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Alcohol-induced ketonemia is associated with lowering of blood glucose, downregulation of gluconeogenic genes, and depletion of hepatic glycogen in type 2 diabetic *db/db* mice

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Abstract

Alcoholic ketoacidosis and diabetic ketoacidosis are life-threatening complications that share the characteristic features of high anion gap metabolic acidosis. Ketoacidosis is attributed in part to the massive release of ketone bodies (e.g., β -hydroxybutyrate; β OHB) from the liver into the systemic circulation. To date, the impact of ethanol consumption on systemic ketone concentration, glycemic control, and hepatic gluconeogenesis and glycogenesis remains largely unknown, especially in the context of type 2 diabetes. In the present study, ethanol intake (36% ethanol- and 36% fat-derived calories) by type 2 diabetic *db/db* mice for 9 days resulted in significant decreases in weight gain (~19.5% ↓) and caloric intake (~30% ↓). This was accompanied by a transition from macrovesicular-to-microvesicular hepatic steatosis with a modest increase in hepatic TG (~37% ↑). Importantly, we observed that ethanol increased systemic β OHB concentration (~8-fold ↑) with significant decreases in blood glucose (~4 fold ↓) and plasma insulin and HOMA-IR index (~3 fold ↓). In addition, ethanol enhanced hepatic β OHB

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Conflict of Interest

The authors declare no conflicts of interest.

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content (~5 fold ↑) and *hmgcs2* mRNA expression (~3.7 fold ↑), downregulated key gluconeogenic mRNAs (e.g., *Pcx*, *Pck1*, and *G6pc*), and depleted hepatic glycogen (~4 fold ↓). Furthermore, ethanol intake significantly decreased mRNA/protein expression and allosteric activation of glycogen synthase (GS) in liver tissues regardless of changes in the phosphorylation of GS, GSK-3β, or Akt. Together, our findings suggest that ethanol-induced ketonemia may occur in concomitance with significant lowering of blood glucose concentration, which may be attributed to suppression of gluconeogenesis in the setting of glycogen depletion in type 2 diabetes.

Keywords

Ethanol; Ketonemia; Glycemic control; Gluconeogenic mRNA; Glycogen synthase; Type 2 diabetes

1. Introduction

Alcoholic ketoacidosis (AKA) is a life-threatening complication [1, 2] accounting for ~7% mortality in subjects with chronic alcohol use [3]. AKA is the most common cause of ketoacidosis after diabetic ketoacidosis (DKA), and it usually occurs in malnourished subjects with a recent history of binge drinking [4–6]. However, it is often unrecognized or misdiagnosed in a clinical setting in conjunction with euglycemic, hypoglycemic, or hyperglycemic conditions [1, 4, 7, 8]. A high anion gap metabolic acidosis seen in AKA or DKA is largely attributed to the massive release of ketone bodies from the liver into the systemic circulation [1, 4, 8, 9]. Of the three ketone bodies [e.g., β-hydroxybutyrate (βOHB), acetoacetate (AcAc), and acetone], the circulating concentration of βOHB is markedly elevated in human subjects, with a plasma βOHB/AcAc ratio as high as 19:1 and 11:1 in AKA and DKA, respectively (cited in [4]). In general, AKA is associated with hyperketonemia (e.g., ↑ βOHB) and normal to low blood glucose concentrations, whereas DKA is associated with hyperketonemia and hyperglycemia [4, 8]. To date, it remains unclear as to how alcohol consumption regulates systemic levels of βOHB and glucose in metabolically compromised conditions such as type 2 diabetes (T2D).

Increased systemic levels of βOHB are primarily attributed to enhanced hepatic ketogenesis, which occurs when acetyl-CoA generated through β-oxidation of fatty acids exceeds cellular energy requirements [10, 11]. As an initial step in the ketogenic pathway, thiolase enzyme catalyzes the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA [10–12]. Subsequently, acetyl-CoA condenses with acetoacetyl-CoA to form hydroxymethylglutaryl-CoA (HMG-CoA) in a reaction catalyzed by HMG-CoA synthase-2 (HMGCS2), the rate-limiting enzyme in ketogenesis. HMG-CoA is sequentially metabolized by HMG-CoA lyase and βOHB dehydrogenase to form AcAc and βOHB, respectively, which are then released into the systemic circulation.

Nearly five decades ago, studies by Williamson *et al.*, Lefevre *et al.*, and Krebs *et al.* have demonstrated the potential for ethanol (EtOH) to enhance plasma ketone concentration, in conjunction with high fat diet or fatty acid utilization in the liver [13–15]. In particular, EtOH plus high fat diet for ~4–6 days enhances ketonemia in human subjects much higher

than that observed with EtOH or high fat diet alone [14]. In addition, EtOH feeding for 3 days in rats led to an increase in plasma ketone and hepatic ketogenesis with a diminution in hepatic glycogen content [14]. Recent studies have shown that in a mouse model of binge drinking, short-term EtOH feeding (2 days or acute single gavage) superimposed on a high fat diet regimen (2–3 days) results in a significant development of hepatic steatosis along with an induction of inflammatory genes [16–18]. However, the regulatory effects of short-term high fat-EtOH diet feeding regimen on plasma ketone and the associated changes in glycemic control have not yet been examined especially in the context of T2D.

A number of studies have shown that EtOH consumption may result in euglycemia or hypoglycemia (cited in [4, 14]), and this occurs as a function of acute/chronic alcohol and dietary nutrients (cited in [19]). Blood glucose concentration is maintained in part by hepatic glucose output, which is regulated by gluconeogenesis and glycogenolysis. In nondiabetic human subjects or rodents, EtOH inhibits hepatic gluconeogenesis with an associated decrease in glycogen content [20–22], which in turn results from its inhibitory effects on glycogen synthase (GS) activity [23–25]. The activation of GS is regulated in part by glycogen synthase kinase-3 β (GSK-3 β) and Akt, key proximal signaling components [26, 27]. Nevertheless, it remains unknown as to how EtOH regulates gluconeogenesis and glycogenesis under ketogenic conditions in T2D.

The objectives of the present study are to determine the effects of short-term high fat-EtOH diet feeding regimen on systemic ketone concentration (e.g., β OHB) and the associated changes in hepatic β OHB in *db/db* mice, an established animal model of T2D with fatty liver. To determine how EtOH regulates systemic glucose concentrations under ketogenic conditions, we examined the changes in hepatic gluconeogenic gene expression, GS expression/ phosphorylation/activation, and GSK-3 β /Akt expression/phosphorylation.

2. Materials and methods

2.1. Materials

Blood glucose strips and glucometer from Bayer Contour (Bayer HealthCare LLC, Mishawaka, IN), blood ketone strips and ketone monitoring system from Abbott Diabetes Care (Alameda, CA) were used. Insulin ELISA kit was purchased from Mercodia Inc. (Cat # 10–1247-011; Uppsala, Sweden). Triglyceride colorimetric assay kit (Cat # 10010303), β -hydroxybutyrate assay kit (Cat # 700740), and glycogen fluorometric assay kit (Cat # 700480) were purchased from Cayman Chemical (Ann Arbor, MI). Direct-Zol RNA Micro prep kit was purchased from Zymo research (Cat # R2061; Irvine, CA). The primer sets for qPCR were obtained from Integrated DNA Technologies, Inc (Coralville, Iowa). The primary antibodies for GS (84 kDa; Cat # 3886), phospho-GS (Ser 641) (85–90 kDa; Cat # 3891), GSK-3 β (46 kDa; Cat # 12456), phospho-GSK-3 β (Ser 9) (46 kDa; Cat #5558), Akt (60 kDa; Cat # 4691), phospho-Akt (Ser473) (60 kDa; Cat # 4060), and β -actin (45 kDa; Cat #4970) were purchased from Cell Signaling Technology (Danvers, MA). UDP-[14 C(U)]-glucose (specific activity: 263.0 mCi/mmol) was purchased from Perkin Elmer (Waltham, MA). All other reagents were from Thermo Fischer Scientific (Hanover Park, IL) or Sigma-Aldrich (St. Louis, MO).

2.2. Animals, Lieber-DeCarli liquid diets, and Experimental protocol

Type 2 diabetic male *db/db* homozygous mice (8 to 9 weeks of age; BKS.Cg-Dock7^m *+/+* Lepr^{db}/J; no. 000642) and age-matched nondiabetic lean *db/m* heterozygous mice (Dock7^m Lepr^{db}) were purchased from Jackson Laboratory (Bar Harbor, ME). Unlike *db/m* mouse, the *db/db* mouse that is deficient in leptin receptor exhibits the characteristic features of T2D including hyperglycemia, hyperinsulinemia, insulin resistance, and obesity [28, 29]. Upon arrival, *db/db* and *db/m* mice were maintained in a humidity- and temperature-controlled animal room with a 12:12-h dark-light cycle. The mice had free access to water and food (regular chow diet) *ad libitum*. All animal experiments were performed in accordance with the Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines and were approved by the committee.

In the present study, *db/db* and *db/m* mice were started on the liquid diets 7 days after arrival at the animal facility. Lieber-DeCarli[®] 82, Shake and Pour, control liquid diet (Product No: F1259SP) and Lieber-DeCarli[®] 82, Shake and Pour, ethanol liquid diet (Product No: F1258SP) were purchased from Bio-Serv (Frenchtown, NJ). Control and ethanol liquid diets were prepared fresh daily according to manufacturer's instructions. Ethanol liquid diet (EtOH LD) consisted of 36% ethanol-derived calories, 36% fat-derived calories, 13% carbohydrate-derived calories, and 15% protein-derived calories. In control liquid diet (control LD), EtOH-derived calories were substituted by equivalent calories from carbohydrates, and the calories derived from all other components were found to be similar to EtOH LD.

Recent studies have shown that in normal chow diet-fed lean mice, short-term [18] or long-term [30] EtOH feeding alone does not result in significant development of steatosis and/or accumulation of triglycerides in the liver. In a different study, short-term EtOH feeding alone on a normal chow diet for 4 days does not induce hepatic steatosis in lean control mice and also does not appear to exaggerate macrovesicular steatosis in *ob/ob* mice (leptin gene-deficient obese phenotype) [31]. As noted in the Introduction, short-term EtOH-high fat diet feeding in mice has recently been used as a model of binge drinking [16–18]. For instance, EtOH feeding (6% v/v) in concomitance with high fat diet for 2 days has been shown to induce steatosis and inflammatory genes in mouse liver [16, 17]. In the present study, we employed a short-term EtOH-high fat diet feeding protocol for up to 9 days to determine its effects on systemic β OH levels and the associated changes in glycemic control in *db/db* versus *db/m* mice. Due to the glucose-lowering effects of EtOH (as shown in Fig. 4B) and the potential hypoglycemic risk, we employed the 9-day feeding protocol in the present study.

As illustrated in Fig. 1, *db/db* mice and *db/m* mice were acclimatized with 1:1 mix of ethanol liquid diet and control liquid diet for 2 days (*n* = 7 mice/group), respectively. Subsequently, the respective mice were maintained on 5% (w/v) Lieber-DeCarli EtOH LD or an isocaloric control LD for 9 days. Body weights of mice were measured every other day from day 1 through day 9. Caloric intakes of liquid diets were measured following the same pattern using the equation; 1 mL = 1 kcal, as shown in Fig. 1.

2.3. Blood β OHB, Glucose, and Intraperitoneal glucose tolerance test (IPGTT)

The mice were fasted for 4h in the mornings on days 1, 3, 5, 7, and 9. In addition, IPGTT was performed on day 5 as described previously [32–34]. The blood samples were collected from the mouse tail after making a 1–2 mm cut at the tail end with a pair of scissors [35]. The concentrations of blood β OHB and glucose were measured using Contour glucometer (Bayer Health Care, Mishawaka, IN) and Precision Xtra ketone meter (Abbott Diabetes Care, Alameda, CA, IL), respectively.

For IPGTT, subsequent blood sampling was done at 15, 30, 60, 120, and 150 min time intervals (after glucose challenge) on day 5 in conscious mice [33]. Area under the curve (AUC) for blood glucose concentration was calculated by applying the trapezoidal rule [36] for the whole time interval (0–150 min) during IPGTT (AUC_{IPGTT}), where the baseline was set at $Y = 0$.

2.4. Plasma insulin concentration and HOMA-IR index

On day 9, after a 4-h of morning fast, blood samples were drawn from the retro-orbital plexus of mice and collected in heparinized microhematocrit tubes, and plasma was separated. Plasma insulin concentrations was measured using ELISA kit (Mercodia Inc.; Cat # 10–1247-011; Uppsala, Sweden), according to the manufacturer's instructions.

Homeostasis model assessment of insulin resistance (HOMA-IR; insulin sensitivity index) was calculated using HOMA 2 computerized model [37].

2.5. Liver and epididymal white adipose tissue (eWAT) weight

On day 9, immediately after recording the fasting levels of β OHB and glucose (12:00 noon - 01:00 pm), mice were euthanized by an overdose of sodium pentobarbital (I.P, 80 mg/ml). Liver and eWAT tissues were then harvested. The tissue weights were normalized to the body weights of mice [38]. The snap-frozen liver tissues were stored at -80°C until further analysis.

2.6. Histology and Oil Red O staining of liver sections

The liver sections were stained with Hematoxylin and Eosin (H&E) and Oil Red O to evaluate lipid accumulation in hepatocytes and examine the extent of steatosis [39, 40]. The images of the liver sections were captured under bright field with Olympus DP72 microscope BX43 using 40X magnification. The total area of lipid droplets was analyzed using image J software (version 1.50i, Bethesda, NIH) [39].

2.7. Triglycerides, β OHB, and Total glycogen levels in the liver

Liver tissues were homogenized in the respective assay buffers, and the supernatants were then used for the determination of triglyceride (Cayman Chemical, Cat # 10010303), β OHB (Cayman Chemical, Cat # 700740) and total glycogen (Cayman Chemical, Cat # 700480), according to manufacturer's instructions. The absorbance/fluorescence was measured using Bio-Tek Synergy HT Multi-Mode microplate reader (Winooski, VT).

2.8. Hepatic glycogen synthase activity

In vitro enzyme assay for glycogen synthase (GS) was performed using radiolabeled UDP- [$^{14}\text{C}(\text{U})$]-glucose, as described previously [23, 41]. The activity assay is based on the principle that involves the incorporation of radiolabeled glucose from UDP- [$^{14}\text{C}(\text{U})$]-glucose into glycogen. The reaction was terminated by spotting 75 μl of the reaction mixture onto 2 cm Whatmann filter paper (No. 31 ET). The filter papers were subjected to washing steps as described by Thomas *et.al* [41], and ^{14}C radioactivity was counted using liquid scintillation counter. The total protein content was measured by using Bio-Rad DC protein assay kit (Bio-Rad Cat # 5000116, Hercules, CA). The absorbance was measured at 750 nm.

2.9. Quantitative real-time PCR analysis

Total RNA was extracted from snap-frozen liver tissues using Direct-Zol RNA Micro prep kit (Zymo research, # R2061, Irvine, CA) and quantified using Nano Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The purity of RNA samples was verified by measurement of absorbance ratios at 260/280 nm and 260/230 nm. To remove contamination due to genomic DNA, the isolated RNAs were treated with RNase-free DNase I (Qiagen, Germantown, MD), as described [42].

800 ng of total RNA was then reverse transcribed to cDNA using GeneAmp RNA PCR Core kit (Thermo Fischer Scientific, # N8080143, Hanover Park, IL). Quantitative real-time PCR (qPCR) analysis was performed using Applied Biosystems 7900HT Fast Real-Time PCR system and Power SYBR Green PCR Master mix (Thermo Fischer Scientific, # A25779, Hanover Park, IL) with the primer sets described in Table 2. The primers for qPCR were designed using algorithm Primer 3. Amplification efficiencies were normalized using the housekeeping gene *Rplpo*. The relative fold change in expression was calculated by Livak method [43]. Each experiment was performed in triplicate.

2.10. Immunoblot analysis

Liver tissue lysates (20 μg protein each) were electrophoresed using precast 4–12% NuPage mini-gels (Life Technologies, Carlsbad, CA). For western blot analysis, the resolved proteins were transferred to PVDF membranes (EMD Millipore, Burlington, MA) as described [42]. The protein bands were normalized using β -actin as the loading control [24, 44], and quantified using Quantity One software (version 4.6.9).

2.11. Statistical analysis

Data are expressed as means $\pm$ SEM. Repeated measures and regular two- way ANOVA followed by Bonferroni and Tukey multiple comparisons test were performed between the control and EtOH treated groups respectively. Values of $p < 0.05$ were considered statistically significant.

3. Results

Overall, in the present study, EtOH feeding in type 2 diabetic mice for 9 days resulted in significant decreases in weight gain ($\sim 19.5\%$ $\downarrow$) and caloric intake ($\sim 30\%$ $\downarrow$) along with a transition from macrovesicular-to-microvesicular hepatic steatosis and a modest increase in

hepatic TG (~37% ↑). Importantly, EtOH-induced elevation in systemic β OHB concentration (~8-fold ↑) was associated with significant lowering of blood glucose (~4 fold ↓), and decreased plasma insulin (~3 fold ↓) and HOMA-IR index (~3 fold ↓). In addition, EtOH intake led to increased hepatic β OHB (~5-fold ↑) and *hmgcs2* mRNA (~4 -fold ↑), downregulation of key gluconeogenic mRNAs (e.g., *Pcx*, *Pck1*, and *G6pc*), and depletion of glycogen (~4 fold ↓). Furthermore, EtOH intake resulted in significant decreases in the mRNA/protein expression and allosteric activation of GS in liver tissues regardless of changes in the phosphorylation of GS, GSK-3 β , or Akt.

3.1. Ethanol decreases weight gain in db/db mice

As shown in Table 1A, EtOH LD feeding in diabetic mice for 9 days resulted in significant decrease in body weight from ~37 g (day 1) to ~35 g (day 9) by ~6% ($p < 0.05$), compared with control LD-fed diabetic mice where body weight increased by ~29% (~34 g on day 1 *versus* ~43 g on day 9). Of note, EtOH LD-fed diabetic mice showed significant decreases in weight gain by 10%, 13%, and 19% on days 5, 7, and 9, respectively, compared with the corresponding time intervals for control LD-fed diabetic mice.

In EtOH LD-fed nondiabetic mice for 9 days, there was a significant decrease in body weight from ~24 g (day 1) to ~20 g (day 9) by ~17% ($p < 0.05$), compared with control LD-fed nondiabetic mice where a significant increase in body weight from ~23 g (day 1) to ~26 g (day 9) by ~13% ($p < 0.05$) was observed. Of note, EtOH LD-fed nondiabetic mice showed significant decreases in weight gain by 11%, 16%, and 24% on days 5, 7, and 9, respectively, compared with the corresponding time intervals for control LD-fed nondiabetic mice.

EtOH consumption for 9 days led to significant decreases in weight gain in diabetic and nondiabetic mice, compared with the respective control groups.

3.2. Ethanol decreases caloric intake in db/db mice

As shown in Table 1B, both EtOH LD- and control LD-fed diabetic mice showed a progressive increase in caloric intake by ~17% and ~25% ($p < 0.05$) from day 1 to day 9 respectively. Of note, EtOH LD-fed diabetic mice showed a significant decrease in caloric intake by 25% and 30% on day 1 and day 9, respectively, compared with the corresponding time intervals for control LD-fed diabetic mice.

Similarly, in both EtOH LD- and control LD-fed nondiabetic mice, there was a progressive increase in caloric intake by ~33 % and ~20% ($p < 0.05$), respectively, from day 1 to day 9. Of note, EtOH LD-fed nondiabetic mice showed a significant decrease in caloric intake by 30% and 33% on day 1 and day 9, respectively, compared with the corresponding time intervals for control LD-fed nondiabetic mice.

Thus, EtOH consumption for 9 days led to significant decreases in caloric intake in diabetic and nondiabetic mice, compared with the respective control groups.

3.3. Ethanol dysregulates hepatic lipid accumulation in type 2 diabetic db/db mice

As shown in Fig. 2A (H&E staining, lower right panel), EtOH feeding in *db/db* mice led to the appearance of microvesicular steatosis in the liver, compared with macrovesicular steatosis seen in control LD-fed *db/db* mice. In addition, Oil Red O staining revealed the accumulation of microvesicular lipid droplets in EtOH-fed *db/db* mice compared with control diet-fed *db/db* mice (Fig. 2B; lower right panel).

Under EtOH LD-fed conditions (Fig. 2A; H&E staining, lower left panel), the liver sections from nondiabetic *db/m* mice showed the development of hepatic steatosis, compared with *db/m* mice on a control LD. In addition, Oil Red O staining revealed a significant accumulation of lipid droplets by ~3-fold in *db/m* mouse liver (Fig. 2B; lower left panel).

Under control LD-fed conditions (Fig. 2A; H&E staining, upper left and right panels), the liver sections from *db/db* mice showed macrovesicular steatosis, compared with nondiabetic *db/m* mice. In addition, Oil Red O staining revealed a significant accumulation of macrovesicular lipid droplets by ~9.4-fold in *db/db* mouse liver compared with *db/m* mouse liver (Fig. 2B; upper left and right panels).

3.4. Ethanol enhances hepatic triglyceride content in db/db mice

As shown in Fig. 3A, in EtOH LD-fed *db/db* mice, a significant increase in hepatic triglyceride content (~37% ↑) was observed compared with control LD-fed *db/db* mice ($p < 0.05$). In parallel, in EtOH LD-fed *db/m* mice, there was an increase in hepatic triglyceride content by ~2 fold compared with control LD-fed *db/m* mice ($p < 0.05$). In addition, under control LD-fed conditions, hepatic triglyceride content was ~3.0-fold higher in *db/db* mice compared with *db/m* mice ($p < 0.05$).

3.5. Hepatic fatty acid metabolism and inflammatory gene expression in EtOH-fed db/db mice

EtOH-induced changes in the mRNA expression of hepatic fatty acid metabolism and inflammatory genes in EtOH LD-fed *db/db* mice were determined by qPCR analysis using specific primers (Table 1).

As shown in Fig. 3B-C, in EtOH LD-fed *db/db* mice, no significant change in mRNA expression levels of *Cd36* and *Fatp 1* (key hepatic genes primarily involved in fatty acid uptake) were observed compared with control LD-fed *db/db* mice. However, in EtOH LD-fed *db/m* mice, there were significant increases in the mRNA expression levels of *Cd36*, *Fatp1* by ~1.7 and ~2.2 fold respectively, compared with control LD-fed *db/m* mice ($p < 0.05$). In addition, under control LD-fed conditions, ~2.5- and ~2.3-fold increases in the mRNA expression of *Cd36* and *Fatp1* were observed in *db/db* mice, compared with *db/m* mice ($p < 0.05$).

In addition, as shown in Fig. 3D-E, in EtOH LD-fed diabetic mice, no significant change in mRNA expression of *Dgat2* (a key hepatic gene involved in triglyceride synthesis) was observed compared with control LD-fed *db/db* mice. However, ~4 fold increase in the mRNA expression of *Atgl* (key hepatic gene primarily involved in lipolysis) was observed in EtOH LD-fed *db/db* mice compared with control LD-fed *db/db* mice ($p < 0.05$).

Furthermore, in control LD-fed *db/db* mice, ~4 fold increase in mRNA expression of *Dgat2* with an accompanying ~2.5 fold decrease in mRNA expression of *Atgl* was observed, compared with control LD-fed *db/m* mice ($p < 0.05$).

As shown in Fig. 3F, in EtOH LD-fed diabetic mice, no significant change in mRNA expression levels of *Hig-2* was observed, compared with control LD-fed *db/db* mice. In EtOH-LD-fed nondiabetic mice, there was an increase in the mRNA expression level of *Hig-2* by ~2 fold compared with control LD-fed nondiabetic mice. Furthermore, under control LD fed conditions, ~3-fold increase in the mRNA expression of *Hig-2* was observed in *db/db* mice, compared with *db/m* mice ($p < 0.05$).

As shown in Fig. 3G-L, in EtOH LD-fed diabetic mice, a significant increase in hepatic mRNA expression level of *Cyp2e1* by ~2 fold was observed, compared with respective control LD fed mice ($p < 0.05$). In EtOH LD-fed nondiabetic mice, there were increases in the mRNA expression level of *Cyp2e1* by ~2.1 fold compared with control LD-fed nondiabetic mice ($p < 0.05$). However, EtOH feeding for 9 days did not show significant changes in the hepatic mRNA expression levels of *Tnfa*, *IL-1 β* , *Mcp1*, *Mip-2* and *Mif* in *db/db* mice and *db/m* mice, respectively. In addition, under control LD-fed conditions in *db/db* mice, ~2- to 4-fold increases in the mRNA expression of key hepatic inflammatory genes were observed, compared with *db/m* mice ($p < 0.05$).

3.6. Ethanol-induced ketonemia is associated with lowering of blood glucose concentration in *db/db* mice

As shown in Fig. 4A, EtOH LD-fed diabetic mice showed significant increases in β OHB concentrations by ~5-, 8-, 8-, and 7-fold on days 3, 5, 7, and 9, respectively, compared with the respective time intervals for control LD-fed *db/db* mice. In control LD-fed diabetic mice, the circulating concentrations of β OHB remained essentially the same with no significant changes from day 1 (0.66 mM) to day 9 (0.65 mM).

In parallel, EtOH LD-fed nondiabetic mice showed significant increases in blood β OHB concentrations by ~2-, 2.5-, 4- and 4.5-fold on days 3, 5, 7, and 9, respectively, compared with control LD-fed nondiabetic mice. In control LD-fed nondiabetic mice, there were no significant changes in blood β OHB concentrations from day 1 (0.55 mM) to day 9 (0.53 mM).

As shown in Fig. 4B, in EtOH LD-fed diabetic mice, there was a significant decrease in the blood glucose concentration from 298 mg/dL (day 1) to 79 mg/dL on day 9 ($p < 0.05$). In control LD-fed diabetic mice, there was a progressive increase in blood glucose concentration from 333 mg/dL (day 1) to 492 mg/dL on day 9 ($p < 0.05$).

In EtOH LD-fed nondiabetic mice, blood glucose concentrations decreased from 115 mg/dL (day 1) to 70 mg/dL and 74 mg/dL on day 3 and day 9, respectively ($p < 0.05$). In control LD-fed non-diabetic mice, blood glucose concentration increased from 116 mg/dL (day 1) to 135 mg/dL on day 9 ($p < 0.05$).

EtOH LD-fed diabetic mice showed significant increases in systemic β OHB concentrations by up to ~7–8-fold along with reduced glucose concentrations (~80–160 mg/dL) on days 7–

9. In parallel, in EtOH LD-fed nondiabetic mice, systemic β OHB concentrations enhanced by ~3–4-fold along with decreased blood glucose concentration to ~74 mg/dL on days 7–9.

3.7. Ethanol improves glucose tolerance and reduces systemic β OHB concentration in db/db mice

In the present study, intraperitoneal glucose tolerance test (IPGTT) was performed on day 5 during the 9-day feeding protocol, as described in Fig. 1. As shown in Fig. 5A, in EtOH LD-fed diabetic mice, there were significant increases in blood glucose concentrations from a baseline value of ~116 mg/dL to values up to ~472 mg/dL at 15 min and > 550 mg/dL at 30–60 min. This was followed by a decline in blood glucose concentrations to ~398 mg/dL and ~185 mg/dL at 120 and 150 min, respectively. In parallel, in control LD-fed diabetic mice, IPGTT revealed elevations in blood glucose concentrations to values greater than the baseline value (~383 mg/dL) at all-time intervals (15–150 min). As shown in Fig. 5B, in EtOH LD-fed diabetic mice, AUC for glucose concentration was significantly lower by 18.2% ($p < 0.05$), compared with control LD-fed diabetic mice.

In EtOH LD-fed nondiabetic mice, glucose tolerance remained unaltered compared with control LD-fed nondiabetic mice.

As shown in Fig. 5C, systemic β OHB levels were significantly decreased during IPGTT in EtOH LD-fed diabetic mice. Notably, blood β OHB concentration decreased from ~4.5 mM (baseline value) to ~3.4 mM (~1.3-fold) and ~2.7 mM (~1.6-fold) at 15 and 150 min time intervals, respectively ($p < 0.05$). In control LD-fed diabetic mice, there were no significant changes in blood β OHB concentrations at 15–150 min during IPGTT.

In parallel, in EtOH LD-fed nondiabetic mice, there was a significant decrease in β OHB from 2 mM to 0.8 mM beginning at the 15 min time point ($p < 0.05$).

Together, EtOH-fed diabetic mice displayed a significant improvement in glucose tolerance, and decline in blood β OHB concentration by up to ~1.6 fold.

3.8. Ethanol decreases plasma insulin concentration and HOMA-IR index in db/db mice

As shown in Fig. 6 (A-B), in EtOH LD-fed diabetic mice, there were significant decreases in plasma insulin concentration (**6A**) and HOMA-IR levels (**6B**) by ~3-fold, compared with control LD-fed diabetic mice ($p < 0.05$) respectively. In EtOH-fed nondiabetic mice, no significant changes in plasma insulin concentration and HOMA-IR levels were observed compared with the respective control.

3.9. Ethanol increases hepatic β OHB content and upregulates Hmgcs2 mRNA expression in db/db mice

As shown in Fig. 7A, an increase in hepatic β OHB by ~5-fold was observed in EtOH LD-fed diabetic mice compared with control LD-fed diabetic mice ($p < 0.05$). In addition, there was ~4-fold increase in EtOH LD-fed nondiabetic mice compared with its respective control ($p < 0.05$).

As shown in Fig. 7B, an increase in *Hmgcs2* mRNA expression by ~4-fold was found in EtOH LD-fed diabetic mice compared with control LD-fed diabetic mice ($p < 0.05$). In addition, there was ~2-fold increase in EtOH-fed nondiabetic mice, compared with its respective control ($p < 0.05$).

3.10. Ethanol diminishes the mRNA expression of hepatic gluconeogenic genes in db/db mice

As shown in Fig. 8A-C, in EtOH LD-fed diabetic mice, significant decreases in the hepatic mRNA expression levels of *Pcx*, *Pck1*, and *G6pc* by ~4 fold, ~3 fold and ~2 fold, respectively, were observed compared with control LD-fed diabetic mice ($p < 0.05$). In EtOH-fed nondiabetic mice, there were decreases in the mRNA expression levels of *Pcx*, *Pck1*, and *G6pc* by ~2-fold, compared with nondiabetic mice on a control LD.

As shown in Fig. 8D, EtOH consumption led to a marginal but statistically insignificant increase in the mRNA expression levels of *srebp1l*, the gene involved in hepatic lipogenesis in diabetic as well as nondiabetic mouse liver.

Thus, EtOH consumption for 9 days led to a significant reduction in the mRNA expression levels of key gluconeogenic genes in diabetic and nondiabetic mouse liver.

3.11. Ethanol depletes hepatic glycogen, decreases glycogen synthase expression, and diminishes allosteric activation of glycogen synthase in db/db mice

As shown in Fig. 9A, in EtOH LD-fed diabetic mice, there was a reduction in liver glycogen content by ~4 fold compared with control LD fed diabetic mice ($p < 0.05$). In EtOH LD-fed nondiabetic mice, the decrease in liver glycogen content occurred to the extent of ~3 fold compared with control LD-fed nondiabetic mice. In addition, in diabetic *db/db* mice, there was an increase in hepatic glycogen content by ~1.9-fold in conformity with previous reports, which demonstrate a 2- to 3-fold increase in hepatic glycogen in *db/db* mice *versus* nondiabetic mice (cited in [29, 45]).

As shown in Fig. 9B, in both EtOH LD-fed diabetic and non-diabetic mice, there was a significant decrease in *Gys2* mRNA by ~2-fold compared with the respective control LD-fed diabetic and non-diabetic mice ($p < 0.05$).

As shown in Fig. 9C, a significant decrease in GS protein expression ($p < 0.05$) in EtOH LD-fed diabetic mice was observed, but it was without any changes in GS protein phosphorylation, compared with control LD-fed diabetic mice. In EtOH LD-fed nondiabetic mice, there was a trend toward a decrease in GS protein expression by ~57.29% but with a significant decrease in the phosphorylation of GS ($p < 0.05$), compared with control LD-fed nondiabetic mice.

As shown in Fig. 9D (left panel), EtOH LD-fed diabetic mice did not display significant changes in hepatic GS activity in the absence of G-6-P, compared with control LD-fed diabetic mice. However, EtOH LD-fed nondiabetic mice showed significant increase in hepatic GS activity by ~ 2-fold compared with control LD-fed nondiabetic mice ($p < 0.05$).

As shown in Fig. 9D (right panel), EtOH LD-fed diabetic mice displayed a significant decrease in hepatic GS activity by ~2-fold in the presence of G-6-P, compared with control LD-fed diabetic mice ($p < 0.05$). In addition, EtOH LD-fed nondiabetic mice showed a significant decrease in hepatic GS activity by ~2.5-fold compared with control LD-fed nondiabetic mice ($p < 0.05$).

Together, the present findings suggest that the diminution in the allosteric activation of GS (by G-6-P) rather than a change in GS phosphorylation plays a major role in the depletion of hepatic glycogen in ethanol-fed diabetic mice.

3.12. Ethanol diminishes GSK-3 β expression and Akt phosphorylation in db/db mice

As shown in Fig. 10A, EtOH LD-fed diabetic mice showed a significant decrease in GSK-3 β protein expression ($p < 0.05$), without any changes in GSK-3 β protein phosphorylation, compared with control LD-fed diabetic mice. There were no significant changes in the expression and phosphorylation of GSK-3 β in EtOH LD-fed nondiabetic mice, compared with its respective control.

As shown in Fig. 10B, no significant change in Akt protein expression was found in EtOH LD-fed diabetic mice. However there was a pronounced decrease in Akt phosphorylation ($p < 0.05$), compared with control LD-fed diabetic mice. In a similar manner, no change in Akt at the level of protein expression was seen in EtOH LD-fed nondiabetic mice but there was a significant reduction in protein phosphorylation ($p < 0.05$).

3.13. Ethanol decreases liver weight and epididymal white adipose tissue weight in db/db mice

As shown in Table 2, EtOH LD-fed diabetic mice showed a significant reduction in liver weight by ~31.4% compared with control LD-fed diabetic mice ($p < 0.05$). In EtOH LD-fed nondiabetic mice, there was a significant decrease in liver weight by ~28% compared with control LD-fed nondiabetic mice.

Furthermore, in EtOH LD-fed diabetic mice, ~15.1% reduction in eWAT weight was observed compared with control LD-fed diabetic mice ($p < 0.05$). In addition, ~24% reduction eWAT tissue was observed in EtOH LD-fed diabetic mice compared with control LD-fed diabetic mice ($p < 0.05$).

In EtOH LD-fed nondiabetic mice, there was a significant reduction in eWAT weight by ~33.7 % compared with control LD-fed nondiabetic mice.

4. Discussion

Previous studies in human subjects have shown that EtOH consumption in concomitance with high fat diet results in a marked increase in plasma ketone concentration [14]. In addition, in rat liver perfusion studies and liver slice preparations, EtOH exposure increases ketone body formation only in the presence of exogenous fatty acids [13, 14]. These seminal findings from earlier studies suggest that under *in vivo* conditions, fatty acids derived from dietary fat and/or adipose tissue lipolysis contribute to EtOH-induced hepatic ketogenesis. In

the present study, short-term EtOH feeding in type 2 diabetic mouse model resulted in a significant increase in the circulating concentration of β OHB. Importantly, hyperketonemia has been observed in conjunction with microvesicular steatosis, triglyceride accumulation, and enhanced mRNA expression levels of fatty acid metabolism and inflammatory genes in diabetic mouse liver.

An increased intramitochondrial acetyl-CoA pool associated with β -oxidation of fatty acids could contribute to exaggerated ketone body formation in the liver [10–12]. From the present findings with liver tissues, it is evident that EtOH enhances *hmgcs2* mRNA expression and β OHB formation in type 2 diabetic mice, which would in part account for the elevated concentration of circulating β OHB as seen in type 2 diabetic mice. However, EtOH-induced increase in β OHB in isolated hepatic tissues appears to be marginal compared with significant increases in circulating β OHB observed over time in type 2 diabetic mice. It is likely that EtOH reduces the utilization of β OHB in peripheral tissues [14], thereby contributing in part to hyperketonemia in T2D. Previous studies have shown that the activity of succinyl-CoA:3-oxoacid-CoA transferase (SCOT), a rate-limiting enzyme for ketone body utilization in peripheral tissues such as kidney and heart, is decreased due to nitration in different experimental conditions including oxidative stress and diabetes [46, 47]. Furthermore, β OHB uptake is markedly impaired in skeletal muscle from diabetic rats [48, 49], suggesting an additional mechanism for hyperketonemia in diabetic state. Future studies should determine the likely regulatory effects of EtOH on ketone utilization (through SCOT activity) and ketone uptake in extrahepatic tissues, including skeletal muscle, kidney, and brain in T2D.

In conjunction with previous reports, an increase in plasma ketone with normal to low blood glucose concentrations in presumably nondiabetic subjects on chronic EtOH [4, 14] suggests the likely existence of this relationship in T2D with EtOH intake. In particular, EtOH-induced ketonemia in type 2 diabetic mice was associated with pronounced lowering of blood glucose from an initial hyperglycemic level on day 1 to euglycemic concentrations from day 3 onwards. In addition, a significant improvement in glucose tolerance was observed in EtOH-fed diabetic mice. The improved blood glucose levels seen in the present study, in conjunction with significant decreases in plasma insulin and HOMA-IR index, may be attributed to impaired hepatic gluconeogenesis as reported in a recent study with fat-specific insulin receptor knockout mice [50]. Importantly, there are no reports that document the measurements of blood ketone (β OHB) in concomitance with blood glucose concentrations during glucose tolerance test. In the present study, concomitant measurements of β OHB during GTT revealed a transient decrease in systemic β OHB, suggesting the potential for dextrose to attenuate ketonemia during the management of ketoacidosis [4].

It is well accepted that EtOH oxidation alters the redox state (e.g., $\uparrow$ NADH/NAD⁺ ratio) in the hepatic mitochondria [51–54]. This would result in enhanced lactate/pyruvate ratio [51, 53], decreased availability of pyruvate (a gluconeogenic substrate) for oxaloacetate formation [51], and inhibition of gluconeogenesis [51, 55, 56]. In addition, EtOH has been shown to downregulate key gluconeogenic mRNAs including PEPCK [57] and glucose-6-phosphatase [57, 58] in mouse liver. In the present study, qPCR analysis of hepatic RNA

extracts from EtOH-fed type 2 diabetic mice revealed significant decreases in the mRNA expression levels of pyruvate carboxylase, PEPCK, and glucose-6-phosphatase, suggesting that the blood glucose-lowering effect of EtOH is attributable in part to the inhibition of hepatic gluconeogenesis in T2D.

Previous studies have shown that EtOH-mediated inhibition of gluconeogenesis is closely correlated with a decrease in glycogen content in the liver [20–22]. In the present study, EtOH consumption led to a significant decrease in hepatic glycogen content in type 2 diabetic mice. Of importance, glycogen turnover is determined by the activation state of glycogen synthase (GS) and glycogen phosphorylase (GP) [59]. Although EtOH did not affect GS phosphorylation, there was a concomitant reduction in the expression levels of GS protein and mRNA, which would account for a decrease in its activity. In this regard, *in vitro* enzyme assays revealed a significant decrease in the allosteric activation of GS in ethanol-fed type 2 diabetic mice. Together, EtOH-mediated depletion of hepatic glycogen in diabetic (present study) or nondiabetic state ([23], present study) is attributable in part to its inhibitory effect on the allosteric activation of GS.

Hepatic GS is also regulated by alterations in the activation state of proximal signaling components such as Akt and GSK-3 β [26, 27]. In particular, Akt activation (phosphorylated form) leads to the phosphorylation/inactivation of its downstream substrate, GSK-3 β . This in turn results in diminished phosphorylation and activation of GS. This conventional viewpoint has recently been challenged by a recent report, according to which activated Akt may enhance GS activity independent of GSK-3 β [60]. In the present study, EtOH diminished Akt phosphorylation without affecting Akt protein expression in EtOH-fed type 2 diabetic mice. This may suggest that alcohol-mediated diminution in Akt activation could contribute to diminished GS activity. However, the observed data on GS protein expression/phosphorylation do not support this notion. This is because GS protein expression but not the phosphorylation was decreased by EtOH as noted above, suggesting that EtOH-mediated inhibition of GS activity occurs independent of Akt. Next, EtOH-fed type 2 diabetic mice led to a marked decrease in the expression level of GSK-3 β protein but without significant changes in GSK-3 β phosphorylation, suggesting that a decrease in GSK-3 β activity is due to decreased GSK-3 β expression. Thus, the present findings suggest that EtOH consumption is associated with diminished activation of Akt and GSK-3 β . Together, EtOH-mediated depletion of hepatic glycogen results from diminished allosteric activation of GS and might occur by mechanisms independent of dysregulated Akt and GSK-3 β *at least* in type 2 diabetic mice.

From a clinical standpoint, it is widely accepted that alcoholic ketoacidosis is characterized by hyperketonemia with normal to low blood glucose concentrations, whereas diabetic ketoacidosis is defined by hyperketonemia and hyperglycemia [4, 8]. Of importance, EtOH-induced ketonemia in T2D may occur in conjunction with the development of glucose intolerance or induction of hypoglycemia [61]. Such differences in glycemic state may be attributed to alterations in nutritional state (e.g., malnutrition) and/or hepatic glycogen reserve [61–64]. For instance, in subjects with T2D, EtOH-mediated increase in plasma ketone (e.g., β OHB) is associated with impaired glucose tolerance as revealed by higher values of hemoglobin A_{1C} and fasting/postprandial plasma glucose [65]. The observed

increase in plasma glucose concentration has been attributed to EtOH-mediated inhibition of insulin action in peripheral tissues rather than its ability to inhibit insulin secretion from pancreatic β -cells [65]. Studies by several other investigators reveal a decrease in plasma insulin level in patients with AKA, and this may be associated with impaired gluconeogenesis and glycogen depletion in the liver with an accompanying increase in the circulating concentration of counter-regulatory hormone (e.g., epinephrine), which can inhibit insulin secretion by pancreatic β -cells (cited in [66]). In perfused rat pancreas, EtOH does not influence basal insulin secretion but it diminishes glucose-stimulated insulin secretion [67]. Thus, even with low plasma insulin and high circulating concentrations of counter-regulatory hormones (e.g., epinephrine and glucagon), EtOH has the potential to induce hypoglycemia by indirectly inhibiting gluconeogenesis [61, 64]. This inhibitory effect of EtOH on gluconeogenesis may occur in conditions (e.g., prolonged fasting) that deplete hepatic glycogen stores [21, 61, 64]. In the present study with type 2 diabetic mice, EtOH consumption for 9 days results in significant lowering of blood glucose concentration in conjunction with hyperketonemia. This effect occurs with diminutions in plasma insulin concentration and HOMA-IR index, suggesting that EtOH-mediated depletion of hepatic glycogen and the associated diminution in gluconeogenic gene expression contribute to its blood glucose-lowering effect.

It is important to acknowledge the limitations of the present study. The EtOH-fed (*db/db* or *db/m* mice) were not pair-fed to their control diet-fed counterparts. In addition, instead of a gradual increase in EtOH concentration during acclimatization with liquid diets, the present study employed a fixed concentration of 3.35% EtOH during the 2-day acclimatization period. It is likely that the observed decrease in caloric intake during short-term alcohol feeding (5% EtOH) for 9 days may in part account for the lowering of blood glucose in *db/db* mice. In this regard, previous studies with pair-fed obese type 2 diabetic rodents have shown that a reduction in food intake contributes to ~53% of the antihyperglycemic effect of liraglutide (a long-acting glucagon-like peptide-1 derivative) [68]. It is noteworthy that on an unrestricted diet, the obese, insulin-resistant rodents (that are also hyperphagic) consume food throughout the 24-h cycle [69, 70]. In contrast, on a restricted diet (pair-fed condition), the obese rodents consume all food within 3 to 10 h that would result in fasting or caloric deprivation for the remaining period in the 24-h cycle [69, 70]. In the present study with type 2 diabetic *db/db* mice (obese/hyperphagic), we thus avoided pair-feeding and also for the reason that *db/db* mice were subjected to 4h fasting periods for blood sample collections to measure glucose and β OHB levels (on days 1, 3, 5, 7 and 9) and to perform GGT test (on day 5) during the 9-day treatment protocol. We employed a morning fast of ~4h to circumvent a catabolic state (e.g., hepatic glycogen depletion) as it may occur with an overnight fast of 14–18 h, as described [33]. Given the fact that the volume and frequency of blood sampling would cause an additional stress during GTT [33], the present study used a minimal blood volume of ~20 μ l (x6) from each mouse for glucose and β OHB measurements. It is likely that the stress of such interventions might have affected the measured parameters including caloric intake, especially in EtOH-fed mice.

In conclusion, the present findings have translational relevance in diabetic state whereupon EtOH has the potential to induce ketonemia in concomitance with significant lowering of blood glucose, which may be attributable to suppression of gluconeogenesis in the setting of

glycogen depletion. It is noteworthy that hepatic glycogen metabolism is subject to regulation by diurnal rhythm as has been reported in studies by several investigators [24, 29, 45]. In particular, EtOH-mediated disruption of diurnal rhythm may contribute to glycogen depletion [24], which is likely to increase the risk of delayed hypoglycemia [71] and ketonemia [72] in type 1 diabetic subjects. Future studies should determine the impact of EtOH on diurnal rhythm, hepatic glycogen, glycemic control, and ketonemia in type 1 *versus* type 2 diabetes. Furthermore, the molecular mechanisms of EtOH-induced metabolic aberrations should be identified for rational therapeutic targeting in type 2 diabetes.

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Fig. 1.

Schematic of the experimental protocol to study the effects of EtOH in type 2 diabetic *db/db* mice *versus* nondiabetic *db/m* mice. Mice were initially acclimatized with liquid diets according to the study group, as described. After acclimatization, mice were fed Lieber-DeCarli ethanol liquid diet (EtOH LD, 5% *w/v*) or control liquid diet (control LD) for 9 days. β OHB, β -hydroxybutyrate; *hmgcs2*, 3-hydroxy-3-methylglutaryl-CoA synthase 2; IPGTT, intraperitoneal glucose tolerance test; GS, glycogen synthase; GSK-3 β , glycogen synthase kinase-3 β ; H&E, haematoxylin and eosin; ORO, Oil Red O; TG, triglyceride; FA, fatty acid.

Fig. 2.

Effects of ethanol on hepatic steatosis in type 2 diabetic *db/db* mice *versus* nondiabetic *db/m* mice. Representative images are shown for (A) H&E and (B) Oil Red O staining of liver sections from *db/db* and *db/m* mice fed an EtOH LD or a control LD. Magnification, 40x; scale bars represent 40 μ m. (C) Quantification of lipid droplet surface area using Oil Red O-stained liver sections. Different letters (a, b, c, and d) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant.

Fig. 3.

Effects of ethanol on triglyceride content and fatty acid metabolism/inflammatory marker mRNA expression levels in the liver from *db/db* mice versus *db/m* mice. (A) Hepatic triglyceride. (B-F) *Cd36*, *Fatp1*, *Dgat2*, *Atgl*, and *Hig-2* (fatty acid metabolism genes); and (G-L) *Cyp2e1*, *Tnfa*, *Il-1β*, *Mcp-1*, *Mip-2*, and *Mif* (inflammatory marker genes). mRNA expression levels were normalized using *Rplp0* gene as an internal control, as described in 'Materials and methods'. Different letters (a, b, c, and d) indicate statistical significance of p < 0.05. Groups sharing a common letter are not statistically significant.

Fig. 4.

Effects of ethanol on the systemic concentrations of β OHB and glucose in type 2 diabetic *db/db* mice *versus* nondiabetic *db/m* mice. β OHB (**A**) and glucose (**B**) concentrations. # $p < 0.05$ compared with *db/db* on control LD and * $p < 0.05$ compared with *db/m* on control LD; $n = 7$ mice/group on days 1, 3, 7 and 9; $n = 4$ mice/group on day 5.

Fig. 5.

Effects of ethanol on the changes in blood glucose and β OHB concentrations after intraperitoneal injection of glucose in type 2 diabetic *db/db* mice versus nondiabetic *db/m* mice. The changes in blood glucose (**A**), AUC (**B**), and β OHB (**C**) are shown. The data shown are the means $\pm$ SEM, $n = 4$. # $p < 0.05$ compared with *db/db* on control LD and * $p < 0.05$ compared with *db/m* on control LD. Different letters (a, b, and c) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant (**B**).

Fig. 6. Effects of ethanol on plasma insulin concentration and HOMA-IR in type 2 diabetic *db/db* mice *versus* nondiabetic *db/m* mice. Plasma insulin concentration (**A**) and HOMA-IR (**B**). Different letters (a, b, and c) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant.

Fig. 7. Effects of ethanol on hepatic β OHB content and *Hmgcs2* mRNA expression in type 2 diabetic *db/db* mice versus nondiabetic *db/m* mice. Hepatic β OHB content (**A**) and *Hmgcs2* mRNA expression (**B**). Different letters (a, b, and c) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant. *Hmgcs2*, 3-hydroxy-3-methylglutaryl-CoA synthase 2.

Fig. 8.

Effects of ethanol on the expression levels of hepatic gluconeogenic mRNAs in type 2 diabetic *db/db* mice versus nondiabetic *db/m* mice. *Pck1* (A), *G6pc* (B), and *Pcx* (C). As controls, *Srebp1f* mRNA expression level (D) was determined in parallel. Different letters (a, b, c, and d) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant. *Pcx*, pyruvate carboxylase; *Pck 1*, phosphoenolpyruvate carboxykinase (PEPCK); *G6pc*, glucose-6-phosphatase; *Srebp1f*, Sterol regulating element binding protein 1c; *Rplp0*, ribosomal protein large P0.

Fig. 9.

Effects of ethanol on hepatic glycogen content and GS phosphorylation/expression/ activity in type 2 diabetic *db/db* mice *versus* nondiabetic *db/m* mice. **(A)** Glycogen content in the liver. **(B)** The changes in the mRNA expression levels of hepatic *GYS2*. **(C)** The changes in the phosphorylation and expression of GS. β -actin was used as a loading control. **(D)** The changes in glycogen synthase activity were determined in the absence or presence of glucose-6-phosphate (G-6-P), an allosteric activator. Different letters (a, b, c, and d) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant.

Fig. 10. Effects of ethanol on hepatic GSK-3β and Akt phosphorylation/expression in type 2 diabetic *db/db* mice versus nondiabetic *db/m* mice. (**A-B**) The changes in the phosphorylation and expression of GSK-3β (**A**) and Akt (**B**). Different letters (a, b, c, and d) indicate statistical significance of $p < 0.05$. Groups sharing a common letter are not statistically significant.

Table 1A.Effects of ethanol on body weight (g) in diabetic *versus* nondiabetic mice

	<i>db/m</i> mice			<i>db/db</i> mice		
	Control LD	EtOH LD	p Value	Control LD	EtOH LD	p Value
Day 1	23.02 ± 0.40	23.8 ± 0.25	0.38	33.8 ± 0.25	36.5 ± 0.57	0.078
Day 3	23.3 ± 0.48	22.2 ± 0.32	0.29	36.7 ± 0.23	35.5 ± 0.64	0.16
Day 5	24.1 ± 0.70	21.2 ± 0.22	0.007 [*]	39.5 ± 0.45	35.4 ± 0.62	0.016 [#]
Day 7	24.7 ± 0.75	20.7 ± 0.48	0.003 [*]	41.5 ± 0.64	35.1 ± 0.40	0.007 [#]
Day 9	26.0 ± 0.70	19.8 ± 0.48	0.0012 [*]	43.5 ± 0.62	34.5 ± 0.40	0.0023 [#]

Table 1B.Effects of ethanol on caloric intake (kcal/mouse/day) in diabetic *versus* nondiabetic mice

	<i>db/m</i> mice			<i>db/db</i> mice		
	Control LD	EtOH LD	p value	Control LD	EtOH LD	p value
Day 1	12.5 ± 0.12	7.5 ± 0.12	0.0074 [*]	20.0 ± 0.12	15.0 ± 0.11	0.008 [#]
Day 3	12.5 ± 0.11	7.5 ± 0.10	0.0076 [*]	20.0 ± 0.11	15.0 ± 0.12	0.0078 [#]
Day 5	15.0 ± 0.12	7.5 ± 0.11	0.0052 [*]	22.5 ± 0.13	15.0 ± 0.10	0.005 [#]
Day 7	15.0 ± 0.11	10.0 ± 0.12	0.0068 [*]	25.0 ± 0.11	17.5 ± 0.11	0.0012 [#]
Day 9	15.0 ± 0.10	10.0 ± 0.11	0.0062 [*]	25.0 ± 0.12	17.5 ± 0.14	0.001 [#]

After acclimatization, mice were fed 5% (w/v) EtOH LD or control LD for 9 days, as described in Fig. 1. The data shown are the means ± SEM; n = 7 mice/group on days 1, 3, 7 and 9; n = 4 mice/group on day 5.

^{*} p < 0.05 compared with control LD-fed mice in the respective group, using repeated measures two-way ANOVA followed by Bonferroni multiple comparisons test.

[#] p < 0.05 compared with control LD-fed mice in the respective group, using repeated measures two-way ANOVA followed by Bonferroni multiple comparisons test.

Table 2.

Primer sequences used for qPCR analysis of hepatic genes.

Gene	Forward Primer (5'–3')	Reverse Primer (5'–3')	Product Size, bp
<i>Cd36</i>	CCAGTCGGAGACATGCTTATT	GTACACAGTGGTGCCTGTT	105
<i>Fatp1</i>	GTCATGAAGGATGTGGACAGAG	GTCGAGGCTCAGCACATTTA	105
<i>Dgat2</i>	GCTGACCACCAGGAACATATATC	GCCTCTGTGCTGAAGTTACA	84
<i>Atgl</i>	TAGCTAACAGTTGGGCTTCAC	CAGAGAGAACAGAGCAGCTTAC	82
<i>Hig-2</i>	TGAAGACAGAGGGTGGACTAT	CTGATCATCCCATAGCCCTTTAG	115
<i>Cyp2e1</i>	CTGAGATATGGGCTCCTGATTC	GCCCAATAACCCTGTCAATTTTC	82
<i>Tnfa</i>	CTGAGTTCTGCAAAGGGAGAG	CCTCAGGGAAGAATCTGGAAAG	107
<i>Il-1β</i>	GGTGTGTGACGTTCCCATTA	ATTGAGGTGGAGAGCTTTCAG	106
<i>Mcp-1</i>	GTTCTGACTCCTCTAGCTTTC	GCATCTGGTCCAGCCAATA	122
<i>Mip-2</i>	AGAGGGTGAGTTGGGAACATA	GCCATCCGACTGCATCTATT	97
<i>Mif</i>	GTTCCACCTTCGCTTGAGT	CATCGCTACCGGTGGATAAA	98
<i>Hmgcs2</i>	CCTGTGAAGAGGGAGATGAAAG	GCCCAGAGTCTGAGAATAAGC	113
<i>Pcx</i>	CTCCGACGTGTATGAGAATGAG	CATCTGGTTAGCCTCCACATAG	127
<i>Pck1</i>	GACAGTCATCATCCCAAGAG	CTCAAAGTCCTCTTCCGACATC	97
<i>G6pc</i>	GACTCGCTATCTCCAAGTGAAT	CCAGATGGGAAAGAGGACATAG	106
<i>Gys2</i>	CCAGTGTGACTACAAACCTCTC	GCGTCTGTCCACGATGTAAATA	95
<i>Srebp1</i>	CATCGACTACATCCGCTTCTT	CACCAGGTCCTTCAGTGATTT	103
<i>Rplp0</i>	CGTTGGAGTGACATCGTCTTTA	GGATGATCTTGAGGAAGTAGTTGG	111

Cd36, fatty acid translocase; *Fatp1*, fatty acid transport protein 1; *Dgat2*, diacylglycerol o-acyltransferase 2; *Atgl*, adipose triglyceride lipase; *Hig-2*, hypoxia-inducible protein- 2; *Cyp2e1*, cytochrome P450 2E1; *Tnfa*, tumor necrosis factor-alpha; *Il-1 β* , interleukin 1 beta; *Mcp-1*, monocyte chemoattractant protein-1; *Mip-2*, macrophage inflammatory protein-2; *Mif*, macrophage migration inhibitory factor; *Hmgcs2*, 3-hydroxy-3-methylglutaryl-CoA synthase 2; *Pcx*, pyruvate carboxylase; *Pck 1*, phosphoenolpyruvate carboxykinase (PEPCK); *G6pc*, glucose-6-phosphatase; *Srebp1*, Sterol regulating element binding protein 1c; *Gys2*, Glycogen synthase 2; *Rplp0*, ribosomal protein large P0; bp: base pair.

Table 3.

Effects of ethanol on liver weight and epididymal white adipose tissue (eWAT) weight in diabetic *versus* nondiabetic mice.

	<i>db/m</i> (n = 4)		<i>db/db</i> (n = 4)	
	Control LD	EtOH LD	Control LD	EtOH LD
Liver weight (g)	1.22 ± 0.36	0.87 ± 0.06 *	2.57 ± 0.02	1.67 ± 0.05 #
Liver weight / Body weight ratio	0.05 ± 0.001	0.036 ± 0.001 *	0.070 ± 0.004	0.048 ± 0.001 #
eWAT weight (g)	0.92 ± 0.04	0.61 ± 0.04 *	2.72 ± 0.02	2.31 ± 0.14 #
eWAT weight /Body weight ratio	0.035 ± 0.007	0.031 ± 0.002	0.066 ± 0.004	0.05 ± 0.004 #

The data are expressed as means ± SEM.

p < 0.05 compared with *db/db* mice (control LD) and

* p < 0.05 compared with *db/m* mice (control LD), using regular two-way ANOVA followed by Tukey multiple comparisons test.