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EFFECT OF DIABETES ON RAT LIVER CYTOCHROME P-450

EVIDENCE FOR A UNIQUE DIABETES-DEPENDENT RAT LIVER CYTOCHROME P-450*

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Abstract—Detergent-solubilized hepatic microsomal fractions from alloxan diabetic rats exhibited a 52,000 molecular weight hemeprotein band that was not present in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) protein profiles of identically solubilized hepatic microsomal fractions from normal, 3-methylcholanthrene- or phenobarbital-treated rats. This 52,000 mol. wt hemeprotein band disappeared from the protein profile of insulin-treated diabetic rat liver to yield the SDS–PAGE profile of normal rat liver. When P-450 hemeproteins were purified by lauric acid affinity and hydroxylapatite chromatography from solubilized microsomes, only the diabetic rat had a 52,000 mol. wt P-450. This distinct 52,000 mol. wt diabetes-induced P-450 interacted with type II compounds to yield a 2-fold greater absorbance change than was observed with the purified P-450s from either the normal or the chemically induced rats. The properties of this unique 52,000 mol. wt P-450 suggest that it may be the catalytic component responsible for the increased rate of type II substrate (aniline) metabolism observed in the diabetic rat.

Diabetes in the rat alters in a substrate-specific manner the activity of the hepatic microsomal mixed-function oxidase system which metabolizes both xenobiotics and endogenous compounds [1–11]. Previous results from this laboratory have indicated that the locus of this effect of diabetes is the cytochrome P-450 component of this oxidase system [11, 12]. The present paper presents evidence that diabetes in the male rat induces the appearance of a unique hepatic microsomal cytochrome P-450 population that contains a diabetes-dependent 52,000 mol. wt band. The substrate binding properties of a 52,000 mol. wt cytochrome P-450 purified from this diabetic hemeprotein population are compatible with the hypothesis that this unique P-450 is the catalytic component contributing to the selective increase in the metabolism of aniline observed in diabetic rats.

MATERIALS AND METHODS

Chemicals. Alloxan and corn oil were obtained from the Eastman Kodak Co. (Rochester, NY), and aminopyrine and aniline hydrochloride from the Aldrich Chemical Co. Inc. (Milwaukee, WI), phenobarbital was from Mallinckrodt, Inc. (Paris, KY). Metyrapone and SKF 525-A (β-diethylaminoethyldiphenylpropylacetate) were gifts from the CIBA Pharmaceutical Co. (Summit, NJ) and Smith Kline & French (Philadelphia, PA) respectively. Emulgen 911 was a gift from the Kao-Atlas Chemical Co. Ltd (Tokyo, Japan). All materials for electrophoresis, Bio-Beads SM-2, hydroxylapatite, and Coomassie brilliant blue R-250 were obtained from the Bio-Rad Laboratories (Richmond, CA). AH-Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), lauric acid from the Sigma Chemical Co. (St. Louis, MO), 3-methylcholanthrene from Tridom Chemical, Inc. (Hauppauge, NY), and isophane insulin from E. R. Squibb & Sons, Inc. (Princeton, NJ).

Animals. Male Sprague–Dawley rats (160–180 g) were obtained from SASCO, Inc. (Omaha, NE) and maintained on standard laboratory chow and water *ad lib*. Diabetes was induced with alloxan (45 mg/kg, intravenously in 0.9% saline). The rats were considered diabetic only if their blood glucose concentration exceeded 300 mg/dl as measured with a commercially available glucose oxidase method (GOD-Perid, Boehringer/Mannheim, Indianapolis, IN). Animals were diabetic at least 12 days prior to use. 3-Methylcholanthrene, 25 mg/kg in corn oil, was injected intraperitoneally 72 and 48 hr before the animals were killed and phenobarbital (80 mg/kg in 0.9% saline) was given daily for 3 days by intraperitoneal injection prior to sacrifice. Diabetic animals treated with insulin were given 4.5 I.U./kg by subcutaneous injection for 8 days prior to killing them. This treatment schedule restored weight gain and blood glucose concentration to normal.

Cytochrome P-450 solubilization. Rats were decapitated, and their livers were perfused *in situ* with ice-cold 0.15 M KCl to eliminate hemoglobin contamination [13]. The livers were removed, and microsomes were prepared by standard methodology as previously described [11]. The washed microsomes

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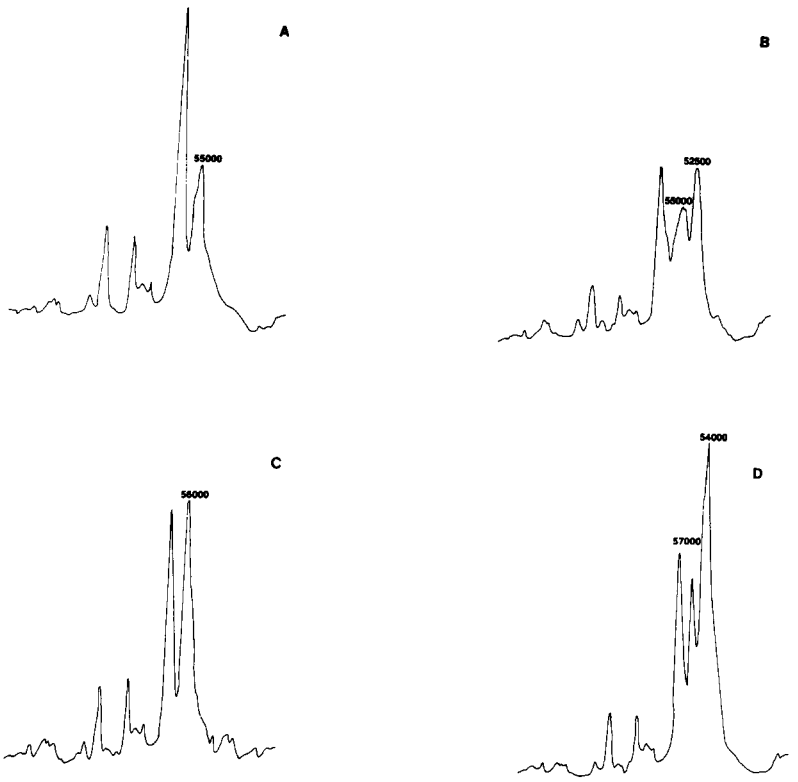

Fig. 1. Absorbance at 550 nm of the Coomassie-blue stained protein bands after SDS–PAGE of Emulgen 911 solubilized fractions from liver microsomes of normal (A), alloxan-diabetic (B), 3-methylcholanthrene-pretreated (C) and phenobarbital-pretreated (D) male rats. Direction of migration was from left to right.

were resuspended in 0.25 M sucrose to a protein concentration of 20 mg/ml and Emulgen 911 solubilization of the microsomal cytochrome P-450 content was conducted as described by Gibson and Schenkman [14]. Cytochrome P-450 solubilized in this manner is stable for at least several months when frozen at −65°.

Cytochrome P-450 separation and purification. The solubilized cytochrome P-450 containing solutions were applied to 1.6 × 20 cm laurate AH-Sepharose 4B columns and separated by affinity chromatography as described by Gibson and Schenkman [14] into two cytochrome P-450 fractions designated fractions I and II. These cytochrome P-450 fractions were treated under N_2 for 30 min with a minimum of 0.15 g/ml Bio-Beads SM-2 in order to remove excess detergent [15]. The fractions were concentrated to 20 ml total volume each in an Amicon filtration cell fitted with a PM-30 membrane. The second and consistently higher specific content cytochrome P-450 containing fractions, fraction II, were further purified by hydroxylapatite chromatography by a modification of the method of Wang *et al.* [16]. In this procedure, the cytochrome P-450 fractions were diluted 2-fold with 20 mM sodium phosphate buffer (pH 7.25) containing 25% glycerol, applied to 1.6 × 15 cm hydroxylapatite columns and eluted stepwise with 38, 112, and 225 mM sodium phosphate buffers (pH 7.25) containing 25% glycerol and 0.2% Emulgen 911. The fractions which eluted with 112 and 225 mM buffer contained cytochrome P-450 and were designated fractions IIA and IIB respectively. These fractions were treated to remove detergent and concentrated as before. All preparations were stored at −65°.

Assays. Protein concentration was determined by the method of Lowry *et al.* [17] with appropriate considerations for interfering substances [18]. Total cytochrome P-450 concentrations were determined from the dithionite reduced CO-difference spectra using the extinction coefficient of 91 $mM^{-1}cm^{-1}$ as described by Omura and Sato [19]. All spectral measurements were made with a Varian Cary 219 spectrophotometer in the automatic baseline correction mode. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the Laemmli system modified for tubular gels and constant power operation as described in detail by Past and Cook [12]. After staining for protein with Coomassie brilliant blue R-250, the gels were visually compared and then scanned under carefully controlled conditions at 550 nm in a Varian Cary 219 spectrophotometer with gel scanning attachment. Molecular weights were determined as described by Shapiro *et al.* [20] from the electrophoretic mobilities of the protein bands observed as peaks on the scans compared to the mobilities (scan peaks) of molecular weight standards: lysozyme, 14,400; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; and phosphorylase B, 92,500 mol. wt from Bio-Rad Laboratories and pyruvate kinase,

Table 1. Rates of drug metabolism by hepatic microsomes from diabetic, insulin-treated diabetic, and normal rats

Microsomes	Aminopyrine metabolism* (formaldehyde produced) [nmoles·min^{-1}·(mg protein)$^{-1}$]	% of Normal	Aniline metabolism* (*p*-aminophenol produced) [nmoles·min^{-1}·(mg protein)$^{-1}$]	% of Normal
Normal	7.21 ± 0.44 (7)	100	0.80 ± 0.04 (7)	100
Diabetic	3.44 ± 0.08† (4)	48	1.74 ± 0.18† (6)	218
Insulin-treated diabetic	7.25 ± 0.19 (5)	101	0.87 ± 0.05 (5)	109

* Each value is the mean ± S.E.M.; the number of observations is given in parentheses.
† $P < 0.01$ vs normal (control) microsomes.

57,000 mol. wt from the Millipore Corp., Bedford, MA. Substrate–cytochrome P-450 difference spectra were obtained using equal concentrations of the cytochrome P-450s (2.0 nM) and 1.1 mM aniline, 1.0 mM metyrapone, 2.6 mM aminopyrine and 85 nM SKF 525-A substrate concentrations. Microsomal mixed-function oxidase reactions with aminopyrine and aniline as substrates and appropriate statistical comparisons were conducted exactly as previously described [11].

RESULTS

Since inter-laboratory comparisons of cytochrome P-450 preparations are often confusing [21], the separation and properties of diabetic cytochrome P-450s have been examined in parallel with the more widely studied cytochrome P-450s obtained from normal (untreated), 3-methylcholanthrene-pretreated and phenobarbital-pretreated rats.

Effects of diabetes. Initially, differences in the hemeprotein composition of the normal and variously treated rat livers were examined by subjecting the detergent solubilized microsomal cytochrome P-450 fractions to SDS–PAGE and observing the stained protein bands. Figure 1 shows that the diabetic fraction yielded a unique protein profile in the cytochrome P-450 molecular weight hemeprotein region [21] compared to the normal fraction or fractions from either of the other experimental groups.

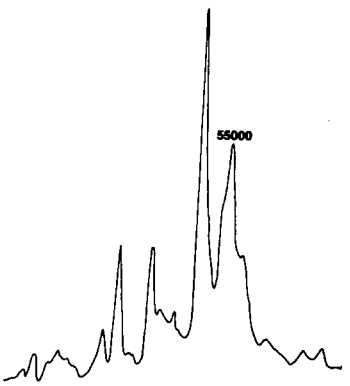

Fig. 2. Absorbance at 550 nm of the Coomassie-blue stained protein bands after SDS–PAGE of an Emulgen 911 solubilized liver microsomal fraction from an insulin-treated diabetic male rat. Direction of migration was from left to right.

The unique diabetic cytochrome P-450 profile (Fig. 1B) has been observed to persist in diabetic rats for at least 44 days (data not shown). This change in apparent cytochrome P-450 hemeprotein composition was accompanied by an increase in the solubilized cytochrome P-450 specific content from 1.49 in normal to 2.20, 2.16, and 3.18 nmoles/mg protein (each an average of duplicate experiments from pooled livers of nine to twenty-two animals) in diabetic, 3-methylcholanthrene-pretreated and phenobarbital-pretreated rats respectively.

Insulin-treated diabetes. If the unique hemeprotein profile shown in Fig. 1B is truly diabetes-dependent, then amelioration of the diabetic state should eliminate this distinct characteristic. The effect of insulin treatment of diabetic rats on this characteristic diabetic hemeprotein profile is shown in Fig. 2. It can be seen that insulin treatment caused the hemeprotein profile of the detergent solubilized microsomal fraction of diabetic rats (Fig. 1B) to return to that of normal rats (Fig. 2 compared to Fig. 1A). This insulin mediated return to a normal hemeprotein pattern was accompanied by a corresponding decrease in the diabetes-elevated cytochrome P-450 content to an amount comparable to that observed in normal solubilized microsomes, 1.49 ± 0.14 nmoles/mg protein (N = 5). Conversion of the diabetic hemeprotein profile and diabetic cytochrome P-450 specific content to normal by insulin treatment indicates that the alterations in cytochrome P-450 content and composition caused by alloxan diabetes were the result of a deficiency of insulin (i.e. diabetes) and not an effect of the diabetogenic agent used. Similarly, we have observed, as have others [2, 6, 10], that insulin treatment returns the divergent effects of diabetes on the metabolism of type I and type II compounds [22] toward normal values (Table 1).

Chromatographic separation of the cytochrome P-450s. To determine which of the specific hemeprotein components in the cytochrome P-450 population were affected by diabetes compared to normal and chemically induced cytochrome P-450s, the solubilized cytochrome P-450 fractions were purified by laurate affinity and hydroxylapatite column chromatography. The results of a typical set of these separation procedures are summarized in Table 2. The specific contents of the cytochrome P-450s reported in Table 2 are based on protein determinations of the purified hemeproteins by the Lowry method and may be underestimated by as much as 50% compared

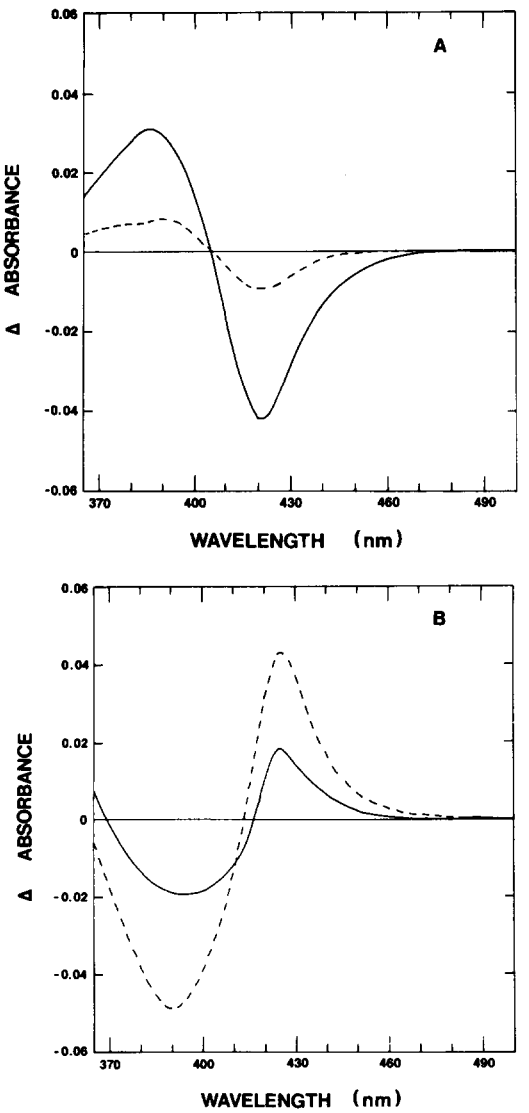

Fig. 3. Difference spectra of 85 nM SKF 525-A (A) and 1.0 mM metyrapone (B) with 2.0 nM normal cytochrome P-450 IIA (——) and 2.0 nM diabetic cytochrome P-450 IIB (---).

to specific content estimations based on protein content determined from amino acid analysis [23]. All of the isolated hemeproteins including the two diabetic fractions yielded a reduced CO-absorbance difference maximum at 450 nm except for the expected 448 nm maxima exhibited by the two 3-methylcholanthrene induced hemeproteins. Each cytochrome P-450 fraction yielded a single major protein band (except as indicated in the molecular weight column) by the SDS–PAGE method of Laemmli, a method considered to give the best resolution of cytochrome P-450 hemeproteins [24]. When co-chromatographed in the SDS–PAGE system with diabetic P-450 IIB, the other P-450 fractions in Table 2 did not co-migrate with diabetic P-450 IIB (data not shown). The molecular weight of diabetic cytochrome P-450 IIA was consistently similar to that of the major normal cytochrome P-450 IIA. The molecular weight of the other diabetic cytochrome P-450, IIB, was less than that of any of the other cytochromes isolated and corresponded to the molecular weight of the diabetes-induced hemeprotein band observed in the solubilized microsomal fraction (Fig. 1B).

Substrate binding affinity. The interactions of the major cytochrome P-450 containing fractions with type I and type II substrates were determined by observing the substrate–cytochrome P-450 difference spectra. The binding spectra of normal cytochrome P-450 IIA, compared to that of diabetic cytochrome P-450 IIB with both SKF 525-A and metyrapone, a type I and type II substrate, respectively, are shown in Fig. 3. As can be seen, these cytochrome P-450s gave typical binding spectra with both types of substrates. However, compared to the normal, an equal concentration of the diabetic cytochrome P-450 yielded a smaller absorbance change (peak to trough) with the type I substrate and a much greater absorbance change with the type II substrate. The increased spectral change and symmetry observed in the type II substrate–diabetic cytochrome P-450 IIB difference spectra indicates the diminished type I binding character [25] of the diabetic compared to the normal hemeprotein. Typical substrate binding spectra were also obtained with the other cytochrome P-450s. Since aminopyrine and aniline have been widely used

Table 2. Characteristics of cytochrome P-450 hemeprotein fractions isolated from solubilized hepatic microsomes by column chromatography

Fraction	Cytochrome P-450		
	Specific content (nmoles/mg protein)	% of Total*	Molecular weight†
Normal IIA	11.6	14	54,000
IIB	16.5	6	54,000
Diabetic IIA	9.4	13	54,000
IIB	14.8	17	52,000
3-Methylcholanthrene IIA	16.9	14	53,500
IIB	20.7	24	55,000
Phenobarbital IIA	11.4	1	54,000
IIB	11.4	23	56,000, 54,000

* Compared to the nmoles of P-450 in the original solubilized microsomal fraction taken as 100%.

† Estimated by SDS–PAGE as described in Materials and Methods.

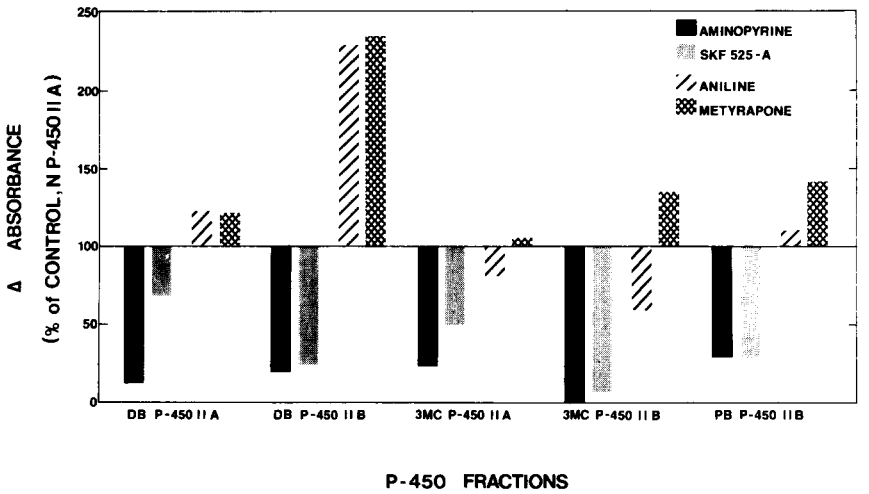

Fig. 4. Absorbance changes ($ABS_{max} - ABS_{min}$) determined from the difference spectra of 2.0 nM each of the major cytochrome P-450s (Table 2) with type I substrates (SKF 525-A, aminopyrine) and type II substrates (metyrapone, aniline) compared to the absorbance changes obtained from the binding of the same substrates with 2.0 nM normal cytochrome P-450 IIA under identical conditions. Changes of absorbance $\times 10^3$ for normal cytochrome P-450 IIA under these conditions were 195 with aminopyrine, 755 with SKF 525-A, 122 with aniline, and 384 with metyrapone.

as examples of type I and type II substrates in contrasting the effects of diabetes on the rates of rat liver microsomal mixed-function oxidase reactions, substrate–cytochrome P-450 binding spectra were also obtained with these substrates. A quantitative comparison of the absorbance changes generated by the binding of experimentally induced cytochrome P-450s to different substrates compared to the binding of the same substrates to normal cytochrome P-450 IIA is shown in Fig. 4. This comparison clearly shows that the diabetic cytochrome P-450 IIB was bound to the type II substrates to give absorbance changes that were over two times greater than that observed with the normal reference cytochrome P-450 and approximately double that of any of the other isolated cytochrome P-450s.

DISCUSSION

The divergent effects of diabetes on the rates of metabolism of type I and type II compounds shown in Table 1 have been well documented in both the microsomal system and in perfused liver [2–4, 6, 8, 10, 11]. The mechanisms through which diabetes exerts these effects on drug metabolism have not been established. However, it has been shown that cytosolic factors are not involved [11]. On the other hand, as we have observed and as reported by others [6], diabetes has been shown to increase the total spectrally measurable level of the terminal component of the mixed-function oxidase system—cytochrome P-450. Indeed, previous comparison of the protein profiles of normal and diabetic hepatic microsomes obtained by SDS–PAGE indicated that specific cytochrome P-450 hemeproteins were distinctly increased and/or decreased by diabetes, and it was suggested that such changes mediated the substrate-specific alterations in drug metabolism in diabetic rat liver [12].

The results presented in this report show that indeed there is a unique P-450 isoenzyme in the diabetic compared to normal or chemically induced solubilized hepatic microsomal fractions. The appearance of this 52,000 mol. wt P-450 in diabetic rat liver and its comparatively high binding affinity for type II substrates correlate with the increased catalytic activity of diabetic hepatic microsomes with these types of substrates ([2–4, 6, 8, 11] and Table 1).

In contrast to the well-characterized chemical induction of cytochrome P-450 by phenobarbital, 3-methylcholanthrene, and other chemical inducers, diabetes is a physiopathological inducer of P-450, or more specifically, a consequence of the uncontrolled disease state in an animal model of the naturally occurring human pathological condition of insulin-dependent diabetes. The present study indicates that the induction of hepatic P-450 by diabetes appears to be persistent, lasting for at least months. This persistence contrasts with the transient induction of P-450 by treatment with phenobarbital or other chemical inducers [26]. Other laboratories have reported isolation of 52,000 mol. wt P-450s from normal rat liver as well as from rats treated with a variety of inducers including Aroclor 1254, pyrazole, and isosafrole [23, 27, 28]. However, intra-laboratory SDS–PAGE or similar comparisons of these induced forms of P-450 and the 52,000 mol. wt diabetic P-450 would have to be conducted in order to obtain truly comparative molecular weights. A more definitive method for the demonstration of unique P-450 hemeproteins would be to assess the cross-reactivity of various forms of P-450 with specific antibodies prepared against purified P-450s [21]. Consequently, an antibody specific to the 52,000 mol. wt diabetic P-450 is currently being prepared in this laboratory.

Although the present study has examined only

model drug substrates, it is likely that the cytochrome P-450 mediated metabolism of many other substances is also altered as a result of the unique hepatic cytochrome P-450 hemeprotein population present in the diabetic rat. For example, it has been shown that diabetic rats are extremely susceptible to liver injury by carbon tetrachloride (CCl_4) [29, 30]. Recent evidence has indicated that a 52,000 mol. wt cytochrome P-450 is responsible for metabolism of CCl_4 to the trichloromethyl radical ($\cdot CCl_3$), a suspected active hepatic toxin [31, 32]. Therefore, the 52,000 mol. wt diabetes-dependent cytochrome P-450 described in this report may be responsible for potentiating the hepatotoxic effect of CCl_4. Likewise, diabetes has also been shown to increase and decrease position-specific hydroxylation of androstenedione in the same manner as it increases and decreases aniline and aminopyrine metabolism respectively [33]. Assessment of hepatic drug metabolism in diabetic humans has been limited. However, in a recent study, it was shown that hepatic cytochrome P-450 levels were increased significantly in human diabetics with normal livers compared to control patients with normal livers [34].

The specific effects of the presence of a unique diabetic hepatic cytochrome P-450 on the metabolism of steroids, fatty acids, and environmental xenobiotics remain to be examined with purified diabetic cytochrome P-450. This laboratory is currently comparing the catalytic activity of the unique diabetic P-450 with the catalytic activities of the normal and chemically induced P-450s in a reconstituted system.

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