Biochemical Pharmacology, Vol. 37, No. 18, pp. 3505–3509, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

CYTOCHROME P-450 ALTERATIONS IN THE BB/Wor SPONTANEOUSLY DIABETIC RAT

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(*Received* 27 *November* 1987; *accepted* 7 *March* 1988)

Abstract—The hepatic content of two individual cytochrome P-450 enzymes was analyzed in spontaneously diabetic (BB/Wor) male rats. The major male-specific form, RLM5, was found to be slightly decreased in livers of male rats shortly after the onset of diabetes. In contrast, the level of RLM6 was elevated in livers of diabetic rats that had not received insulin and had become ketotic. These results confirm that the changes in some individual forms of cytochrome P-450 after chemical (streptozotocin) induction of diabetes are also seen in the spontaneously diabetic animal. The earlier observed alterations in cytochrome P-450 are therefore due to the state of diabetes and not to inductive or depressive effects of streptozotocin.

Cytochrome P-450 is a term employed to designate a class of heme-containing enzymes found in most tissues. Hepatic microsomal cytochrome P-450 is primarily responsible for the metabolism of exogenous compounds but also functions in the metabolism of several endogenous compounds such as steroid hormones [1–6], prostaglandins [7], and fatty acids [8, 9], as well as other compounds such as acetone and acetol [10]. Under certain pathophysiological conditions, such as chemically-induced diabetes, hepatic microsomal cytochrome P-450-dependent drug metabolism is either depressed or enhanced depending on the nature of the substrate employed [11–13]. Insulin treatment of diabetic rats is generally able to reverse these changes, suggesting that the effects are due to the pathophysiological state of diabetes and not to inductive or repressive effects of the chemical employed to produce diabetes. Further indication that this is the case is that some of the same changes in drug metabolism are also seen in the spontaneously diabetic rat [14]. This strain of rat, the BioBreeding/Worcester (BB/Wor) rat, demonstrates many of the same changes seen in the disease for which it is a model, insulin-dependent (Type I) diabetes in humans [15, 16].

Recently, we demonstrated alterations in hepatic microsomal content of individual hepatic cytochrome P-450 enzymes in streptozotocin-induced diabetes using monospecific antibodies raised to five forms of this enzyme found in the untreated male rat [17, 18]. We now report that some of the major changes seen in hepatic cytochrome P-450 in this model system are also observable in the BB/Wor rat model.

MATERIALS AND METHODS

Animals. Spontaneously diabetic (BB/Wor) male rats were obtained from the NIH contract colony maintained at the University of Massachusetts Medical Center, Worcester, MA. Two types of rats were utilized, diabetes resistant (DR) and diabetes prone (DP). Rats in our experiments were designated as diabetes resistant (DR), diabetes prone (the 6-week-old DP rats), newly diabetic (DP rats newly diagnosed as diabetic, as judged by elevated serum glucose levels) or chronically diabetic but receiving a low dose of insulin daily. Prone rats were approximately 6 weeks of age; they develop diabetes between 60 and 120 days of age (mean 92 days) and must receive insulin to survive. This group received 1–2 units of U40 protamine zinc (Eli Lilly, Indianapolis) insulin daily in order to eliminate ketone bodies in the urine as well as to avoid weight loss. Some of these animals were withdrawn from insulin treatment (diabetic minus insulin) 4 days before they were killed.

Preparation of microsomes. Rats received food and water *ad lib.* and were killed by decapitation between 9:00 and 10:00 a.m. Livers were excised, chilled in 0.25 M sucrose on ice, and perfused with ice-cold 0.9% sodium chloride. Hepatic microsomes from individual rats were prepared by the rapid calcium-aggregation procedure of Schenkman and Cinti [19]. The washed microsomes were resuspended in sucrose at a concentration of 15–25 mg/ml and stored in 1- to 1.5-ml aliquots at −80°.

Purification of cytochrome P-450 enzymes and preparation of monospecific antibodies. Cytochromes P-450 RLM5† and RLM6 were purified from

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† Several different laboratories have developed independent nomenclatures for the different forms of rat liver microsomal cytochromes P-450. The corresponding names for those forms discussed in this paper include: RLM5, M.1, P-450h, 2c, UT-A [20–24]; RLM6, P-450j, P-450ac [18, 25, 26]. The nomenclature used by our laboratory indicates the enzyme source, e.g. Rat Liver Microsomes. The relative migration in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the 45–61 kD region, from anode to cathode, is designated by the number. Thus, RLM6 is the sixth band upward from 45 kD toward the cathode. RLM6 is orthologous to a form of rabbit liver microsomal cytochrome P-450, isozyme 3a [27], and to a form of human liver cytochrome P-450, HLj [28, 29].

Table 1. Comparison of serum glucose, serum acetone and microsomal enzyme activities in BB/Wor rats

Strain or treatment of BB/Wor rat	Specific content (nmol P-450/ mg protein)	Serum glucose (mg/dl)	Serum acetone (mM)	Aminopyrine demethylase (nmol product/mg protein/min at 37°)	Aniline hydroxylase (nmol product/mg protein/min at 37°)
Prone	0.64 ± 0.07	157 ± 25	0.35 ± 0.09	6.6 ± 0.8	0.35 ± 0.01
Resistant	0.76 ± 0.11	133 ± 15	0.29 ± 0.06	7.8 ± 0.6	0.37 ± 0.02
Newly diabetic	0.86 ± 0.08	340 ± 98	0.40 ± 0.11	6.8 ± 0.66*	0.40 ± 0.08
Diabetic + insulin	0.58 ± 0.11	311 ± 86	0.39 ± 0.18	4.0 ± 0.73†	0.37 ± 0.11
Diabetic − insulin	0.89 ± 0.28	662 ± 201†	4.69 ± 2.6†	6.1 ± 1.3‡	0.80 ± 0.12†

Prone rats were 6 weeks old when killed. All other rats were between 12 and 16 weeks of age when killed. Values (mean ± SD) for each variable represent determinations from six animals, except for diabetic rats deprived of insulin for 3 or 4 days where twelve rats were employed. Newly diabetic rats were killed on the day elevated levels of urinary glucose were detected. Rats described as "Diabetic + insulin" were maintained on low dose insulin for 2–4 weeks. Rats described as "Diabetic − insulin" were maintained on low dose insulin for 2–4 weeks, then deprived of insulin for 4 days before being killed.

*–‡ P values were obtained using Student's *t*-test, comparing diabetic with diabetes resistant values: *$P < 0.1$, †$P < 0.01$, and ‡$P < 0.02$.

untreated or diabetic male CD rats by published methods [18, 21]. Polyclonal antibodies were raised to these two cytochrome preparations and made monospecific by previously described procedures [17, 18].

Assay procedure. Serum glucose was analyzed by the glucose oxidase-peroxidase method with a kit from the Sigma Chemical Co. After incubation, the absorbance at 405 nm was determined and compared to a standard solution of glucose. Serum acetone was determined by a modification of the method of Jain [30]. Blood was collected in sealable vials from the decapitated rats for serum glucose and acetone measurements. The blood was allowed to clot and was then centrifuged to separate the serum. Acetone and glucose were measured within 24 hr and the samples were kept frozen at −80°. Studies indicated that no change in glucose or acetone occurred in these samples for at least 1 month. 6 ft × 2 mm glass column was packed with 80/100 Carbopack C/0.2% CW 1500 (Supelco) and incubated at 180° overnight in a Varian model 3700 gas chromatograph. For acetone analysis, the injection temperature was set at 160° and the oven temperature was 70°. Serum acetone levels were determined by comparison of data to a standard curve of known concentrations of acetone. Hepatic microsomal aminopyrine demethylase and aniline hydroxylase assay procedures have been described [31]. Microsomal protein was determined by the biuret method, purified detergent-free cytochrome P-450 was determined by the Lowry procedure, and cytochrome P-450 was determined from the difference spectrum of the sodium dithionite reduced-carbon monoxide adduct versus the reduced cytochrome as described before [32].

Western blots were performed by the method of Towbin *et al.* [33] with modifications [17]. Electrophoretic transfer onto nitrocellulose paper (Schleicher & Schuell) was performed for 45 min at 100 V using a model TE 51 Transphor power supply (Hoefer Scientific Instruments). Alkaline phosphatase-conjugated goat anti-rabbit or rabbit anti-goat antibody (Sigma) diluted in 3% bovine serum albumin in phosphate-buffered saline was employed as the secondary antibody, and immunodetectable bands were produced as previously described [18].

RESULTS

Table 1 demonstrates that microsomal drug-metabolizing activities were altered in the diabetic rats in much the same way as was seen in the alloxan- and streptozotocin-induced diabetic rats [17, 34]. For example, aminopyrine demethylase was decreased slightly in the newly diagnosed diabetic and in chronically diabetic rats either receiving insulin or deprived of insulin. In contrast, the aniline hydroxylase activity appeared to be elevated in chronically diabetic rats not receiving insulin but not in the newly diabetic rat. Aniline hydroxylase levels were somewhat depressed in the chronically diabetic rat receiving a low dose of insulin. The latter finding resembles the results seen with the streptozotocin-induced diabetic rat receiving insulin [12, 14, 17]. Acetone levels were elevated only slightly in the newly diabetic BB/Wor rat compared to the non-diabetic resistant rats. In contrast, acetone levels were extremely high (10-fold elevated) only in the diabetic rats deprived of insulin for 3 or 4 days (Table 1). Similar acetone levels have been obtained in animals made diabetic by streptozotocin or alloxan [34, 35].

When microsomes prepared from livers of the BB/Wor animals were analyzed with monospecific antibody to RLM5 (Fig. 1), the levels of this cytochrome appeared to be slightly lower in the 6-week-old diabetes prone (DP) rats (lanes 1–3) than in the 12-week-old DR rats (lanes 4–6). This is not surprising since RLM5 is a developmentally induced isozyme and is not fully expressed at the age of 6 weeks [36]. In the age-matched newly diabetic animals, the concentration of RLM5 appeared to be no different after the onset of diabetes (Fig. 1, lanes 7–9). After 3 weeks, while the animals received low dose insulin treatment, there was a noticeable, yet slight decrease in RLM5 (Fig. 1, lanes 10–12). After insulin withdrawal for 4 days (Fig. 1, lanes 13–15),

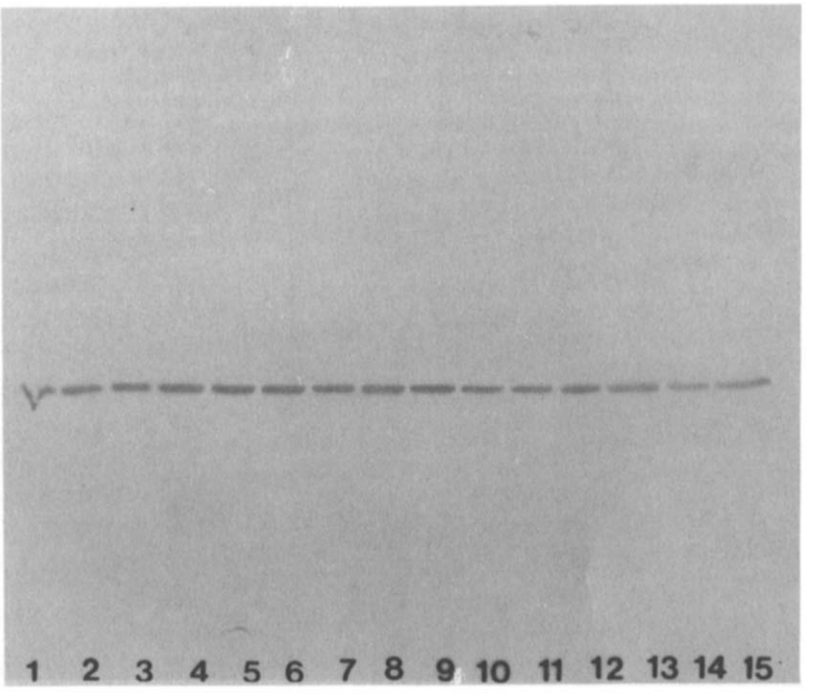

Fig. 1. Western blot analysis for RLM5 of microsomal fractions from BB/Wor rats. Microsomal protein (12.5 μg) from the following types of BB/Wor rats was electrophoresed: lanes 1–3, DP (prone); lanes 4–6, DR (resistant); lanes 7–9, (newly diabetic); lanes 10–12, diabetic receiving low dose insulin; and lanes 13–15, diabetic rats to which low dose insulin treatment was withdrawn 3 or 4 days before they were killed. See Results for a more complete description. After transfer, nitrocellulose sheets were probed with monospecific antibody to RLM5 as described in Materials and Methods.

diabetic rats demonstrated a further decrease in the levels of RLM5 relative to levels seen in the diabetes resistant rats (lanes 4–6). This decrease was not as dramatic as that seen in the streptozotocin-induced diabetic rat after 7 days without insulin [17, 37]. When the same microsomal samples were analyzed with monospecific antibody to RLM5a, there was consistently no apparent change seen in the levels of this cytochrome in any of the different populations of animals (data not shown), in agreement with results from streptozotocin-induced diabetic rats [37]. In contrast to the changes seen for RLM5, the concentration of RLM6 appeared to be not much different in the DP rats than in the DR rats (Fig. 2, lanes 1–3 and 4–6 respectively). When, at about 9 weeks of age, diabetes became detectable (by elevated urinary glucose levels), the levels of RLM6 appeared to become slightly higher (lanes 7–9) in the newly diabetic rats, but after 3 weeks of low dose insulin treatment the levels were restored to normal (lanes 10–12). When insulin treatment was subsequently withdrawn in the diabetic rats, there was a rapid elevation in the levels of RLM6 (lanes 13–15) discernible within 3–4 days. These results demonstrate that diabetes, which appears spontaneously in these animals, is like streptozotocin-induced diabetes and results in elevated levels of RLM6 and decreased levels of RLM5 [17, 18]. Low dose insulin treatment for 3 weeks was sufficient to maintain RLM6 at control levels bud did not reverse completely the decreased levels of RLM5. Complete withdrawal from insulin was required before a full induction of RLM6 occurred and was probably, as seen with streptozotocin [37], time dependent. A reported difference in the kinetics of RLM5 and RLM6 changes in diabetes and during insulin reversal of the effects of diabetes [37] further suggests differences in the mechanisms responsible for regulation of these two cytochromes.

DISCUSSION

The study of the role of drug-metabolizing enzymes in altered physiology has not been addressed at the level of individual cytochromes until recently [17, 18, 38]. The present study demonstrates that the levels of at least two individual constitutive cytochromes P-450 can be altered under the influence of a spontaneous pathophysiological condition such as diabetes, indicating that the levels of these cytochromes are under homeostatic control. We have demonstrated previously altered levels of at least four constitutive cytochromes, including the two demonstrated herein, in chemically-induced diabetes [17, 18, 37].

Since a genetic model of human spontaneously occurring diabetes exists (the BB/Wor rat), we have also monitored levels of several constitutive cytochromes P-450 in these rats. The rationale here is 2-fold: first, the absence of streptozotocin administration eliminates any potential problems with hepatotoxicity due to the drug; and, second, the use of

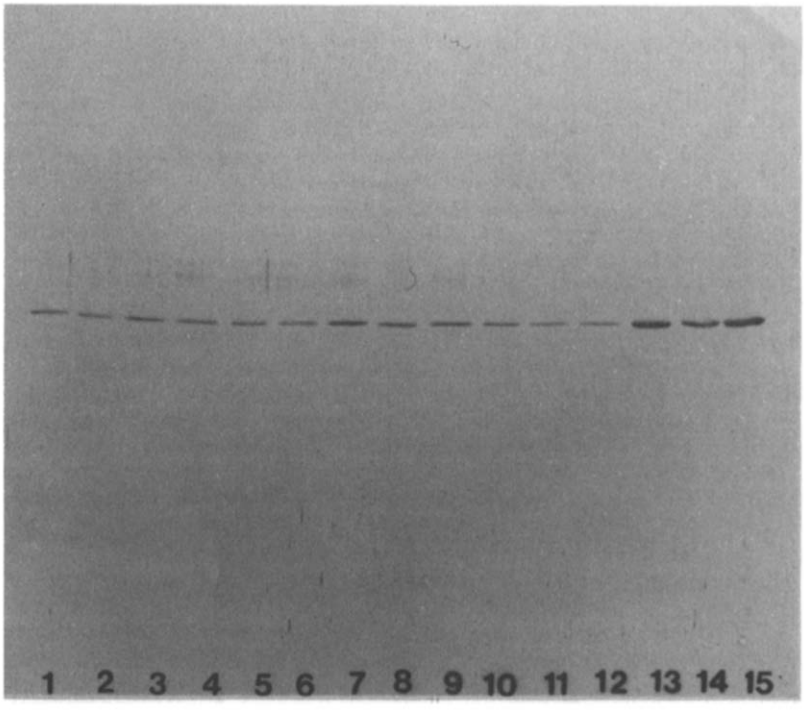

Fig. 2. Western blot analysis for RLM6 of microsomal fractions from BB/Wor rats. Samples applied to each well were the same as in Fig. 1. Blots were probed with monospecific antibody to RLM6 as described in Materials and Methods.

the spontaneous diabetic rat eliminates the possibility that changes observed were due to the presence of a drug. The changes observed in the spontaneous diabetic rat were similar to those seen in the streptozotocin-induced diabetic rat [37], and this is a further indication that the effects seen were due to the disease and not to the agent used to elicit the diabetic state. The BioBreeding/Worcester (BB/Wor) spontaneously diabetic rat strain is subdivided into two different populations of animals [16]. There is a resistant (DR) sub-population that does not develop diabetes and a sub-population that is prone (DP) to diabetes but has not yet demonstrated overt signs of diabetes; 50–85% of prone rats develop diabetes between 60 and 120 days and are subsequently maintained on low doses (1–2 I.U./day) of insulin in order to allow these rats to survive. These chronically diabetic, but insulin-treated rats, had only slightly elevated blood glucose levels (200–400 mg/dl compared to the streptozotocin-induced rat not receiving insulin, i.e. 400–700 mg/dl), but no apparent elevation in serum acetone levels (Table 1).

As in chemically-induced diabetes [17, 18], genetically-induced diabetes results in decreased levels of RLM5 and elevated levels of RLM6. Although the level of RLM5 had not decreased as dramatically in the newly diabetic rat as in the streptozotocin diabetic rat, it must be remembered that the diabetic rat had to be maintained on a low insulin dose within 4 days in order to survive. Studies with streptozotocin-induced diabetic rats indicate that complete loss of RLM5 occurs by three weeks after induction of diabetes [37]. Therefore, the residual level of RLM5 seen in the insulin-treated BB/Wor rat may more appropriately be compared to the level of RLM5 seen in the streptozotocin-diabetic rat receiving insulin treatment. Four days without insulin in the diabetic rat may not be sufficient time to allow a major loss of this cytochrome. The level of RLM6 in the DP rat was not detectably different from the level seen in the DR rat. This is probably because serum acetone levels were not greater than in the resistant rat (Table 1). As suggested earlier [18], alterations in RLM6 levels probably reflect changes in plasma acetone levels. In the insulin-treated chronically diabetic rat, the level of RLM6 did not increase but resembled levels seen in the resistant rat and in the streptozotocin-induced diabetic rat maintained on insulin [18]. It is possible that the low dose of insulin used was sufficient to prevent the induction of RLM6 (perhaps by decreasing plasma levels of ketone bodies). In BB/Wor rats newly diagnosed as diabetic, there was no sizable elevation in RLM6 levels until 3 or 4 days after the rise in blood glucose. This was consistent with the small elevation in aniline hydroxylase levels by 4 days after insulin withdrawal (Table 1, and Ref. 14). As in chemically-induced diabetes, after the onset of spontaneously occurring diabetes, RLM5 levels are decreased and RLM6 levels are increased [37]. While treatment with low dose insulin was sufficient to reverse the elevation of RLM6 in these rats, RLM5 levels were not completely restored to control levels. The greater responses of the two cytochromes in streptozotocin-diabetic rats stem from the lack of an

absolute requirement for insulin in order for these rats to survive for 3–15 weeks. It takes about 3 weeks for changes in RLM5 and RLM6 to become maximal [37].

The regulation of some constitutive cytochrome P-450 enzymes in altered pathophysiological states appears to be under complex physiological and hormonal control. For example, the presence of high levels of RLM5 in male rats has been shown to be dependent on growth hormone [39]. The absence of RLM5 in streptozotocin-induced male diabetic rats has been postulated to be due to a change in growth hormone in these rats [37], since the intermittent male-secretion pattern of growth hormone has been shown to be lost in streptomycin-induced diabetes [40]. While it is clear that certain cytochrome P-450 enzymes undergo major or minor changes in concentration in the liver endoplasmic reticulum after the onset of chemically-induced or spontaneous diabetes, it is not known how these changes occur at the molecular level. The ability of insulin to reverse all of these changes in cytochrome P-450 content, although at different rates, suggests that this hormone is somehow involved in some step common to both of the postulated regulatory pathways.

Acknowledgements—The authors would like to thank Dr. Carl Malchoff for critical discussions involving this work. We would also like to thank Dr. John E. Mole for providing the use of his laboratory for some of these experiments. The expert technical assistance of Wendy J. VanSickle in the production of antisera is acknowledged. This work was supported in part by US Public Health Service Grant GM26114.

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