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Hepatic Cytochrome P-450j Induction in the Spontaneously Diabetic BB Rat

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SUMMARY

Hepatic microsomal cytochrome P-450j has been studied using the male spontaneously diabetic BB rat as a model for insulin-dependent diabetes. This approach avoids any direct hepatotoxic effects from chemical diabetogenic agents. Both diabetic rats maintained on insulin and nondiabetic littermates were used as controls. Levels of cytochrome P-450j were increased approximately 3-fold in the diabetics 4 days after the cessation of insulin therapy. In addition, cytochrome P-450j-catalyzed enzymatic activities, aniline hydroxylation, and *N*-nitrosodimethylamine *N*-demethylation were increased by the diabetic state at this same time period. Cytochrome P-450f remained at control levels in all groups of animals. In order to test the hypothesis that ketone bodies are involved in the increase in cytochrome P-450j in the diabetic state, plasma β-hydroxybutyrate levels were monitored. Hepatic aniline hydroxylation, *N*-nitrosodimethylamine *N*-demethylation, and cytochrome P-450j levels in individual animals were found to correlate with plasma β-hydroxybutyrate levels ($r = 0.59–0.71$ $p < 0.001$). In contrast, no significant correlation between levels of cytochrome P-450j and plasma glucose, insulin, or cholesterol was observed in individual animals ($r = 0.07–0.23$, $p > 0.4$). We conclude that cytochrome P-450j is induced in the livers of spontaneously diabetic rats, and that this induction may be associated directly or indirectly with elevated plasma ketone levels.

It has been known for some years that chemically induced diabetes affects the hepatic microsomal cytochrome P-450 monooxygenases (1–8). Certain enzyme activities, such as benzo[*a*]pyrene hydroxylase and aminopyrine *N*-demethylase, are decreased in male diabetic rats (1–8). Other activities, such as aniline hydroxylase, (2, 3, 5, 7, 8) are increased in both male and female diabetic rats. More recently it has been found that NDMA demethylase is also elevated in the diabetic state (9). We have shown that most, if not all, NDMA demethylation at 1 mM substrate concentration is catalyzed by a single cytochrome P-450 isozyme (P-450j) in rats (10).[1] This isozyme also catalyzes the hydroxylation of aniline (12, 13) and is elevated by treatment with ethanol (10, 13, 14), isoniazid (10, 12) dimethyl sulfoxide, pyrazole, and 4-methylpyrazole (10), as well as fasting (15) and chemically induced diabetes (10). Therefore, we set out to determine whether cytochrome P-450j was induced[2] in diabetic rats which were not exposed to a toxic diabetogenic chemical such as streptozotocin or alloxan. The animal model chosen was the spontaneously diabetic BB rat (8, 16, 17). This is an inbred strain of Wistar rat in which approximately 50% of the animals spontaneously develop insulin-dependent diabetes at about 100 days of age.

It is known that many of the conditions in which cytochrome P-450j and *N*-nitrosodimethylamine demethylation are increased occur when plasma ketones are elevated (10, 18). We have previously postulated that the change in cytochrome P-450 isozyme pattern in diabetic rats may be due in part to the ketosis produced (8). Therefore, we studied the correlation between levels of cytochrome P-450j and plasma β-hydroxybutyrate.

Materials and Methods

Chemicals. Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Aniline hydrochloride was obtained from Eastman Kodak Co. (Rochester, NY). Protamine zinc insulin (Connaught Laboratories, Willowdale, Ontario, Canada) was used to maintain the diabetic animals where indicated. [^{14}C]NDMA was purchased from New England Nuclear Research Products (Boston, MA). All other chemicals and reagents were of analytical grade purity.

Animals. Male spontaneously diabetic BB rats were obtained from the Health Protection Branch, Health and Welfare, Canada, courtesy of Dr. P. Thibert. Diabetic rats were maintained on 9 units/kg/day

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[1] According to a recently recommended nomenclature system for cytochromes P-450 (11), rat P-450f and P-450j are encoded by the genes designated *II C7* and *II E1*, respectively.

[2] For the purposes of this paper, we define induction as an increased level of cytochrome due to increased *de novo* synthesis and/or stabilization of preexisting protein.

ABBREVIATIONS: NDMA, *N*-nitrosodimethylamine; SDS, sodium dodecyl sulfate; IgG, immunoglobulin fraction purified from preimmune sera.

TABLE 1

Enzyme activities and levels of cytochrome P-450 isozymes in hepatic microsomes from male spontaneously diabetic BB rats

The control group consisted of nondiabetic littermates. The insulin-treated group consisted of diabetics administered 9 units/kg/day protamine zinc insulin subcutaneously. Values are means ± standard errors. Due to the small group size ($n = 4$) of diabetic animals off insulin therapy for 2 days, the analysis of variance and Newman-Keuls Multiple Range Test were not used for statistical analysis of these data in Tables 1 and 2. However, the results obtained from this group were significantly different from the values obtained for insulin-treated or control animals by Student's t test for all parameters except cytochrome P-450f, cholesterol, and insulin (control versus 2 days off insulin).

Group	N	Aniline hydroxylation	NDMA Demethylation	Cytochrome P-450j	Cytochrome P-450f
		nmol/min/mg microsomal protein		*μg/mg microsomal protein*	
Control	10	0.57 ± 0.04	0.36 ± 0.02	4.59 ± 0.36	10.82 ± 0.72
Insulin-treated	8	0.41 ± 0.02	0.27 ± 0.02	3.04 ± 0.17	12.94 ± 0.93
Two days off insulin	4	0.94 ± 0.09	0.92 ± 0.12	10.30 ± 1.16	12.13 ± 2.68
Four days off insulin	18	1.01 ± 0.11[a,b]	1.20 ± 0.16[a,b]	13.49 ± 1.69[a,b]	13.0 ± 1.39

[a] Significantly different from control group, $p < 0.05$.
[b] Significantly different from insulin-treated group, $p < 0.05$.

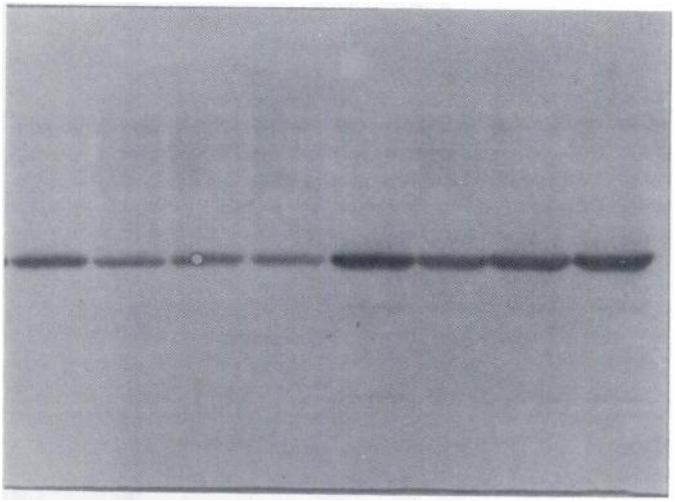

Fig. 1. Immunoblot of rat hepatic microsomes probed with anti-P-450j. SDS-gel electrophoresis, electrophoretic transfer of proteins to nitrocellulose, and immunoblotting were performed as described (10). Each well of the gel contained 7 μg of microsomal protein. Wells *1* and *2*, nondiabetic controls; wells *3* and *4*, insulin-treated diabetics; wells *5–8*, diabetics (4 days).

protamine zinc insulin, subcutaneously, for 2–5 weeks following the development of glucosuria. For the experimental groups, no insulin was given for either 2 or 4 days. Nondiabetic littermates served as controls. All animals were maintained on Lobund grade corncob bedding (Paxton Processing, Paxton, IL) at 22°, lights on 6:00 am to 8:00 pm. The urine of untreated diabetic animals had greater than 2% urinary glucose as measured by Testape. Animals were approximately 4 months of age at the time of the experiment. They were stunned and decapitated, and liver microsomes were prepared as previously described (19).

Assays. Aniline hydroxylation was measured as previously described (20, 21). NDMA demethylation was assayed by the method of Hawke and Welch (22), using conditions as described by us previously (23). The preparation of monospecific polyclonal antibodies to cytochromes P-450f and P-450j was carried out by the methods of Bandiera *et al.* (24) and Thomas *et al.* (10), respectively. Antibodies to cytochromes P-450f and P-450j were used in a competitive enzyme-linked immunosorbent assay to quantitate these cytochromes as described previously (10, 24). Conditions for SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose, and immunoblot analysis of the proteins have been described (10). Insulin was assayed utilizing the radioimmunoassay kit of Amersham Corp. (Arlington Heights, IL); Diagnostica Assay Kits for cholesterol and glucose were obtained from Boehringer-Mannheim GmbH (Mannheim, West Germany). β-Hydroxybutyrate was measured according to the method of Persson (25).

Statistics. Analysis of variance was performed, along with the Newman-Keuls Multiple Range Test and calculation of Pearson correlation coefficients (r) and coefficient of determination (r^2). Results were considered significant at $p < 0.05$.

Results

As shown in Table 1, hepatic microsomes from nondiabetic rats and diabetic BB rats treated with insulin metabolized

TABLE 2

Plasma levels of β-hydroxybutyrate, glucose, insulin, and cholesterol in the male spontaneously diabetic BB rat

The control group consisted of nondiabetic littermates. Insulin-treated rats were diabetics given 9 units/kg/day protamine zinc insulin, subcutaneously. Values are means ± standard errors. See Table 1 legend for explanation of statistical analyses.

Group	N	β-Hydroxybutyrate	Glucose	Insulin	Cholesterol
		mM	*mg/dl*	*units/ml*	*mg/dl*
Control	10	0.20 ± 0.04	151.8 ± 10.2	62.8 ± 13.0	88.0 ± 2.3
Insulin-treated	8	0.32 ± 0.09	150.6 ± 18.8	283.9 ± 67.9[a,b]	98.1 ± 5.3
Two days off insulin	4	1.80 ± 0.25	422.5 ± 7.2	37.5 ± 6.9	80.5 ± 4.0
Four days off insulin	18	7.25 ± 1.41[a,c]	445.3 ± 29.3[a,c]	27.6 ± 3.2	109.8 ± 9.2

[a] Significantly different from the control group, $p < 0.05$.
[b] Significantly different from the 4 days off insulin group, $p < 0.05$.
[c] Significantly different from the insulin-treated group, $p < 0.05$.

TABLE 3

Effect of anti-P-450j on NDMA demethylation in hepatic microsomes

Microsomal protein (0.2 mg) was preincubated with 0.4 mg of anti-P450j IgG for 10 min at room temperature. Following preincubation, metabolism of NDMA (1 mM) was measured as described (22, 23). As shown previously (10), addition of control IgG has no effect on enzymatic activity.

Group	NDMA demethylase		% Inhibition of NDMA demethylation
	Control IgG	Anti-P-450j	
	nmol/min/mg protein		
Nondiabetic	0.33	0.05	85
Insulin-treated	0.27	0.05	81
	0.25	0.03	88
Two days off insulin	1.15	0.12	90
	1.23	0.11	91
Four days off insulin	2.53	0.20	92
	1.23	0.12	90
	2.48	0.25	90
	1.43	0.12	92
	2.89	0.22	92
	1.65	0.12	93
Total			89.4 ± 1.1

aniline and NDMA with equivalent efficiency and had approximately the same content of cytochromes P-450j and P-450f. When insulin was withheld from spontaneously diabetic animals for 2 days, a 2- to 3-fold increase in both catalytic activity and cytochrome P-450j content was observed. Only modest additional increases in catalytic activity and cytochrome P-450j were observed if insulin treatment of diabetic rats was withdrawn for 4 days (Table 1). By contrast, cytochrome P-450f remained at control levels under these same conditions. Cytochrome P-450f has been shown to be relatively resistant to induction by a wide variety of chemicals that are known inducers of certain other cytochrome P-450 isozymes (24). Hence, immunoquantitation of cytochrome P-450f was performed in this study to demonstrate that the diabetic state does not lead to an increase in all isozymes of cytochrome P-450.

Further proof of induction of cytochrome P-450j was obtained from an immunoblot of an SDS-gel probed with anti-P-450j (Fig. 1). Clearly, there are greater amounts of the cytochrome present in hepatic microsomes of diabetic animals, (wells *5–8*, Fig. 1) compared to either the nondiabetic controls (wells *1* and *2*, Fig. 1) or the insulin-treated diabetics (wells *3* and *4*, Fig. 1).

In the diabetic rats, plasma β-hydroxybutyrate was elevated 9-fold at 2 days and 36-fold at 4 days without insulin (Table 2). Plasma glucose was elevated at 2 days and remained at that level during the 4-day period. Plasma insulin decreased to less than half the control values at the 4-day time period. This is consistent with previous experiments with spontaneously diabetic BB rats (8). Cholesterol remained at control levels in all groups at these time periods. Insulin treatment of diabetic rats resulted in control values of all parameters measured, with the exception of insulin itself (Tables 1 and 2).

In order to evaluate NDMA demethylation as a measure of cytochrome P-450j content in the spontaneously diabetic rat, monospecific antibody to cytochrome P-450j was used to inhibit this catalytic activity (Table 3). Representative microsome samples from individual animals in each treatment group were analyzed for hepatic demethylase activity with the addition of anti-P450j. Greater than 80% of the enzyme activity in each sample was found to be due to cytochrome P-450j. The variability among the control values in the diabetic group without insulin for 4 days may be a reflection of the range of severity of diabetes in the animals.

Excellent correlations were found between cytochrome P-450j and aniline hydroxylation ($r^2 = 0.95$), or NDMA demethylation ($r^2 = 0.86$; Fig. 2). Significant correlations were also seen between plasma β-hydroxybutyrate and hepatic microsomal cytochrome P-450j ($r^2 = 0.355$; Fig. 2), aniline hydroxylation ($r^2 = 0.35$), and NDMA demethylation ($r^2 = 0.51$). However, no significant correlations were obtained between any of the above parameters and cytochrome P-450f, plasma insulin, cholesterol, and glucose. It should be noted that, with glucose, there is a maximal renal reabsorptive capacity which must be exceeded before glucose appears in the urine. Therefore, this

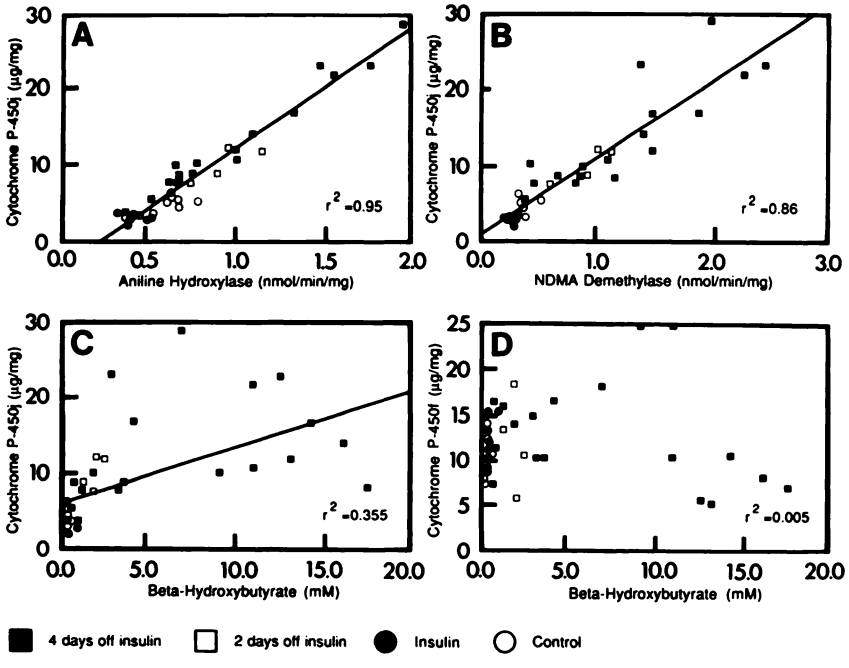

Fig. 2. Correlations of cytochromes P-450j or P-450f with hepatic enzyme activity or plasma β-hydroxybutyrate levels.

complicates any correlations using plasma levels and accounts for there being only two sets of values: control or insulin-treated levels of approximately 150 mg/dl, and the plateau diabetic levels of 425–445 mg/dl.

Discussion

The spontaneously diabetic BB rat provides an excellent model of insulin-dependent diabetes (17). No exogenous chemicals are required to produce the diabetic state; therefore, a clear interpretation of data is possible. In these animals, diabetes is manifested by the usual increases in plasma and urinary glucose. After 4 days without insulin administration, endogenous insulin levels decrease to approximately half that found in the nondiabetic littermates. A severe ketosis occurs, as indicated by the increased β-hydroxybutyrate levels in plasma. Under these conditions, the diabetic animals survive only up to a week or two, depending on the severity of the diabetes.

We found a significant induction of hepatic microsomal cytochrome P-450j in the diabetic animals when insulin was withheld for 2–4 days. The activity of NDMA demethylase, approximately 90% of which was shown to be due to cytochrome P-450j, was similarly increased under these conditions. Several isozymes of cytochrome P-450 have previously been shown to be responsible for aniline hydroxylase activity, including cytochrome P-450j (12). We found a significant increase in this activity after 2 and 4 days of untreated diabetes. Under all conditions tested, levels of cytochrome P-450f remained constant, which provided a control, indicating that normal levels of this isozyme were maintained in the diabetic state. There was a greater variability in cytochrome P-450f and P-450j levels noted among the BB diabetic animals compared to their nondiabetic littermates. This increased variability may be due to different degrees of pathology occurring in the BB rats, and is similar to that seen in human patients (17).

As expected, an excellent correlation was found between the levels of cytochrome P-450j and aniline hydroxylation or NDMA demethylation. Although the correlation of β-hydroxybutyrate with these parameters was not as impressive, it was still highly significant ($p < 0.001$). Variations in the degree of pathology of the disease state in individual diabetic animals may account for the decreased correlation coefficient. Miller and Yang (18) have shown that NDMA demethylation is induced in the fasted rat, a metabolic condition also associated with ketosis. Fasted rats had a plasma β-hydroxybutyrate level elevated 10-fold over control, accompanied by a 3-fold increase in NDMA activity. Therefore, there may be a common determinant to the induction observed in these two animal models. Our results indicate that cytochrome P-450j is induced in the spontaneously diabetic rat, and that elevated ketone bodies in these animals may be involved, either directly or indirectly, in this induction.

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