whose activity was slightly higher (Table 1). The results were similar to those observed previously [14, 15].

In immunochemical quantitation studies, levels of

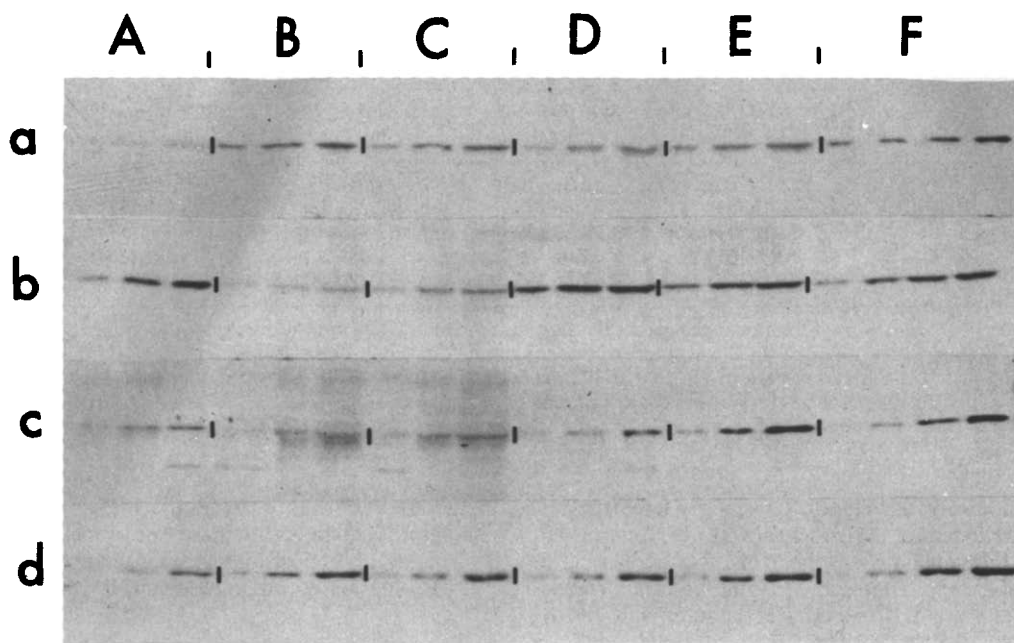


Fig. 1. Immunoblots of rat liver microsomal P-450 enzymes. (a) P-450ac: Each microsomal sample was applied to three wells (from left to right, 1.33, 2.67, and 5.33 μ g of protein per well, respectively, in each track). Designations of tracks are: A, control; B, severe diabetes; C, moderate diabetes; D, fasting 39 hr; E, fasting 87 hr; and F, purified P-450ac standard. (b) P-450 UT-A: Tracks contained the following samples (numbers in parentheses indicate μ g of protein applied per well): A, control (1.33, 2.67, 5.33); B, severe diabetes (5.33, 10.7, 21.4); C, moderate diabetes (5.33, 10.7, 21.4); D, fasting 39 hr (2.67, 5.33, 10.7); E, fasting 87 hr (2.67, 5.33, 10.7); F, microsomes with predetermined levels of P-450 UT-A. (c) P-450 PCN-E: Tracks contained the following samples: A, control (1.33, 2.67, 5.33); B, severe diabetes (5.33, 10.7, 21.4); C, moderate diabetes (5.33, 10.7, 21.4); D, fasting 39 hr (0.67, 1.33, 2.67); E, fasting 87 hr (0.67, 1.33, 2.67); F, microsomes with predetermined levels of P-450 PCN-E. (d) P-450 UT-F: Tracks contained the following samples: A, control; B, severe diabetes; C, moderate diabetes; D, fasting 39 hr; E, fasting 87 hr; F, microsomes with predetermined levels of P-450 UT-F. For tracks A-E, the three wells contained 0.67, 1.33, and 2.67 μ g protein respectively.

Table 2. Effects of diabetes and fasting on immunochemically determined levels of hepatic P-450 enzymes

Treatment group	Enzyme level (pmol/mg protein)				
	P-450ac	P-450 UT-A	P-450 PCN-E	P-450 UT-F	P-450 PB-C
C	65 (100)	295 (100)	151 (100)	115 (100)	355 (100)
D1	261 (402)	27 (9)	59 (39)	129 (112)	261 (73)
D2	326 (502)	30 (10)	57 (38)	171 (148)	264 (74)
F1	201 (309)	158 (53)	184 (122)	134 (116)	340 (96)
F2	307 (472)	157 (53)	314 (208)	180 (156)	375 (106)

Designations of treatment groups are the same as in Table 1. Data are the averages of two to three determinations. Variations were within 15% of the averages. The data are also shown (in parentheses) as percentages of the values of the control group.

P-450ac, P-450 UT-A, P-450 PCN-E, P-450 UT-I, P-450 PB-C, and P-450 UT-F were measured. P-450ac, an acetone-inducible form responsible for the low K_m form of NDMA demethylase [1, 4, 23], increased about 5-fold in rats with severe diabetes or fasting and about 3- or 4-fold in rats with moderate diabetes or fasting (Fig. 1a, Table 2). P-450 UT-A, a male specific form, was decreased to about 10 and 50% of

the control levels in diabetic and fasting rats respectively (Fig. 1b, Table 2). On the other hand, P-450 PCN-E, a 16α -cyanopregnenolone/dexamethasone inducible form, responded differently in that it decreased (to 40%) in diabetic rats but increased in fasting rats (up to 2-fold) (Fig. 1c, Table 2). In the diabetic groups, more protein was applied to the wells, and this may account for the higher back-

Table 3. Effects of diabetes and fasting on microsomal enzyme activities

Treatment group	Enzyme activity (nmol HCHO formed/min/mg microsomal protein)			
	NDMA demethylase	Benzphetamine demethylase	<i>p</i> -Nitroanisole demethylase	Erythromycin demethylase
C	1.42 (100)	14.32 (100)	4.34 (100)	0.445 (100)
D1	2.34 (165)	5.68 (40)	2.27 (52)	0.320 (72)
D2	3.02 (213)	5.63 (39)	2.18 (50)	0.244 (54)
F1	2.17 (152)	7.08 (49)	1.38 (32)	0.481 (108)
F2	2.81 (198)	7.55 (53)	1.92 (44)	0.818 (184)

Designations of treatment groups are the same as in Table 1. Data are the averages of two samples for groups F1 and F2, or the averages of three determinations of a single sample for groups C, D1, and D2. Variations were within 10% of the averages. The data are also shown (in parentheses) as percentages of the values of the control group.

ground observed (Fig. 1c). This factor, nevertheless, should not affect the conclusion of this analysis. P-450 UT-F, a female specific form, was increased 1.5-fold in severe diabetes and fasting, but the increases were less clear in moderate diabetes and fasting (Fig. 1d, Table 2). The levels of P-450 PB-C, a phenobarbital-inducible form, were decreased slightly by diabetes but were not changed appreciably by fasting (Table 2). A female P-450 form, P-450 UT-I, was increased slightly in diabetic and fasting treatments (data not shown). Because of the low magnitude of changes in P-450s UT-F, PB-C, and UT-I, their biological significances are not known.

It should be noted that the sum of the amounts of all P-450 enzymes determined immunochemically in a microsomal sample (Table 2) was, on average, about 40% higher, than the total P-450 determined spectrophotometrically (Table 1). This may be due to either or both of the following reasons: (a) the presence of apoenzyme or denatured P-450, and (b) an over-estimation of P-450 enzymes by the immunochemical technique. This problem, however, is not believed to affect the interpretations for the large changes in P-450ac, P-450 UT-A, and P-450 PCN-E observed herein.

The increases in microsomal P-450ac were accompanied by increases of NDMA demethylase activities in all treated groups. However, the extent of the increases of NDMA demethylase activity, 1.5- to 2-fold (Table 3), was less than that of the P-450ac protein induction. This may be due to the presence of P-450 apoenzyme or denatured enzyme which is recognized by anti-P-450ac IgG but catalytically inactive. However, more definitive evidence is needed for this postulate. The activities of benzphetamine demethylase and *p*-nitroanisole demethylase were decreased in both diabetic and fasting rats to 30–50% of the control levels. The decrease of the former activity is possibly a reflection of the lowered P-450 UT-A levels. The activity of erythromycin demethylase, which is known to be

metabolized efficiently by P-450 PCN-E [26], was decreased by diabetes but increased by fasting (Table 3). This result is consistent with the changes in P-450 PCN-E determined immunochemically.

DISCUSSION

During the past several years, our laboratories have studied the regulation of different P-450 enzymes. We have investigated whether the induction of P-450ac by fasting and diabetes is related to the ketone bodies generated under these conditions [16], because acetone and isopropanol are also potent inducers of this hemoprotein [27]. However, ketosis alone has been shown to be insufficient to account for the induction of P-450ac in fasting [16]. In addition to ketosis, hormonal factors appear to be important in the regulation of P-450ac. It has been demonstrated that P-450ac mRNA is elevated in fasting and diabetic rats [19, 20], but not during the induction of P-450ac by acetone, isopropanol, and pyrazole [21, 28]. Recent results suggest that the elevation of P-450 mRNA in diabetes is probably due to mRNA stabilization [20] and that acetone treatment may retard the rate of degradation of P-450ac*. It is possible that, in diabetes, the "induction" of P-450ac is due both to an elevation in P-450ac mRNA as a result of mRNA stabilization and to an enhanced protein stabilization due to the high level of acetone produced. Previous results indicated that both actinomycin D, an RNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor, inhibit the induction of NDMA demethylase activity by fasting and acetone [14, 27]. The possibility that enhanced transcriptional and translational rates contribute to the induction of P-450ac by diabetes, fasting, and acetone treatment requires further investigation.

The male specific P-450 form, P-450 UT-A, and the female specific P-450 form, P-450 UT-I, have been shown to be regulated by male and female hormones through a hypothalamic-pituitary-gonadal control mechanism [2, 29, 30]. In the present study, we found that P-450 UT-A decreased drastically in diabetic rats and, to a lesser extent, in fasting rats. The result is consistent with a previous report that testosterone 16 α -hydroxylase activity is decreased

* Gonzalez FJ, Skoda R, Hardwick JP, Song BJ, Umeno M, McBride OW, Kozak C, Matsunaga E, Matsunaga T, Kimura S, Park SS, Yang CS, Nebert DW, Gelboin HV and Meyer UA, Presentation at the Seventh International Symposium on Microsomes and Drug Oxidations, August 17–21, 1987, Adelaide, South Australia.

by diabetes [10] and the present observation that benzphetamine demethylase activity was decreased by fasting in male rats. P-450 UT-A is known to exhibit both of these activities. Several studies have revealed that serum testosterone levels decreased in diabetic and fasting states [31, 32]. Such decreases may be partially responsible for the observed changes in P-450 UT-A and P-450 UT-I. On the other hand, diabetes also decreases growth hormone receptor in rat liver [33], which may affect the expression of P-450 enzymes.

Although diabetes and fasting have similar actions in the induction of P-450ac and suppression of P-450 UT-A, they produce divergent effects on a third P-450 enzyme, P-450 PCN-E, which was increased in fasting rats and decreased in diabetic rats. Recent studies revealed that there are at least two forms of P-450 PCN-E and they are regulated differently [34, 35]. During fasting, glucocorticoid hormones are increased, which may lead to the induction of P-450 PCN-E [34]. The mechanism by which diabetes decreases P-450 PCN-E is not known at the present time. The possibility of a direct inhibitory effect of streptozotocin on P-450 PCN-E cannot be excluded.

In studying chemically-induced diabetes, there is concern as to whether the effect is caused by the diabetic condition or merely by the chemical treatment. The presently observed results appear to be due to the diabetic condition because similar effects were observed in studies with spontaneously diabetic male rats [36] (BB/Wor rats obtained from the University of Massachusetts, Worcester, MA). In comparison to the diabetic-resistant rats, the chronic diabetic rats had a higher level of P-450ac but a lower level of P-450 UT-A. These alterations were reversed by the administration of insulin.

The presently observed changes in P-450 enzymes provide a basis for the interpretation of some of the previous observations concerning the effects of fasting and diabetes on drug metabolism [5–15]. The results should also help to predict the metabolism pattern of many xenobiotics and endobiotics *in vivo*. It is not known whether these changes can be observed in humans, and this topic remains to be investigated further.

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