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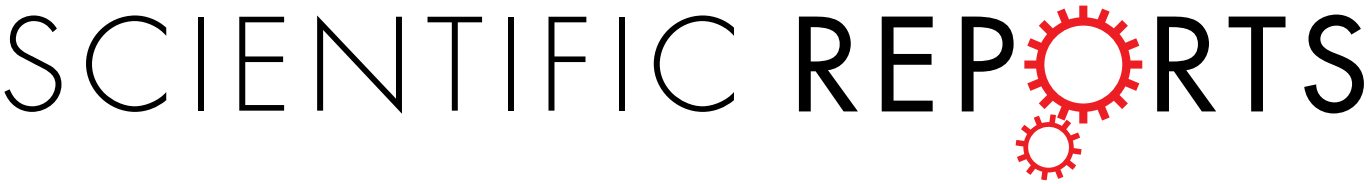

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Bile acid TUDCA improves insulin clearance by increasing the expression of insulin-degrading enzyme in the liver of obese mice

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Disruption of insulin secretion and clearance both contribute to obesity-induced hyperinsulinemia, though reduced insulin clearance seems to be the main factor. The liver is the major site for insulin degradation, a process mainly coordinated by the insulin-degrading enzyme (IDE). The beneficial effects of taurine conjugated bile acid (TUDCA) on insulin secretion as well as insulin sensitivity have been recently described. However, the possible role of TUDCA in insulin clearance had not yet been explored. Here, we demonstrated that 15 days treatment with TUDCA reestablished plasma insulin to physiological concentrations in high fat diet (HFD) mice, a phenomenon associated with increased insulin clearance and liver IDE expression. TUDCA also increased IDE expression in human hepatic cell line HepG2. This effect was not observed in the presence of an inhibitor of the hepatic membrane bile acid receptor, S1PR2, nor when its downstream proteins were inhibited, including IR, PI3K and Akt. These results indicate that treatment with TUDCA may be helpful to counteract obesity-induced hyperinsulinemia through increasing insulin clearance, likely through enhanced liver IDE expression in a mechanism dependent on S1PR2-Insulin pathway activation.

Obesity is the primary cause of hyperinsulinemia, which increases the risk for cancer and cardiovascular diseases[1,2] and potentiates insulin resistance that may trigger type 2 diabetes (T2D)[3]. High levels of plasma insulin concentration can be attributed to increased insulin secretion and/or decreased insulin clearance[4,5]; however, there is evidence that reduced insulin clearance is likely the primary factor in obesity-induced hyperinsulinemia[6].

Insulin clearance occurs mainly in the liver by the action of insulin-degrading enzyme (IDE), which degrades approximately 50% of insulin in its first passage through the hepatic portal system[7,8]. IDE is a zinc metalloproteinase which degrades not only insulin but also other amiloidogenic peptides such as glucagon[9], amylin[10] and amyloid β[11]. Thus, malfunction of this enzyme is associated with T2D and Alzheimer's Diseases (AD)[12,13].

While there is a consensus that increasing IDE function in AD patients could be useful to treat this pathology, this same therapeutic approach is uncertain for patients with T2D. Improvement of insulin signaling and glucose tolerance was observed in mice treated with the IDE inhibitor 6bK[14]. However, treatment with BDM44768, another IDE inhibitor, impaired glucose tolerance, despite increasing insulin signaling[15]. In addition, IDE-deficient mice display chronic hyperinsulinemia that induces, over time, glucose intolerance as well as insulin resistance[16], suggesting that the hyperinsulinemic state, due to IDE deficiency, could be a trigger for the development of T2D. In the same way, Goto-Kakizaki rats (a non-obese T2D animal model), which have a defect at the IDE gene, as well as some type 2 diabetic patients, exhibit reduced insulin clearance and augmented plasma insulin concentrations prior to the onset of T2D[17,18]. Therefore, we believe that therapeutic strategies focusing on increased IDE expression and insulin clearance could be helpful in the prevention and/or treatment of T2D, especially when hyperinsulinemia precedes the development of this pathology.

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	CON	CON + TUDCA	HFD	HFD + TUDCA
Body Weight (g)	30.33 ± 1.21	29.68 ± 1.48	41.22 ± 1.18*	33.88 ± 1.27*#
Perigonadal fat pad weight (g)	0.314 ± 0.03	0.276 ± 0.02	1.309 ± 0.10*	0.8746 ± 0.06#
Retroperitoneal fat pad weight (g)	0.154 ± 0.01	0.133 ± 0.01	0.630 ± 0.06*	0.336 ± 0.03*#
Fasted Glycemia (mg/dL)	90 ± 4.32	91.5 ± 10.0	111 ± 5.42*	89.38 ± 5.56
Fed Glycemia (mg/dL)	137 ± 7.78	121 ± 6.02	161 ± 2.00*	138 ± 6.48

Table 1. Final characterization of CON, CON + TUDCA, HFD and HFD + TUDCA mice. (*)Indicates statistic differences compared to CON, and (#)Indicates differences between HFD and HFD + TUDCA (One-way ANOVA followed by Newmans-Keuls posttest, $P < 0.05$). Data are mean ± SEM (n = 4–8).

In this sense, insulin sensitizer agents such as physical exercise, bariatric surgery and pioglitazone treatment have been found to reduce plasma insulin concentrations in obese rodents, through increased insulin clearance and improved glucose homeostasis[19–21]. However, exercise has a low adherence rate[22], bariatric surgery is an invasive procedure[23], and pioglitazone treatment has significant side effects[24]. Thus, the use of endogenous molecules that increase insulin clearance, without the side effects or adherence concerns, could be a potential treatment for hyperinsulinemia.

In this context, the taurine conjugated bile acid tauroursodeoxycholic (TUDCA) has emerged as a possible candidate due to its beneficial effect upon glucose homeostasis[25–27]. In the liver, TUDCA improves insulin sensitivity by reducing endoplasmic reticulum (ER) stress[28,29]. Also, TUDCA activates insulin signaling in the liver, by the interaction with the sphingosine-1-phosphate receptor 2 (S1PR2), resulting in PI3K/Akt pathway activation[30]. However, the effect of TUDCA upon insulin clearance, as well as hepatic IDE expression remains unclear.

Here, using high fat diet (HFD) mice as an experimental model of hyperinsulinemia, we demonstrated that treatment with TUDCA normalizes their plasma insulin concentrations by increasing insulin clearance. This effect is probably due to increased IDE expression in the liver. *In vitro* experiments, using hepatic human cell line HepG2, demonstrated that TUDCA also increases IDE expression, by a mechanism dependent on the interaction of TUDCA with the S1PR2 receptor, via the insulin signaling pathway. These findings suggest treatment with TUDCA as a promising therapeutic intervention for the control of hyperinsulinemia in obese pre-diabetic individuals.

Results

TUDCA reduced body weight, fat pad weight and blood glucose in HFD mice. As expected, body weight was significantly increased in HFD, compared with CON mice (Table 1). This effect was accompanied by higher perigonadal and retroperitoneal fat pad weight, as well as higher fed/fasted blood glucose concentrations. TUDCA treatment reduced body and fat pad weight in the HFD + TUDCA mice (Table 1) and also returned fed/fasted blood glucose concentrations to levels similar to the CON mice (Table 1). However, TUDCA treatment did not alter all these parameters in CON + TUDCA mice, corroborating previous studies[29,31].

TUDCA improved glucose tolerance and insulin sensitivity in HFD mice. To investigate the effects of TUDCA on glucose homeostasis, we performed intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT). After the glucose load, during ipGTT, all groups had a maximal glucose peak at 15–30 min (Fig. 1A). However, HFD mice presented higher blood glucose concentrations indicating an impairment of glucose tolerance, as judged by the higher AUC of blood glucose, compared with the other groups (Fig. 1B). Interestingly, HFD + TUDCA mice presented an improved glucose tolerance (Fig. 1A), as observed by the lower AUC of blood glucose, during ipGTT (Fig. 1B). During the ipITT, HFD mice displayed higher blood glucose, compared with CON mice (Fig. 1C), suggesting impaired insulin sensitivity in these HFD mice, as judged by the glucose disappearance rate (KITT) (Fig. 1D). The treatment with TUDCA restored insulin sensitivity in HFD + TUDCA mice (Fig. 1D), increasing the KITT values (Fig. 1D). Finally, we assessed plasma insulin levels in the fed and fasted state and we observed that HFD increased plasma insulin concentrations in both states (Fig. 1E and F) and the treatment with TUDCA restored this parameter in HFD + TUDCA to levels similar to those found in CON mice (Fig. 1E and F).

TUDCA increased insulin clearance and IDE expression, but not IDE activity, in the liver of HFD mice. Plasma insulin concentration is controlled by insulin secretion and clearance. To measure insulin secretion, we assessed the plasma concentration of C-peptide during ipGTT. After glucose administration, the HFD group presented increased C-peptide levels during the test (Fig. 2C), indicating higher insulin secretion in this group compared to CON mice (Fig. 2D). Also, plasma insulin concentration was increased in the HFD mice (Fig. 2A and B), reducing the C-peptide:insulin ratio. Insulin and C-peptide are co-secreted by the pancreatic β cells (ratio 1:1); however C-peptide has a longer half-time than insulin. Thus, reduction in the C-peptide:insulin ratio indicates a reduced insulin clearance, as we observed in the HFD mice (Fig. 2E and F). The TUDCA treatment, in HFD mice, did not alter the higher insulin secretion, as we observed by the elevated plasma C-peptide concentration during the ipGTT (Fig. 2C and D); however, it reduced plasma insulin concentration (Fig. 2A and B), restoring the C-peptide:insulin ratio to similar levels of CON group (Fig. 2E and F), indicating a reestablishment of insulin clearance in these HFD + TUDCA mice. To elucidate the mechanism whereby TUDCA restored insulin clearance in HFD mice, we also investigated the IDE expression and activity in the liver of these mice. As expected, IDE expression (Fig. 3A) and activity (Fig. 3B and C) are reduced in HFD mice, supporting the lower

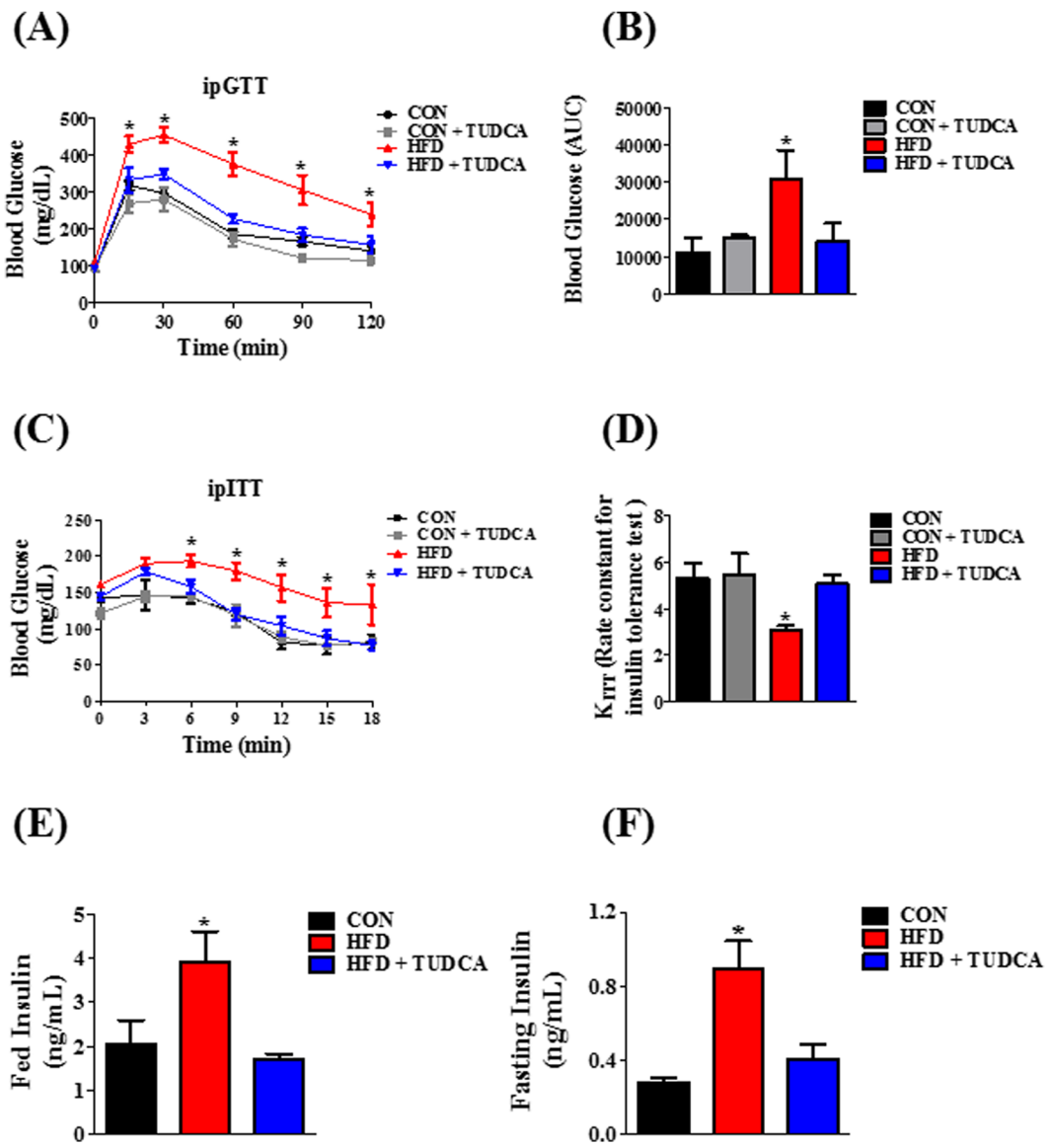

Figure 1. TUDCA treatment improves glucose tolerance, insulin sensitivity and insulinemia in HFD mice. Blood glucose during ipGTT (**A**) and ipITT (**C**). Area under the curve (AUC) of total blood glucose concentration during ipGTT (**B**) and glucose disappearance rate during ipITT (KITT) (**D**). Plasma insulin in fed (**E**) and fasting (**F**) state. Mice were fed a control diet (CON and CON + TUDCA) or high fat diet (HFD and HFD + TUDCA) for 12 weeks, and received or not i.p. 300 mg/kg TUDCA during 15 days, as indicated. Data are mean ± SEM (n = 4–8). *P ≤ 0.05 *vs* CON.

insulin clearance in this group. Although TUDCA treatment did not alter IDE activity (Fig. 3B and C), it restored the IDE protein expression in the HFD + TUDCA to levels similar to that of CON mice (Fig. 3A), explaining at least in part the reestablishment of insulin clearance in these mice.

TUDCA increased IDE expression in HepG2 cells by an S1PR2–IR pathway dependent mechanism. To assess the direct effect of TUDCA on IDE expression, we performed *in vitro* experiments using human liver cell line HepG2. First, these cells were exposed to different concentrations of TUDCA; after 24 h incubation, we observed that TUDCA increased IDE expression at 50, 100 and 200 μM (Fig. 4A). Thus, in the subsequent experiments, we used 100 μM TUDCA. It is known that the activation of the insulin pathway increases IDE expression in neurons[32] and the bile acid TUDCA activates the insulin pathway mainly by the S1PR2 receptor in the liver[30]. Figure 4B shows that in the presence of the S1PR2 inhibitor, JTE-013, TUDCA failed to increase the expression of IDE. In addition, TUDCA also failed to increase IDE expression in the cells incubated with S196 (an IR inhibitor)[33], MK-2206 (an Akt inhibitor) or Wortmannin (a PI3K inhibitor)[34] (Fig. 4C and D, respectively).

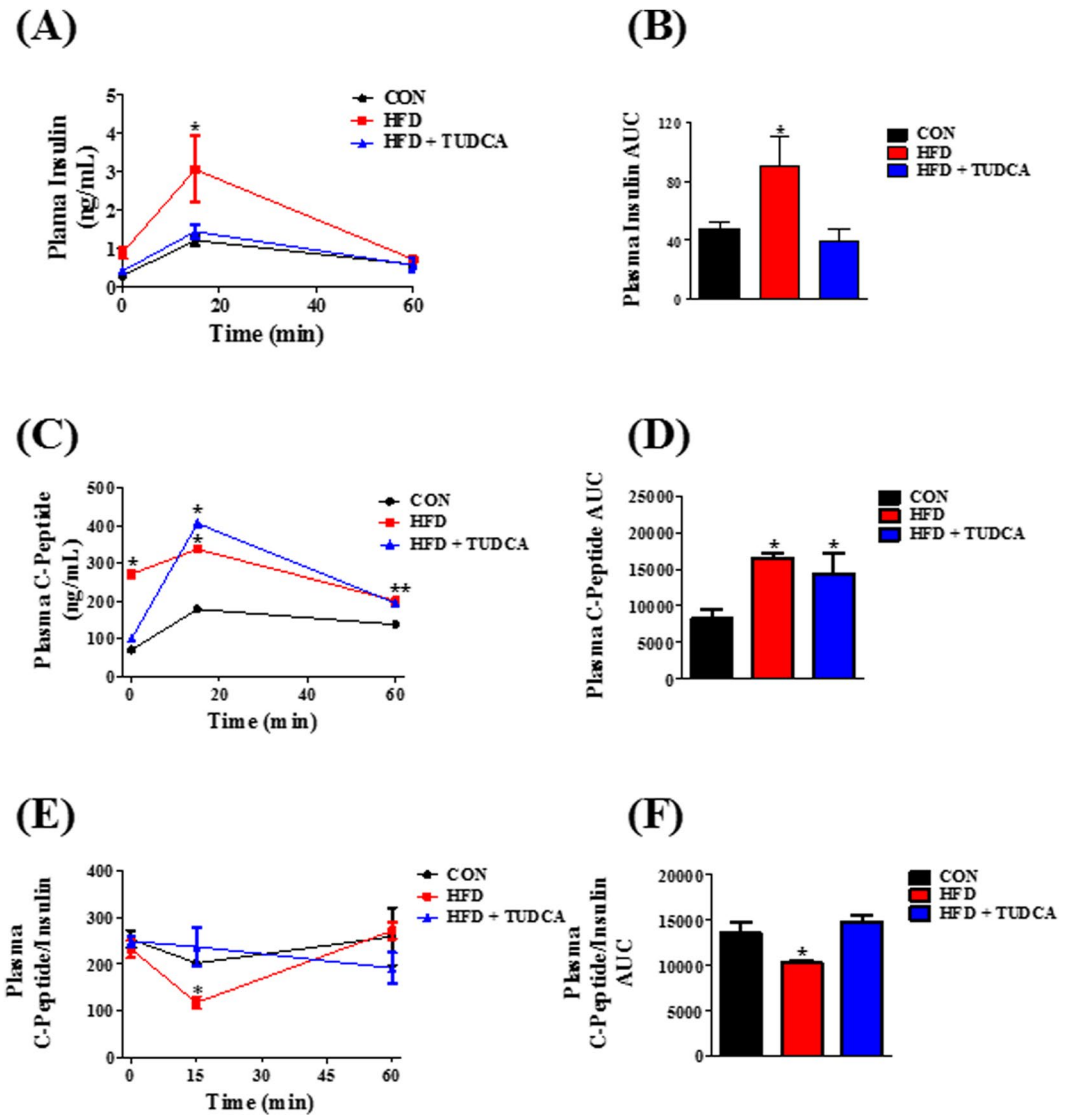

Figure 2. TUDCA treatment increases insulin clearance in HFD mice. Plasma levels of insulin (**A**), C-peptide (**C**) and the C-peptide/Insulin ratio (**E**). AUC of plasma insulin concentration (**B**), C-peptide (**D**) and C-peptide/Insulin ratio (**F**). Mice were fed a control diet (CON) or high fat diet (HFD and HFD + TUDCA) for 12 weeks, and received or not i.p. 300 mg/kg TUDCA during 15 days, as indicated. Data are mean ± SEM (n = 4–8). *P ≤ 0.05 *vs* CON.

Discussion

Plasma insulin concentration is controlled by the interaction between insulin secretion and insulin clearance[7,8]. Prolonged hyperinsulinemia is associated with reduced IR tyrosine kinase activity, and the normalization of plasma insulin concentrations recovers insulin signaling[3]. In addition, non-obese mice, with over-expression of the insulin gene, present high insulin levels associated with impaired insulin sensitivity and, consequently, T2D[35]. Therefore, therapeutic interventions targeting insulin clearance could be relevant to treatment and/or prevention of this pathology. Here, we demonstrate that 15 days of TUDCA treatment ameliorates insulin clearance in HFD mice, probably by increasing IDE expression in the liver. In addition, we also demonstrate that TUDCA increases IDE expression in HepG2 cells, through a mechanism dependent on activation of the S1PR2/Insulin pathway.

As already shown[31], we observed that TUDCA treatment decreased body weight due to a reduction in fat pad deposits, which was associated with improved glucose tolerance and insulin sensitivity. Similarly, 30 days of treatment with TUDCA also improved insulin signaling in the liver of ob/ob mice[29]. This improvement could be explained at least in part by the reduction of ER stress induced by TUDCA, which acts as a potent chemical chaperone enhancing ER capacity and protein folding activity. The improvement of ER stress induced by TUDCA also occurs in the hypothalamus, which in turn, decreased the body weight of high fat diet fed mice, by increased their energy expenditure through a stimulation of UCP1 protein expression in the white and brown adipose tissue, without alterations on food intake[36].

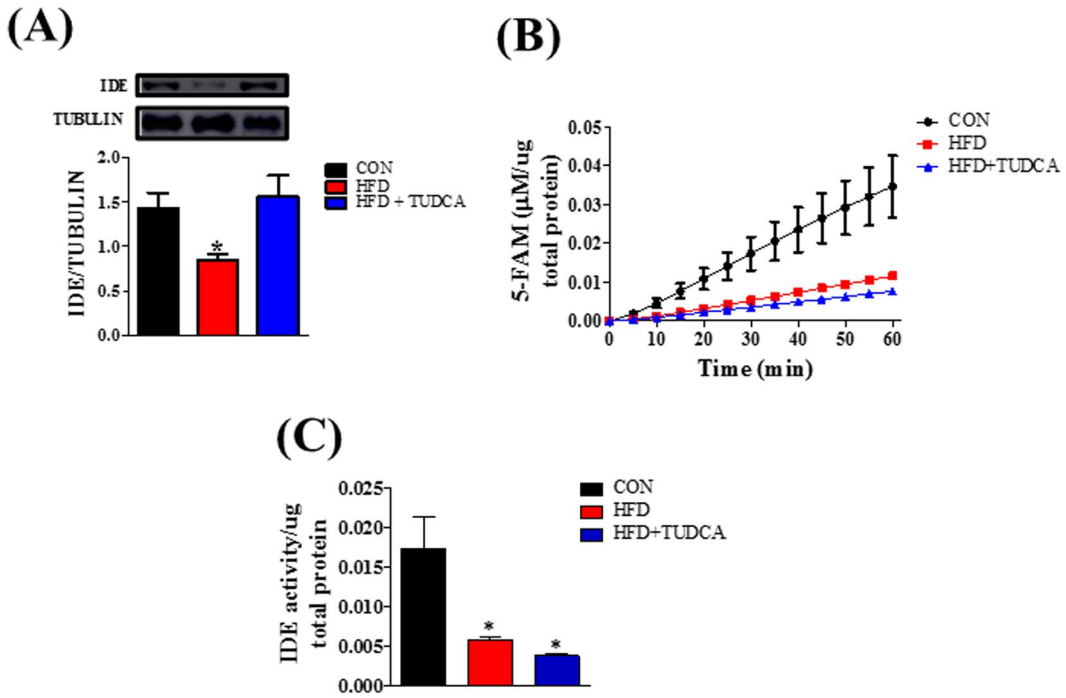

Figure 3. TUDCA treatment increases IDE expression, but not activity in HFD mice. Protein expression of IDE in the liver and its representative immunoblotting images (**A**). Kinetic of IDE activity assay (**B**) and total IDE activity (**C**) in the liver of mice. Fluorescent intensity at Ex/Em = 490/520 nm was recorded, every 5 min, during 60 min. 5-FAM concentration was calculated using a standard curve and normalized per µg of total protein. Mice were fed a control diet (CON) or high fat diet (HFD and HFD + TUDCA) for 12 weeks, and received or not i.p. 300 mg/kg TUDCA during 15 days, as indicated. Data are mean ± SEM (n = 4–8). Data are mean ± SEM (n = 4–8). *P ≤ 0.05 *vs* CON.

In both the HFD and HFD + TUDCA groups, plasma C-peptide concentrations were increased. In HFD mice, this increase indirectly reflects augmented insulin secretion, probably as a consequence of insulin resistance[37]. In the HFD + TUDCA mice, which had lower insulin resistance than HFD mice, the increased insulin secretion may be a direct effect of TUDCA on pancreatic islets[38].

The mechanism whereby TUDCA increases insulin secretion has been investigated. In pancreatic islets of pigs, TUDCA reduced ER stress induced by thapsigargin and increased insulin secretion, suggesting this bile acid as an important enhancer of islet function[39]. Furthermore, in pancreatic islets of mice incubated with a cAMP competitor or a PKA inhibitor, the increase of insulin secretion induced by TUDCA was blunted, indicating the involvement of this pathway in the mechanism whereby TUDCA increases the secretion of this hormone[38].

Although the effect of TUDCA upon insulin clearance had not been investigated before, previous data indicated a possible role of this bile acid in the degradation of this hormone. It was observed that 30 days of treatment with TUDCA reduced plasma insulin concentration in ob/ob mice[29]. Considering that TUDCA increased insulin secretion in pancreatic islets[38,39], the lower insulinemia found in those obese mice could possibly be explained by changes in insulin clearance. Indeed, we demonstrated that treatment with TUDCA increases insulin removal in HFD mice, suggesting this bile acid as an important insulin clearance booster. We believe that this effect of TUDCA could be due to increased protein expression of IDE, the main enzyme responsible for insulin degradation[7], since the activity of this enzyme did not change in the liver of HFD + TUDCA mice.

In an attempt to explore the direct effect of TUDCA upon IDE expression, we incubated the human hepatic cell line HepG2, with or without TUDCA, and we confirmed that TUDCA *per se* increases IDE expression in these cells. These data reinforce our premise that this bile acid may improve insulin clearance by increasing IDE expression in the liver, contributing to the normalization of plasma insulin levels in hyperinsulinemic pre-diabetic mice.

In addition to the well-known effects of bile acids as regulators of lipid digestion and absorption in the small intestine[40], it was suggested that they also act as hormones, dependent on the bile acid type binding with specific receptors[38–42]. In the hepatic cell TUDCA is a ligand of a G-protein coupled protein receptor called S1PR2[30]. The binding of TUDCA and other conjugated bile acids to S1PR2 activates the insulin pathway at the IR-PI3K-Akt level. Here, we show that TUDCA increases IDE expression in hepatic cells and this effect seems to be dependent on S1PR2-IR-PI3K-Akt activation pathway.

Previous studies have demonstrated that different interventions that improve insulin signaling are associated with increased insulin clearance and IDE expression in obese mice[19,20]. In fact, neural cells exposed to insulin showed increased IDE expression, whereas insulin pathway inhibition, at the PI3K level, avoided the

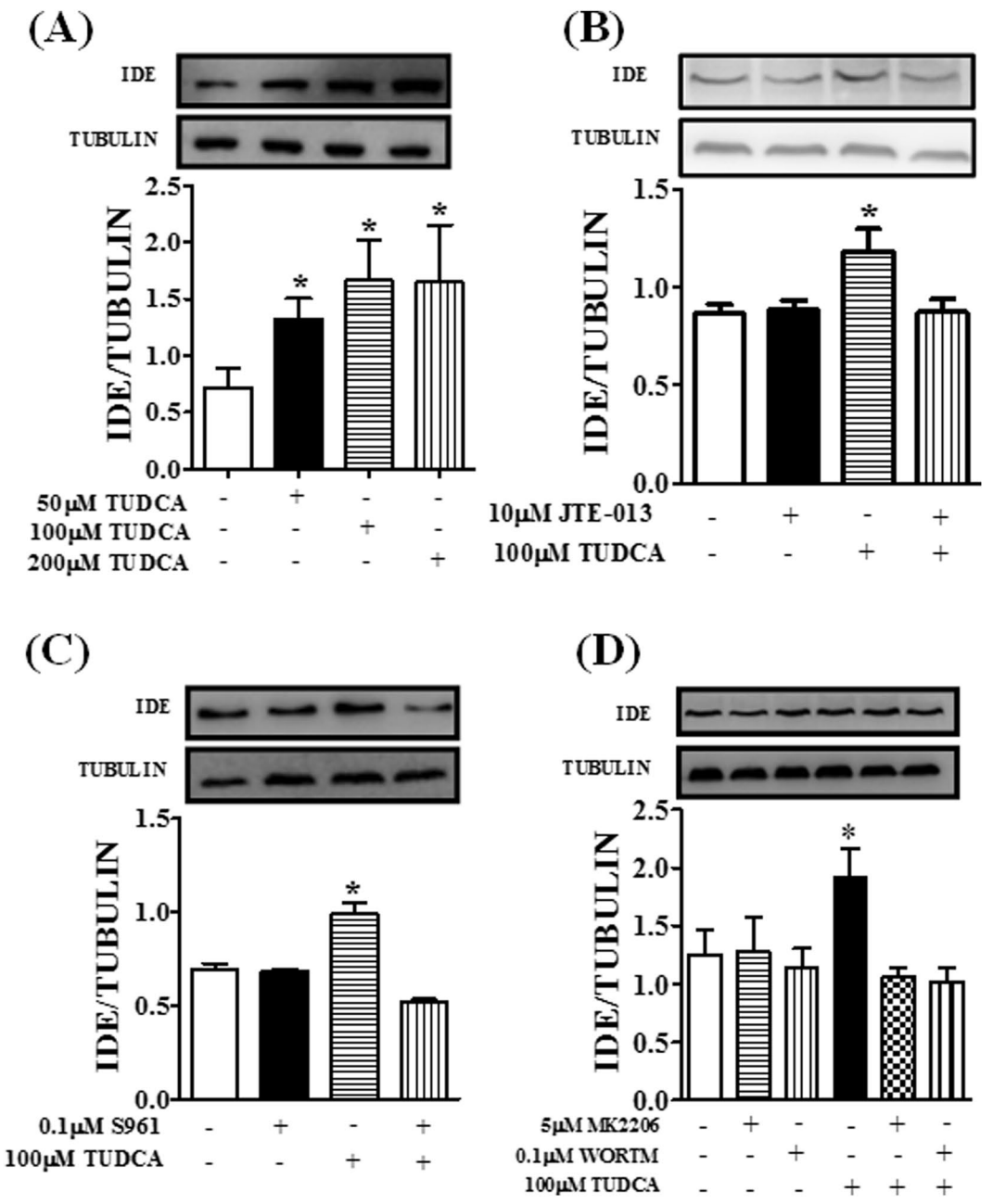

Figure 4. TUDCA modulates IDE expression in HepG2 cells by a S1PR2 -IR receptor pathway. Protein expression of IDE in HepG2 cells treated or not with different concentrations of TUDCA for 24-h (**A**). Effect of TUDCA on IDE expression in the presence of 10 μM sphingosine-1-phosphate receptor 2 inhibitor (JTE-013) (**B**), 0.1 μM insulin receptor inhibitor (S961) (**C**) and 5 μM of Akt inhibitor (MK2206) or 0.1 μM of PI3k inhibitor (Wortmannin) (**D**). Data are mean ± SEM (n = 4–6). *P ≤ 0.05 *vs* control conditions.

insulin-induced IDE expression[32]. This evidence supports our findings about the possible role of the insulin pathway over IDE expression.

Although this evidence indicates that increased insulin-signaling might induce IDE expression, others studies have interpreted the evidence differently, suggesting that IDE expression could also affect the insulin pathway[43]. Human subjects, with a polymorphism at the *IDE* gene, develop insulin resistance and T2D[44]. Likewise, Goto-Kakizaki rats, which have a defect at the *IDE* gene, develop hyperinsulinemia, insulin resistance and, ultimately, T2D[17]. Also, IDE knockout mice are hyperinsulinemic, which contributes to chronic insulin signaling stimulation, which in turn results in insulin resistance through reduced IR expression in skeletal muscle, adipose tissue and the liver[16].

Our data confirm the important role of TUDCA in the regulation of insulin signaling and secretion, and further elucidates the function of this bile acid on insulin metabolism and its importance in pathological conditions such as obesity and T2D. Although elevated serum bile acids were observed in T2D patients[45], improved glucose metabolism

in obese patients was related to the increase of serum bile acids after bariatric surgery[46]. This phenomenon seems to be due to the higher expression of enzymes responsible for bile acid synthesis and conjugation, such as cholesterol 7α hydroxylase (CYP7A1), bile acid: CoA synthase (BACS) and bile acid-CoA: amino acid N-acyltransferase (BAAT)[47]. Indeed, overexpression of CYP7A1, which increased bile acid synthesis, prevented high fat diet-induced obesity and also insulin resistance in mice[48], pointing bile acids as an interesting target to fight these pathologies.

In conclusion, our outcomes provide evidence that TUDCA may be a therapeutic strategy to counteract obesity-induced hyperinsulinemia. We found that TUDCA increased insulin clearance in HFD mice, probably through increased IDE expression in the liver, reestablishing their plasma insulin levels. Our results also indicated that TUDCA-induced IDE expression seems to be mediated by the S1PR2-Insulin signaling pathway.

Materials and Methods

Reagents. TUDCA was purchased from Calbiochem (Sao Paulo, Brazil, cat. 580549) and Insulin and C-Peptide Elisa Kits were acquired from Millipore (Darmstadt, Germany, cat. #EZRMI-13K and #EZRMCP2-21K, respectively). Western Blot reagents were purchased from Bio-Rad (Madrid, Spain) and antibodies were acquired from Abcam (Cambridge, UK) and Sigma Aldrich (St Louis, MO, USA). The remaining reagents were purchased from Sigma Aldrich.

Animals. The experiments involving animals were approved by the Animal Care Committee at UNICAMP (license number: 3815-1) and were conducted in accordance to the last revision of the National Institutes of Health (NIH) guide for the care and use of laboratory animal. Male 21-days old C57Bl/6 mice were obtained from the breeding colony at UNICAMP and maintained at 22 ± 1 °C, on a 12-h light–dark cycle. After 1 month, the mice were fed a standard diet (CON) or a high fat diet with 35% fat (HFD) during 12 weeks. On the last 15 days of this period, the mice received, i.p., PBS (groups CON and HFD) or 300 mg/kg TUDCA (groups CON + TUDCA and HFD + TUDCA). The mice were killed in a CO_2 chamber and decapitated for blood collection and removal of the liver for posterior analyses.

Intraperitoneal Glucose and Insulin Tolerance Tests. At the end of treatment with TUDCA, the mice were subjected to 12-h fasting to perform the ipGTT. The fasting blood glucose level were measured (time 0) by a glucometer. After, the mice received an i.p. glucose load of 2 g/kg body weight and the glycemia was measured at 15, 30, 60 and 120 min after the glucose load. For the ipITT, the mice were subjected to a 2-h fasting and the glycemia was measured before (time 0) and 3, 6, 9, 12, 15 and 18 min after the i.p. administration of 0.75 U/kg insulin load. The KITT was calculated as previously described[49].

Plasma Insulin and C-peptide measurements. Mouse insulin and C-peptide Elisa Kits (Darmstadt, Germany, cat. #EZRMI-13K and #EZRMCP2-21K, respectively) were used to measure plasma insulin and C-peptide. The plasma samples were obtained by centrifugation of blood samples at 1100 g, 15 min, 4 °C. The assays were performed as indicated on kit protocol. The blood samples for insulin measurements were collected on fed and fasted state, as well as at the ipGTT times 0, 15 and 60 min. The C-peptide was measured in these same plasma samples of ipGTT, used for insulin measurements.

Cell culture and treatment. HepG2 liver cell line were cultured in DMEN (Vitrocell, Campinas, SP, Brazil), enriched with 10% (vol./vol.) fetal bovine serum (FBS) for 3 days, under a humidified condition with 5% CO_2 at 37 °C. After that, the cells were incubated in the presence, or not, of different TUDCA concentrations (T50, T100 and T200 μM) during 24-h. The concentration of 100 μM was adopted for the following experiments. The inhibitors of insulin pathway and bile acid receptors were added when necessary, as describe on figure legends. In the experiments with S961, MK2206 or Wortmannin, the cells were submitted to a 6-h serum starved before treatment.

Western blot analysis. Liver samples were collected and homogenized with 500 μL of Cell Lysis Buffer. For HepG2 Western blot, after treatment, the cells were collected in trypsin/EDTA, washed with PBS, and homogenized in urea anti-protease/anti-phosphatase buffer. For SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis, all samples were treated with a Laemmli buffer containing dithiothreitol. After heating to 95 °C for 5 min, proteins were separated by electrophoresis in a 10% polyacrylamide gel. The transfer to nitrocellulose membranes was performed in a Trans Blot transfer for 2-h in 100 V, with Tris/Glycine buffer. After, the membranes were blocked with 5% non-fat dry milk buffer (5% milk, 10 mM TRIS, 150 mM NaCl and Tween 20 0.02%) during 1-h, and then, they were incubated with a polyclonal antibody against IDE (Abcam, cat. ab32216). Tubulin (Sigma Aldrich, cat. 6074) was used as control of the experiment. Visualization of specific protein bands was performed by incubating the membranes with appropriate secondary antibodies. Protein bands were visualized using the Amersham Imager 600 (GE Healthcare Life Sciences, Buckinghamshire, UK) which detects the chemiluminescence. The band intensities were quantified with the Image J software (National Institutes of Health, Bethesda, MD, USA).

IDE activity. Liver IDE activity measurement was performed using the SensoLyte 520 IDE Activity Assay Kit (AnaSpec, Fremont, CA, USA, cat. AS-72231) following the manufacturer's instructions. The total IDE activity was calculated as previously described[19] and normalized per μg of total protein, which was determined using Bradford reagent. The kinetic concentration of 5-FAM was also normalized per μg of total protein.

Statistical analysis. The data were presented as means ± standard errors media (SEM) for 4–8 animals, or obtained for 3 different cells experiments, each one in triplicate. The comparisons between all groups were performed by one-way ANOVA analysis followed by Newman-Keuls test. When the comparisons were determined between two groups Student´s t-test was adopted. The difference between the groups were considered statistically significant if $P \leq 0.05$.

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Author Contributions

J.F.V., J.M.C.J. and M.A.K. contributed to research design and J.F.V., M.A.K., G.M.S., P.C.B., S.M.F., R.C.S.B. and L.S.L.M. conducted the experiments and acquired data. A.C.B. and E.M.C. provided all reagents. J.F.V. contributed to data analysis and J.F.V., M.A.K. and J.M.C.J. to data interpretation. J.F.V., J.M.C.J. and M.A.K. wrote the manuscript. A.C.B., J.M.C.J. and E.M.C. revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Additional Information

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