Potentiation of Carbon Tetrachloride Hepatotoxicity and Lethality in Type 2 Diabetic Rats

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ABSTRACT

There is a need for well characterized and economical type 2 diabetic model that mimics the human disease. We have developed a type 2 diabetes rat model that closely resembles the diabetic patients and takes only 24 days to develop robust diabetes. Nonlethal doses of allyl alcohol (35 mg/kg i.p.), CCl₄ (2 ml/kg i.p.), or thioacetamide (300 mg/kg i.p.) yielded 80 to 100% mortality in diabetic rats. The objective of the present study was to investigate two hypotheses: higher CCl₄ bioactivation and/or inhibited compensatory tissue repair were the underlying mechanisms for increased CCl₄ hepatotoxicity in diabetic rats. Diabetes was induced by feeding high fat diet followed by a single dose of streptozotocin on day 14 (45 mg/kg i.p.) and was confirmed on day 24 by hyperglycemia, normoinsulinemia, and oral glucose intolerance. Time course studies (0–96 h) of CCl₄ (2 ml/kg i.p.) indicated that although initial liver injury was the same in nondiabetic and diabetic rats, it progressed only in the latter, culminating in hepatic failure, and death. Hepatic microsomal CYP2E1 protein and activity, lipid peroxidation, glutathione, and ¹⁴C-CCl₄ covalent binding to liver tissue were the same in both groups, suggesting that higher bioactivation-based injury is not the mechanism. Inhibited tissue repair resulted in progression of injury and death in diabetic rats, whereas in the nondiabetic rats robust tissue repair resulted in regression of injury and survival after CCl₄ administration. These studies show high sensitivity of type 2 diabetes to model hepatotoxicants and suggest that CCl₄ hepatotoxicity is potentiated due to inhibited tissue repair.

Several animal models resembling type 2 diabetes either occur spontaneously or can be induced experimentally. Most of the commonly used models of type 2 diabetes are genetic and have the disadvantage of prohibitive costs, unavailability, and failure to represent etiology of human disease. Consumption of high fat diet leads to insulin resistance and is considered to be a major predisposing factor for type 2 diabetes (Kraegen et al., 1986). Models based on high fat diet take 3 months or longer to develop diabetes and yield only moderate hyperglycemia (Surwit et al., 1988; Pascoe et al., 1992; Reed et al., 2000). Thus, there is a need for a robust type 2 diabetes model.

To address this need, we have refined and characterized an existing model based on high fat diet and a single dose of streptozotocin (STZ, 45 mg/kg i.p.). The principle behind the development of type 2 diabetes is simple. High fat diet elicits insulin resistance, and the rats maintain normoglycemia due to compensatory hyperinsulinemia. Administration of STZ (45 mg/kg i.p.) decreases insulin levels, destroying a population of pancreatic β-cells such that the insulin-resistant rats are now unable to maintain normal glucose levels and develop hyperglycemia, even though insulin levels in these rats are comparable with normal diet-fed normoglycemic rats. This is exactly what is seen in human diabetes where insulin resistance precedes hyperglycemia, thereby making this model a good representative of human type 2 diabetic (DB) condition.

ABBREVIATIONS: STZ, streptozotocin; DB, diabetic; NDB, nondiabetic; ND + STZ, normal diet-fed rats injected streptozotocin; TA, thioacetamide; AA, allyl alcohol; SD, Sprague-Dawley; "H-T, tritiated thymidine; HFD, high fat diet-fed rats injected citrate buffer; HFD + STZ, high fat diet-fed rats injected streptozotocin; ND, normal diet fed rats injected citrate buffer; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PCNA, proliferating cell nuclear antigen; MES, 2-(N-morpholino)ethanesulfonic acid.
A recent epidemiological study showed that the DB patients are twice as likely to suffer from hepatic failure compared with non-diabetic (NDB) patients (El-Serag and Everhart, 2002). Severe life-threatening liver injuries are not uncommon in diabetic patients receiving medication, including anti-diabetic drugs such as troglitazone and acarbose (Ballet, 1977; Andrade et al., 1996; Neuschwander-Tetri et al., 1998). Evidence with animal studies suggest that type 1 diabetic rats are more sensitive to model hepatotoxicants such as thioacetamide (TA) and CCl₄ (Wang et al., 1997; Mak and Ko, 1997; Wang et al., 2000). Increased hepatic bioactivation enzymes (Watkins et al., 1988), decreased detoxification systems (Mak and Ko, 1997), and inhibited tissue repair (Wang et al., 2000) are examples of mechanisms that lead to higher sensitivity. Although there is ample evidence that type 1 diabetes increases sensitivity to hepatotoxicants, such sensitivity of type 2 diabetes has not been investigated. This is more important because type 2 diabetes afflicts 95% of all the DB patients (Portha et al., 1991). Having refined and characterized a robust type 2 DB rat model, we wished to test its sensitivity to model hepatotoxicants. We hypothesized that type 2 DB rats are sensitive to hepatotoxic and lethal actions of diverse model hepatotoxicants, by enhanced bioactivation and impaired compensatory liver repair. Three hepatotoxicants [CCl₄, TA, and allyl alcohol (AA)] were used for lethality studies. For mechanistic studies, CCl₄ was chosen as the model hepatotoxicant.

Our studies indicate that type 2 diabetes is highly sensitive to hepatotoxicants. Higher sensitivity does not seem to be due to enhanced bioactivation of CCl₄. It seems that impaired compensatory liver tissue repair leads to progression of liver injury initiated by an ordinarily nonlethal dose of CCl₄, culminating in liver failure and mortality in type 2 DB rats. Future studies on signaling events underlying liver tissue repair in diabetes will be focused on mechanisms of markedly increased sensitivity to hepatotoxicants and on impaired compensatory tissue repair.

Materials and Methods

Animals and Diets

Animal maintenance and research were conducted in accordance with the National Institute of Health Guide for Laboratory Animal Welfare. Male Sprague-Dawley (SD) rats (100–124 g) obtained from our central facility were housed over sawdust bedding (Sani-Chips; Harlan Teklad, Madison, WI) in air-conditioned quarters (21 ± 1°C) with a 12-h photoperiod. Rats had ad libitum access to water and rodent chow [either normal rodent diet consisting of 6% fat, 20% protein, and 53% carbohydrate (Rat Chow no. 7012; Harlan Teklad, Madison, WI) or high fat diet consisting of 20% fat, 20% protein, and 46% carbohydrate (Dyets no. 100795, Dyets Inc., Bethlehem, PA)].

Chemicals

[³H-CH₃]Thymidine (³H-T; 2 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA); scintillation fluid was obtained from Fisher Scientific Co. (Baton Rouge, LA). Rosiglitazone (Avandia, 8-mg tablets; GlaxoSmithKline, Research Triangle Park, NC) was purchased from Rite Aid Pharmacy (West Monroe, LA). All other chemicals and biochemical kits were purchased from Sigma-Aldrich (St. Louis, MO).

Treatments

Induction of Type 2 Diabetes. Beginning on day 0, rats were fed either normal rodent chow or high fat diet. On day 14, rats on the high fat diet were injected with either a single low dose of streptozotocin (HFD + STZ) (STZ, 45 mg/kg i.p., in 0.01 M citrate buffer pH 4.3) to induce diabetes or citrate buffer (1 ml/kg) (HFD) as vehicle control. Rats on the normal rodent diet injected with either a single dose of STZ (ND + STZ) (45 mg/kg i.p., in 0.01 M citrate buffer pH 4.3) or citrate buffer (ND) (1 ml/kg i.p.) served as controls. Subsequent to STZ or citrate buffer treatment, rats had free access to food and water and were continued on their respective diets for the duration of study. Blood sampled from the retroorbital plexus under diethyl ether anesthesia on day 24 was used to measure plasma glucose and insulin concentration. Rats exhibiting plasma glucose >200 mg/dl (kit 315-100; Sigma-Aldrich, St. Louis, MO) and plasma insulin in the range of 0.5 to 3 ng/ml (enzyme-linked immunosorbent assay; Crystal Chemical Co., Downers Grove, IL) were considered to have type 2 diabetes.

Characterization of Type 2 Diabetes Model

On day 24, in the HFD + STZ group, type 2 diabetes was confirmed by measuring nonfasting plasma glucose and insulin levels. Other characteristics such as plasma triglycerides (glycerol phosphate oxidase, Trinder method; catalog no. 343, Sigma-Aldrich) and free fatty acids (catalog no. 994-75409, Wako Chemicals, Richmond, VA) were measured. The degree of uncontrolled DB state was confirmed by measuring glycated hemoglobin (catalog no. 441-B, Sigma-Aldrich). To test the persistence of diabetes, the DB rats were continued on high fat diet for 6 months. After 6 months, diabetes was confirmed by measuring glucose, insulin, triglycerides, free fatty acids, and glycated hemoglobin as mentioned above.

Oral Glucose Tolerance Test. To assess oral glucose tolerance, rats (n = 4 each group) were fasted overnight (12 h) and their plasma glucose and insulin response to oral administration of a bolus glucose solution (20% solution, 5 g/kg) was determined. Blood samples were taken from the retroorbital plexus under diethyl ether anesthesia before (time 0) and 15, 30, 60, 120, 180, 240, and 360 min after glucose administration. Plasma glucose and plasma insulin concentrations were determined.

Effect of Antihyperglycemic Agents. The response of this type 2 diabetes model to metformin and rosiglitazone treatments was tested. DB rats with similar blood glucose concentrations (n = 8) were injected with either vehicle (distilled water) or metformin (350 mg/kg i.p.) twice daily at 10:00 AM and 10:00 PM each day for 5 days. Blood was sampled retroorbitally at 9:50 AM, 4:00 PM, and 9:50 PM. To another set of rats, rosiglitazone (Avandia, 64 mg/kg p.o.) was administered once daily for 14 days at 2:00 PM. Plasma glucose was analyzed once daily (9:50 AM) as mentioned above.

Lethality Study

On day 24, DB and NDB rats (n = 10/group) were treated with CCl₄ (2 ml/kg i.p., in corn oil 1:1) or corn oil (2 ml/kg), TA (300 mg/kg i.p.) or saline (3 ml/kg) and AA (35 mg/kg i.p.) or vehicle control (distilled water, 1 ml/kg). Rats were observed twice daily for 14 days. Survival/mortality was recorded thrice on the 1st day and twice daily thereafter for 14 days.

CCl₄-Induced Liver Injury. On day 24, DB (HFD + STZ) and NDB rats (ND injected citrate buffer, HFD injected citrate buffer, and normal diet group injected STZ (ND + STZ)) were treated with either a single injection of CCl₄ (2 ml/kg i.p., in equal volume of corn oil) or corn oil (2 ml/kg i.p.) alone. The rats treated with CCl₄ were euthanized with diethyl ether for the time course studies (0 to 96 h, n = 4/time point except DB group, n = 12 for 36 and 48 h). The plasma and liver tissue from these rats were collected at 0, 12, 24, 36, 48, 72, and 96 h after dosing. From the DB and NDB rats treated with corn oil, blood and liver samples were collected only at 0, 24, and 96 h after corn oil administration (n = 4/group/time point).

Hepatotoxicity

Plasma Transaminases, Ammonia, and Bilirubin. Plasma was separated by heparinization and centrifugation. Plasma alanine
The liver sections were processed, and then embedded in paraffin. Portions of liver were taken from the left lateral lobes and fixed immediately in 10% phosphate-buffered formalin.

Liver Glycogen Levels. Total hepatic glycogen was estimated as a marker of hepatic energy status, as total nonprotein anthrone-positive sugars (Seifer et al., 1950), and expressed as micrograms of glycogen per gram of liver.

Histopathology. Portions of liver were taken from the left lateral lobes and fixed immediately in 10% phosphate-buffered formalin. The liver sections were processed, and then embedded in paraffin. Liver sections (5 μm in thickness) were stained with H&E for histological examination by light microscopy. Unstained liver sections were prepared for PCNA immunohistochemistry. The extent of liver necrosis was estimated semiquantitatively under a light microscope and lesions were scored as multifocal necrosis. Most affected lobules contained necrotic zones. Scoring was as follows: 0, no necrosis; 1, minimal (occasional necrotic cells/lobule); 2, mild (<1/3 of the lobule necrotic); 3, moderate (>1/3 to <2/3 of the lobule necrotic); 4, severe (<2/3 of lobule affected); and 5, massive (most parenchyma necrotic).

Effect of Diabetes on Hepatic CYP2E1
Preparation of Microsomes. Liver microsomes were prepared by differential ultracentrifugation using the method of Chipman et al. (1979). Protein content of the microsomes was determined by Bradford (1979). Protein content of the microsomes was determined by the method of Chipman et al. (1979). Protein content of the microsomes was determined by the method of Chipman et al. (1979).

CYP2E1 Protein and Enzyme Activity Assay. CYP2E1 protein (Western blot analysis) and enzyme activity (hydroxylation of p-nitrophenol) was measured as described previously (Wang et al., 2001).
Effect of Diabetes on Covalent Binding, Lipid Peroxidation, and Glutathione Levels

In Vivo Covalent Binding of 14C-CCl4 Equivalents to Liver Proteins. To determine whether diabetes altered the covalent binding of 14C-equivalents of liver proteins, 14CCl4 was administered (2 ml/kg i.p., 8 µCl/µg, in 1:1 corn oil) to DB and NDB rats. The rats were killed 2 h after 14CCl4 administration. Livers were excised, frozen in liquid nitrogen, and then kept at −80°C until time for analysis. Two grams of liver tissue was homogenized in 4 ml of ice-cold Tris-HCl buffer (pH 7.4) in a Polytron homogenizer for 30 s at 4°C. Two milliliters of homogenate was added to tubes containing 4 ml of ice-cold 100% ethanol, which precipitated proteins. The tubes were chilled in an ice bath for 15 min, mixed using a vortex mixer, and then centrifuged at 3000g for 30 min. To remove lipids and noncovalently bound 14C equivalents away, the pellets were extracted extensively with a series of washes with 3:1 chloroform/methanol mixture. The pellets (containing covalently bound 14CCl4 derived radiolabel) were air-dried, dissolved in 1 ml of 1 N NaOH, and the radioactivity was estimated by scintillation spectrometry.

In Vivo Lipid Peroxidation. In vivo lipid peroxidation of liver tissue, as assessed by diene conjugation, was measured as described previously (Mehendale and Klingensmith, 1988). Three grams of liver tissue, as assessed by diene conjugation, was measured as described previously (Mehendale and Klingensmith, 1988). Three grams of tissue, as assessed by diene conjugation, was measured as described previously (Mehendale and Klingensmith, 1988). A 0.5-ml aliquot of the homogenate was extracted with 9.5 ml of chloroform/methanol [2:1 (v/v)], filtered, and then centrifuged at 3000g for 15 min at 4°C. The supernatant was deproteinized by adding to it an equal volume of metaphosphoric acid and then centrifuging it at 2000g for 2 min. The supernatant was carefully collected and was ready for total glutathione estimation using the commercially available glutathione assay kit (catalog no. 703002) from Cayman Chemical (Ann Arbor, MI).

Glutathione Estimation. Total glutathione was estimated at 0, 12, and 24 h after CCl4 administration. Approximately 0.5 g of liver was washed with phosphate-buffered saline and homogenized in 1.5 ml of cold buffer (i.e., 50 mM MES, pH 6–7 containing 1 mM EDTA). The homogenate was further centrifuged at 10,000g for 15 min at 4°C. The supernatant was deproteinized by adding to it an equal volume of metaphosphoric acid and then centrifuging it at 2000g for 2 min. The supernatant was carefully collected and was ready for total glutathione estimation using the commercially available glutathione assay kit (catalog no. 703002) from Cayman Chemical (Ann Arbor, MI).

Tissue Repair

3H-T Pulse Labeling Study. S-Phase DNA synthesis was estimated by 3H-T incorporation into hepatonuclear DNA as described by Chang and Looney (1965) and by Chauveau et al. (1956). DNA content was estimated by diphenylamine reaction (Burton, 1956), and incorporation of radiolabeled 3H-T was determined by liquid scintillation spectrometry (PerkinElmer Life Sciences, Boston, MA).

PCNA Assay. Cell cycle progression was estimated by immunohistochemical analysis of PCNA expression as described by Greenwell et al. (1991). For histomorphometric analysis, each section was observed for cells in different stages of the cell cycle in 10 high power fields as reported previously (Wang et al., 2000). One thousand cells/slide were counted, and the number of cells in each stage was recorded.

Statistical Analysis

Data are expressed as means ± S.E. Comparison between two groups at the same time point was made by Student’s t test. Comparison between different time points for each group was made by analysis of variance followed by Tukey’s and Dunnett’s post hoc tests using the SPSS, 11.0 software (SPSS Science Inc., Chicago, IL). The criterion for significance was p ≤ 0.05.
ter and food intake, and increased urine output (14-fold) compared with the NDB rats (data not shown).

Table 1 contains the values obtained for plasma glucose, insulin, leptin, triglycerides, free fatty acids, and glycated hemoglobin in the DB and NDB rats. Average plasma glucose was 3-fold higher in the DB rats (450 ± 66 mg/dl, HFD + STZ group) compared with the NDB rats (123 ± 10 mg/dl, ND group) (Table 1). The plasma insulin levels in the DB rats were in the normal range (0.5–2 ng/ml). Glycated hemoglobin (HbA1c) was 2-fold higher in DB rats, indicating persistent hyperglycemia. The DB rats were also characterized by higher plasma triglycerides (1.6-fold) and free fatty acid levels (2.2-fold) compared with NDB rats (Table 1).

Figure 1 illustrates the blood glucose values (A) and insulin (B) during a time course after oral glucose tolerance test. In the NDB rats fed normal diet (ND), plasma glucose values returned to normal levels 2 h after oral glucose challenge. However, DB rats exhibited high fasting glucose (241 ± 32 mg/dl, indicated at 0-h time point in Fig. 1A) and glucose intolerance as indicated by high glucose levels at 2 h (450 ± 78 mg/dl) (Fig. 1). The HFD rats, in spite of normal glucose levels, exhibited insulin resistance as evidenced by higher insulin levels at 2 h after glucose administration (Fig. 1B). Thus, the oral glucose tolerance test proved three points: 1) feeding high fat diet results in insulin resistance; 2) DB rats exhibited increased fasting glucose; and 3) glucose intolerance was evident in DB rats.

The DB rats responded to treatment with known antidiabetic drugs such as metformin and rosiglitazone. Metformin treatment for 5 d significantly decreased plasma glucose to the normal range in the DB rats by day 3. Table 1 contains the average plasma glucose measured three times a day. Rosiglitazone treatment for 14 days significantly reduced the blood glucose level in DB rats by day 10.

The DB rats that were continued on the high fat diet regimen for 6 months exhibited hyperglycemia, hypertriglyceridemia, high free fatty acids, and high glycated hemoglobin, indicating that the DB state is persistent, as shown in

Table 1. These DB rats also exhibited classical secondary complications of diabetes such as diabetic renal hypertrophy and cataract (data not shown). Therefore, this type 2 DB model seems to resemble closely the human type 2 DB syndrome.

Sensitivity of Type 2 Diabetic Rats to Model Hepatotoxicants

Lethality Studies. Toxicity initiated by CCl₄ (2 ml/kg i.p.) and TA (300 mg/kg i.p.) markedly progressed in the DB rats as indicated by 100% mortality contrasted with 100% survival of the NDB rats. Death occurred between 24 and 48 h after CCl₄ and TA administration only in DB rats (Table 2). Also, AA (35 mg/kg i.p.) toxicity was potentiated in DB rats, leading to 80% mortality in the DB group compared with 100% survival of the NDB group. The DB rats died between 36 and 48 h after AA administration. High sensitivity of DB rats to CCl₄ toxicity was chosen for mechanistic investigation.

Hepatotoxicity. Time-course study of liver injury of CCl₄ can reveal insights regarding the extent of initial injury and progression/regression phase of injury. Therefore, plasma ALT and AST activities were measured over a time course to evaluate liver injury (Fig. 2), whereas bilirubin and ammonia (Fig. 3) were measured to evaluate liver function after CCl₄.
treatment. As indicated above, in the DB group, 12 rats were used at the 36- and 48-h time points. A total of 4 and 0 rats were alive at 36 and 48 h, respectively. The data represent the values for surviving animals at all time points.

Although the initial injury in both DB and NDB rats was the same, DB rats exhibited significantly greater liver injury at 36 h (ALT, 2-fold and AST, 2.7-fold). Liver dysfunction [plasma bilirubin (6.8-fold) and ammonia (7.5-fold)] was high and progressive compared with normal diet fed NDB controls at 36 h, thereby indicating hepatic failure and greater sensitivity of type 2 DB rats to CCl4 hepatotoxicity. In the DB rats, liver injury was progressive and irreversible from 12 h onwards, which peaked at 36 h. The temporal persistence and progression of liver injury are concordant with the time of mortality as seen with lethality studies. In contrast, plasma ALT activity increased in NDB rats until 48 h and then gradually decreased to normal by 96 h (Fig. 2A), suggesting recovery from liver injury consistent with 100% survival observed in the lethality study. Plasma AST also revealed a similar pattern of liver injury (Fig. 2B). In DB and NDB groups receiving corn oil, ALT/AST were measured at 0 (data shown), 12, 24, and 96 h after corn oil administration and no liver injury was evident (data not shown).

Plasma bilirubin increased in NDB rats compared with the control at 12 h, elevated at 36 and 48 h before declining to control level at 72 h after CCl4 administration. Although increased compared with control in the NDB group, bilirubin remained in the normal range (0.1–1.2 mg/dl). In stark contrast, in DB rats, plasma bilirubin (Fig. 3A) markedly increased at 24 and 36 h after CCl4 administration (4–5 mg/dl), indicating progressive liver dysfunction. Plasma ammonia (Fig. 3B) increased in NDB rats only at 48 h and then declined to normal by 96 h, indicating recovery. However, in DB rats elevation of plasma ammonia was substantial and progressive, confirming liver failure. Progressively elevated ammonia and bilirubin levels indicated deteriorating hepatic function culminating in liver failure in DB rats receiving the same dose of CCl4 from which the NDB rats recovered.

**Histopathology.** Liver sections were examined by light microscopy (Fig. 4) for necrosis, vacuolization, and inflammatory cell infiltration, characteristic of CCl4 injury. The induction of diabetes did not cause any liver damage (Fig. 4, A and B). In the liver of NDB rats at 12 h after CCl4 administration, swollen hepatocytes with inflammatory cell infiltration in centrilobular area were minimally evident (Fig. 4D). Vacuolar degeneration and centrilobular necrosis of hepatocytes and mild inflammation were observed at 36 h (Fig. 4F). By 72 h after CCl4 administration, the necrotic cells had disappeared. In contrast, in the liver of DB rats, mild-to-moderate

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**Fig. 5.** Liver glycogen levels over a time course after CCl4 administration. Treatment details are as in Fig. 2. *p* ≤ 0.05.

**Fig. 6.** Effect of type 2 diabetes on hepatomicrosomal CYP2E1 expression and enzyme activity. Rats were made DB as mentioned in Fig. 1. On day 24, liver samples were collected under diethyl ether euthanasia and microsomes were prepared (*n* = 4). A representative CYP2E1 blot is shown (top). A and B, microsomal CYP2E1 protein was detected by Western blot and quantified by densitometry; CYP2E1 activity was measured by para-nitrophenol-hydroxylase assay (C).
coagulative necrosis was evident at 12 h (Fig. 4C). These effects were progressive by 36 h, manifesting severe-to-massive coagulative necrosis with extensive bridging of necrotic zone affecting all lobules (Fig. 4E). The liver injury was semiquantitatively estimated (Table 3) through a scoring system as described under Materials and Methods. Mean necrosis scores were 2-fold higher in DB rats at 24 and 36 h, compared with NDB rats receiving the same dose of CCl₄.

Liver Glycogen Levels. Liver injury results in breakdown of glycogen, causing significant reduction in hepatic glycogen stores. In both DB and NDB rats, the pattern of glycogen depletion after CCl₄ administration until 36 h (Fig. 5) was similar. There were no survivors in the DB group at 48 h. Glycogen levels rose in all NDB rat livers at 48 h, reaching normal levels by 72 h.

Is Higher Sensitivity of DB Rats Due to Enhanced Bioactivation of CCl₄?

Effect of Diabetes on Hepatic CYP2E1. Western blot analysis showed that hepatic microsomal CYP2E1 protein in DB rats was not different from the NDB rats (Fig. 6, A and B). Microsomal CYP2E1 enzyme activity (Fig. 6C) measured as p-nitrophenol hydroxylation was also unchanged in the DB rats relative to NDB rats, confirming that neither hepatic microsomal CYP2E1 protein nor the enzyme activity is changed in the type 2 DB rats.

Covalent Binding. ¹⁴CCl₄-derived covalently bound radiolabel to liver macromolecules is a reliable measure of the extent of CCl₄ bioactivation. As shown in Fig. 7A, the amount of label covalently bound to liver proteins was not different in the DB rats compared with NDB rats at 2 h after CCl₄ administration.

Lipid Peroxidation. Lipid peroxidation is a critical event after bioactivation of CCl₄. The effect of diabetes on lipid...
peroxidation was investigated by estimating diene conjugation of hepatic lipids. Although lipid peroxidation did increase in all groups (Fig. 7B) after CCl\textsubscript{4} administration, that in the DB group was not different from any other NDB group.

**Hepatic Glutathione.** Diabetes did not decrease hepatic glutathione content. Hepatic glutathione was decreased (Fig. 7C) in the DB rats at 6 h after CCl\textsubscript{4} administration but was restored by 12 h. In the NDB groups, there was no significant depletion in glutathione levels after CCl\textsubscript{4} administration.

**Tissue Repair**

\textsuperscript{3}H-T Pulse Labeling Study. Stimulation of the S phase of cell division cycle is an obligatory step in cell division and tissue repair. The \textsuperscript{3}H-T pulse labeling study (Fig. 8) revealed that DNA synthesis was significantly increased in CCl\textsubscript{4}-treated rats compared with rats not exposed to CCl\textsubscript{4}. In the NDB rats, S-phase DNA synthesis was increased as early as 12 h after CCl\textsubscript{4} administration, which persisted throughout the time course. In the DB group, whereas S-phase stimulation did occur at 12 h, it was 60\% lower and remained lower until death. DB rats were never able to mount sufficient S-phase stimulation in response to challenge by CCl\textsubscript{4}.

PCNA Assay. Immunohistochemical staining of PCNA (Figs. 9 and 10) was conducted with two objectives. First, to verify the findings of S-phase stimulation determined by \textsuperscript{3}H-thymidine pulse labeling study. Second, to assess cell cycle progression. In both DB and NDB rats, most hepatocytes (92–95\%) rested in G\textsubscript{0} phase at 0 h and began entering the replication cycle soon after CCl\textsubscript{4} administration. In the livers of NDB rats, significantly greater number of hepatocytes were in G\textsubscript{1} phase (30 and 35\%, at 12 and 24 h, respectively) compared with DB rats (18 and 20\%). A large number of hepatocytes were seen in S phase (40\%) in NDB rats compared with 10\% in DB rats at 36 h. It should be noted that the wave of S phase was apparent as early as 12 h and persisted throughout the time course in NDB rats. In contrast, S phase was suppressed and delayed in DB rats. The results in the PCNA analysis were consistent with \textsuperscript{3}H-thymidine pulse labeling results. In NDB rats, by 72 h, significant numbers of cells were in S phase, and most of the cells had progressed to M phase. Thus, in the NDB rats, stimulated cell cycle progression lead to recovery and restoration of liver function. However, the delayed and inhibited cell cycle progression evident in DB rats is consistent with the progression of liver injury, liver failure, and mortality.

**Discussion**

The pathophysiology of type 2 diabetes is due to both insulin inaction and insufficient insulin secretion (LeRoith, 2002). Prediabetes is a condition characterized by insulin resistance and hyperinsulinemia to maintain normal glucose levels. The transition from the prediabetic to DB state occurs when the β-islet cells in the pancreas are exhausted and fail to secrete enough insulin, leading to frank hyperglycemia (Warram et al., 1990; Groop et al., 1997). Genetic and environmental factors are thought to be responsible for this progression from prediabetic to DB state (Reed et al., 2000). Although several laboratory animal models of type 2 diabetes are available, they are not without some drawbacks. For example, C57 BL/6j obese (ob/ob) and C57 BLKS/J DB (db/db) mice, both of which are genetic models, have transient hyperglycemia, and extreme degree of hyperinsulinemia (Bailey and Flatt, 1991), characteristics not exhibited in diabetic patients. A significant lacuna in this field has been the unavailability of a satisfactory DB animal model that adequately represents the etiology and symptomatology of human type 2 diabetes. Added factors are the time and high expense in developing the available animal models of type 2 diabetes.

The present study was initiated to refine and characterize a type 2 DB rat model to test whether it closely resembles the pathophysiology of diabetic patients. Also, there was a need to decrease the time to develop the model and decrease its cost. Our studies show that feeding high fat for 24 days in conjunction with by a single dose of STZ (45 mg/kg i.p.) on day 14 results in a 3-fold increase in plasma glucose while maintaining normal insulin levels. This DB rat model also exhibits oral glucose intolerance and elevated fasting glucose, which are characteristic of DB patients. Persistent hyperglycemia is indicated by elevated glycated hemoglobin levels. Furthermore, the DB rats exhibited high triglycerides and free fatty acid levels, characteristic abnormalities of lipid metabolism seen in human diabetics (Albrink, 1974). The DB state was controlled by treatment with known antidiabetic
drugs that control human diabetes. Metformin and rosiglitazone, work via independent mechanisms, thereby confirming the metabolic similarity to human type 2 diabetes. Therefore, this rat model seems to represent the human type 2 diabetes. Long-term (6-month) studies confirm the persistence of the diabetic state and related physiological dysfunction.

Hepatotoxicity of model hepatotoxicants is increased in type 1 DB rats (Hanasono et al., 1975; Watkins et al., 1988; Mak and Ko, 1997; Maritim et al., 2000; Wang et al., 2000). We wanted to test whether our model could be used to investigate potential high sensitivity to hepatotoxicants due to type 2 diabetes. Consistent with our hypothesis, administration of ordinarily nonlethal doses of three structurally and mechanistically dissimilar model hepatotoxicants, CCl₄, TA, and AA, resulted in significant mortality. These findings address a long-standing question as to whether type 2 diabetes also exhibits high sensitivity to hepatotoxicants.

The high sensitivity of type 2 DB rats to CCl₄ was chosen for more detailed mechanistic studies. The lethal outcome in type 2 DB rats to CCl₄ could be due to increased bioactivation-based liver injury. Hepatocarcinoma CYP2E1 protein and enzyme activity are markedly increased in type 1 DB rats (Wang et al., 2000). Contradictory evidence has been reported about the status of CYP2E1 activities in DB patients (Wang et al., 2003; Lucas et al., 1998). We found that in the type 2 DB rats neither CYP2E1 protein nor its enzyme activity (Fig. 6, B and C) were increased. It is possible that the lack in change in CYP2E1 in DB rats is in part due to normal insulin levels, as insulin itself is thought to decrease CYP2E1 expression (Novak and Woodcroft, 2000), although this has not been demonstrated in vivo. Insulin signaling pathway(s) involving PI3, p70, S6, and Src kinases seem to mediate the suppression of CYP2E1 in primary cultured rat hepatocytes (Woodcroft et al., 2002). The lack of any change in CYP2E1 expression observed in our study is consistent with our finding that initial liver injury of CCl₄ in DB rats did not differ from that observed in NDB rats. Our findings are consistent with the unchanged CYP2E1 mRNA and protein in ob/ob mice and Zucker rats as reported previously (Novak and Woodcroft, 2000).

The uncontrolled DB state is characterized by increased oxidative stress (Dincer et al., 2002) leading to increased lipid peroxidation. Covalent binding and lipid peroxidation play a key role in CCl₄-induced liver injury. Our studies showed that lipid peroxidation and ¹⁴CCl₄-derived covalently bound ¹⁴C was the same in DB rats as in NDB rats, suggesting increased bioactivation cannot be the mechanism for potentiation of hepatotoxicity.

Lethality studies indicate TA and AA hepatotoxicities were also enhanced, leading to mortality in the DB rats. TA, a centrilobular hepatotoxin, is also bioactivated by CYP2E1 to reactive intermediate TA-S-dioxide, which covalently binds, causing hepatic necrosis, hepatic failure, and death (Wang et al., 2000). Allyl alcohol, a periportal hepatotoxin, is bioactivated by alcohol dehydrogenase, forming a reactive

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**Fig. 10.** Histomorphometric analysis of cells in various gap phases in livers from DB and NDB rats after CCl₄ administration. Percentage of cells in different gap phases were calculated from a total of 1000 counted hepatocytes per slide. Treatment details are as in Fig. 2. *, significantly different from respective 0-h time point; †, significantly different from the NDB normal diet-fed control (ND). *p* ≤ 0.05.
metabolite that binds to macromolecules and initiates lipid peroxidation (Butterworth et al., 1978).

These three compounds are structurally and mechanistically different, indicating the possibility of a common mechanism of potentiation of injury in DB rats. A considerable body of evidence suggests the important role of tissue repair in determining the final outcome of toxicity (Mangipudy et al., 1995, 1996; Rao et al., 1997; Soni et al., 1999; Ramaiah et al., 2000; Wang et al., 2001). Inhibiting CYP2E1 failed to protect type 1 DB rats from an ordinarily nonlethal dose of TA due to inhibited compensatory tissue repair response (Wang et al., 2001). Mangipudy et al. (1995) reported that a low dose of TA (50 mg/kg) administered 36 h before administration of lethal dose of TA (600 mg/kg i.p.) offers protection from lethality. The mechanism underlying this protection was found to be due to preplaced hepatocellular division and tissue repair stimulated by the low-dose treatment. Antimotic intervention with colchicine resulted in a significantly diminished repair response leading to lethality even from the ordinarily nonlethal dose of 300 mg/kg i.p. (Mangipudy et al., 1996).

In our studies, we found that in NDB rats the timely and adequate liver tissue repair led to recovery from liver injury and animal survival. On the other hand, liver tissue repair was inhibited as early as 12 h in DB rats, explaining that even though the initial injury was the same, the persistent unrepaired injury led to hepatic failure and death in the DB rats. Although the mechanism of progression of liver injury when compensatory tissue repair is impaired is not completely understood, recently new developments in this area suggest a role for “death proteins” spilling out of necrotic cells in this tissue destructive process (Limaye et al., 2003). Our findings indicating suppression of tissue repair in type 2 DB rats are consistent with the findings of Yang et al. (2001) showing that liver regeneration is impaired in ob/ob mice after partial hepatectomy. However, the partial hepatectomy model lacks the dynamic interaction of hepatocellular injury after hepatocellular injury and stimulates liver regeneration with cell division and tissue repair as seen in hepatic disorders.

We report the development and characterization of a robust and economical type 2 DB rat model based on high fat diet, that mimics human type 2 diabetes and responds to known antidiabetic drugs. Furthermore, CCl4 hepatotoxicity and lethality are potentiated in type 2 DB rats. Bioactivation of CCl4 is not increased in this potentiation. Stimulated liver tissue repair that normally occurs in rats receiving the same dose of CCl4 is substantially blunted in DB rats. Consequently, liver injury progresses causing hepatic failure and death in DB rats.

Studies on molecular mechanisms underlying diminished tissue repair response will shed light on the specific cellular signaling mechanisms that are affected in this impaired tissue repair in type 2 diabetes.

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References


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