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

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A) **Running Title:** Enhanced CCl$_4$- Hepatotoxicity in Type 2 Diabetes
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D) **Abbreviations:** AA, allyl alcohol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DB, diabetic; db/db, C57 BLKS/J diabetic mice; GK, Goto kakizaki; H & E, hematoxylin and eosin; HFD, high fat diet fed rats injected citrate buffer; HFD + STZ, high fat diet fed rats injected streptozotocin; $^3$H-T, tritiated thymidine; MES, M 2-(N-morpholino) ethanesulphonic acid; ND, normal diet fed rats injected citrate buffer; NDB, nondiabetic; ND + STZ, normal diet fed rats injected streptozotocin; ob/ob, C57 BL/6J obese mice; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy; PI3 Kinase, phosphatidylinositol kinase; PNP, para-nitrophenol; SD, Sprague Dawley; SDS, sodium dodecyl sulphate; STZ, streptozotocin; TA, thioacetamide.

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# Abstract

There is a need for well-characterized and economical type 2 diabetic model that mimicks 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, ip), $CCl_4$ (2 ml/kg, ip), or thioacetamide (300 mg/kg, ip) yielded 80 to 100 % mortality in diabetic rats. The objective of the present study was to investigate two hypotheses: higher $CCl_4$ bioactivation and/or inhibited compensatory tissue repair were the underlying mechanisms for increased $CCl_4$ 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, ip) and was confirmed on day 24 by hyperglycemia, normoinsulinemia and oral glucose intolerance. Time course studies (0-96 h) of $CCl_4$ (2 ml/kg, ip) 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. Hepatomicrosomal CYP2E1 protein and activity, lipid peroxidation, glutathione and $^{14}CCl_4$ -covalent binding to liver tissue was 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, while in the nondiabetic rats robust tissue repair resulted in regression of injury and survival after $CCl_4$ administration. These studies show high sensitivity of type 2 diabetes to model hepatotoxicants and suggest that $CCl_4$-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 fail 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, ip). 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, ip) 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 to 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.

A recent epidemiological study showed that the DB patients are twice as likely to suffer from hepatic failure compared to nondiabetic (NDB) patients (El-Serag HB and Everhart JE, 2002). Severe life threatenting liver injuries are not uncommon in diabetic patients receiving medication including antidiabetic drugs such as troglitazone and acarbose (Ballet F, 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 $CCl_4$ (Watkins et al., 1988, Mak and Ko, 1997; Wang et al., 2000). Increased hepatic-biotransformation 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. While 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 since 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 [$CCl_4$, TA and allyl alcohol (AA)] were employed for lethality studies. For mechanistic studies $CCl_4$ was chosen as the model hepatotoxicant.

Our studies indicate that type 2 diabetes is highly sensitive to hepatotoxicants. Higher sensitivity does not appear to be due to enhanced bioactivation of $CCl_4$. It appears that impaired compensatory liver tissue repair leads to progression of liver injury initiated by an ordinarily nonlethal dose of $CCl_4$ 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 ($21 \pm 1^{o}$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 (Harlan Teklad, Rat Chow # 7012, Madison, WI) or high fat diet consisting of 20% fat, 20% protein, and 46% carbohydrate (Dyets Inc., Dyets # 100795, Bethlehem, PA)].

### Chemicals

[$^3$H-CH$_3$]-Thymidine ($^3$H-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, Glaxo Smith Kline, Research Triangle Park, NC) was purchased from Rite Aid Pharmacy (West Monroe, LA). All other chemicals and biochemical kits were purchased from Sigma Chemical Co. (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, ip, 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, ip, in 0.01 M citrate buffer pH 4.3) or citrate buffer (ND) (1ml/kg, ip) 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 (Sigma Kit no. 315-100) and plasma insulin in the range of 0.5 to 2 ng/ml (ELISA, Crystal Chem 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 non-fasting plasma glucose and insulin levels. Other characteristics such as plasma triglycrides (glycerol phosphate oxidase [GPO]- Trinder method, Sigma catalog # 343) and free fatty acids (Cat # 994-75409, Wako Chemicals, Richmond, VA) were measured. The degree of uncontrolled DB state was confirmed by measuring glycated hemoglobin (Sigma catalog # 441-B). To test the persistence of diabetes, the DB rats were continued on high fat diet for six 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, ip) twice daily at 10 am and 10 pm each day for five days. Blood was sampled retroorbitally at 9:50, 16:00, and 21:50 hours. To another set of rats, rosiglitazone (Avandia®, 64 mg/kg, po) was administered once daily for 14 days at 2 pm. Plasma glucose was analyzed once daily as mentioned above.

**Lethality Study**

On day 24, DB and NDB rats ($n = 10$ per group) were treated with $CCl_4$ (2 ml/kg, ip, in corn oil 1:1) or corn oil (2 ml/kg), thioacetamide (TA, 300 mg/kg, ip) or saline (3 ml/kg) and allyl alcohol (AA, 35 mg/kg, ip) or vehicle control (distilled water, 1 ml/kg). Rats were observed twice daily for 14 days. Survival/ mortality was recorded thrice on the first day and twice daily thereafter for 14 days.

**$CCl_4$-induced Liver Injury:** On day 24, DB (HFD + STZ group) and NDB rats [normal diet (ND) injected citrate buffer, high fat diet (HFD) injected citrate buffer, and normal diet group injected STZ (ND+STZ)] were treated with either a single injection of

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CCl$_4$ (2 ml/kg, ip, in equal volume of corn oil) or corn oil (2 ml/kg, ip) alone. The rats treated with CCl$_4$ 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 h 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 aminotransferase (ALT, EC 2.6.2.2) and aspartate aminotransferase (AST, EC 2.6.1.1) were measured as biomarkers of liver injury using Sigma kit # 59 UV and kit # 158 UV, respectively. Total bilirubin and ammonia were measured as biomarkers of liver function using Sigma kit # 550-A and kit # 171-UV, respectively.

*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 thick) were stained with Hematoxylin and Eosin (H & E) for histological examination by light microscopy. Unstained liver

sections were prepared for proliferating cell nuclear antigen (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); 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's method using the Bio-Rad protein assay (Bio-Rad Laboratories, CA). Microsomes were stored at $-80^{\circ}$C until needed.

*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 $^{14}CCl_4$-Equivalents to Liver Proteins*: To determine if diabetes altered the covalent binding of $^{14}$C-equivalents of liver proteins, $^{14}CCl_4$ was administered (2 ml/kg, ip, 8 µCi/ rat ~ 32 µCi/ kg, in 1:1 corn oil) to DB and NDB rats. The rats were killed 2 h after $^{14}CCl_4$ administration. Livers were excised, frozen in liquid

nitrogen, and then kept at $-80^{\circ}$C until time for analysis. Two grams of liver tissue were homogenized in 4 ml ice-cold Tris-HCl buffer (pH 7.4) in a polytron homogenizer for 30 sec at $4^{\circ}$C. Two ml of homogenate was added to tubes containing 4 ml 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 3,000 X $g$ for 30 min. To remove lipids and non-covalently bound $^{14}$C-equivalents away, the pellets were extracted extensively with a series of washes with 3:1 chloroform: methanol mixture. The pellets (containing covalently bound $^{14}$CCl$_4$-derived radiolabel) were air-dried, dissolved in 1 ml 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 were homogenized in 2 vol. of ice-cold 0.3 M sucrose containing 3 mM EDTA. A 0.5 ml aliquot of the homogenate was extracted with 9.5 ml chloroform: methanol (2:1 v/v), filtered, and then brought to a total volume of 10 ml. The extract was washed once with 2 ml saline and twice with 0.5 ml of upper phase (25 ml water + 95 ml of chloroform: methanol). The lower phase was used as the reference blank for spectrophotometry. Methanol (200 µl) was added to each tube and the samples were scanned from 230 nm to 280 nm using UV-spectrophotometer.

***Glutathione Estimation:*** Total glutathione was estimated at 0, 12, and 24 h following CCl$_4$ 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 10,000 $g$ for 15 mins at 4$^o$C. The supernatant was deproteinated by adding to it an equal volume of metaphosphoric acid and then centrifuging it at 2000 $g$ for 2 min. The supernatant was carefully collected and was ready for total glutathione estimation using the commercially available glutathione assay kit (cat # 703002) from Cayman Chemical Company, Ann Arbor, MI.

**Tissue Repair**

***$^3$H-T Pulse Labeling Study:*** S-Phase DNA synthesis was estimated by $^3$H-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 $^3$H-T was determined by liquid scintillation spectrometry (Packard Instruments Co., Meriden, CT).

***Proliferating Cell Nuclear Antigen (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 were 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 ANOVA followed by Tukey's and Dunnet's Post-Hoc tests using the

SPSS, 11.0 software (SPSS Incorporated, Chicago, IL). The criterion for significance was

$p \leq 0.05$.

## Results

**Induction and Characterization of Type 2 Diabetes.** There was no lethality among the DB rats and they appeared healthy. They exhibited weight loss but without any change in liver to body weight ratio, significantly higher water and food intake, and increased urine output (14-fold) compared to the NDB rats (data not shown).

Table 1 contains the values obtained for plasma glucose, insulin, triglycerides and glycated hemoglobin in the DB and NDB rats. Average plasma glucose was 3-fold higher in the DB rats ($450 \pm 66$ mg/dl, HFD + STZ group) compared to the NDB rats ($123 \pm 10$ mg/dl, ND group) (Table 1). The plasma insulin levels in the DB rats were in the normal range (0.5 to 2 ng/ml). Glycated hemoglobin ($HbA_{1c}$) 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 to NDB rats (Table 1).

Figure 1 illustrates the blood glucose values (panel A) and insulin (panel B) during a time course after oral glucose tolerance test. In the NDB rats fed normal diet, plasma glucose values returned to normal levels 2 h after oral glucose challenge. However, DB rats exhibited high fasting glucose ($241 \pm 32$ mg/dl, indicated at 0 h time point in Fig. 1A) and glucose intolerance as indicated by high glucose levels at 2 h ($450 \pm 78$ mg/dl) (Fig. 1). The HFD rats, inspite 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 five days 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 which 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 appears to resemble closely the human type 2 DB syndrome.

**Sensitivity of Type 2 Diabetic Rats to Model Hepatotoxicants.**

**Lethality Studies.** Toxicity initiated by $CCl_4$ (2 ml/kg, ip) and TA (300 mg/kg, ip) 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 $CCl_4$ and TA administration only in DB rats. Also, AA (32 mg/kg, ip) toxicity was potentiated in DB rats leading to 80% mortality in the DB group compared to 100% survival of the NDB group. The DB rats died between 36 and 48 h after AA administration. High sensitivity of DB rats to $CCl_4$ toxicity was chosen for mechanistic investigation.

**Hepatotoxicity.** Time course study of liver injury of $CCl_4$ 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 while bilirubin and ammonia were measured to evaluate liver function after $CCl_4$ 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 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 to normal diet fed NDB controls at 36 h, thereby indicating hepatic failure and greater sensitivity of type 2 DB rats to $CCl_4$ 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 till 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 to the control at 12 h, elevated at 36 and 48 h before declining to control level at 72 h after $CCl_4$ administration. Although increased compared to control in the NDB group, bilirubin remained in the normal range (0.1 to 1.2 mg/dl). In stark contrast, in DB rats, plasma bilirubin (Fig. 3A) markedly increased at 24 and 36 h after $CCl_4$ administration (4 to 5 mg/dl), indicating progressive

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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 $CCl_4$ 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 $CCl_4$ injury. The induction of diabetes did not cause any liver damage (Fig. 4A & B). In the liver of NDB rats at 12 h after $CCl_4$ 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 $CCl_4$ administration, the necrotic cells had disappeared. In contrast, in the liver of DB rats, mild to moderate 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 to NDB rats receiving the same dose of $CCl_4$.

**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

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glycogen depletion after $CCl_4$ administration till 36 h (Fig. 5) was similar. While there were no survivors in the DB group, 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 $CCl_4$?**

   **1.** *Effect of Diabetes on Hepatic CYP2E1.* Western blot analysis showed that hepatic microsomal CYP2E1 protein was not different from the NDB rats (Fig. 6A & B). Microsomal CYP2E1 enzyme activity (Fig. 6C) measured as *p*-nirophenol 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.

   **2. *Covalent Binding:*** $^{14}CCl_4$-derived covalently bound radiolabel to liver macromolecules is a reliable measure of the extent of $CCl_4$ bioactivation. As shown in Fig. 7A, the amount of label covalently bound to liver proteins was not different in the DB rats compared to NDB rats at 2 h after $CCl_4$ administration.

   **3. *Lipid Peroxidation:*** Lipid peroxidation is a critical event following bioactivation of $CCl_4$. The effect of diabetes on lipid peroxidation was investigated by estimating diene conjugation of hepatic lipids. While lipid peroxidation did increase in all groups (Fig. 7B) after $CCl_4$ administration, that in the DB group was not different from any other NDB group.

*4. Hepatic Glutathione:* Diabetes did not decrease hepatic glutathione content. Hepatic glutathione was decreased (Fig. 7C) in the DB rats at 6 h following $CCl_4$ administration but was restored by 12 h. In the NDB groups, there was no significant depletion in glutathione levels after $CCl_4$ administration.

**Tissue Repair:**

[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 ^{3}H-T pulse labeling study (Fig. 8) revealed that DNA synthesis was significantly increased in $CCl_4$- treated rats compared to rats not exposed to $CCl_4$. In the NDB rats, S-phase DNA synthesis was increased as early as 12 h after $CCl_4$ administration, which persisted throughout the time course. In the DB group, while 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 $CCl_4$.

*PCNA Assay.* Immunohistochemical staining of PCNA (Fig. 9 & 10) was conducted with two objectives. First, to verify the findings of S-phase stimulation determined by [3][H]-thymidine pulse labeling study. Second, to assess cell-cycle progression. In both DB and NDB rats, most hepatocytes (92 to 95%) rested in $G_0$ phase at 0 h and began entering the replication cycle soon after $CCl_4$ administration. In the livers of NDB rats, significantly greater number of hepatocytes were in $G_1$-phase (30 and 35%, at 12 and 24 h, respectively) compared to DB rats (18 and 20%). A large number of hepatocytes were seen in S-phase (40%) in NDB rats compared to 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 [$^3$H]-thymidine pulse labeling results. In NDB rats, by 72 h significant number 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 et al., 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). While 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 if 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, ip) on day 14 results in a three-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, a 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-months) studies confirm the persistence of the diabetic state and related physiological dysfunction.

Hepatotoxicity of model hepatotoxicants is increased in type 1 DB rats (Watkins et al., 1988; Mak and Ko, 1997; Maritum 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, $CCl_4$, 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 $CCl_4$ was chosen for more detailed mechanistic studies. The lethal outcome in type 2 DB rats to $CCl_4$ could be due to increased bioactivation-based liver injury. Hepatomicrosomal 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. 6B & 6C) 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 et al., 2000), although this has not been demonstrated *in vivo*. Insulin signaling pathway(s) involving PI3, p70, S6 and Src kinases appear 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 $CCl_4$ 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 et al., 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 $CCl_4$ –induced liver injury. Our studies showed that lipid peroxidation and $^{14}CCl_4$-derived covalently bound $^{14}C$ was the same in DB rats as in NDB rats, suggesting increased bioactivation cannot be the mechanism for potentiation of hepatotoxicity.

Lethality studies indicate thioacetamide (TA) and allyl alcohol (AA) hepatotoxicities were also enhanced leading to mortality in the DB rats. TA, a centrilobular hepatotoxicant is also bioactivated by CPY2E1 to reactive intermediate TA-S-dioxide, which covalently binds, causing hepatic necrosis, hepatic failure and death (Wang et al., 2000). Allyl alcohol, a periportal hepatotoxicant, is bioactivated by alcohol dehydrogenase, forming a reactive 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; Mangipudy et al., 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 single low dose of thioacetamide (TA, 50 mg/kg) administered 36 h prior to administration of lethal dose of TA (600 mg/kg, ip) 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. Antimitotic intervention with colchicine resulted in a significantly diminished repair response leading to lethality even from the ordinarily nonlethal dose of 300 mg/kg, ip (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 following partial hepatectomy (PH). However, the PH model lacks the dynamic interaction of hepatocellular injury following hepatotoxicant challenge 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 mimicks human type 2 diabetes and responds to known antidiabetic drugs. Further, $CCl_4$ hepatotoxicity and lethality are potentiated in type 2 DB rats. Bioactivation of $CCl_4$ is not increased in this potentiation. Stimulated liver tissue repair that normally occurs in rats recovering the same dose of $CCl_4$ 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.

## Acknowledgements

# References

Albrink MJ (1974) Dietary and drug treatment of hyperlipidemia in diabetes. *Diabetes* **23:**913-918.

Andrade RJ, Lucena MI, and Rodriguez-Mendizabal M (1996) Hepatic injury caused by acarbose. *Ann. Intern. Med.* **124**:931.

Bailey CJ and Flatt PR (1991) Animal models of non-insulin dependent diabetes mellitus, in *Textbook of Diabetes*. (Pickup J. Williams G eds): pp 228-239 Blackwell Scientific, London UK.

Ballet F (1977) Hepatotoxicity in drug development: detection, significance and solutions. *J. Hepatol.* **26**:26-36.

Burton K (1956) A study of the conditions and mechanisms of the diphenylamine reaction for positive colorimetric estimation of DNA. *Biochem J* **62:**315-323.

Butterworth KR, Carpanini FMB, Dunnington D, Grasso P and Pelling D (1978) The production of periportal necrosis by allyl alcohol in the rat. *Br J of Pharm* **63**:353-354.

Chang LO and Looney WB (1965) A biochemical and autoradiographic study of the *in vivo* utilization of tritiated thymidine in regenerating rat liver. *Cancer Res* **25:**1817-1822.

Chaveau J, Moule Y and Roullier CH (1956) Isolation of pure and unaltered liver nuclei, morphology and biochemical composition. *Exp Cell Res* **11:**317-321.

Chipman JK, Kurukgy M and Walker CH (1979) Comparative metabolism of a dieldrin analogue: hepatic microsomal systems as models for metabolism in the whole animal. *Biochem Pharmacol* **28:**69-75.

Dincer Y, Akcay T, Alademir Z and Ilkova H (2002) Assessment of DNA base oxidation and glutathione level in patients with type 2 diabetes. *Mutat Res* **505**:75-81.

El-Serag HB and Everhart JE (2002) Diabetes increases the risk of acute hepatic failure. *Gastroenterology* **122**:1822-1828.

Greenwell A, Foley JF and Maronpot RR (1991) An enhancement method for immunohistochemical staining of proliferating cell nuclear antigen in archival rodent tissues. *Cancer Lett* **59:**251-256.

Groop L, Forsblom C and Lehtovirta M (1997) Characterization of the prediabetic state. *Am J Hypertens* **10**:172S-180S.

Kraegen EW, James DE, Storlien LH, Burleigh KM and Chisholm DJ (1986) *In vivo* insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration. *Diabetologia* **29:**192-198.

LeRoith D (2002) Beta-cell dysfunction and insulin resistance in type 2 diabetes: role of metabolic and genetic abnormalities. *Am J Med* **113:**3S-11S.

Limaye PB, Apte UM, Shankar K, Bucci TJ, Warbritton A, and Mehendale HM (2003) Calpain released from dying hepatocytes mediates progression of acute liver injury by model hepatotoxicants. *Toxicol Appl Pharmacol* **191**:211-226.

Lucas D, Farez C, Bardou LG, Vaisse J, Attali JR and Valensi P (1998) Cytochrome P450 2E1 activity in diabetic and obese patients as assessed by chlorzoxazone hydroxylation. *Fundam Clin Pharmacol* **12**:553-558.

Mak DH and Ko KM (1997) Alterations in susceptibility to carbon tetrachloride toxicity and hepatic antioxidant/detoxification system in streptozotocin-induced short-term

diabetic rats: effects of insulin and Schisandrin B treatment. *Mol Cell Biochem* **175**:225-232.

Mangipudy RS, Chanda S and Mehendale HM (1995) Tissue repair response as a function of dose in thioacetamide hepatotoxicity. *Environ Health Perspect* **103**:260-267.

Mangipudy RS, Rao PS and Mehendale HM (1996) Effect of an antimitotic agent colchicine on thioacetamide hepatotoxicity. *Environ Health Perspect* **104:**744-749.

Maritim AC, Moore BH, Sanders RA and Watkins JB III (2000) Effect of melatonin on phase I and II biotransformation enzymes in streptozotocin-induced diabetic rats. *Internat J Toxicol* **19**:277-283.

Mehendale HM and Klingensmith JS (1988) *In vivo* metabolism of CCl$_4$ by rats pretreated with chlordecone, mirex, or phenobarbital. *Toxicol Appl Pharmacol* **93**:247-256.

Neuschwander-Tetri BA, Isley WL, Oki JC, Ramrakhini S, Quiason SG, Philips NJ, and Brunt EM (1998) Troglitazone-induced hepatic failure leading to liver transplantation: a case report. *Ann. Intern. Med*. **129**:38-41.

Novak RF and Woodcroft KJ (2000) The alcohol-inducible form of cytochrome P450 (CYP 2E1): role in toxicology and regulation of expression. *Arch Pharm Res* **23**:267-282.

Pascoe WS, Jenkins AB, Kusunoki M and Storlien LH (1992) Insulin action and determinants of glycemia in a rat model of type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* **35**:208-215.

Portha B, Serradas P, Bailbe D, Suzuki K, Goto Y and Giroix MH (1991) Beta-cell insensitivity to glucose in the GK rat, a spontaneous nonobese model for type 2 diabetes. *Diabetes* **40:**486-491.

Ramaiah SK, Apte U, and Mehendale HM (2000) Diet restriction as a protective mechanism in noncancer toxicity outcomes: a review. *Internat J Toxicol* **19**:413-424.

Rao PS, Mangipudy RS and Mehendale HM (1997) Tissue injury and repair as parallel and opposing responses to $CCl_4$ hepatotoxicity: a novel dose-response. *Toxicology* **118**:181-193.

Reed MJ, Meszaros K, Entes LJ, Claypool MD, Pinkett TM, Gadbois TM and Reaven GM (2000) A new rat model of Type 2 diabetes: the fat-fed, streptozotocin-treated rat. *Metabolism* **49**:1390-1394.

Seifer S, Dayton S, Novic B and Muntwyler E (1950) Estimation of glycogen with anthrone reagent. *Arch Biochem* **25**:191-200.

Soni MG, Ramaiah SK, Mumtaz MM, Clewell H, and Mehendale HM (1999) Toxicant-inflicted injury and stimulated tissue repair are opposing toxicodynamic forces in predictive toxicology. *Regul Toxicol Pharmacol* **29**:165-174.

Surwit, RS, Kuhn CM, Cochrane C, McCubbin JA and Feinglos MN (1988) Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* **37**:1163-1167.

Wang T, Fonetenot RD, Soni MG, Bucci TJ and Mehendale HM (2000) Enhanced hepatotoxicity and toxic outcome of thioacetamide in streptozotocin-induced diabetic rats. *Toxicol Appl Pharmacol* **166:**92-100.

Wang T, Shankar K, Ronis MJJ and Mehendale HM (2001) Diallyl sulfide inhibition of CYP2E1 does not rescue diabetic rats from thioacetamide-induced mortality. *Toxicol Appl Pharmacol* **173**:27-37.

Wang Z, Hall SD, Maya JF, Li L, Asghar A and Gorski JC (2003) Diabetes mellitus increases the in vivo activity of cytochrome P450 2E1 in humans. *Br J Clin Pharmacol* **55**:77-85.

Warram JH, Martin BC, Krolewski AS, Soeldner JS and Kahn CR (1990) Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann Intern Med* **113**:909-915.

Watkins JB III, Sanders RA and Beck LV (1988) The effect of long-term streptozotocin-induced diabetes on the hepatotoxicity of bromobenzene and carbon tetrachloride and hepatic biotransformation in rats. *Toxicol Appl Pharmacol* **93:**329-338.

Woodcroft KJ, Hafner MS and Novak RF (2002) Insulin signaling in the transcriptional and posttranscriptional regulation of CYP2E1 expression. *Hepatology* **35**:263- 273.

Yang SQ, Lin HZ, Mandal AK, Huang J and Diehl AM (2001) Disrupted signaling and inhibited regeneration in obese mice with fatty livers: implications for nonalcoholic fatty liver disease pathophysiology. *Hepatology* **4:**694-706.

**Footnotes**

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**Legends for Figures**

**Fig. 1.** Effect of diabetes on oral glucose tolerance. Male SD rats were fed either normal diet or high fat diet for 2 weeks and on day 14 were injected either STZ (45 mg/kg, ip, in 0.01 M citrate buffer, pH 4.3) or citrate buffer (1 ml/kg). Injecting STZ to high fat diet fed rats (HFD + STZ) induced type 2 diabetes. On day 24, DB and NDB rats were administered glucose solution (20 % glucose solution, 5 g/kg, po). Blood samples were collected under diethyl ether anesthesia at various time points (0, 15, 30, 60, 120, 180, 240, and 300 min) after glucose administration. Glucose content of plasma from these samples was estimated as described under the methods section. *, significantly different from respective 0 h time point; !, significantly different from the NDB normal diet fed control injected citrate buffer (ND) at the same time point. #, significantly different from the NDB normal diet fed control injected STZ (ND + STZ) at the same time point. $\theta$, significantly different from the NDB high diet fed control injected citrate buffer (HFD) at the same time point. $p \leq 0.05$.

**Fig. 2.** Effect of type 2 diabetes on liver injury by $CCl_4$ in male SD rats. Panel A. Plasma alanine aminotransferase (ALT) and Panel B. aspartate aminotransferase (AST) activities over a time course after $CCl_4$ administration. Rats were made DB as described in Fig. 1. On day 24, DB and NDB rats received $CCl_4$ (2 ml/kg, ip, in 1:1 corn oil). Blood samples were collected under diethyl ether anesthesia at various time points (0 - 96 h) after $CCl_4$ administration ($n = 4$ / time point / group except for diabetic group $n = 12$ at 36 and 48 h time point to allow survival of enough rats for time course studies). *, significantly

different from respective 0 h time point; !, significantly different from the NDB normal diet fed control injected citrate buffer (ND) at the same time point. $p \leq 0.05$.

**Fig. 3.** Effect of type 2 diabetes in male SD rats on total plasma bilirubin (Panel A) and ammonia (Panel B) levels over a time course after $CCl_4$ administration. Treatment details are as in Fig. 2. *, significantly different from respective 0 h time point; !, significantly different from the NDB normal diet fed control injected citrate buffer (ND) at the same time point. $p \leq 0.05$.

**Fig. 4.** Effect of type 2 diabetes on $CCl_4$-induced hepatic necrosis in male SD rats. Typical photomicrographs of liver sections from NDB rat (ND) (right) and DB rats (left) (HFD + STZ) at 0, 12 h and 36 h after $CCl_4$ administration. Panel A, C, & E: DB rats 0, 12, & 36 h post treatment with $CCl_4$. Panel B, D, & F: NDB (ND) rats 0, 12, & 36 h post-treatment with $CCl_4$. C, central vien; N, areas of necrosis. H & E original magnification 200X.

**Fig. 5.** Liver glycogen levels over a time course after $CCl_4$ administration. Treatment details are as in Fig. 2. *, significantly different from respective 0 h time point. $p \leq 0.05$.

**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 in the upper panel, A; B, microsomal CYP2E1

protein was detected by Western blot and quantified by densitometry; CYP2E1 activity was measured by PNP – hydroxylase assay (C).

**Fig. 7.** Covalent binding (A), lipid peroxidation (B), and total glutathione levels (C) over a time course after $CCl_4$ administration to male SD rats. Treatment details are as in Fig. 2. *, significantly different from respective 0 h time point; !, significantly different from the NDB normal diet fed control injected citrate buffer (ND) at the same time point. $p \leq 0.05$.

**Fig. 8.** [3][H]-Thymidine incorporation into hepatonuclear DNA over a time course after $CCl_4$ administration to male SD rats. Treatment details are as in Fig. 2. *, significantly different from respective 0 h time point; !, significantly different from the NDB normal diet fed control injected citrate buffer (ND) at the same time point. $p \leq 0.05$.

**Fig. 9.** PCNA assay in liver sections of rats over a time course after $CCl_4$ administration. Treatment details are as in Fig. 2. Typical Photomicrographs of liver sections from NDB rat (ND) (right) at 12, 36, and 72 h and DB rats (HFD + STZ) (left) at 12, 24, and 36 h after $CCl_4$ administration. $G_0$ cell with blue nuclear staining; $G_1$ cell with light-brown nuclear staining; S-cell with dark-brown nuclear staining; $G_2$ cell with cytoplasmic staining and with or without a speckeled nuclear staining. Panel A, C, & E: DB rats 12, 24, and 36 h post-treatment with $CCl_4$, Panel B, D, & F: NDB (ND) rats 12, 36, and 72 h post-treatment with $CCl_4$. Original magnification 400X.

**Fig. 10.** Histomorphometric analysis of cells in various gap phases in livers from DB and NDB rats after CCl$_4$ administration. Percent 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). $p \leq 0.05$.

TABLE 1

Induction and Characterization of Type 2 Diabetes[a]

| Parameters | Normal diet fed rats | | | | High fat diet fed rats | | | |
|---|---|---|---|---|---|---|---|---|
| | (ND) | | + STZ (ND + STZ) | | (HFD) | | + STZ (HFD + STZ) | |
| | Nondiabetic rats | | | | | | Diabetic rats | |
| | 24 day | 6 month | 24 day | 6 month | 24 day | 6 month | 24 day | 6 month |
| Plasma Glucose (mg/ dl) | $123 \pm 10$ | $150 \pm 20$ | $164 \pm 42$ | $140 \pm 32$ | $142 \pm 18$ | $130 \pm 10$ | $450 \pm 66$[b] | $530 \pm 40$[b] |
| Plasma Insulin (ng/ dl) | $1.6 \pm 0.4$ | $2.6 \pm 0.5$ | $1.4 \pm 0.3$ | $2.4 \pm 0.3$ | $2.3 \pm 0.2$ | $2.9 \pm 0.2$ | $1.8 \pm 0.4$ | $0.7 \pm 0.2$[b] |
| Plasma Leptin (ng/dl) | $1.4 \pm 0.1$ | $3.1 \pm 0.3$ | $2.8 \pm 0.25$ | $2.9 \pm 0.4$ | $2.4 \pm 0.2$ | $2.5 \pm 0.2$ | $0.7 \pm 0.05$[b] | $0.3 \pm 0.08$[b] |
| Plasma Triglycerides (mg/ dl) | $68 \pm 10$ | $67 \pm 12$ | $70 \pm 6$ | $72 \pm 13$ | $141 \pm 15$ | $138 \pm 14$ | $1141 \pm 20$[b] | $1267 \pm 38$[b] |
| Plasma free fatty acids (mEq/ L) | $54 \pm 4$ | $55 \pm 10$ | $52 \pm 5$ | $54 \pm 12$ | $73 \pm 18$ | $71 \pm 23$ | $124 \pm 14$[b] | $234 \pm 13$[b] |
| Glycated Hb (%) | $2 \pm 0.02$ | $1.8 \pm 0.05$ | $2 \pm 0.05$ | $1.9 \pm 0.06$ | $2 \pm 0.02$ | $1.91 \pm .05$ | $4.3 \pm 0.23$[b] | $5.22 \pm 0.3$[b] |
| Metformin treatment [c] (Plasma Glucose, mg/dl) | | | | | | | $175 \pm 35$[d] | |
| Rosiglitazone treatement [c] (Plasma Glucose, mg/dl) | | | | | | | $135 \pm 34$[d] | |

[a]Male Sprague-Dawley rats were fed either normal diet or high fat diet for 2 weeks and on day 14 were injected once with either STZ (45 mg/kg, ip, in 0.01 M citrate buffer, pH 4.3) or citrate buffer (1 ml/kg). High fat diet fed rats injected with STZ (HFD + STZ) developed type 2 diabetes.  On day 24 and on 6 month, blood samples were collected from the dorsal aorta from the DB and NDB rats ($n = 4$ per group) under diethyl ether anesthesia.

[b] Significantly different from the respective NDB normal diet fed controls (ND). $p< 0.05$

[c] DB rats with similar glucose concentrations were injected with either vehicle (distilled water) or metformin (350 mg/kg, ip) twice daily each day for five days at 10 am and 10 pm, and blood was sampled retroorbitally. Rosiglitazone (64 mg/kg, po) was administered once daily for 14 days at 2 pm. Plasma glucose was analyzed once daily as described previously.

[d] Significantly different from the DB untreated rats (HFD + STZ). $p< 0.05$

TABLE 2

Lethality Study After Single Administration of $CCl_4$, TA and AA[a]

| Toxicant | Normal diet fed rats (ND) | Normal diet fed rats + STZ (ND + STZ) | High fat diet fed rats (HFD) | High fat diet fed rats + STZ (HFD + STZ) |
|---|---|---|---|---|
| | Nondiabetic rats | | | Diabetic rats |
| **% Mortality following toxicant administration** | | | | |
| Carbon tetrachloride ($CCl_4$) (2 ml/kg, ip) | 0 | 0 | 0 | 100[b] |
| Thioacetamide (TA) (300 mg/kg, ip) | 0 | 0 | 0 | 100[b] |
| Allyl alcohol (AA) (35 mg/kg, ip) | 0 | 0 | 0 | 80[c] |

[a]Diabetes was induced as mentioned in Table 1. On day 24 the DB and NDB rats, received a nonlethal doses of carbon tetrachloride ($CCl_4$) (2 ml/kg, in 1: 1 corn oil), thioacetamide (TA) (300 mg/kg, ip, in saline), and allyl alcohol (AA) (35 mg/kg, ip, in distilled water) ($n = 10$ per toxicant challenge). Rats were observed twice daily for 14 days.

[b] All deaths occurred between 24 and 48 h after toxicant challenge.

[c] All deaths occurred between 36 and 60 h after toxicant challenge.

TABLE 3

Effect of Diabetes on CCl$_4$ –induced Hepatic Necrosis[a]

| Hours after CCl$_4$ treatment | Necrosis Score | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Normal diet fed rats | | | | High fat diet fed rats | | | |
| | ND | | ND + STZ | | HFD | | HFD + STZ | |
| | | Mean | | Mean | | Mean | | Mean |
| 0 | 0, 0, 0, 0 | 0 | 0, 0, 0, 0 | 0 | 0, 0, 0, 0 | 0 | 0, 0, 0, 0 | 0 |
| 12 | 0, 0, 0, 0 | 0 | 0, 0, 0, 0 | 0 | 0, 0, 0, 0 | 0 | 1, 1, 1, 1 | 1 |
| 24 | 1, 1, 1, 1 | 1 | 1, 1, 1, 1 | 1 | 1, 1, 1, 1 | 1 | 2, 3, 2, 2 | 2.25 |
| 36 | 1.5, 1.5, 2, 2 | 1.75 | 2, 2, 1, 2 | 1.75 | 2, 1.5, 1.5, 2 | 1.75 | 4, 4, 5, 4 | 4.25 |
| 72 | 0, 0, 0, 0 | 0 | 0, 0, 0, 0 | | 0, 0, 0, 0 | 0 | No survivors | |

*Note.* Experimental protocol is as described in Fig. 2.

[a] Lesions scored as multifocal necrosis and hepatitis. 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); 5 = massive (most parenchyma necrotic).

Legend (top right):
- ND
- ND + STZ
- HFD
- HFD + STZ (Diabetic)

**A** — y-axis: ALT (U/L X 100)

**B** — y-axis: AST (U/L X 100)

x-axis: Hours after CCl$_4$ administration

0   12   24   36   48   72   96

Figure A: Bilirubin (mg/dL) vs Hours after CCl$_4$ administration. Groups: ND, ND + STZ, HFD, HFD + STZ (Diabetic).

Figure B: Ammonia (mmol/L) vs Hours after CCl$_4$ administration.

X-axis: Hours after CCl$_4$ administration: 0, 12, 24, 36, 48, 72, 96

**DB (HFD+STZ)**          **NDB (ND)**

Legend:
- ND
- ND+STZ
- HFD
- HFD+STZ Diabetic

Y-axis: Glycogen ($\mu$g/g of liver)

X-axis: Hours after $CCl_4$ administration

**A**

ND   HFD+STZ (DB)   HFD   ND+STZ

**B**

Relative CYP2E1 Protein

**C**

PNP hydroxylation (nmol/mg/min)
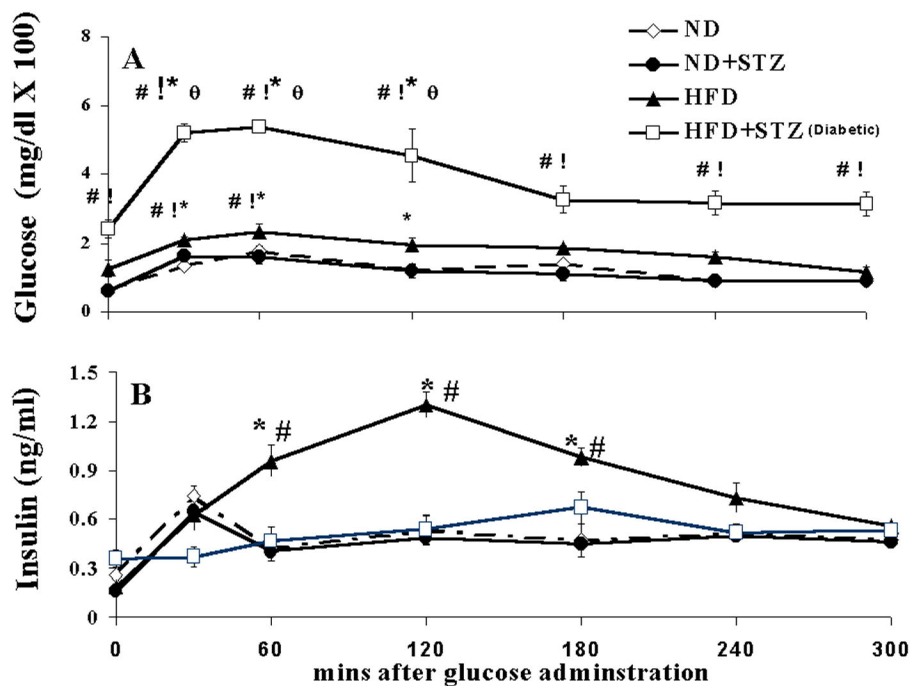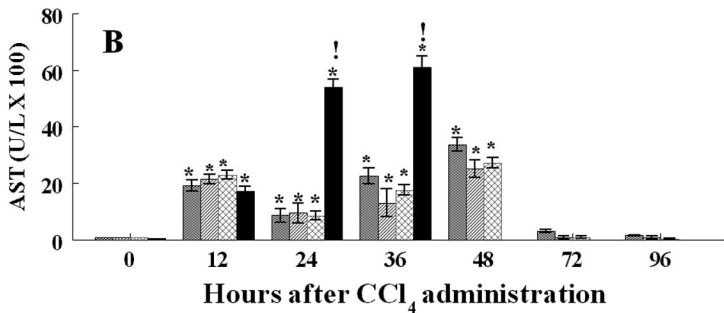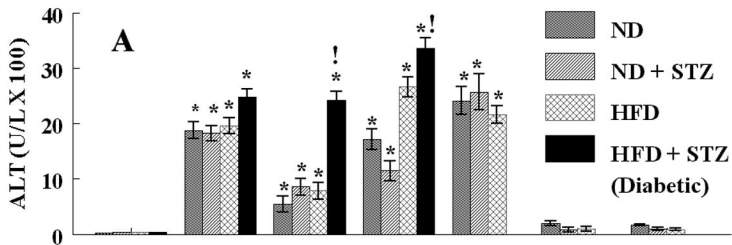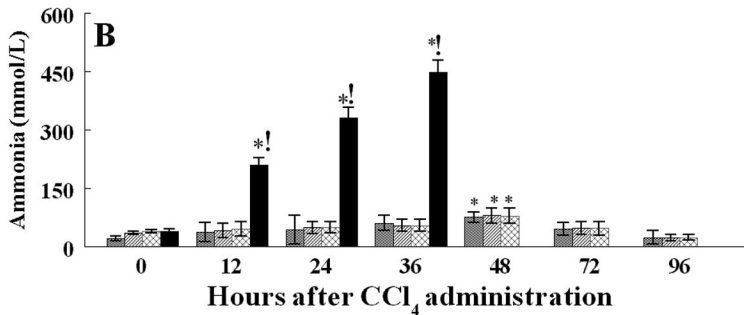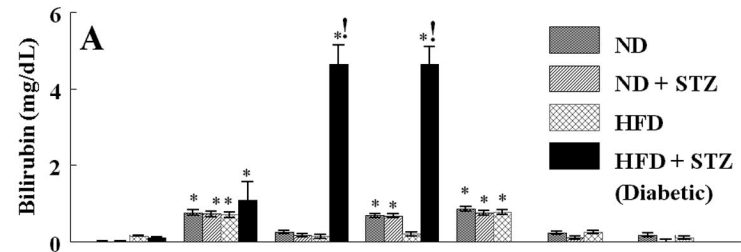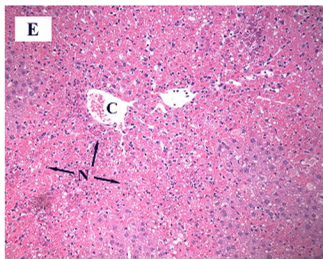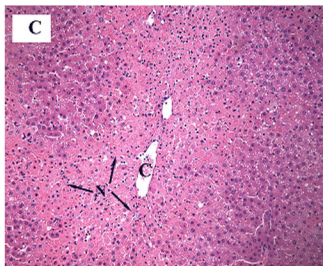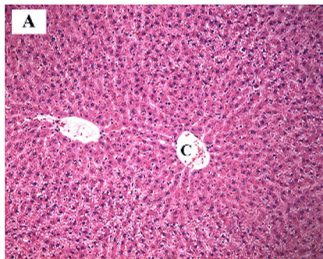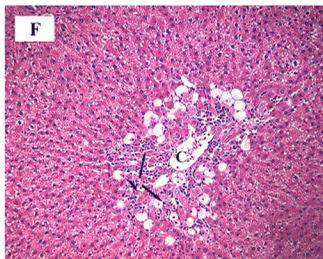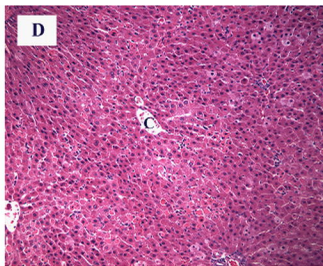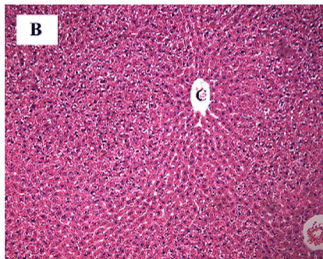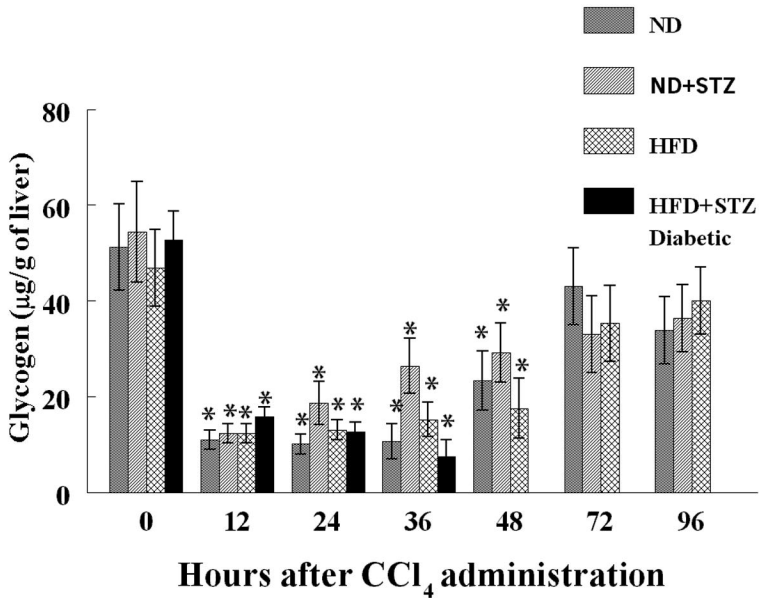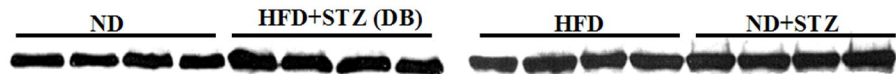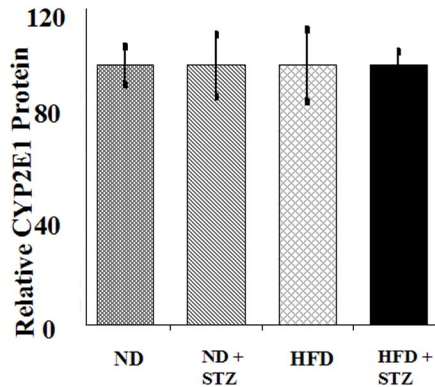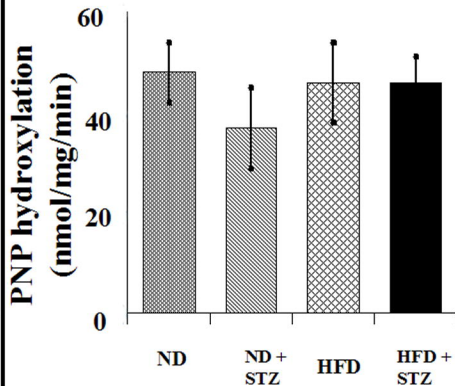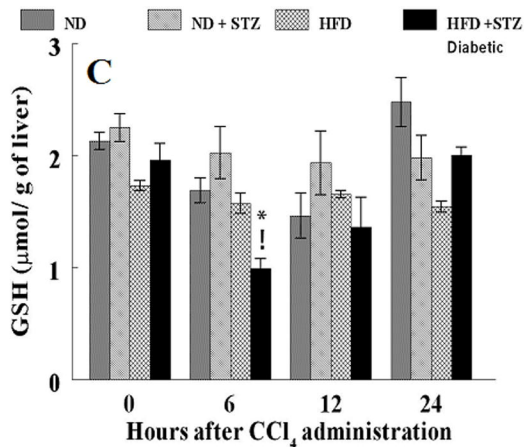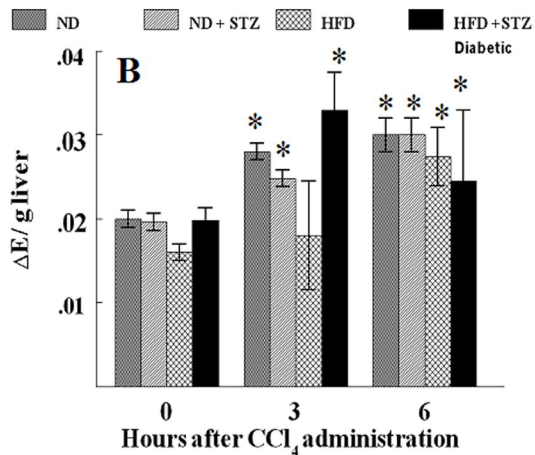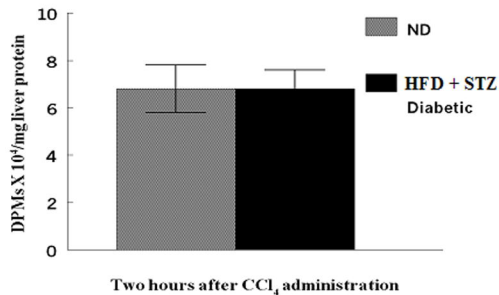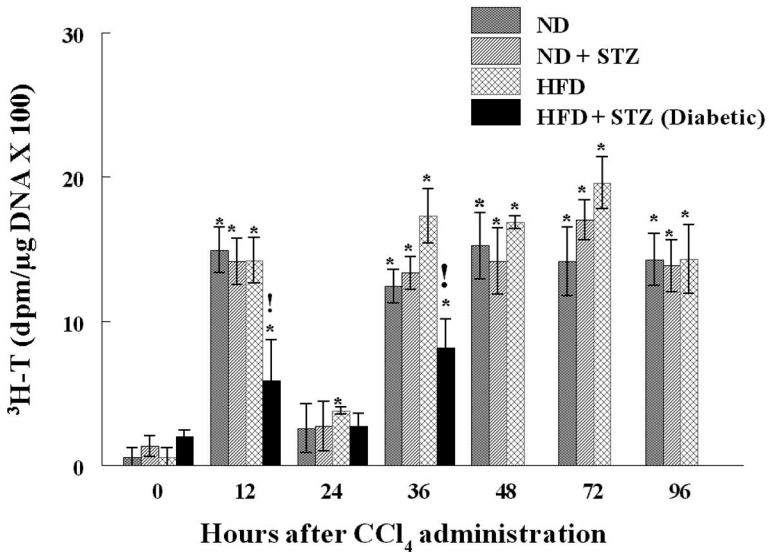
DB (HFD+STZ)      NDB (ND)
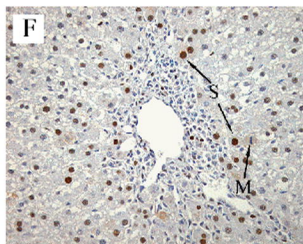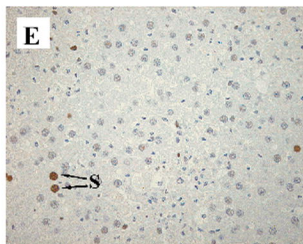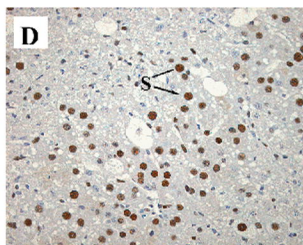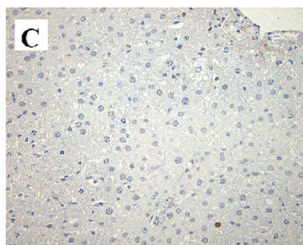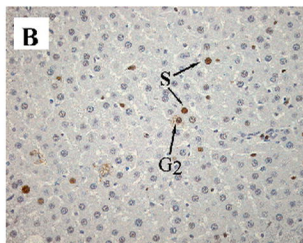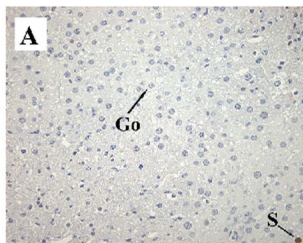
**% of cells in cell cycle phase**

**Hours after $CCl_4$ administration**
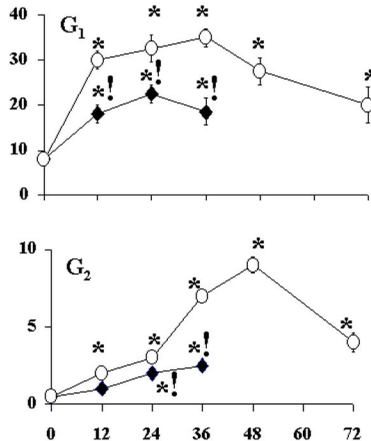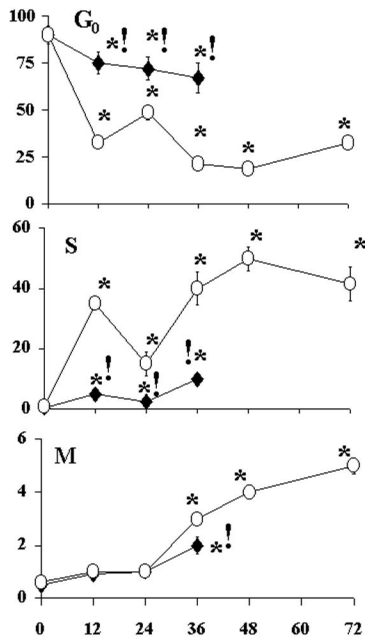
◆ HFD + STZ (Diabetic)

○ ND (non-Diabetic)