Chronic Ethanol and Triglyceride Turnover in White Adipose Tissue in Rats

INHIBITION OF THE ANTI-LIPOLYTIC ACTION OF INSULIN AFTER CHRONIC ETHANOL CONTRIBUTES TO INCREASED TRIGLYCERIDE DEGRADATION

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Chronic ethanol consumption disrupts whole-body lipid metabolism. Here we tested the hypothesis that regulation of triglyceride homeostasis in adipose tissue is vulnerable to long-term ethanol exposure. After chronic ethanol feeding, total body fat content as well as the quantity of epididymal adipose tissue of male Wistar rats was decreased compared with pair-fed controls. Integrated rates of in vivo triglyceride turnover in epididymal adipose tissue were measured using 2H2O as a tracer. Triglyceride turnover in adipose tissue was increased due to a 2.3-fold increase in triglyceride degradation in ethanol-fed rats compared with pair-fed controls with no effect of ethanol on triglyceride synthesis. Because increased lipolysis accompanied by the release of free fatty acids into the circulation is associated with insulin resistance and liver injury, we focused on determining the mechanisms for increased lipolysis in adipose tissue after chronic ethanol feeding. Chronic ethanol feeding suppressed β-adrenergic receptor-stimulated lipolysis in both in vivo and ex vivo assays; thus, enhanced triglyceride degradation during ethanol feeding was not due to increased β-adrenergic-mediated lipolysis. Instead, chronic ethanol feeding markedly impaired insulin-mediated suppression of lipolysis in conscious rats during a hyperinsulinemic-euglycemic clamp as well as in adipocytes isolated from epididymal and subcutaneous adipose tissue. These data demonstrate for the first time that chronic ethanol feeding increased the rate of triglyceride degradation in adipose tissue. Furthermore, this enhanced rate of lipolysis was due to a suppression of the anti-lipolytic effects of insulin in adipocytes after chronic ethanol feeding.

These pathophysiological effects of ethanol can be modeled in rodents fed diets containing ethanol; chronic ethanol feeding to rats induces hepatic steatosis coupled with the development of hyperlipidemia, characterized by elevated plasma cholesterol and triglyceride concentrations (2). These data suggest that the disruption of lipid homeostasis by ethanol is likely a mediator of alcohol-related disease progression. However, the effects of chronic ethanol feeding on lipid metabolism in adipose tissue, the biggest storage pool of lipids, are unknown.

Adipose tissue is a specialized connective tissue that functions as the major storage site for fat in the form of triglycerides. Serving as an energy reserve, adipose tissue synthesizes triglycerides when energy intake exceeds energy output. During fasting or in response to infection and inflammation, adipose tissue mobilizes free fatty acids and glycerol, providing other tissues with metabolites and energy substrates (3). Mobilization of fatty acids and glycerol from triglycerides in adipose tissue, also termed lipolysis, is tightly regulated by a number of hormones. The primary hormones regulating lipolysis are catecholamines, which initiate lipolysis by the stimulation of β-adrenergic receptors, and insulin, which inhibits catecholamine-induced lipolysis (4). Chronic ethanol exposure disrupts both G protein- and insulin-dependent signal transduction in a variety of cell types, including adipocytes (5–8). For example, we have demonstrated that ethanol feeding for 4 weeks decreases β-adrenergic receptor-stimulated lipolysis (9) and suppresses insulin-stimulated glucose uptake in isolated adipocytes (10, 11). However, the effect of chronic ethanol feeding on the anti-lipolytic response of adipocytes to insulin has not been examined.

In this study we investigated the effects of chronic ethanol feeding over a 2-week period on the integrated rates of in vivo triglyceride turnover in epididymal adipose tissue by the use of 2H2O (12). After the administration of 2H2O, 2H in body water equilibrates with the carbon-bound hydrogens of glycerol 3-phosphate, and the rates of triglyceride synthesis and degradation are determined by measuring the incorporation/washout of 2H to/from carbon 1 of triglyceride-bound glycerol (13). The rate of triglyceride degradation during 2 weeks of ethanol feeding was increased by 2.3-fold. Because increased rates of lipolysis are associated with the development of insulin resistance and fatty liver in other model systems, we therefore investigated the mechanisms by which chronic ethanol increased triglyceride degradation. We find that the ethanol-
induced increase in lipolysis in adipose was due to a loss of the anti-lipolytic actions of insulin rather than an increase in stimulation of lipolysis by β-adrenergic receptor activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Wistar rats (150–160g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The Lieber-DeCarli high fat ethanol diet was purchased from Dyets (Bethlehem, PA). Maltose dextrins were obtained from BioServ (Frenchtown, NJ). The ethanol-L3K assay kit was purchased from Diagnostic Chemicals Ltd (Oxford, CT). 2H2O (99.9 atom percent excess) and [3H]glycerol (98 atom percent excess) were purchased from Isotec (Miamisburg, OH), ion-exchange resins were from Bio-Rad, gycerokinase was from Roche Applied Science, bis(trimethylsilyl)trifluoroacetamide with 10% trimethylchlorosilane was from Regis Technologies Inc. (Morton Grove, IL), and gas chromatography-mass spectrometry (GC-MS) supplies were from Agilent Technologies (Wilmington, DE). NEFA C kit for the measurement of plasma free fatty acid concentration was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Cilostamide, a phosphodiesterase 3B (PDE3B)-selective inhibitor, was from BIOMOL (Plymouth Meeting, PA). [3H]cAMP was from Amersham Biosciences. Antibodies were from the following sources: anti-extracellular signal-regulated kinase, Upstate, Charlottesville, VA; anti-PDE3B, Santa Cruz Biotechnology, Santa Cruz, CA. A blood glucose meter and blood glucose test strips were from CVS (Woonsocket, RI), human insulin was from Eli Lilly (Indianapolis, IN), rat insulin enzyme-linked immunosorbant assay reagents were from Merckodia Inc. (Winston Salem, NC), and all other reagents were from Sigma.

**Animal Protocol for Chronic Ethanol Feeding**—Rats were allowed free access to the Lieber-DeCarli liquid diet containing ethanol as 35% of total calories or pair-fed an isocaloric diet which substituted maltose dextrins for ethanol for 4 weeks as previously described (9). Rats were housed in individual wire-bottom cages under controlled temperature and humidity with 12 h light-12 h dark (7:00 p.m.-7:00 a.m.) cycle. In studies to determine 2H2O-based triglyceride turnover rate, rats were given an intraperitoneal injection of 2H2O-saline (0.9 g of NaCl in 1000 ml of 99.9% 2H2O, 16.25 μl/g body weight) after 2 weeks of feeding. 2H2O was then included in the diets (enriched to 5% of 2H) for 5 days; after that, 2H2O was switched to tap water. A total of 30 rats were euthanized after 48 h, 2 H rats per group on days 0, and 2 rats per group on days 3, 5, 7, 9, 11, and 14.

At the end of the feeding protocol, rats were anesthetized by intraperitoneal injection of 0.075 ml for ethanol-fed rats or 0.12 ml for pair-fed rats per 100 g of body weight of a mixture containing 10 mg/ml acemprazine, 100 mg/ml ketamine, and 20 mg/ml xylazine. The lower dose of anesthetic for ethanol-fed rats was used because of an increased sensitivity to the anesthetic mixture after ethanol feeding. However, it is unlikely that the different doses had any impact in our assay, as ketamine and xylazine at these doses either have no effects or equivalent effects on lipogenic and lipolytic activities of adipose tissue (14, 15). Under anesthesia, blood was collected, and epididymal adipose tissue was frozen in liquid nitrogen and stored at −80 °C. Plasma samples were prepared by centrifugation at 16,100 × g for 2 min, and plasma ethanol concentration was measured immediately by the ethanol-L3K kit. The rats used in these studies were not fasted; all studies were carried out at 10:30 a.m., except the hyperinsulinemic-euglycemic clamps, which were performed at 12:00 noon (as time 0 of the clamps). Procedures involving animals were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University or the Cleveland Clinic.

**Body Composition Analysis**—Percent body fat was determined in ethanol- and pair-fed rats by magnetic resonance imaging at the Small Animal Imaging Core in the Case Western Reserve University Cancer Center. Rats were anesthetized with isoflurane and placed in an eight-channel human head coil on a Bruker/Siemens Medspec 4T magnetic resonance imaging scanner. Coronal, proton density weighted, spin echo images (TR/TE = 5910 ms/7 ms, resolution = 860 μm × 860 μm × 2 mm, matrix = 128 × 256) were obtained for each animal. Twenty images per rat were obtained with and without water suppression to enable the lipid calculations. Data were analyzed using the Amira image processing and visualization software (Mercury Computer Systems, Inc.) to determine total body fat volume to total body volume ratios.

**The 2H-Labeling of Body Water**—The 2H-labeling of body water was assayed by exchange with acetone as described by Yang et al. (16) and as modified previously (12, 13). Briefly, known 2H atom percent excess standards were prepared by mixing naturally labeled water and 99.9% 2H2O. Assays were performed using 40 μl of plasma or standard, 2 μl of 10 N NaOH, and 4 μl of a 5% (v/v) solution of acetone in acetonitrile. After overnight incubation, the solution was extracted with 600 μl of chloroform and dried with Na2SO4. The 2H-labeling of acetone was then determined by GC-MS. Ions of mass-to-charge ratios (m/z) 58–60 were monitored.

**The 2H-Labeling of Triglyceride-bound Glycerol**—Frozen epididymal adipose tissue was hydrolyzed with 1 N KOH in 90% ethanol at 70 °C for 2 h. After evaporation of ethanol, free glycerol was recovered as previously described (12, 13). H2O (3 ml) was added, and the solution was acidified to ~pH 1. After extraction of fatty acids by diethyl ether (3× with 4 ml), the aqueous solution was neutralized to ~pH 7, and free glycerol in the aqueous solution was reacted with 0.2 ml ATP and 5 units of gycerokinase at 37 °C for 2.5 h. The formed glycerol 3-phosphate was then purified by passing the solution over an AG 1-X8 resin (formate form), washing the column with water, and eluting the column with 4 N NaOH and 4 μl of a 5% (v/v) solution of acetone in acetonitrile. After overnight incubation, the solution was extracted with 600 μl of chloroform and dried with Na2SO4. The 2H-labeling of acetone was then determined by GC-MS. Ions of mass-to-charge ratios (m/z) 445–446 and 357–358 were monitored.

**Mathematical Modeling**—The rates of triglyceride synthesis and degradation were determined by mathematically modeling the change in adipose mass (Fig. 1A). 2H-labeling of body water

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2 The abbreviations used are: GC-MS, gas chromatography-mass spectrometry; PDE, phosphodiesterase; Ra, the rate of appearance.
Then the incorporation of $^2\text{H}$ from body water into lipids was modeled using a single-compartment model assuming that the $^2\text{H}$-labeling of plasma water reflects the $^2\text{H}$-labeling of water in adipose tissue. The parameters of interest, the rates of triglyceride synthesis and degradation, were then estimated from the data by using nonlinear least-squares fitting. In previous reports, triglyceride turnover was modeled by first using the change in triglyceride-glycerol mass (12) rather than adipose mass.

$\beta_3$-Adrenergic Receptor-stimulated in Vivo Lipolysis—After four weeks of pair- or ethanol-feeding, rats were anesthetized by inhalation of an isoflurane and oxygen mixture. Under anesthesia, rats were given an intraperitoneal injection of the $\beta_3$-adrenergic receptor agonist, CL316,243, at a dose of 0.1 mg/kg body weight. Blood samples were collected before and at 8, 16, 30, and 60 min after the injection from the tail vein. Plasma was separated, and plasma glycerol and free fatty acid concentrations were then determined as indices of lipolysis.

Isolation of Adipocytes and ex Vivo Lipolysis Assay—After 4 weeks of feeding, rats were anesthetized by an intraperitoneal injection of a mixture as described above, and epididymal and/or subcutaneous adipose tissue was removed. Ex vivo lipolysis was measured as glycerol released into the cell medium over 1 h as described before (9). Briefly, adipocytes were isolated by collagenase digestion (9), and cell concentration was adjusted to $1 \times 10^6$ cells/ml. 200-μl aliquots of cells were placed into 5-ml polypropylene tubes, and 1 μM isoproterenol, a $\beta$-adrenergic receptor agonist, and/or increasing concentrations of insulin were added. Adipocytes were incubated for 1 h at 37°C in a shaking water bath (100 rpm), and glycerol concentration in the cell medium was measured using free glycerol reagent (GPO Trinder reagent).

PDE3B Expression and Activity—Total RNA was isolated from fat pads by using the RNAeasy lipid tissue mini kit (Qiagen, Valencia, CA), and DNA digestion was performed by using RNase-free DNase I set (Qiagen) according to the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed by using the RETROscript kit (Ambion, Austin, TX) with random primers according to manufacturer’s protocol. Real-time PCR were performed by using the SYBR Green Core reagents (Applied Biosystems, Warrington, UK) and sets of primers specific for PDE3B (forward, 5′-AGT GCC AAG ATG TTC AGG AG-3′; reverse, 5′-AGT CCC AGA GAA TC-3′) and $\beta$-actin (forward, 5′-CGG TCA GGT CAT CAC TAT CG-3′; reverse, 5′-TTC CAT ACC CAG GAA AG-3′). Activity of PDE3B and Western blot analysis of PDE3B protein in lysates of adipocytes was carried out as previously described (9).

Hyperinsulinemic-Euglycemic Clamp—After 3 weeks of pair- or ethanol-feeding, rats were anesthetized by inhalation of an isoflurane and oxygen mixture, and the left carotid artery and the right jugular vein were catheterized for blood sampling and intravenous infusion during the clamp, respectively. All rat surgeries were done in the Mouse Metabolic and Phenotyping Center at Case Western Reserve University. Rats were allowed a week to recover from the surgery while maintained on their respective diets. Hyperinsulinemic-euglycemic clamps were then performed on one rat at a time as previously described (17), with minor modifications. Data on the effects of chronic ethanol on glucose disposal during these specific clamp studies have been previously reported (18). Briefly, rats were transported from the Animal Resource Center and allowed at least 90 min to stabilize before commencement of the glucose clamp. $[^{2}\text{H}_5]$Glycerol ($-1$ μmol/kg/min) was continuously infused from the jugular vein catheter for a 90-min basal period and 2-h clamp period. Base-line levels of blood glucose, plasma insulin, plasma glycerol, and plasma free fatty acid, and the plasma appearance rate of glycerol (Ra) were determined as the means of values obtained in blood samples collected at $-30$ and $-5$ min. At time 0, a primed (60 milliunits/kg)/continuous (4 milliunits/kg/min) infusion of human insulin was started and continued for 2 h. The blood glucose concentration was clamped at a euglycemic level by a variable rate infusion of 20% glucose. Blood glucose was monitored with a blood glucose meter, and the rate of glucose infusion was adjusted every 10 min. Blood samples for determination of plasma insulin, plasma glycerol concentration, plasma free fatty acid concentration, and plasma $[^{2}\text{H}_2]$glycerol enrichments were obtained at $-30$, $-5$, 90, 100, 110, and 120 min. There were no differences in the hematocrit between pair- and ethanol-fed rats either before or after the clamp (pair-fed, from 35 ± 2% to 30 ± 2%; ethanol-fed, from 35 ± 5% to 26 ± 3%).

Appearance Rate of Glycerol in Plasma (Ra)—The plasma glycerol Ra was used as an index for systemic lipolysis, as calculated by the equation $Ra = \frac{(ENR_{inf}/ENR_{pl}) - 1}{F}$, where $ENR_{inf}$ is the isotopic enrichment of the infusate, $ENR_{pl}$ is the isotopic enrichment of plasma, and $F$ is the rate of the isotope infusion (19). The $^2\text{H}$-labeling of plasma glycerol was determined as described below. 20 μl of plasma was deproteinized with 200 μl of methanol by centrifugation for 10 min at 16,100 × g. The fluid fraction was then evaporated to dryness and reacted with 50 μl of bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane for 20 min at 75°C. Isotope enrichment was determined by GC-MS. Ions of mass-to-charge ratios ($m/z$) 205–208 were monitored.

Statistical Analyses—For experiments to determine the rate of triglyceride turnover using $^2\text{H}_2\text{O}$, individual data points are shown (Fig. 1). The general linear model procedure on SAS for personal computers was used to compare the change in wet weight of epididymal adipose tissue by linear regression between pair- and ethanol-fed rats (Fig. 1A). For all other experiments data are expressed as the means ± S.E. Dose-response curves to insulin in isolated adipocytes were estimated by non-linear regression (GraphPad Prism® 4; San Diego, CA). Statistical analyses were performed using the general linear model procedure followed by least square means tests using SAS for personal computers.

RESULTS

Characteristics of Ethanol-fed Rats—To study the effects of chronic ethanol feeding on lipid metabolism in adipose tissue, rats were fed a liquid diet with or without ethanol for 4 weeks. Rats gained weight over time on both pair- and ethanol-liquid
Chronic Ethanol Increases Triglyceride Turnover

TABLE 1
Rat body weights, plasma ethanol concentrations, and epididymal fat weights

Data are expressed as mean ± S.E. for body weight data (n = 13), plasma ethanol (n = 4–5), and epididymal fat weight data (n = 7 for week 2, n = 6 for week 3, and n = 4 for week 4). ND, not detectable.

<table>
<thead>
<tr>
<th>Weeks on diet</th>
<th>Body weight (g)</th>
<th>Plasma ethanol (mM)</th>
<th>Epididymal fat weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pair-fed</td>
<td>EtOH-fed</td>
<td>Pair-fed</td>
</tr>
<tr>
<td>0</td>
<td>168 ± 8</td>
<td>186 ± 6</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>234 ± 5</td>
<td>237 ± 6</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>259 ± 7</td>
<td>255 ± 6</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>272 ± 5</td>
<td>280 ± 6</td>
<td>4.8 ± 0.4</td>
</tr>
</tbody>
</table>

$^a p < 0.05$ pair-fed versus EtOH-fed.

![Image](http://www.jbc.org/)

**FIGURE 1.** 2H-Labeling of body water and triglyceride-bound glycerol isolated from epididymal adipose tissue in rats. A, the changes in epididymal adipose tissue wet weight over time were analyzed by linear regression. Pair-fed (n = 16 points); y = 0.172x + 2.69, $r^2 = 0.73$; EtOH-fed (n = 17 points); y = 0.081x + 2.10, $r^2 = 0.60$. Each data point represents a single animal. The slopes and y intercepts were different between pair- and ethanol-fed rats, $p < 0.05$. B, 2H-labeling of body water was assayed by the method of hydrogen exchange with acetone in plasma. Isotope enrichment was determined by GC-MS. Ions of mass-to-charge ratios (m/z) 58–60 were monitored. Each data point represents a single animal (n = 15 per group). C, the labeling of 2H bound to carbon 1 of triglyceride-glycerol in epididymal adipose tissue was determined as described under “Experimental Procedures,” and non-linear regression analysis was used to generate a best-fit curve describing triglyceride turnover in epididymal adipose tissue. Each data point represents a single animal (n = 15 per group).

**TABLE 2**
Rates of triglyceride synthesis and degradation

The rates of triglyceride synthesis and degradation were determined by mathematically modeling the change in adipose mass (Fig. 1A) and 2H-labeling of body water (Fig. 1B) and triglyceride-bound glycerol (Fig. 1C) using a procedure that was modified from previous reports (12, 13) as described under “Experimental Procedures.” The upper and lower limits of the 99% confidence interval are shown.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Triglyceride synthesis (g/day) (99% confidence limits)</th>
<th>Triglyceride degradation (g/day) (99% confidence limits)</th>
<th>Net triglyceride kinetics (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair-fed</td>
<td>0.293 ± 0.050 (0.142 ± 0.050)</td>
<td>0.142 ± 0.050 (0.268 ± 0.016)</td>
<td>0.151</td>
</tr>
<tr>
<td>EtOH-fed</td>
<td>0.404 ± 0.071 (0.357 ± 0.071)</td>
<td>0.322 ± 0.071 (0.505 ± 0.139)</td>
<td>0.082</td>
</tr>
</tbody>
</table>

diets, and there were no differences in body weights between pair- and ethanol-fed rats after feeding for 2–4 weeks (Table 1). Plasma ethanol concentration was 15–17 mM in ethanol-fed rats and not detectable in pair-fed rats (Table 1). The weight of epididymal adipose tissue was lower in rats after ethanol feeding for 2, 3, and 4 weeks compared with pair feeding (Table 1). The ratio of total fat volume to total body volume, assessed by magnetic resonance imaging analysis, was 35% lower after chronic ethanol feeding for 4 weeks compared with pair-fed rats. The ratio of total fat volume/total body volume was 0.11 ± 0.01 (n = 5) in ethanol-fed rats compared with 0.17 ± 0.02 (n = 6, $p < 0.05$) in pair-fed rats, indicating that total body lipid content was decreased by chronic ethanol feeding.

**Triglyceride Turnover Rates**—Rates of in vivo triglyceride synthesis and degradation in epididymal adipose tissue were determined during the last two weeks of ethanol feeding using $^2$H$_2$O as described under “Experimental Procedures” (12, 13). Epididymal fat weight increased over time in both pair- and ethanol-fed rats; the rate of increase was lower during chronic ethanol feeding (Fig. 1A). Fig. 1B shows the 2H-labeling curves of body water in pair- and ethanol-fed rats. The initial intraperitoneal injection of $^2$H$_2$O enriched body water to an ~2.1 mol % excess in both pair- and ethanol-fed rats. Five-day maintenance on $^2$H$_2$O in the diets increased the labeling to 3.4 mol % excess. When rats were switched from $^2$H$_2$O to tap water, 2H was washed out of body water. The calculated half-life, $t_{1/2}$, of body water did not differ between pair-fed (2.1 days) and ethanol-fed (2.2 days) rats (Fig. 1B).

To determine the kinetics of triglyceride turnover, the labeling of $^2$H bound to C1 of triglyceride-glycerol was measured in epididymal adipose tissue from both pair- and ethanol-fed rats. Although rats were maintained on $^2$H$_2$O, the labeling of triglyceride-glycerol increased in both groups; after switching to tap water, the labeling of triglyceride-glycerol was maintained (Fig. 1C). A mathematical model was then generated to fit the data for the effects of ethanol feeding on epididymal adipose tissue mass (Fig. 1A), 2H-labeling of body water (Fig. 1B), and the 2H-labeling of triglyceride-glycerol (Fig. 1C). These models were then used to calculate the rates of triglyceride turnover (Table 2). Ethanol feeding increased the rate of triglyceride synthesis by 1.4-fold compared with pair-fed, but this increase was not different at the 99% confidence level. In contrast, ethanol feeding increased triglyceride degradation by 2.3-fold compared with pair-fed controls, which was different at the 99% confidence level (Table 2). The net accumulation rates of triglycerides in epididymal adipose tissue was lower during ethanol feeding, at 0.082 g/day in ethanol-fed rats compared with 0.151
g/day in pair-fed (Table 2). Taken together, these data suggest that ethanol feeding for 2–4 weeks stimulated the degradation of triglycerides in epididymal adipose tissue.

$\beta_3$-Adrenergic Agonist-stimulated in Vivo Lipolysis—Hydrolysis of triglycerides mobilizes free fatty acids into the circulation. Because increased concentrations of free fatty acids can contribute to the development of a number of pathophysiological conditions associated with chronic alcohol consumption, including insulin resistance, diabetes, and hepatic steatosis (20), we next investigated the mechanisms by which chronic ethanol increased the rate of triglyceride degradation. Hydrolysis of triglycerides in adipocytes is primarily regulated by the activity of the sympathetic nervous system and by plasma insulin levels (4). Catecholamines stimulate lipolysis by activating $\beta$-adrenergic receptors, whereas insulin acts as a counter-regulator to inhibit lipolysis through the insulin receptor (4).

Because chronic ethanol increased the rate of triglyceride degradation in epididymal adipose tissue, we hypothesized that ethanol activated the lipolytic action of $\beta$-adrenergic receptors and/or suppressed the anti-lipolytic action of insulin. However, we have recently reported that chronic ethanol feeding actually decreases $\beta$-adrenergic receptor-stimulated lipolysis in isolated adipocytes (9). To validate these ex vivo experiments in the intact animals, we measured in vivo lipolysis stimulated by CL316,243, an agonist specific for $\beta_3$-adrenergic receptors, the primary isoform of $\beta$-adrenergic receptors expressed in adipose tissue (21). Base-line concentrations of plasma glycerol and free fatty acids were not different between pair- and ethanol-fed rats (Fig. 2). Intraperitoneal injection of CL316,243 increased plasma glycerol and free fatty acid concentration in both pair- and ethanol-fed rats. However, the $\beta_3$-agonist-mediated elevation of plasma glycerol and free fatty acid concentration was suppressed in ethanol-fed rats compared with pair-fed rats (Fig. 2). Thus, these data, consistent with previous data in isolated adipocytes (9), demonstrated that chronic ethanol feeding to rats decreases $\beta$-adrenergic receptor-stimulated lipolysis and suggested that the increase in the rate of triglyceride degradation observed in vivo in chronically ethanol-fed rats was not mediated by an increased lipolytic response of adipocytes to $\beta$-adrenergic activation.

Anti-lipolytic Action of Insulin in Vivo—In contrast to the role of $\beta$-adrenergic agonist in the activation of lipolysis, insulin is the key inhibitor of lipolysis. Because insulin signaling pathways are also targets of chronic ethanol, we next examined the effect of chronic ethanol on insulin-mediated inhibition of lipolysis making use of the hyperinsulinemic-euglycemic clamp technique (19). Plasma insulin levels were maintained at a steady state in both pair- and ethanol-fed rats during 90–120 min of the clamp. Glucose infusion rate during this time period was also constant (data not shown). Therefore, the last 30 min of the clamp (90–120 min) was assumed to be at steady state, and the means of the 90-, 100-, 110-, and 120-min values were used for steady state values (19).

Body weights of rats used in the hyperinsulinemic-euglycemic clamps did not differ between pair- and ethanol-fed rats (Table 3). There were no differences between pair- and ethanol-fed rats in basal blood glucose, mean blood glucose at 90–120 min of the glucose clamp, and mean plasma insulin levels at 90–120 min achieved during insulin infusion (Table 3). However, basal plasma insulin was lower in rats after a 4-week ethanol feeding compared with pair feeding (Table 3).
To test the sensitivity of systemic lipolysis to insulin, we first measured plasma glycerol and free fatty acid concentrations during 90–120 min of the clamps. Basal plasma glycerol and free fatty acid concentrations were not different between pair- and ethanol-fed rats (Table 3). In response to insulin, plasma glycerol concentration was decreased by 56% from basal level in pair-fed rats compared with only 20% in ethanol-fed rats at the steady state (Fig. 3, A and C). Plasma free fatty acid concentrations were decreased by 64% in pair-fed rats compared with 36% in ethanol-fed rats at the steady state (Fig. 3, B and D). Plasma glycerol Ra was then determined as an index for systemic lipolysis (19). Basal glycerol Ra did not differ between pair- and ethanol-fed rats; however, at the steady state of the clamp, plasma glycerol Ra was decreased by 28% in pair-fed rats with no change in ethanol-fed rats (Fig. 4). These data suggest that chronic ethanol feeding impaired the ability of insulin to inhibit systemic lipolysis, which may contribute to the increased rate of triglyceride degradation observed in vivo after ethanol exposure.

Anti-lipolytic Action of Insulin ex Vivo—To further investigate the mechanisms for impaired insulin-mediated suppression of lipolysis, we examined the effects of chronic ethanol feeding on hormone-regulated lipolysis in an ex vivo model of isolated adipocytes. Consistent with our previous study (9), ethanol feeding for 4 weeks decreased isoproterenol-stimulated lipolysis in isolated epididymal and subcutaneous adipocytes without changing basal rates of lipolysis (Fig. 5). Insulin at concentrations from 0.1 to 100 nM dose-dependently decreased isoproterenol-stimulated lipolysis in epididymal (Fig. 5A) and subcutaneous (Fig. 5B) adipocytes isolated from pair-fed rats. In contrast, at these physiological concentrations, insulin did not inhibit lipolysis in adipocytes isolated from ethanol-fed rats (Fig. 5). Maximal inhibition of isoproterenol-stimulated lipolysis was observed with 10 μM insulin and did not differ between adipocytes isolated from epididymal (Fig. 5A) or subcutaneous (Fig. 5B) adipose depots from either pair- and ethanol-fed rats.

FIGURE 3. Plasma glycerol and free fatty acid concentrations during the hyperinsulinemic-euglycemic clamp. Hyperinsulinemic-euglycemic clamps were performed as described under “Experimental Procedures.” A and C, plasma glycerol and free fatty acid (FFA) concentrations during the hyperinsulinemic-euglycemic clamp were normalized to basal concentrations of plasma glycerol and free fatty acids within each group. B and D, mean plasma glycerol and free fatty acid (FFA) concentrations were shown as the means of the 90, 100, 110, and 120 min values as the steady state values. Data represent the mean ± S.E. (n = 7 for pair-fed group and 6 for ethanol-fed group). p < 0.05 (*), pair-fed versus EtOH-fed.

FIGURE 4. Chronic ethanol feeding inhibits the anti-lipolytic response of adipocytes to insulin in vivo. Plasma glycerol Ra at the steady state during 90–120 min of the hyperinsulinemic-euglycemic clamp was measured as an index for systemic lipolysis. A, plasma glycerol Ra was calculated as described under “Experimental Procedures.” B, mean plasma glycerol Ra was shown as the mean of the 90, 100, 110, and 120 min values as the steady state values. Data represent the mean ± S.E. (n = 7 for pair-fed group and 6 for ethanol-fed group). *, p < 0.05 compared with own basal within each group; †, p < 0.05 pair-fed versus EtOH-fed.
Previous studies have demonstrated that chronic ethanol feeding impairs insulin-stimulated glucose uptake in isolated adipocytes (10, 11). This impairment in insulin-stimulated responses was not due to impaired activation of phosphatidylinositol 3-kinase or phosphorylation of Akt but, rather, was due to a loss of Cbl/TC10 activation (8, 11). Because the suppression of lipolysis by insulin involves the activation of PDE3B by Akt/protein kinase B, leading to a decrease in cAMP concentration and subsequently inactivation of hormone-sensitive lipase, we next investigated the effects of chronic ethanol on PDE3B expression and activity in isolated adipocytes. Chronic ethanol feeding had no effect on total PDE3B mRNA or protein in isolated adipocytes (Fig. 6, A and B). Furthermore, the total PDE3B activity at base line and in response to insulin was not affected by chronic ethanol feeding (Fig. 6 C).

**DISCUSSION**

Chronic ethanol consumption disrupts lipid homeostasis in the liver as well as at the whole-body level in both humans and animal models (2). Although it is clear that the regulation of lipid homeostasis in adipose tissue plays an important role in maintaining whole-body lipid homeostasis, the effects of chronic ethanol on lipid metabolism in adipose tissue have not been studied. Here we report that chronic ethanol feeding to rats increased the degradation of triglycerides in epididymal adipose tissue. Furthermore, the total PDE3B activity at base line and in response to insulin was not affected by chronic ethanol feeding (Fig. 6 C).
Chronic ethanol feeding increased the rate of triglyceride degradation, we hypothesized that chronic ethanol either increased the stimulatory response to β-adrenergic receptors and/or decreased the inhibitory response to insulin. However, in isolated adipocytes, we have previously reported that β-adrenergic receptor-stimulated lipolysis was actually suppressed rather than enhanced after chronic ethanol feeding (9). This suppression was due to an impaired β-adrenergic receptor-stimulated accumulation of cAMP after chronic ethanol feeding associated with increased expression of PDE4 (9). Lower cAMP accumulation led to decreased cAMP-dependent protein kinase activation and decreased phosphorylation of hormone-sensitive lipase and perilipins (9). Based on these data from isolated adipocytes (9), we hypothesized that it was unlikely that increased β-adrenergic receptor activity after chronic ethanol contributed to increased lipolysis in vivo. This hypothesis was confirmed in vivo by showing that β2-adrenergic receptor-stimulated systemic lipolysis was suppressed after chronic ethanol exposure (Fig. 2).

Because chronic ethanol exposure did not increase β-adrenergic receptor-stimulated lipolysis, we next investigated the effects of chronic ethanol on the anti-lipolytic action of insulin using both ex vivo model of primary adipocytes as well as an in vivo model utilizing a hyperinsulinemic-euglycemic clamp. Chronic ethanol feeding impaired the ability of insulin to inhibit lipolysis both ex vivo and in vivo. Although hyperinsulinemic-euglycemic clamps are commonly used to assess the sensitivity of glucose disposal to insulin (17), this clamp technique is also used to estimate the sensitivity of systemic lipolysis to insulin using plasma glycerol Ra as an index (19). During the steady state of the clamp, plasma glycerol Ra was decreased by 28% from base line in pair-fed rats, with no change in ethanol-fed rats (Fig. 4). Consistent with the lack of decrease in glycerol Ra, the ability of insulin to decrease plasma glycerol and free fatty acid concentrations during the insulin clamp was also suppressed in ethanol-fed rats compared with pair-fed rats (Fig. 3).

Chronic ethanol feeding impacts on a number of insulin-regulated metabolic pathways. In addition to the loss of insulin-mediated inhibition of lipolysis after chronic ethanol reported here, chronic ethanol feeding to rats also decreases whole-body glucose utilization during the hyperinsulinemic-euglycemic clamp (18, 30, 31) as well as insulin-stimulated glucose uptake in isolated rat adipocytes (10, 11). In humans, short term exposure to ethanol decreases peripheral glucose utilization, assessed either with hyperinsulinemic-euglycemic clamps (32, 33) or stable isotope analysis of gluconeogenic flux during ethanol infusion (34). Studies in individual tissues and cell types have found that ethanol impairs the insulin signaling pathway in a variety of cell types, including cerebellar neurons (6), hepatocytes (7), and adipocytes (8), suggesting that chronic ethanol-induced insulin resistance likely results from impaired insulin signaling. In adipocytes, chronic ethanol feeding impairs insulin-stimulated activation of the Cbl/TC10 pathway but does not disrupt insulin-stimulated phosphatidylinositol 3-kinase or Akt activation (11). Akt/protein kinase B is known to be upstream of PDE3B activation by insulin. Here we find that
stimulation of PDE3B activity by insulin is not decreased after chronic ethanol feeding (Fig. 6), suggesting that ethanol disrupts insulin-mediated suppression of lipolysis downstream of Akt/protein kinase B → PDE3B activation by insulin.

In addition to the reduced sensitivity to insulin observed after chronic ethanol feeding, basal concentrations of plasma insulin were also decreased (Table 3). This decrease may be due to an impaired function of pancreatic β cells by ethanol, as evidenced by the inability of rats to maintain a second pulse of insulin release during a glucose tolerance test after chronic exposure to ethanol (35–37). Taken together, these data suggest that both the lower basal plasma insulin concentration as well as the reduced ability of insulin to inhibit lipolysis in adipocytes contribute to the increased rate of triglyceride degradation in adipose tissue after chronic ethanol feeding.

Because chronic ethanol feeding increased the rate of triglyceride degradation in adipose tissue, it would also be expected that plasma free fatty acid concentrations would be increased. However, we found no differences in the base-line concentrations of plasma free fatty acids between pair- and ethanol-fed rats (Fig. 2B and Table 3). Maintenance of normal base-line concentrations of free fatty acids despite an increased rate of triglyceride degradation suggests that the free fatty acids released during triglyceride degradation were either rapidly re-esterified in adipose tissue and/or rapidly taken up by other tissues once released into the circulation. This rate of uptake may actually be increased after chronic ethanol, as chronic ethanol consumption increases the hepatocellular uptake of long chain fatty acids (38). Interestingly, chronic ethanol feeding did increase plasma-free fatty acid concentrations during the steady state of the hyperinsulinemic-euglycemic clamp (Fig. 3, C and D), suggesting that chronic ethanol may elevate free fatty acids in the circulation under certain conditions such as hyperinsulinemia.

In summary, we have demonstrated that chronic ethanol feeding to rats increased the in vivo rates of triglyceride degradation in epididymal adipose tissue. Increased lipolytic capacity of adipose tissue after ethanol was not due to an increased sensitivity to β-adrenergic activation but, instead, due to a suppression of the anti-lipolytic response of adipocytes to insulin. These data, thus, identify for the first time the mechanisms by which chronic ethanol consumption disrupts lipid homeostasis in adipose tissue. The dysregulation of adipose tissue metabolism, thus, likely contributes to the pathophysiological effects of ethanol.

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