Tauroursodeoxycholic acid improves glucose tolerance and reduces adiposity in normal protein and malnourished mice fed a high-fat diet

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ABSTRACT

Early childhood malnutrition may facilitate the onset of obesity and diabetes mellitus in adulthood which, when established, makes it more resistant to therapeutic interventions. The beneficial effects of tauroursodeoxycholic acid (TUDCA) in glucose homeostasis and body fat accumulation were analyzed in protein-restricted mice fed a high-fat diet (HFD). C57BL/6 mice were fed a control (14% protein [C]) or a protein-restricted (6% protein [R]) diet for 6 weeks. Afterward, mice received an HFD or not for 12 weeks (C mice fed an HFD [CH] and R mice fed an HFD [RH]). In the last 15 days of this period, half of the mice fed a HFD received i.p. PBS (groups CH and RH) or 300 mg/kg TUDCA (groups CH and RH). RH mice developed obesity, as demonstrated by the increase in fat accumulation, liver steatosis, and metabolic inflexibility. Additionally, showed glucose intolerance and insulin hypersecretion. TUDCA reduced adiposity and improve metabolic flexibility through increased HSL phosphorylation and CPT1 expression in eWAT and BAT, and reduced ectopic fat deposition by activating the AMPK/HSL pathway in the liver. Also, improved glucose tolerance and insulin sensitivity, normalizing insulin secretion by reducing GDH expression and increasing insulin peripheral sensitivity by greater expression of the IRβ in muscle and adipose tissue and reducing PEPCK liver expression. Our data indicate that TUDCA reduces global adiposity and improves glucose tolerance and insulin sensitivity in protein malnourished mice fed a HFD. Therefore, this is a possible strategy to reverse metabolic disorders in individuals with the double burden of malnutrition.

1. Introduction

Despite the global efforts, undernutrition is still a major burden in developing countries, affecting about 151 million children and contributing to about 45% of deaths in children under-5 years of age worldwide (Dalvi et al., 2018; Wells et al., 2020). In addition, studies indicate that low birth weight induced by intrauterine or postnatal protein malnutrition is a risk factor for the development of chronic non-communicable diseases such as obesity, hypertension and type 2 diabetes mellitus (DM2) in adulthood (Hales & Barker, 2013; Vaag et al., 2012; Victora et al., 2008). In contrast, obesity is also a public health problem, affecting around 600 million adults worldwide, mainly in
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Developed but also in low-income and middle-income countries (LMICs) (Symposium, n.d.; WHO, 2020). The growth in the number of overweight and obese adults in LMICs has given rise to the phenomenon of double burden of malnutrition (DBM).

DBM is characterized by the simultaneous manifestation of undernutrition and overweight/obesity and affects most LMICs, being recognized by WHO and present in 3–35% of families across the globe, varying from country to country (Barry M., Camila C., Laurence M., 2020; Demaio & Branca, 2018). DBM is currently recognized as a form of nutritional alteration and obesity that has a different genesis and treatment to the obesity phenotype arising only from the consumption of high-calorie diets (Dalvi et al., 2018). It was demonstrated that the protein restriction during pregnancy and lactation exacerbated insulin resistance induced by a high-fat diet (HFD) in adult rodents (Holness et al., 2020). Furthermore, some studies have also shown that protein restriction followed by an HFD induces obesity, hepatic steatosis, glucose intolerance, peripheral insulin resistance, insulin hypersecretion and impaired mitochondrial metabolism in pancreatic islet (Batista et al., 2013; Leite et al., 2016; Vettorazzi et al., 2014). However, it is important to note that this obesity type showed to be resistant to conventional treatments.

It was demonstrated that vagotomy was only effective in reducing body weight, adiposity and basal glycemia in obese mice that were not fed a low-protein diet in adolescence (Lubaczewska et al., 2017). In addition, it has been demonstrated that supplementation with taurine, an amino acid that already demonstrated several physiological properties, reduced adiposity, insulin hypersecretion, and improved glucose tolerance and insulin action in obese mice fed a HFD (Freitas et al., 2019; Kim et al., 2019; Ribeiro et al., 2012). However, taurine supplementation did not lead to these same effects in obese mice that undergo protein malnutrition (Batista et al., 2013; Branco et al., 2017; Camargo et al., 2015; Cappelli et al., 2014; Vettorazzi et al., 2014). This indicates that DBM model rodents are more resistant to the therapeutic strategies already tested, suggesting the use of other targets to improve the damages in glycemic homeostasis caused by obesity after protein restriction.

The use of some bile acids (BA) in the treatment of metabolic diseases has been described for thousands of years in Chinese medicine (Wang et al., 2014). Among these, tauroursodeoxycholic acid (TUDCA), formed from the association of ursodeoxycholic acid (UDCA) and the amino acid taurine, has been gaining prominence. TUDCA is a endogenous component produced from primary BAs that are metabolized in the small intestine by intestinal bacteria and return to the liver, by the enterohepatic circulation, for conjugation with the amino acid taurine (Kusaczuk, 2019). This conjugation makes this BA more polar and soluble, reducing its toxicity and increasing its therapeutic efficiency (Vettorazzi et al., 2016). Currently, the use of TUDCA for the treatment of primary biliary diseases is approved by the Food Drug Administration (Kusaczuk, 2019), however, experimental and clinical trial evidence has emphasized the involvement of TUDCA in improving glucose homeostasis and reducing adiposity (Cha et al., 2014; Legry et al., 2014; Macho et al., 1999; Ozcan et al., 2006; Xia et al., 2017), suggesting a key role as a therapeutic agent for obesity, insulin resistance and DM2.

TUDCA treatment has already been shown to reduce endoplasmic reticulum stress in several tissues (Kusaczuk, 2019), in addition to increase the viability in pancreatic β cell treated with fatty acids (Zhu et al., 2013) and to increase insulin secretion in mouse islets by a mechanism mediated by the cAMP/PKA/CREB pathway (Vettorazzi et al., 2016). In relation to the insulin action, was demonstrated that TUDCA treatment improved liver and muscle insulin sensitivity in obese humans, due to an increase of the IRS/IRS-1 and IRS-2/p-Akt pathway (Kars et al., 2010). Furthermore, TUDCA also acts on lipid metabolism, increasing lipolysis in adipocyte culture treated with TNF-α (Xia et al., 2017) and reducing hepatic steatosis in obese rodents (Legry et al., 2014). However, TUDCA action on adiposity and glycemic homeostasis in malnourished mice fed a HFD still unknown. Therefore, in this study, we investigated new insights about the damages in energy metabolism and glycemic homeostasis in DBM mice and the benefits of treatment with TUDCA upon these parameters. Our results, showed TUDCA as a potential therapeutic agent for the treatment of comorbidities associated with obesity programmed by a low-protein diet in adolescence.

2. Methods

2.1. Animal model

Male 21-day-old C57BL/6 mice were obtained from the breeding colony at UNICAMP and were maintained at 22 ± 1 °C on a 12-h light-dark cycle, with free access to food and water. Mice were initially randomly grouped as follows: mice fed a regular protein diet (14% protein) (control diet (C) group) or mice fed a 6% protein-restricted diet (R) group. After 6 weeks, the C and R groups were subdivided and received or not an HFD (35% fat) for 12 weeks [C + HFD (CH) and R + HFD (RH), respectively]. On the last 15 days of this period, half of the mice fed a HFD received, i.p., PBS (groups CH and RH) or 300 mg/kg TUDCA (Tauroursodeoxycholic Acid, Sodium Salt - CAS 14605–22–2 – Calbiochem; groups CHT and RHT). The dose of TUDCA (300 mg/kg body weight) was adopted following the protocol published by Vettorazzi et al., 2017. At 18 weeks of treatment, mice were euthanized in a chamber with CO2 and the blood was collected and centrifuged and the plasma samples were stored at −80 °C. Liver, epididymal white adipose tissue (eWAT), brown adipose tissue (BAT) and gastrocnemius muscle samples (Gastroc) were also collected, snapped frozen, and stored at −80 °C. All experiments involving animals were approved by the Animal Care Committee at UNICAMP (No. 5283-1). Diet compositions were described in Table S1 (Supplementary material) and the experimental models described in Fig. 1. Total mice used: C (7), CH (7), CHT (6), R (7), RH (7) and RHT (6) were expressed also in the captions of the figures. Other mice were used, additionally, for liver histology experiments after reviews C (2), CH (2), CHT (2), R (2), RH (2) and RHT (2).

2.2. Nutritional status assessment

During the 18 weeks of the experimental period, all groups of mice were weighed weekly. Food consumption was measured once a week and feed efficiency were obtained by the ratio from the total BW gained divided by the total food consumption in the experimental period. At 20 weeks of age, mice from 6 experimental groups were weighed and the nasoanal lengths were measured to obtain the Lee index [BW (g)1/3/ nasoanal length (cm) × 1000]. Fasted mice were euthanized in a chamber with CO2, and the retroperitoneal, epididymal and brown adipose tissues were weighed. Blood was collected, and plasma was stored at −20 °C. Plasma cholesterol (CHOL), triglycerides (TG) and total proteins levels were measured using colorimetric standard commercial kits (CHOL: Biolcin K0083-3; TG: Biolcin K177-3 and total proteins: Biolcin K031-1) according to the manufacturer’s instructions.

2.3. Intraperitoneal glucose (ipGTT) and insulin tolerance tests (ipITT)

At 20 weeks of age, 12 h-fasted mice received an intraperitoneal (ip) injection of glucose (2 g/kg BW). Blood was collected from the tip of the tail vein, and glycemia was measured using an automatic glucometer (Accu-Chek Performa, Roche Diagnostic, USA), before (time (T) 0) and 15, 30, 60, 120 and 180 min after glucose injection. Insulinemia was also measured at 0 and 30 min by rat/mouse insulin ELISA kit (ZMBI-13 K: Millipore-Sigma, St. Charles, MO, USA). To ipITT test, after fasting for two hours, the baseline glycemia was measured by a glucometer and the mice received, intraperitoneally, 0.75 U/Kg body weight of human recombinant insulin (Humulin, Indianapolis, USA). Blood glucose was monitored at 10, 15, 30, 45 and 60 min after insulin administration.
2.4. Islet isolation and static insulin secretion

Islets were isolated by collagenase digestion of the pancreas. For static insulin secretion, pancreatic islets (4 islets per well) were incubated for 1 h with Krebs-bicarbonate buffer (KBB; (in mmol/L) 115 NaCl, 5 KCl, 2.56 CaCl2, 1 MgCl2, 10 NaHCO3, 15 HEPES), supplemented with 5.6 mmol/L glucose and 0.3% BSA and equilibrated with a mixture of 95% O2/5% CO2 to regulate the pH at 7.4. After pre-incubation time, this medium was discarded and replaced by fresh KBB, and the islets were incubated for 1 h with 2.8 and 11.1 mM glucose. After 1 h of incubation time, the medium was removed and stored at −20 °C. Insulin levels were measured by rat/mouse insulin ELISA kit (EZRM1-13 K; Millipore-Sigma, St. Charles, MO, USA). Total islet protein was assayed using the Bradford dye method (BRADFORD, 1976) with BSA as the standard curve.

2.5. Western blot analysis

For the SDS-PAGE and Western blot analysis, the total protein extract from liver, muscle, eWAT, and groups of 250 isolated islets were homogenized with Cell Lysis Buffer (10 mM EDTA, 100 mM TRIS, 100 mM Sodium Pyrophosphate, 100 mM Sodium Fluoride, 100 mM Sodium Orthovanadate, 2 mM PMSF, 0.1 mg/ml Aprotinin and 10% Triton). After that, the protein quantification was performed by Bradford (BRADFORD, 1976). Total islet protein was separated by electrophoresis in a 10% SDS-PAGE gel and the islet samples were electrophoretically transferred onto a PVDF membrane in Tris-glycine buffer. Membranes were then incubated with a polyclonal antibody against pAKβ (Cell Signaling, Catalog no 9217, 1:1000); AKT (Cell Signaling, Catalog no 2021, 1:1000); GLUT4 (Santa Cruz, Catalog no 3122, 1:1000); CPT1A (Cell Signaling, Catalog no 2765, 1:1000); and GAPDH (Sigma-Aldrich, Catalog no 6724, 1:1000) as loading controls. Detection of the protein signal was made after 1 h incubation with a secondary property antibody by the chemiluminescence method. Chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, ThermoFisher, Catalog no 34096) was detected by digital imaging system (Amersham Imager 600, GE Healthcare). Optical density was quantified using ImageQuant TL software (GE Healthcare).

2.6. Histological analysis of liver and adipose tissue

After euthanasia, liver and eWAT fragments were collected and fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 24 h at room temperature. Samples were washed 3x with PBS and maintained in 70% etOH at 4 °C. eWAT samples were embedded in paraffin, sectioned and stained with hematoxylin-eosin. Adipocyte perimeter was determined using the ImageJ software (National Institutes of Health, MD, USA) by averaging counts from approximately 70 cells (±20 cells)/section, 3 sections per sample. Liver samples were then included in embedding medium (Tissue Plus O.C.T. compound, Scigen Scientific, Gardena, Houston, USA) and frozen at −80 °C for 24 h. Sections of 7 μm in thickness were obtained at −15 °C in a cryostat. These sections were fixed with 4% formaldehyde for 10 min and submitted to staining with Oil Red O (O.R.O; (Mehlem et al., 2013), counterstained with hematoxylin, and were then mounted with Canada balsam and cover slips for image capture. Ten random fields of 2 liver sections for each group were used to measure the positive area percentage for O.R.O staining, per field analyzed, with the aid of Image J software. Adipocyte and liver images were captured under 20X objective lens in an optical microscope using an optical microscope (Olympus BX51).
2.7. Hepatic lipid content

Liver samples were also collected to extract hepatic lipids through the Folch’s method (JORDI FOLCH, M. LEES, 1957). The extract was evaporated and diluted in isopropanol. Then, the measurement of triglycerides and cholesterol was performed according to the manufacturer’s instruction (Roche, Mannheim, Germany).

2.8. Respiratory quotient and energy expenditure

One week before euthanasia, a group of mice was individually placed in sealed metabolic cages, and after 24 h of acclimatization, O\textsubscript{2} consumption and CO\textsubscript{2} production were measured for 24 h (Oxylet system; Pan Lab/Harvard Instruments, Barcelona, Spain). Respiratory quotient (RQ) and Energy Expenditure (EE) were then calculated using the O\textsubscript{2} and CO\textsubscript{2} data by Metabolism\textsuperscript{®} Software (Pan Lab/Harvard Instruments).

2.9. Statistical analysis

Results are presented as mean ± SEM for the number of determinations (n) indicated. Data were first analyzed using the Shapiro-Wilk normality test and subsequently submitted to parametric (One-Way ANOVA followed by Bonferroni) or non-parametric (Kruskal-Wallis followed by Dunns) unpaired tests, using the GraphPad Prism software, and the level of significance was set at P < 0.05. Graphs were also performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, San Diego, CA, US). Some data were directed to Two-way ANOVA followed by Bonferroni.

3. Results

3.1. TUDCA treatment reduces the weight gain and fat accumulation in CHT and RHT mice

Initially, we investigated whether the hypoproteic and hyperlipidic diet would be effective in the development of undernutrition and obesity, and also characterized the effects of TUDCA treatment upon mice features. As shown in Fig. 2 (D and I), mice fed on a low-protein diet developed undernutrition as indicated by a reduction in the body weight (BW) and total plasma protein levels in R compared with C mice. After 8 weeks on high-fat diet, CH and RH mice showed an increase in body weight compared with C and R groups (Fig. 2A-B). Furthermore, both the CH and RH groups developed obesity, as displayed an increase in feed efficiency, final BW, Lee index, plasma cholesterol levels and epididymal, retroperitoneal, and interscapular brown fat accumulation. TUDCA treatment reduced feed efficiency, final BW, Lee index, plasm cholesterol levels, and fat pad weight in CHT and RHT mice (Fig. 2). As shown in Fig. S1 (supplementary material) TUDCA treatment reduced food efficiency without changing food intake. This demonstrates that this difference was linked with the reduction in body weight observed after treatment. No difference was found in plasma triglycerides and total protein levels between the obese groups and those treated with TUDCA. Therefore, TUDCA was effective in reducing adiposity in both obese groups.

3.2. TUDCA treatment improves glucose tolerance, insulin sensitivity and reduces insulin secretion in CHT and RHT mice

To investigate the effects of TUDCA on glucose homeostasis, we performed intraperitoneal glucose and insulin tolerance tests (ipGTT and ipITT) and measure insulin secretion. As shown in Fig. 3 undernutrition mice reduced fasting blood glucose, insulinemia (0 and 30 min after glucose load) (Fig. 3D-G), and insulin secretion stimulated by 11.1 mM of glucose when compared with C mice (Fig. 3K). HFD diet induced glucose intolerance and hyperinsulinemia before and after glucose load in CH and RH mice. However, only RH mice presents increased basal glycemia (Fig. 3D). Besides that, pancreatic islets of CH and RH mice secreted more insulin stimulated by 11.1 mM and showed an increase in protein expression of glutamate dehydrogenase (GDH) (Fig. 3K-L). GDH is an important component in the amplifying pathway (AMP PATH) to insulin secretion and data have already shown that its content and activity are increased in islets of RH mice (Leite et al., 2016). TUDCA treatment improves glucose tolerance, as observed by the lower AUC of blood glucose during ipGTT, reduced the insulinemia before and after a glucose intraperitoneal injection, and the insulin secretion and GDH expression in CHT and RHT mice. As shown in Fig. 3 (H-J) undernutrition led to increased insulin sensitivity, represented by the lower AUC during the pITT, when compared to C. Moreover, before the test CH and RH mice had higher glucose compared to C and R. TUDCA treatment reduced the insulin resistance, as showed by the lower AUC of glycemia during ipITT, in the CHT and RHT mice (Fig. 3H-J).

3.3. TUDCA treatment increases insulin action in peripheral tissue of CHT and RHT mice

Next, we investigated whether improvement of glucose homeostasis in CHT and RHT mice was also associated with increase insulin action in muscle, liver, and adipose tissues. For this, we assessed the expression of the β subunit of the insulin receptor (IR\textbeta) as well as the basal phosphorylation of protein kinase B/Akt (p-AKT), one of its down-stream effectors. As shown in Fig. 4, protein restriction led to increased protein expression of IR\textbeta in adipose tissue and gastrocnemius muscle. The HFD diet reduced IR\textbeta levels in all evaluated tissues and the p-AKT/AKT ratio expression in BAT only in RH mice. In RHT mice, the TUDCA treatment increased IR\textbeta, p-AKT/AKT ratio and Akt substrate of 160 kDa (p-AS160) expression in muscle (Fig. 4A-B), IR\textbeta and GLUT4 in eWAT (Fig. 4D and F), IR\textbeta, p-AKT/AKT ratio and GLUT4 levels in BAT (Fig. 4G-I) and IR\textbeta expression in liver (Fig. 4J). Furthermore, the liver of RHT mice showed a reduction in the protein expression of PEPCK (Fig. 4L), an important enzyme for gluconeogenesis, indicating possible participation of TUDCA in the control of hepatic glucose production. Taken together, these results suggest that TUDCA improves glucose tolerance by reducing insulin secretion and increasing the action of insulin on the liver, muscle, and adipose tissue of both obese groups.

3.4. TUDCA treatment alters lipid metabolism in liver and adipose tissue of CHT and RHT mice

To determine whether the reduction in fat accumulation by TUDCA treatment in CHT and RHT mice was also associated with increased insulin action in muscle, liver, and adipose tissues. For this, we assessed the expression of the β subunit of the insulin receptor (IR\textbeta) as well as the basal phosphorylation of protein kinase B/Akt (p-AKT), one of its down-stream effectors. As shown in Fig. 4, protein restriction led to increased protein expression of IR\textbeta in adipose tissue and gastrocnemius muscle. The HFD diet reduced IR\textbeta levels in all evaluated tissues and the p-AKT/AKT ratio expression in BAT only in RH mice. In RHT mice, the TUDCA treatment increased IR\textbeta, p-AKT/AKT ratio and Akt substrate of 160 kDa (p-AS160) expression in muscle (Fig. 4A-B), IR\textbeta and GLUT4 in eWAT (Fig. 4D and F), IR\textbeta, p-AKT/AKT ratio and GLUT4 levels in BAT (Fig. 4G-I) and IR\textbeta expression in liver (Fig. 4J). Furthermore, the liver of RHT mice showed a reduction in the protein expression of PEPCK (Fig. 4L), an important enzyme for gluconeogenesis, indicating possible participation of TUDCA in the control of hepatic glucose production. Taken together, these results suggest that TUDCA improves glucose tolerance by reducing insulin secretion and increasing the action of insulin on the liver, muscle, and adipose tissue of both obese groups.
Fig. 2. TUDCA treatment reduces body weight, fat stores, and plasma triglycerides in previously protein malnourished obese mice. A, B: Weekly record of body weight for 18 weeks from C (n = 7), CH (n = 7), CHT (n = 6), R (n = 7), RH (n = 7) and RHT mice (n = 6). C: Mean ± SEM of feed efficiency, D: final body weight (BW), E: Lee’s index, F: the weight of perigonadal adipose tissue, G: retroperitoneal adipose tissue and H: brown adipose tissue (BAT), and I: plasma total proteins, J: cholesterol and K: triglycerides of male mice obese, previously malnourished, treated or not with 300 mg/kg of TUDCA. Different letters over the bars indicate statistically significant differences. Data A-B were analyzed by Two-way ANOVA followed by Bonferroni. Data C-K were analyzed by One-Way ANOVA followed by Bonferroni (P < 0.05).
Fig. 3. TUDCA treatment restores glucose tolerance, insulin sensibility and reduces insulin secretion by GDH pathway modulation in previously protein malnourished obese mice. A-B: Changes in blood glucose and C mean ± SEM of the total plasma glucose concentrations during the ipGTT, expressed by the AUC in C mice (n = 7), CH (n = 7), CHT (n = 6), R (n = 7), RH (n = 7) and RHT (n = 6) in the eighteenth week of treatment. D-E: Mean ± SEM of glycemia and F-G: insulinemia during time 0 and 30 min of ipGTT. H-I: Glycemic profile during ipITT and J: mean ± SEM of AUC of total glycemia observed in C, CH, CHT, R, RH, and RHT mice. K: Glucose-induced insulin secretion in islets isolated of all experimental groups. Groups of 4 islets were incubated for 1 h with different glucose (G) concentrations. Each bar represents the mean ± SEM of 6–7 independent experiments. L: Representative blots and densitometric analysis of glutamate dehydrogenase (GDH) in islets pancreatic of the different experimental groups. Different letters over the bars indicate statistically significant differences. ns: no significant difference. Data C-G were analyzed by One-Way ANOVA followed by Bonferroni; Data A-B and H-I were analyzed by Two-way ANOVA followed by Bonferroni and J-L were analyzed by Kruskal-wallis followed by Dunns (P < 0.05).
Fig. 4. TUDCA treatment increases insulin action in peripheral tissues from previously protein malnourished obese mice. IRβ, pAKT/AKT ratio, pAS160, GLUT4 and PEPCK protein expressions in peripheral tissues from C (n = 7), CH (n = 7), CHT (n = 6), R (n = 7), RH (n = 7) and RHT (n = 6) mice. Fragments obtained from the skeletal muscle (A-C), white adipose tissue (D-F), brown adipose tissue (G-I) and liver (J-L) were used for immunoblotting experiments. The bars represent mean ± SEM of densitometric values. Different letters represent statistically significant differences. Data A-C, E-F, H-I and K-L were analyzed by One-Way ANOVA followed by Bonferroni; Data D, G and J were analyzed by Kruskal-wallis followed by Dunns (P < 0.05).
together, these data demonstrate that TUDCA reduces fat accumulation and ectopic fat deposition by increasing lipolysis in eWAT and the liver.

3.5. TUDCA treatment improves metabolic flexibility in CHT and RHT mice

The next step in this work was to evaluate whether the reduction in adiposity observed in the CHT and RHT group could also be related to the modulation of energy expenditure (EE) and oxidative capacity of BAT. As showed in Fig. 7, protein malnutrition increased EE and maintained metabolic flexibility in R mice. Analysis of EE over 24 hr showed that HFD diet reduced EE in both the light and dark cycles only in CH mice. However, the analyzes of respiratory quotient (RQ) values showed that CH and RH mice were metabolically inflexible, as judged by their incapacity to increase RQ values (Fig. 7G), evidencing a lower carbohydrate oxidation capacity. The treatment with TUDCA did not modify the energy expenditure in the CHT and RHT groups, however, it improved metabolic flexibility in both obese groups, as observed by increased RQ values during the dark cycle, indicating higher carbohydrate oxidation. This may be due to the increased protein expression of the pAMPK, pHSL, CPT1 and uncoupling protein 1 (UCP1) in BAT from obese mice treated with TUDCA, being more effective in RHT mice (Fig. 7H-J).

4. Discussion

Population studies point to low birth weight induced by intrauterine or postnatal malnutrition as a risk factor for the development of chronic non-communicable diseases such as obesity, hypertension, and DM2 in adulthood (Aubin et al., 2012; Ganfarani et al., 1999; Hales & Barker, 2013; Sawaya et al., 2003). Moreover, the association between protein malnutrition and obesity, a phenotype known as the DBM, is a form of nutritional alteration and obesity, having a different genesis and treatment of obesity originating only from high-calorie diets (Wells et al., 2020). In animal models, ingestion of a high-fat diet by previously malnourished rodents led to increased body weight, fat accumulation, hyperleptinemia, glucose intolerance, insulin resistance, higher hepatic glucose production, metabolic inflexibility, and insulin hypersecretion (Batista et al., 2013; Branco et al., 2017; Camargo et al., 2015; Cappelli et al., 2014; Leite et al., 2016; Vettorazzi et al., 2014).

Here, for the first time, we provide evidence that fasting hyperglycemia along with glucose intolerance and insulin resistance observed in RH mice is associated with reduced expression of IRβ in peripheral insulin responsive tissues and increased insulin secretion by the GDH pathway. IRβ is an important protein in the peripheral insulin pathway since the insulin binding to the extracellular domain of its receptor allows IRβ to assume tyrosine kinase activity and autophosphorylate in various tyrosine residues phosphorylating the substrates of IR into tyrosine. This takes the activation of the PI-dependent protein kinase,
and subsequently to AKT. AKT activation and phosphorylation stimulates glucose uptake in muscle and adipocytes via translocation of GLUT4 vesicles to the plasma membrane and inhibits hepatic glucose production by phosphorylation of forkhead box protein O1 (Boucher et al., 2014). The downregulation of the IRβ protein may be associated with basal hyperinsulinemia observed in RH mice, and already demonstrated in DBM models (Lubaczeuski et al., 2017; Venci et al., 2020), working in a negative feedback mechanism dose-and time dependent. The hyperinsulinemia observed in the RH group was associated, in addition to resistance to the hormone, with increased insulin secretion. In this work, we demonstrated that RH mice secrete more insulin stimulated by high glucose concentration due, at least in part, to increased protein expression of GDH, an important protein in the insulin secretion amplification pathway (Vetterli et al., 2012). It is known that

Fig. 6. TUDCA treatment improves hepatic steatosis via AMPK/pHSL pathway in previously protein malnourished obese mice. A-B: Oil Red O stained liver sections (20x magnification; PS = portal space), C: weight, D-E: the content of triglycerides and cholesterol, representative blots and densitometric analysis of F: pAMPK and G: pHSL in the liver from C (n = 9), CH (n = 9), CHT (n = 8), R (n = 9), RH (n = 9) and RHT mice (n = 8). The bars represent mean ± SEM of densitometric values. Different letters represent statistically significant differences. Data A-C, E and G were analyzed by One-Way ANOVA followed by Bonferroni; Data D and F were analyzed by Kruskal-wallis followed by Dunns (P < 0.05). Arrows indicate larger lipid droplets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
islets from RH mice present mitochondrial dysfunction leading to an increased contribution of the amplification pathway to insulin secretion (Leite et al., 2016).

Bile acids TUDCA has been already used in the treatment of several liver diseases (Amaral et al., 2009; Vang et al., 2014), however, recently it has been shown to extend beyond hepatobiliary disorders. Experimental evidence has emphasized the involvement of TUDCA in protecting against endoplasmic reticulum stress, acting as a molecular chaperone restoring glycemic homeostasis, and thus playing a key role in the pathogenesis of obesity, insulin resistance, and DM2 (Ozcan et al., 2006; Ozcan, et al., 2009). A study with obese humans showed improvement in insulin sensitivity after 1.75 mg of daily TUDCA intake for 4 weeks. The muscle and liver tissue of these individuals recovered more than 30% of insulin sensitivity, via activation of the p-IRS/AKT pathway, rivaling current anti-diabetic pharmaceutical products (Kars et al., 2010). However, the precise cellular mechanisms responsible for the improvement of peripheral insulin sensitivity are not yet clear.

TUDCA and other taurine conjugated bile acids have a hydrophilic profile, which gives them greater affinity by nuclear farnesoid X receptor (FXR) as well as membrane Takeda G protein receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2) (Wan & Sheng, 2018). It is known that TUDCA activates the ERK1/2 and AKT signaling pathways primarily via S1PR2 in primary rodent hepatocytes (Fang et al., 2007). Also, it was already demonstrated that the FXR activation by conjugated bile acids in hepatocytes, increased the AKT phosphorylation and repressed the gluconeogenic genes, as PEPCK in a Gαi independent manner (Cao et al., 2010). Regarding glucose uptake, it was demonstrated that the treatment for 3 weeks with TUDCA increased, through the inhibition of histone deacetylases, the protein expression of GLUT4 in the gastrocnemius muscle of rats exposed to ethanol during pregnancy (Yao et al., 2014). Confirming these findings, in this work, TUDCA treatment improved glucose tolerance and insulin sensitivity in both groups of obese mice. These facts were associated with an increase in the IRβ/GLUT4 pathway in peripheral tissues, which allowed greater glucose uptake, reduction in hepatic glucose production via IRβ/PEPCK, but also by a reduction in insulin secretion via the negative regulation of GDH.

Recent studies have shown that TUDCA has important actions on the pancreatic β cell function, modulating the viability and insulin secretion. It was demonstrated that TUDCA improved cell viability by reducing the ATF4/CHOP pathway, rivaling current anti-diabetic pharmaceutical products (Kars et al., 2010). However, the precise cellular mechanisms responsible for the improvement of peripheral insulin sensitivity are not yet clear.

TUDCA and other taurine conjugated bile acids have a hydrophilic profile, which gives them greater affinity by nuclear farnesoid X receptor (FXR) as well as membrane Takeda G protein receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2) (Wan & Sheng, 2018). It is known that TUDCA activates the ERK1/2 and AKT signaling pathways primarily via S1PR2 in primary rodent hepatocytes (Fang et al., 2007). Also, it was already demonstrated that the FXR activation by conjugated bile acids in hepatocytes, increased the AKT phosphorylation and repressed the gluconeogenic genes, as PEPCK in a Gαi independent manner (Cao et al., 2010). Regarding glucose uptake, it was demonstrated that the treatment for 3 weeks with TUDCA increased, through the inhibition of histone deacetylases, the protein expression of GLUT4 in the gastrocnemius muscle of rats exposed to ethanol during pregnancy (Yao et al., 2014). Confirming these findings, in this work, TUDCA treatment improved glucose tolerance and insulin sensitivity in both groups of obese mice. These facts were associated with an increase in the IRβ/GLUT4 pathway in peripheral tissues, which allowed greater glucose uptake, reduction in hepatic glucose production via IRβ/PEPCK, but also by a reduction in insulin secretion via the negative regulation of GDH.
channels and increased insulin secretion in islets of mice (Düfer et al., 2012). In our study, TUDCA normalized insulin secretion in the CHT and RHT groups, possibly by reducing GDH expression. It is known that the GDH activity can be negatively regulated by FXR activation in the liver (Renga et al., 2011), however, our study reports the possible action of TUDCA in the GDH modulation and insulin secretion control.

In addition to glucose intolerance, we demonstrated that RH mice also developed obesity linked to white adipocyte hypertrophy, hepatic steatosis, metabolic inflexibility with reduced pAMPK / HSL pathway in adipose and hepatic tissues. AMPK is a protein kinase essential for the metabolism of glucose and fatty acids. The general effect of AMPK is the exchange of ATP-consuming mechanisms such as lipogenesis and gluconeogenesis with ATP-producing mechanisms such as the oxidation of glucose and fatty acids (Kahn et al., 2005). In adipocytes, AMPK also shows an antilipolytic action by phosphorylation of HSL in the Ser 565 residue, decreasing its activation by protein kinase A (Sullivan et al., 1994). Besides, AMPK mediates glucose transport in resting skeletal muscle through AS160 phosphorylation and inhibition, which increases the translocation of GLUT4 to the membrane (Treebak et al., 2006).

In our study, treatment with TUDCA reduced the white adipocyte perimeter and hepatic steatosis, effect associated with increased protein expression of AMPK/HSL pathway in the liver and adipose tissues of the RHT group. The greater activation of AMPK in the RHT group may be involved in the greater preventive action of TUDCA against the accumulation of fat, since AMPK phosphorylates and inactivates acetyl-CoA carboxylase (ACC), which reduces the values of malonyl-CoA, an inhibitor of carnitine palmitoyl transferase and allows the oxidation of fatty acids (Winder & Hardie, 1996). In the liver, adipose tissue and muscle, AMPK inhibits glycerol phosphate acyltransferase by reducing the synthesis of triglycerides (TG) (Park et al., 2002). Also, AMPK increases expression of PGC-1α that elevates mitochondrial biogenesis and oxidative phosphorylation (Nisoli et al., 2003). Thus, this kinase is involved in both processes that are mediated by the hormone insulin, as well as in the oxidation of energy substrates, and its modulation by TUDCA can have a protective effect against the energy imbalance that occurs in obesity associated with protein malnutrition.

The characterization of TUDCA action on BAT thermogenesis, as well as its possible effector pathways, are still unknown. It was demonstrated that treatment with TUDCA increased oxygen consumption in the brown adipose tissue of mice, also related to the gene expression of UCP1, PGC-1α and GLUT4 in a primary brown adipocyte culture. These effects were partially lost after deletion of the type 2 deiodinase gene of these adipocytes, suggesting a possible TUDCA pathway in thermogenesis (Da-Silva et al., 2011). Besides, it was found that intracerebroventricular treatment with TUDCA increased body temperature and UCP1 expression in obese rodents (Contreras et al., 2017). It is noteworthy that TUDCA treatment also increased the protein expression of UCP1 in the BAT and improved the metabolic flexibility in the RHT group. This may be associated with the increased oxidation of fatty acids observed after treatment with TUDCA (via AMPK/HSL), but also with the higher rate of glucose oxidation (via IRβ/GLUT4) in BAT. Thereby, we characterized the action of TUDCA in increasing glucose uptake and oxidation of fatty acids and, therefore, improving metabolic flexibility in obesity even when it is not derived from protein malnutrition.

**Fig. 8. Graphical Abstract: TUDCA improves glucose tolerance and reduce adiposity from protein malnourished mice fed on a high-fat diet.** TUDCA treatment had implications in the adiposity in obese protein malnourished mice, increasing lipolysis in eWAT, reducing the perimeter of adipocytes and promoting greater oxidation of substrates in BAT restoring the metabolic flexibility. Furthermore, reversed hepatic steatosis via activation of AMPK /pHSL pathway in the liver of these rodents. TUDCA also improved glucose homeostasis, increasing the insulin action in peripheral tissues, promoting greater glucose uptake and reducing glucose hepatic production, besides normalized insulin secretion via negative GHβ regulation. These data sets highlight TUDCA as an effective therapeutic molecule for reversing the pathophysiological changes observed in individuals with DBM.
Although TUDCA is not yet used as a therapy in the treatment of obesity-associated comorbidities, a single study has demonstrated the effects of treatment with TUDCA in increasing insulin sensitivity in the liver and skeletal muscle of obese humans (Kars et al., 2010). However, the mechanisms responsible for these effects are still not understood. In our work, we demonstrated that TUDCA reduces adiposity (via AMPK/HSL) and improves glycemic homeostasis (via IRB/GLUT4) in the model of obesity preceded by undernutrition (see Fig. 8). These findings describe molecular mechanisms that contribute to the effect of TUDCA on energy and glucose metabolism during obesity. Also, highlights the importance of studies looking for alternatives to ensure greater systemic bioavailability of this bile acid, so that it can be tested and used as an oral antiobesogenic supplement. Therefore, our results support preclinical studies in evaluating the benefits of TUDCA in reversing obesity-associated comorbidities, when derived or not from undernutrition, and open paths for conducting clinical studies.

5. Conclusion

Our data demonstrated, for the first time, that the association of obesity with protein malnutrition results in hypertrophy of white adipocyte, hepatic steatosis, metabolic inflexibility, and glucose intolerance, associated with insulin resistance and hypersecretion. Also, we revealed that TUDCA treatment reduced adiposity and fat ectopic deposition via AMPK/HSL pathway and improved glucose homeostasis, normalizing insulin secretion via GDH and increasing glucose uptake via IRB/GLUT4 upregulation in RHT mice. These data highlight TUDCA as a highly effective therapeutic strategy for reversing disorders in lipid and glucose metabolism in DBM.

CRediT authorship contribution statement

Thiago dos Reis Araujo: Conceptualization, Methodology, Data curation, Validation, Investigation, Formal analysis, Software, Writing – original draft, Writing – review & editing. Mariana Roberta Rodrigues Muniz: Data curation, Validation, Investigation, Formal analysis, Software, Writing – review & editing. Bruna Lourenço Alves: Data curation, Validation, Investigation, Writing – review & editing. Lohanna Monali Barreto dos Santos: Data curation, Validation, Investigation, Writing – review & editing. Maressa Fernandes Bonfim: Data curation, Validation, Investigation, Writing – review & editing. Joel Alves da Silva Junior: Data curation, Validation, Investigation, Writing – review & editing. Jean Francisco Vettorazzi: Conceptualization, Methodology, Writing – review & editing. Claudio Cesar Zoppí: Writing – review & editing. Everardo Magalhães Carneiro: Conceptualization, Methodology, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Disclosures

None.

Author contributions


Appendix A. Supplementary material

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