Tauroursodeoxycholic acid may improve liver and muscle but not adipose tissue insulin sensitivity in obese men and women

Marleen Kars[1], Ling Yang[2], Margaret F. Gregor[2], B. Selma Mohammed[1], Terri A. Pietka[1], Brian N. Finck[1], Bruce W. Patterson[1], Jay D. Horton[3], Bettina Mittendorfer[1], Gökhan S. Hotamisligil[2], Samuel Klein[1]

[1]Center for Human Nutrition and Atkins Center of Excellence in Obesity Medicine, Washington University School of Medicine, St. Louis, Missouri, 63110
[2]Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA 02115
[3]Department of Internal Medicine, UT Southwestern Medical Center, Dallas, TX 75390

Short running title: TUDCA and insulin action

Correspondence:
Samuel Klein, MD
E-mail: sklein@wustl.edu

Objective: Insulin resistance is commonly associated with obesity. Studies conducted in obese mouse models found that endoplasmic reticulum (ER) stress contributes to insulin resistance and treatment with tauroursodeoxycholic acid (TUDCA), a bile acid derivative that acts as a chemical chaperone to enhance protein folding and ameliorate ER stress, increases insulin sensitivity. The purpose of this study was to determine the effect of TUDCA therapy on multi-organ insulin action and metabolic factors associated with insulin resistance in obese men and women.

Research Design and Methods: Twenty obese subjects (age: 48 ± 11 y, body mass index: 37 ± 4 kg/m^2; means $\pm$ SD) were randomized to 4 weeks of treatment with TUDCA (1750 mg/day) or placebo. A two-stage hyperinsulinemic-euglycemic clamp procedure in conjunction with stable isotopically labeled tracer infusions and muscle and adipose tissue biopsies were used to evaluate *in vivo* insulin sensitivity, cellular factors involved in insulin signalling, and cellular markers of ER stress.

Results: Hepatic and muscle insulin sensitivity increased by ~30% after treatment with TUDCA, but did not change after placebo therapy (P <0.05). In addition, therapy with TUDCA, but not placebo, increased muscle insulin signalling (phosphorylated IRS^{Tyr} and Akt^{Ser473} levels) (P <0.05). Markers of ER stress in muscle or adipose tissue did not change after treatment with either TUDCA or placebo.

Conclusion: These data demonstrate that TUDCA might be an effective pharmacological approach for treating insulin resistance. Additional studies are needed to evaluate the target cells and mechanisms responsible for this effect.

The ability of insulin to decrease hepatic glucose production, suppress adipose tissue lipolytic rate, and stimulate skeletal muscle glucose uptake is critical for normal metabolic function. Obesity is an important cause of multi-organ insulin resistance (1-3), and insulin sensitivity decreases linearly with increasing body mass index (4;5). Insulin resistance has important clinical implications, because it is involved in the pathogenesis of many of the metabolic complications associated with obesity. The precise mechanisms responsible for the link between obesity and insulin resistance are not known, but likely involve alterations in fatty acid metabolism, excess triglyceride accumulation in the liver and muscle (6-11), and systemic low-grade inflammation (12-14).

Recently, endoplasmic reticulum (ER) stress has been identified as a contributor to insulin resistance associated with obesity in experimental models (15;16). The ER is responsible for the synthesis, folding and trafficking of secretory and membrane proteins. Disruption of ER homeostasis results in an adaptive unfolded protein response (UPR), which aims to restore ER folding capacity and mitigate stress. ER stress can also inhibit insulin signalling, at least in part, by activating the c-Jun NH(2) terminal kinase (JNK) pathway through inositol-requiring enzyme–1 (IRE-1) (15;17-20) or RNA-dependent protein kinase (PKR)-mediated mechanisms (21). Increased ER stress is associated with impaired insulin action in obese mice (15), and chemical or genetic amelioration of this stress improves insulin sensitivity and glucose homeostasis (18). Increased ER stress in liver and adipose tissue and insulin resistance are also associated with obesity in humans (22;23), whereas weight loss decreases ER stress and improves insulin sensitivity (22).

Tauroursodeoxycholic acid (TUDCA) is a bile acid derivative that has been used in Europe to treat cholelithiasis and cholestatic liver disease. TUDCA can also act as a chemical chaperone to enhance protein folding and protect cells against ER stress (18). In obese mice, parenteral TUDCA treatment reduces ER stress, improves systemic insulin resistance, and decreases intrahepatic triglyceride (IHTG) content (18). Although data from studies conducted in animal models and cell systems demonstrate beneficial metabolic effects, the effect of TUDCA on insulin action has not been studied in human subjects.

The purpose of the present study was to determine whether chemical interventions targeting the ER stress pathway results in metabolic benefits in people. Accordingly, we conducted a randomized controlled trial in insulin-resistant, obese subjects to evaluate the effect of treatment with TUDCA on insulin sensitivity in the liver (glucose production), muscle (glucose uptake) and adipose tissue (lipolysis). We hypothesized that treatment with TUDCA would improve multi-organ insulin signalling and sensitivity and other metabolic factors associated with insulin resistance. The hyperinsulinemic-euglycemic clamp procedure, in conjunction with stable isotopically labeled tracer infusions, was used to determine *in vivo* insulin sensitivity, and adipose tissue and skeletal muscle biopsies were obtained to assess ER stress markers, phosphorylation of JNK and components of the insulin signalling pathway before and after 4 weeks of treatment with TUDCA or placebo.

RESEARCH DESIGN AND METHODS
Study Subjects. Twenty obese adults (8 men, 12 women; age: 48 ± 11 y, body mass index [BMI]: 37 ± 4 kg/m^2; means $\pm$ SD) participated in this single-blinded, randomized, placebo controlled trial

(ClinicalTrials.gov number NCT00771901). All subjects were insulin resistant, defined as a homeostasis model assessment of insulin resistance (HOMA-IR) value of ≥ 3.0 (24). Subjects completed a comprehensive medical evaluation, including a detailed history, physical examination, blood tests and a 2-h oral glucose tolerance test. Those who had diabetes, chronic liver disease other than non-alcoholic fatty liver disease (NAFLD), severe hypertriglyceridemia (fasting serum triglyceride concentrations > 400 mg/dl), and those who smoked cigarettes or were taking medications known to alter glucose or lipid metabolism were excluded. We purposely studied obese subjects who were insulin-resistant but did not have diabetes to provide the best chance for detecting an improvement in insulin sensitivity by TUDCA therapy, without the potential confounding influences of treatment with diabetes medications and differences in glucose control among study subjects. All subjects were sedentary (regular exercise < 1 h/week and ≤ 1 time/week) and weight stable (< 2% weight change) for at least 3 months before the study. Subjects provided written informed consent before participating in this study, which was approved by Human Research Protection Office of Washington University School of Medicine in St. Louis, MO.

Experimental protocol: *Body composition.* Body composition analyses were performed approximately one week before the hyperinsulinemic-euglycemic clamp procedure was performed. Body fat mass (FM) and fat-free mass (FFM) were determined by using dual-energy X-ray absorptiometry (DEXA, QDR 4500; Hologic, Waltman, MA). Abdominal subcutaneous adipose tissue and intra-abdominal adipose tissue volumes were determined by using magnetic resonance imaging; the sum of 10 axial images of 1-cm thickness, beginning at the L4-L5 interspace and extending proximally, was used to determine each fat depot volume. Intrahepatic triglyceride content was determined by using magnetic resonance spectroscopy (3T Siemens Magnetom Trio Scanner, Siemens, Erlanger, Germany); three 15 x 15 x 15-mm voxels were examined in each subject, and the values were averaged to provide an estimate of the percent of total liver volume comprised of triglyceride (25).

Hyperinsulinemic-euglycemic clamp procedure. Subjects were admitted to the Clinical Research Unit (CRU) at Washington University School of Medicine in the afternoon on the day before the clamp procedure. At 1800 h, they consumed a standard meal containing 12 kcal per kg FFM, with 55% of total energy provided as carbohydrates, 30% as fat, and 15% as protein. Subjects then fasted, except for water, until completion of the clamp procedure the next day. At 0500 h the following morning, a catheter was inserted into a forearm vein to infuse stable isotopically-labeled tracers (purchased from Cambridge Isotope Laboratories, Andover, MA), dextrose and insulin. A second catheter was inserted into the contralateral radial artery to obtain blood samples. Radial artery cannulation was not successful in 4 subjects, so a catheter was inserted into a hand vein, which was heated to 55°C by using a thermostatically controlled box to obtain arterialized blood samples (26). At 0600 h, a primed-continuous infusion of [6,6-^{2}H$_2$]glucose (priming dose 22.5 μmol/kg body weight; infusion rate 0.25 μmol/kg body weight/min) was started and maintained for 9.5 hours. At 0800 h, a continuous infusion of [2,2-^{2}H$_2$]palmitate (infusion rate 0.035 μmol/kg body weight/min) bound to 25% human albumin was started and maintained for 7.5 hours. At 0930 h, 3.5 h after starting the glucose tracer infusion, a two-stage hyperinsulinemic-euglycemic clamp procedure was started and continued for 6 h. During stage 1 of the clamp procedure (3.5 to

6.5 h), insulin was infused at a rate of 7 mU/m^2 body surface area [BSA]/min (initiated with a priming dose of 28 mU/m^2 BSA/min for 5 min and then 14 mU/m^2 BSA/min for another 5 min) for 3 h. During stage 2 of the clamp procedure (6.5 to 9.5 h), the rate of insulin infusion was increased to 50 mU/m^2 BSA/min (initiated with a priming dose of 200 mU/m^2 BSA/min for 5 min and then 100 mU/m^2 BSA/min for another 5 min). These insulin infusion rates were chosen to evaluate adipose tissue insulin sensitivity (low-dose insulin infusion to submaximally suppress lipolysis of adipose tissue triglycerides) and skeletal muscle insulin sensitivity (high-dose insulin infusion to stimulate muscle glucose uptake) (27). Euglycemia was maintained at a blood concentration of approximately 5.6 mM (100 mg/dl) by infusing 20% dextrose enriched to 2.5% with [6,6-^{2}H$_2$]glucose. The infusion rate of [6,6-^{2}H$_2$]glucose was reduced by 50% of basal during stage 1, and by 75% of basal during stage 2 of the clamp procedure to account for the expected decline in endogenous glucose production. The infusion rate of [2,2-^{2}H$_2$]palmitate was reduced by 50% of basal during stage 1 to account for the expected decline in lipolytic rate.

Blood samples were obtained before the start of the tracer infusions to determine background plasma tracer-to-tracee ratios (TTRs) of glucose and palmitate, and every 10 min during the final 30 min of the basal period, and stages 1 and 2 of the clamp procedure to determine glucose, free fatty acid (FFA) and insulin concentrations, and substrate kinetics. Blood samples were collected in chilled heparinized tubes to determine glucose and insulin concentration. All other blood samples were collected in chilled tubes containing EDTA. Samples were placed on ice, and plasma was separated by centrifugation within 30 min of collection. Plasma samples were stored at -80°C until final analyses were performed.

Subcutaneous abdominal adipose tissue and muscle tissue (vastus lateralis portion of the quadriceps femoris) were obtained during the basal period to determine the expression and regulation of ER stress markers. Additional muscle tissue was obtained at ~30 minutes after starting stage 2 of the clamp procedure to determine the levels and the extent of phosphorylation of JNK and elements of the insulin signalling pathway. The biopsy sites were cleaned and draped, and the skin and underlying tissues were anesthetized with lidocaine. A small (~0.5 cm) skin incision was made with a scalpel; adipose tissue was aspirated through a 4 mm liposuction cannula and muscle tissue was obtained by using Tilley-Henkel forceps (Sontec Instruments, Inc., Centennial, CO). Muscle and adipose tissue samples were immediately rinsed in ice-cold saline, frozen in liquid nitrogen and stored at -80°C until final analyses were performed.

Intervention. After the baseline clamp procedure was completed, each subject was randomized to 4 weeks of oral treatment with either TUDCA (1750 mg/day) or placebo. Both TUDCA and placebo were kindly provided by Bruschettini S.r.l, Genova, Italy. During the 4-week intervention period, subjects were seen every week to review any study-related issues, reinforce treatment compliance, check body weight and assess vital signs. After 4 weeks of treatment, the body composition analyses and clamp procedure performed at baseline were repeated. Stage 2 of the clamp procedure was not completed in 2 of the 10 subjects who received TUDCA treatment because of technical difficulties in obtaining blood samples. Treatment with drug or placebo was continued until all evaluations were finished.

Sample processing and analyses. Plasma glucose concentration was determined by using an automated glucose analyzer (YSI 2300 STAT Plus, Yellow Springs Instruments Co., Yellow Springs, OH). Plasma FFA

concentrations were quantified by using gas chromatography (HP 5890 Series II GC; Hewlett-Packard, Palo Alto, CA) (28). Plasma insulin concentration was measured by using a chemiluminescent immunometric assay (Immulite 1000; Diagnostic Products Corp, Los Angeles, CA). Plasma CRP and IL-6 concentrations were measured by using commercially available high-sensitivity immunoassays (R&D Systems, Inc., Minneapolis, MN). Total and high-molecular weight (HMW) adiponectin concentrations were determined by using FPLC (AKTA FPLC system, GE Healthcare) and fluorescent Western blotting (LI-COR Biotechnology, Lincoln, NE), as previously described (29). Plasma glucose and palmitate TTRs were determined by using gas chromatography/mass spectroscopy (MSD 5973 system with capillary column; Hewlett-Packard), as previously described (30).

To determine the activation of the insulin signalling and the JNK pathways in muscle and adipose tissue, we measured the site-specific phosphorylation of IRS-1, Akt, and JNK. Tissue samples were cryopulverized and the powdered tissue transferred to a tube containing cell lysis solution (Cell Signaling, Beverly, MA) and homogenized by using a polytron (PowerGen 125, Fisher, Pittsburgh, PA). Homogenates were spun for 15 min at 2000 x g at 4°C to pellet insoluble material. The total protein concentration in the supernatant was measured (DC Protein Assay, Bio-Rad, Hercules, CA) and 25 to 50 µg protein were electrophoresed by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with polyclonal antibodies directed against total Akt, Akt^{Ser473}, total JNK, and JNK^{Thr183} (all Cell Signaling, Beverly, MA) in Western analyses. To evaluate IRS-1 tyrosine phosphorylation, blots were concurrently probed with a rabbit polyclonal antibody against IRS-1 (gift of Mike Mueckler – Washington University School of Medicine, St Louis, MO) and a mouse

monoclonal phospho-tyrosine antibody (Cell Signaling, Danvers, MA) and then incubated with secondary antibodies tagged with red (anti mouse) or green (anti rabbit) fluorophores. Detection was performed with the LiCor dual color system (Li-Cor Biosciences Lincoln, Nebraska). All band intensities were visualized and quantified by using the LiCor system and Odyssey 3.0 software and phosphorylation levels were expressed as a function of total protein levels. Real-time (RT) quantitative PCR was performed as previously described to determine the mRNA expression of ER stress markers (glucose-regulated protein 78 [Grp78], spliced X-box binding protein-1 [XBP-1], and C/EBP homologous protein [CHOP]) in adipose tissue (22). Frozen adipose tissue samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis was performed by using 1 µg of sample RNA reverse transcribed with high capacity cDNA archive system (Applied Biosystems, Foster City, CA); quantitative RT-PCR was performed by using SybrGreen reagent in ABI 7300 RT PCR system (Applied Biosystems, Foster City, CA). The mRNA expression of ER stress markers were normalized to 18S ribosomal RNA. The protein expression of homocysteine-induced endoplasmic reticulum protein (HERP) and the concentrations of eukaryotic elongation initiation factor 2α (eIF2α) phosphorylated at serine 52 and JNK phosphorylated at threonine 183/185 in adipose tissue were determined by Western analyses using rabbit polyclonal anti-p-eIF2α (Invitrogen, Carlsbad, CA) and mouse monoclonal anti-p-JNK (Cell Signaling, Danvers, MA) antibodies as previously described (22). Anti-HERP antibody was a gift from Dr. Yasuhiko Hirabayashi of Tohoku University, Japan. The band intensities of p-(eIF2α), p-JNK, and HERP were normalized to actin.

Calculations. Isotopic steady state conditions were achieved during the final 30 min of the

basal period and stages 1 and 2 of the clamp procedure, so Steele's equation for steady-state conditions was used to calculate glucose rate of appearance (Ra), glucose rate of disappearance (Rd) and palmitate Ra (31). The hepatic insulin sensitivity index was determined as the reciprocal of the hepatic insulin resistance index, which was calculated as the product of the basal hepatic glucose production rate (in μmol/min) and basal plasma insulin concentration (in μU/ml) (32). Adipose tissue insulin sensitivity was assessed by calculating the relative decrease from basal in palmitate Ra into plasma during stage 1 of the clamp procedure. Skeletal muscle insulin sensitivity was assessed by calculating the relative increase from basal in glucose Rd from plasma during stage 2 of the clamp procedure. The HOMA-IR score was calculated as the product of fasting plasma insulin (in mU/l) and glucose (in mmol/l) concentrations divided by 22.5 (24).

Statistical Analyses. Statistical analyses were performed by using SPSS for Windows (version 16.0, SPSS Inc., Chicago, IL). Results are reported as means ± SD (normally distributed data sets) or medians and quartiles (skewed data sets). Two-way repeated measures analysis of variance (ANOVA) was used to determine whether the changes in outcomes (normally distributed data sets) in response to either TUDCA or placebo treatment were different. Skewed data sets were evaluated by using non-parametric analyses. A P-value ≤ 0.05 was considered statistically significant. Results are reported as mean ± SD (normally distributed data sets) or medians and quartiles (skewed data sets). Based on data on glucose kinetics we obtained in obese subjects previously (33), we estimated that a sample size of 10 participants per group would allow us to detect a 25% difference in insulin sensitivity after treatment between groups, with an *alpha* value of 0.05 and power of 80% (*beta* = 0.2). A difference in insulin sensitivity of this magnitude is clinically meaningful because it represents the lower end of the observed effect of current treatment strategies for insulin resistance, such as moderate weight loss (33), or pharmacotherapy (e.g., metformin and thiazolidinediones) (34-41).

RESULTS

Subject characteristics, metabolic variables, and body composition. Subjects randomized to receive TUDCA and placebo were similar in age, sex, BMI and body composition (Table 1). Body weight, total body fat and fat distribution (intra-abdominal fat volume and IHTG content) did not change after 4 weeks of treatment with either TUDCA or placebo (Table 1). Baseline (before treatment) plasma concentrations of glucose, insulin, FFA, triglyceride, aspartate aminotransferase, alanine aminotransferase, total adiponectin, high molecular weight adiponectin, and markers of inflammation were not different between groups, and did not change after either TUDCA or placebo treatment (Table 1). The HOMA-IR score did not change after placebo treatment, but was ~20% lower after TUDCA treatment (Table 1); however, the decrease in HOMA-IR after TUDCA therapy was not statistically significant.

Insulin sensitivity assessed by using the hyperinsulinemic euglycemic clamp technique. During the hyperinsulinemic-euglycemic clamp procedure, euglycemia was maintained at approximately 100 mg/dl in all subjects (average plasma glucose concentration: 100.0 ± 2.1 mg/dl during stage 1 and 102.0 ± 2.7 mg/dl during stage 2 of the clamp procedure) and plasma insulin concentration increased to 29 ± 17 μU/ml during stage 1 and to 81 ± 19 μU/ml during stage 2.

The hepatic insulin sensitivity index increased by ~30% after TUDCA treatment, but was not affected by placebo therapy (Figure 1A). Glucose Rd increased by ~150 % (P <0.001) from basal values during stage 2 of the clamp

procedure in both the TUDCA and placebo groups before treatment. Compared with baseline (pre-treatment) values, the increase in glucose Rd above basal values during stage 2 was 34 ± 23 % greater after treatment with TUDCA (P <0.05), but did not change after treatment with placebo (Figure 1B). Insulin infusion suppressed palmitate Ra by ~50% in both TUDCA and placebo groups before treatment, and this remained the same after treatment with either TUDCA or placebo (Figure 1C).

Consistent with our kinetic data, TUDCA therapy increased insulin signalling in muscle but not adipose tissue. Compared with placebo treatment, TUDCA treatment increased insulin-stimulated phosphorylation of IRS^{Tyr} and Akt^{Ser473} in muscle (both P <0.05; Figure 2). In contrast, TUDCA therapy did not alter insulin-stimulated phosphorylation of IRS^{Tyr} and Akt^{Ser473} in adipose tissue (data not shown). No difference in skeletal muscle phosphorylation of JNK^{Thr183}, was detected between subjects treated with placebo or TUDCA (Figure 2).

Endoplasmic reticulum stress markers in muscle and adipose tissue. Adipose tissue mRNA expression of spliced XBP-1, Grp78 and CHOP (Figure 3A) and protein levels of HERP, $eIF2\alpha^{Ser52}$, and $JNK^{Thr183/183}$ (Figure 3B) were not affected by either placebo or TUDCA treatment. Spliced XBP-1, Grp78 and CHOP mRNA expression in skeletal muscle were very low (below the limit of reliable detection for spliced XBP-1) and did not change after TUDCA treatment (data not shown).

DISCUSSION

Data from recent studies conducted in both rodent models and human subjects have demonstrated that obesity is associated with liver and adipose tissue, but not muscle, ER stress (15;18;22;23). Treating obese mice with TUDCA, which acts as a chemical chaperone that reduces ER stress in liver and adipose tissue, results in improved insulin sensitivity in liver, muscle, and adipose tissue (18). However, the use of agents that can decrease ER stress to treat obesity-associated insulin resistance has not been evaluated in people. Accordingly, we conducted a randomized controlled trial to determine the effect of 4 weeks of treatment with TUDCA on multi-organ insulin sensitivity and factors involved in regulating insulin action in obese subjects with insulin resistance. Our data demonstrate that TUDCA therapy increases hepatic and muscle insulin action *in vivo* with a concomitant increase in the phosphorylation of components of the muscle insulin signalling pathway. Moreover, the magnitude of the improvement in hepatic and muscle insulin sensitivity (both ~30%) is similar to the insulin-sensitizing effects of currently available diabetes medications, such as thiazolinediones and metformin (35-41). However, we did not detect an effect of TUDCA on adipose tissue insulin sensitivity or ER stress, making it unlikely that a reduction in adipocyte ER stress was responsible for the effect on insulin action in this trial. These data suggest that TUDCA therapy might provide a novel pharmacological approach for improving glucose homeostasis in insulin-resistant obese people.

The precise cellular mechanisms responsible for the improvement in hepatic insulin sensitivity after TUDCA therapy are not clear. Data from studies conducted in human hepatocyte cultures have shown that TUDCA activates Akt and its downstream targets via a G-protein coupled receptor-dependent mechanism (42;43). In addition, activation of the nuclear farnesoid X receptor (FXR) by bile acids or FXR agonists improves insulin sensitivity *in vitro* in cell systems and *in vivo* in animal models (44;45). However, TUDCA, unlike some other bile acids, is a relatively poor substrate for FXR (46). In animal models and cultured liver cells,

TUDCA treatment can suppress ER stress and increase insulin signalling (18). Therefore, it is possible that TUDCA therapy affected hepatic ER stress in our subjects, but we did not obtain liver tissue in our subjects to directly evaluate this possibility.

The mechanisms responsible for the TUDCA-induced improvement in skeletal muscle insulin sensitivity and insulin signalling in our subjects and in the previous study conducted in mice (18) are also not clear. In contrast with adipose tissue and liver, ER stress indicators are not increased in skeletal muscle from obese mice (15;18) or obese people (23), and TUDCA treatment did not affect muscle ER stress markers in our study. In addition, muscle does not express the FXR (46), which precludes the possibility of an FXR-mediated improvement in muscle insulin action. However, G-protein receptors are expressed ubiquitously, so some of the effects of TUDCA in muscle might be mediated by G-protein receptor activation of Akt.

Although we found TUDCA therapy improved hepatic and muscle insulin sensitivity, we did not find a significant effect of TUDCA therapy on HOMA-IR and many of the metabolic variables associated with insulin resistance, such as plasma glucose, insulin, and FFA concentrations. It is likely that these measures were not adequate to detect an effect of TUDCA, which required a more sensitive assessment of insulin action by using stable isotopically labelled tracers and the hyperinsulinenmic-euglycemic clamp procedure.

Unlike data obtained from mouse models (18), we did not detect an effect of TUDCA therapy on adipose tissue insulin sensitivity or signalling, or the expression of adipose tissue ER stress markers. Several factors might be responsible for the apparent difference observed after TUDCA therapy in mice and our study in human subjects. First, it is possible that the amount of TUDCA we gave our subjects, which is the maximum dose used to treat biliary disease, was insufficient to generate changes in adipose tissue ER stress; in the previous study, ~30 times more TUDCA relative to body weight was given to mice than to our subjects. Second, TUDCA was given intraperitoneally in the mice but orally to our subjects. It is likely this difference in route of administration further limited the systemic availability of TUDCA in our subjects, because TUDCA is effectively metabolized by the liver and there is minimal splanchnic escape of bile acid conjugates into the systemic circulation (47). Finally, the expression of transporters responsible for TUDCA tissue uptake is very low in most extrahepatic tissues (48;49), so large plasma concentrations might be needed to achieve adequate tissue uptake to affect intracellular function. These pharmacokinetic factors raise the possibility that adipose tissue did not get sufficient exposure to orally administered TUDCA to affect ER stress in our subjects. Future studies with different dosing regimens are needed to address this issue.

In summary, insulin resistance is involved in the pathogenesis of the key metabolic disorders associated with obesity (1-3;6-14). The results from the present study demonstrate that TUDCA therapy increases liver and muscle insulin sensitivity in obese, insulin-resistant subjects. Additional studies are needed to determine the specific cellular mechanisms responsible for this effect, and to determine the therapeutic potential of this class of compounds for obese people with insulin resistance.

Author contributions. MK, BM, GSH, and SK were involved in designing and conducting the infusion studies, processing the study samples, collecting data, performing the final data analyses, and writing the manuscript. BSM was involved in conducting the infusion studies. MFG, LY, TAP, BNF,

REFERENCES
1. Marchesini,G, Bugianesi,E, Forlani,G, Cerrelli,F, Lenzi,M, Manini,R, Natale,S, Vanni,E, Villanova,N, Melchionda,N, Rizzetto,M: Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 37:917-923, 2003
2. Ioannou,GN, Weiss,NS, Boyko,EJ, Mozaffarian,D, Lee,SP: Elevated serum alanine aminotransferase activity and calculated risk of coronary heart disease in the United States. *Hepatology* 43:1145-1151, 2006
3. Adams,LA, Lymp,JF, St Sauver,J, Sanderson,SO, Lindor,KD, Feldstein,A, Angulo,P: The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 129:113-121, 2005
4. Ferrannini,E, Natali,A, Bell,P, Cavallo-Perin,P, Lalic,N, Mingrone,G: Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR). *J Clin Invest* 100:1166-1173, 1997
5. Esteghamati,A, Khalilzadeh,O, Anvari,M, Ahadi,MS, Abbasi,M, Rashidi,A: Metabolic syndrome and insulin resistance significantly correlate with body mass index. *Arch Med Res* 39:803-808, 2008
6. Neschen,S, Morino,K, Hammond,LE, Zhang,D, Liu,ZX, Romanelli,AJ, Cline,GW, Pongratz,RL, Zhang,XM, Choi,CS, Coleman,RA, Shulman,GI: Prevention of hepatic steatosis and hepatic insulin resistance in mitochondrial acyl-CoA:glycerol-sn-3-phosphate acyltransferase 1 knockout mice. *Cell Metab* 2:55-65, 2005
7. Savage,DB, Choi,CS, Samuel,VT, Liu,ZX, Zhang,D, Wang,A, Zhang,XM, Cline,GW, Yu,XX, Geisler,JG, Bhanot,S, Monia,BP, Shulman,GI: Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J Clin Invest* 116:817-824, 2006
8. Korenblat,KM, Fabbrini,E, Mohammed,BS, Klein,S: Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology* 134:1369-1375, 2008
9. Deivanayagam,S, Mohammed,BS, Vitola,BE, Naguib,GH, Keshen,TH, Kirk,EP, Klein,S: Nonalcoholic fatty liver disease is associated with hepatic and skeletal muscle insulin resistance in overweight adolescents. *Am J Clin Nutr* 88:257-262, 2008

10. Gastaldelli,A, Kozakova,M, Hojlund,K, Flyvbjerg,A, Favuzzi,A, Mitrakou,A, Balkau,B: Fatty liver is associated with insulin resistance, risk of coronary heart disease, and early atherosclerosis in a large European population. *Hepatology* 49:1537-1544, 2009

11. Seppala-Lindroos,A, Vehkavaara,S, Hakkinen,AM, Goto,T, Westerbacka,J, Sovijarvi,A, Halavaara,J, Yki-Jarvinen,H: Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocrinol Metab* 87:3023-3028, 2002

12. Hotamisligil,GS: Inflammation and metabolic disorders. *Nature* 444:860-867, 2006

13. Cai,D, Yuan,M, Frantz,DF, Melendez,PA, Hansen,L, Lee,J, Shoelson,SE: Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 11:183-190, 2005

14. Shoelson,SE, Herrero,L, Naaz,A: Obesity, inflammation, and insulin resistance. *Gastroenterology* 132:2169-2180, 2007

15. Ozcan,U, Cao,Q, Yilmaz,E, Lee,AH, Iwakoshi,NN, Ozdelen,E, Tuncman,G, Gorgun,C, Glimcher,LH, Hotamisligil,GS: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306:457-461, 2004

16. Gregor,MF, Hotamisligil,GS: Thematic review series: Adipocyte Biology. Adipocyte stress: the endoplasmic reticulum and metabolic disease. *J Lipid Res* 48:1905-1914, 2007

17. Hirosumi,J, Tuncman,G, Chang,L, Gorgun,CZ, Uysal,KT, Maeda,K, Karin,M, Hotamisligil,GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333-336, 2002

18. Ozcan,U, Yilmaz,E, Ozcan,L, Furuhashi,M, Vaillancourt,E, Smith,RO, Gorgun,CZ, Hotamisligil,GS: Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 313:1137-1140, 2006

19. Aguirre,V, Uchida,T, Yenush,L, Davis,R, White,MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275:9047-9054, 2000

20. Urano,F, Wang,X, Bertolotti,A, Zhang,Y, Chung,P, Harding,HP, Ron,D: Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664-666, 2000

21. Nakamura, T, Furuhashi,M, Li,P, Cao,H, Tuncman,G, Sonenberg,N, Gorgun,CZ, Hotamisligil,GS: Double-Stranded RNA-Dependent Protein Kinase Links Pathogen Sensing with Stress and Metabolic Homeostasis. *Cell* 140(3):338-348, 2010

22. Gregor,MF, Yang,L, Fabbrini,E, Mohammed,BS, Eagon,JC, Hotamisligil,GS, Klein,S: Endoplasmic reticulum stress is reduced in tissues of obese subjects after weight loss. *Diabetes* 58:693-700, 2009

23. Sharma,NK, Das,SK, Mondal,AK, Hackney,OG, Chu,WS, Kern,PA, Rasouli,N, Spencer,HJ, Yao-Borengasser,A, Elbein,SC: Endoplasmic reticulum stress markers are associated with obesity in nondiabetic subjects. *J Clin Endocrinol Metab* 93:4532-4541, 2008

24. Matthews,DR, Hosker,JP, Rudenski,AS, Naylor,BA, Treacher,DF, Turner,RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28:412-419, 1985

25. Frimel,TN, Deivanayagam,S, Bashir,A, O'Connor,R, Klein,S: Assessment of intrahepatic triglyceride content using magnetic resonance spectroscopy. *J Cardiometab Syndr* 2:136-138, 2007

26. Jensen,MD, Heiling,VJ: Heated hand vein blood is satisfactory for measurements during free fatty acid kinetic studies. *Metabolism* 40:406-409, 1991

27. Groop,LC, Bonadonna,RC, DelPrato,S, Ratheiser,K, Zyck,K, Ferrannini,E, DeFronzo,RA: Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *J Clin Invest* 84:205-213, 1989

28. Patterson,BW, Zhao,G, Klein,S: Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. *Metabolism* 47:706-712, 1998

29. Schraw,T, Wang,ZV, Halberg,N, Hawkins,M, Scherer,PE: Plasma adiponectin complexes have distinct biochemical characteristics. *Endocrinology* 149:2270-2282, 2008

30. Patterson,BW, Zhao,G, Elias,N, Hachey,DL, Klein,S: Validation of a new procedure to determine plasma fatty acid concentration and isotopic enrichment. *J Lipid Res* 40:2118-2124, 1999

31. Steele,R: Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann NY Acad Sci* 82:420-430, 1959

32. Matsuda,M, DeFronzo,RA: Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22:1462-1470, 1999

33. Kirk,E, Reeds,DN, Finck,BN, Mayurranjan,SM, Patterson,BW, Klein,S: Dietary fat and carbohydrates differentially alter insulin sensitivity during caloric restriction. *Gastroenterology* 136:1552-1560, 2009

34. Schrauwen-Hinderling,VB, Mensink,M, Hesselink,MK, Sels,JP, Kooi,ME, Schrauwen,P: The insulin-sensitizing effect of rosiglitazone in type 2 diabetes mellitus patients does not require improved in vivo muscle mitochondrial function. *J Clin Endocrinol Metab* 93:2917-2921, 2008

35. Nolan,JJ, Ludvik,B, Beerdsen,P, Joyce,M, Olefsky,J: Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med* 331:1188-1193, 1994

36. Frias,JP, Yu,JG, Kruszynska,YT, Olefsky,JM: Metabolic effects of troglitazone therapy in type 2 diabetic, obese, and lean normal subjects. *Diabetes Care* 23:64-69, 2000

37. Kruszynska,YT, Yu,JG, Olefsky,JM, Sobel,BE: Effects of troglitazone on blood concentrations of plasminogen activator inhibitor 1 in patients with type 2 diabetes and in lean and obese normal subjects. *Diabetes* 49:633-639, 2000

38. Yu,JG, Javorschi,S, Hevener,AL, Kruszynska,YT, Norman,RA, Sinha,M, Olefsky,JM: The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* 51:2968-2974, 2002

39. Shadid,S, Jensen,MD: Pioglitazone increases non-esterified fatty acid clearance in upper body obesity. *Diabetologia* 49:149-157, 2006

40. Giugliano,D, De Rosa,N, Di Maro,G, Marfella,R, Acampora,R, Buoninconti,R, D'Onofrio,F: Metformin improves glucose, lipid metabolism, and reduces blood pressure in hypertensive, obese women. *Diabetes Care* 16:1387-1390, 1993

41. Mannucci,E, Ognibene,A, Cremasco,F, Bardini,G, Mencucci,A, Pierazzuoli,E, Ciani,S, Messeri,G, Rotella,CM: Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects. *Diabetes Care* 24:489-494, 2001

42. Dent,P, Fang,Y, Gupta,S, Studer,E, Mitchell,C, Spiegel,S, Hylemon,PB: Conjugated bile acids promote ERK1/2 and AKT activation via a pertussis toxin-sensitive mechanism in murine and human hepatocytes. *Hepatology* 42:1291-1299, 2005

43. Fang,Y, Studer,E, Mitchell,C, Grant,S, Pandak,WM, Hylemon,PB, Dent,P: Conjugated bile acids regulate hepatocyte glycogen synthase activity in vitro and in vivo via Galphai signaling. *Mol Pharmacol* 71:1122-1128, 2007

44. Cariou,B, van Harmelen,K, Duran-Sandoval,D, van Dijk,TH, Grefhorst,A, Abdelkarim,M, Caron,S, Torpier,G, Fruchart,JC, Gonzalez,FJ, Kuipers,F, Staels,B: The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice. *J Biol Chem* 281:11039-11049, 2006

45. Cipriani,S, Mencarelli,A, Palladino,G, Fiorucci,S: FXR activation reverses insulin resistance and lipid abnormalities and protects against liver steatosis in Zucker (fa/fa) obese rats. *J Lipid Res* 2009

46. Nguyen,A, Bouscarel,B: Bile acids and signal transduction: role in glucose homeostasis. *Cell Signal* 20:2180-2197, 2008

47. Hylemon,PB, Zhou,H, Pandak,WM, Ren,S, Gil,G, Dent,P: Bile acids as regulatory molecules. *J Lipid Res* 50:1509-1520, 2009

48. Bouscarel,B, Ceryak,S, Gettys,TW, Fromm,H, Noonan,F: Alteration of cAMP-mediated hormonal responsiveness by bile acids in cells of nonhepatic origin. *Am J Physiol* 268:G908-G916, 1995

49. Li,N, Hartley,DP, Cherrington,NJ, Klaassen,CD: Tissue expression, ontogeny, and inducibility of rat organic anion transporting polypeptide 4. *J Pharmacol Exp Ther* 301:551-560, 2002

FIGURE LEGENDS

Figure 1. Liver, muscle and adipose tissue insulin sensitivity before (white bars) and after (gray bars) 4 weeks of placebo or TUDCA treatment. A: hepatic insulin sensitivity index. B: glucose rate of disappearance (Rd); data represent only 8 of the 10 subjects who received TUDCA treatment because of technical difficulties in obtaining blood samples in 2 subjects. C: palmitate rate of appearance. Values are means ± SD. * Value significantly different from corresponding value before treatment, P <0.05. [†] Main effect of insulin, P <0.0001.

Figure 2. Effect of placebo (white bars) or TUDCA (gray bars) treatment on skeletal muscle IRS[Tyr], Akt[Ser473] and JNK[Thr183] levels. Values are means ± SD and expressed relative to values before treatment, which were set to 1 for each person. * Value significantly different from corresponding placebo value, P <0.05.

Figure 3. Endoplasmic reticulum stress markers before (white bars) and after (gray bars) placebo or TUDCA therapy. A: gene expression (relative to 18S ribosomal RNA). B: protein content (relative to actin). Values are medians and quartiles.

Table 1. Subjects' characteristics, metabolic variables, and body composition

	Placebo		TUDCA	
	Before	After	Before	After
N (M/F)	10 (4/6)	-	10 (4/6)	-
Age (y)	49 ± 14	-	47 ± 9	-
Body mass index (kg/m^2)	37 ± 5	38 ± 5	35 ± 3	35 ± 3
Total body mass (kg)	109 ± 18	110 ± 17	100 ± 12	100 ± 12
Fat mass (%)	39 ± 7	39 ± 7	39 ± 8	39 ± 8
Subcutaneous abdominal fat volume (cm^3)	5087 ± 1749	4999 ± 1672	4803 ± 1202	4716 ± 1188
Intra-abdominal fat volume (cm^3)	2146 ± 789	2172 ± 716	2724 ± 846	2818 ± 924
Intrahepatic triglyceride content (%)	14.0 ± 10.7	13.7 ± 9.6	8.2 ± 5.5	9.3 ± 7.2
HOMA-IR	4.2 ± 1.8	4.3 ± 1.7	4.4 ± 2.7	3.8 ± 3.4
Plasma concentrations				
Glucose (mg/dl)	97 ± 9	97 ± 10	96 ± 9	94 ± 5
Insulin (μU/ml)	18 ± 7	18 ± 7	19 ± 12	16 ± 15
Free fatty acids (mmol/l)	0.59 ± 0.13	0.63 ± 0.15	0.62 ± 0.15	0.62 ± 0.19
Triglycerides (mg/dl)	154 ± 52	145 ± 77	136 ± 67	141 ± 66
AST (IU/l)	25.9 ± 10.3	27.9 ± 14.5	22.4 ± 5.9	22.5 ± 6.1
ALT (IU/l)	30.3 ± 24.7	35.6 ± 34.9	26.9 ± 9.6	24.6 ± 8.1
CRP (mg/l)	5.67 ± 5.69	5.00 ± 3.87	5.40 ± 4.11	4.43 ± 2.94
IL-6 (pg/ml)	2.60 ± 0.95	2.44 ± 0.86	2.32 ± 0.65	2.60 ± 1.17
Total adiponectin (mg/l)	6.7 ± 3.8	6.9 ± 4.6	8.3 ± 3.1	8.3 ± 3.1
HMW adiponectin (mg/l)	2.2 ± 2.6	2.2 ± 2.9	3.1 ± 1.9	3.4 ± 2.4

Values are means ± SD. CRP: C-reactive protein; IL: interleukin; HMW: high molecular weight. HOMA-IR: homeostasis model assessment of insulin resistance; AST: aspartate aminotransferase; ALT: alanine aminotransferase.
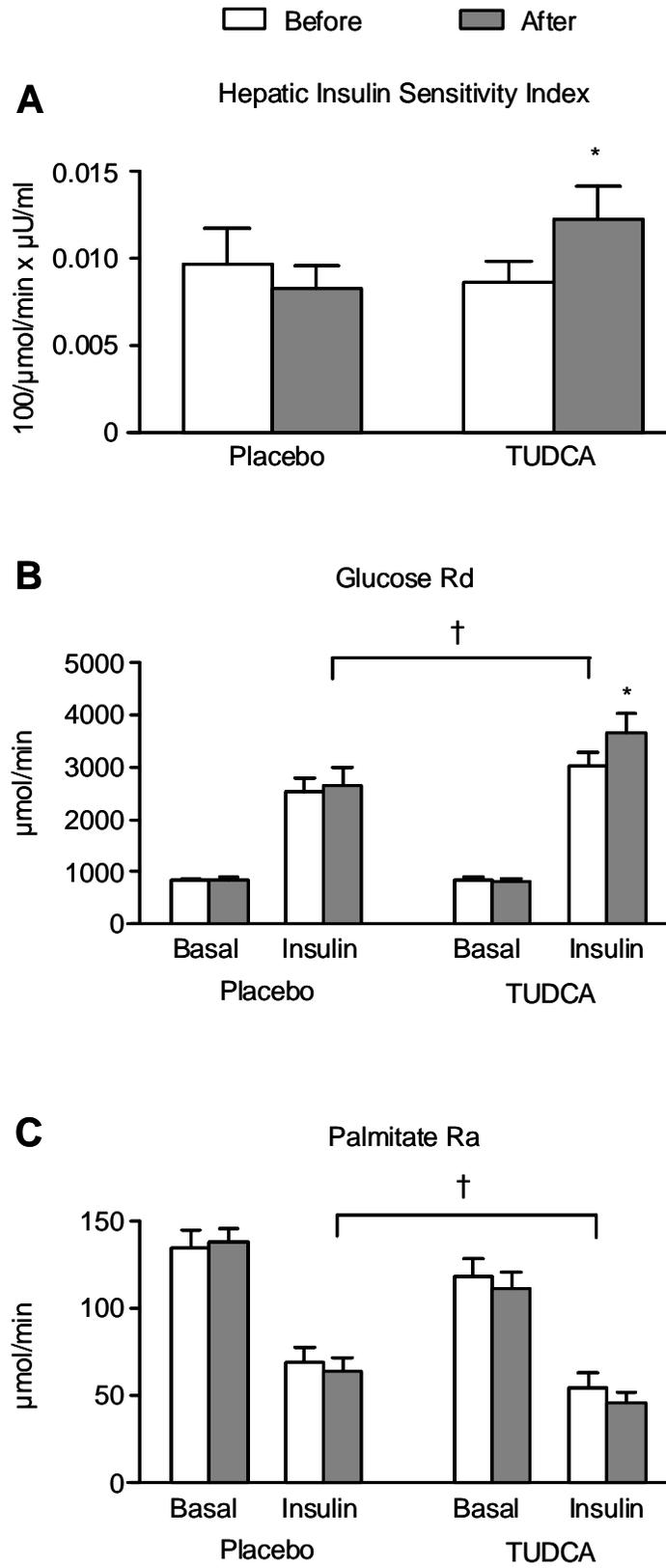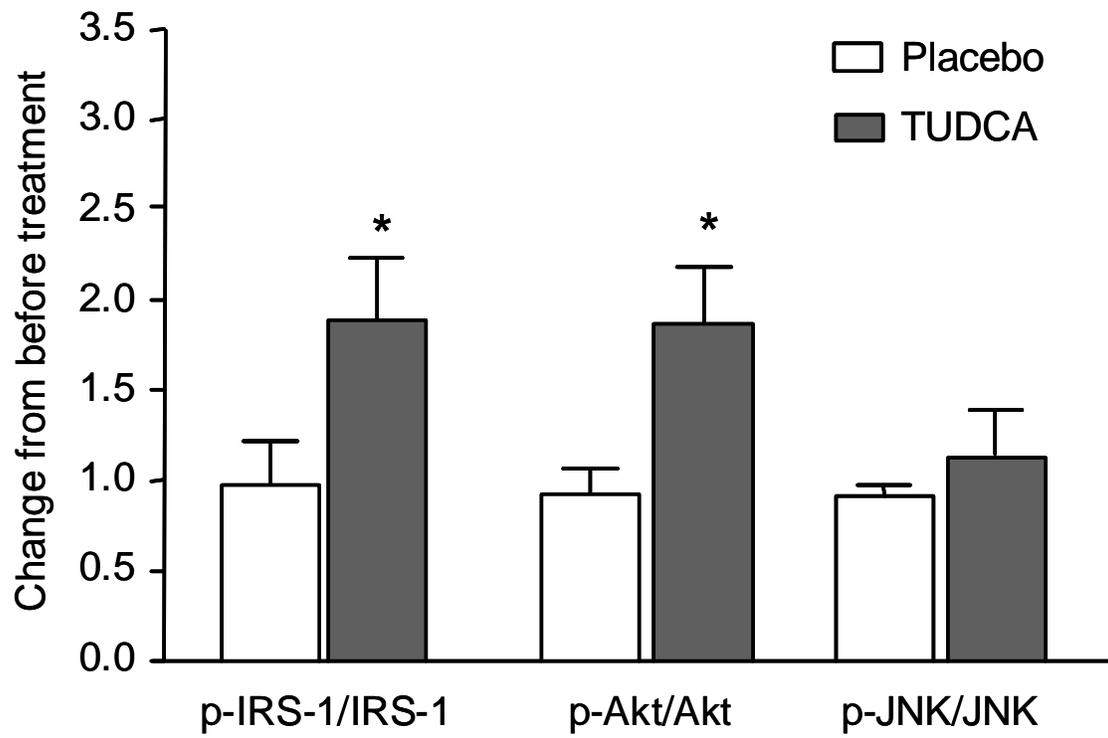
Figure 1

A   Hepatic Insulin Sensitivity Index

B   Glucose Rd

C   Palmitate Ra

Figure 2

Figure 3