RESEARCH ARTICLE

Taurine supplementation improves liver glucose control in normal protein and malnourished mice fed a high-fat diet

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Scope: Poor nutrition during the perinatal period is associated with an increased risk for metabolic syndrome in adulthood. Taurine (TAU) regulates β-cell function and glucose homeostasis. Here, we assessed the effects of TAU supplementation upon adiposity and glucose control in malnourished mice fed a high-fat diet (HFD).

Methods and results: Weaned male C57BL/6J mice were fed a control (14% protein - C) or a protein-restricted (6% protein - R) diet for 6 weeks. Afterwards, mice received or not an HFD for 8 weeks (CH and RH). Half of the HFDmice were supplemented with 5% TAU after weaning (CHT and RHT). Protein restriction led to typical malnutrition features. HFD increased body weight, adiposity, and led to hyperleptinemia, hyperphagia, glucose intolerance, and higher liver glucose output in RH and CH groups. Fasted R mice showed higher plasma adiponectin levels and increased phosphorylation of the AMP-activated protein kinase (p-AMPK) in the liver. These parameters were reduced in RH mice and increased p-AMPK persisted in RHT. TAU prevented obesity and improved glucose tolerance only in CHT, but liver glucose control was ameliorated in both supplemented groups. Better CHT liver glucose control was linked to increased Akt (thymoma viral proto-oncogene/protein kinase B) phosphorylation.

Conclusion: Malnourished mice fed an HFD developed obesity, glucose intolerance, and increased liver glucose output. TAU preserved only normal liver glucose control in RHT mice, an effect associated with increased liver p-AMPK content.

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1 Introduction

Insulin resistance is a risk factor for type 2 diabetes mellitus (T2DM). It is characterized by the impaired action of insulin on the inhibition of liver glucose output and decreased

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Abbreviations: Akt, thymoma viral proto-oncogene/protein kinase B; **AMPK**, AMP-activated protein kinase; **AUC**, area under curve; **BCAA**, branched-chain amino acids; **BCATm**, mitochondrial branched chain amino acid aminotransferase; **BW**, body weight; **CHOL**, cholesterol; **GITT**, glucagon tolerance test; **GTT**, glucose tolerance test; **GLUT-4**, glucose transporter 4; **HFD**, high-fat diet; **ITT**, insulin tolerance test; **LKB1**, liver kinase B1; **L-NAME**, L-NG-nitroarginine methyl ester; **NEFA**, nonesterified fatty acid; **NO**, nitric oxide; **ONOO⁻**, peroxynitrite; **PI3K**, phosphoinositide 3-kinase; **PTT**, pyruvate tolerance test; **TAU**, taurine; **TG**, triglyceride; **T2DM**, type 2 diabetes mellitus

adipocyte and muscle glucose uptake [1]. Several studies have shown a correlation between low birth weight and risk of insulin resistance and T2DM in later life [2–4]. This correlation is supported by reports of abnormal function of phosphoinositide 3-kinase (PI3K), including lower Akt phosphorylation and glucose transporter (GLUT)-4 protein expression in the muscles of low birth-weight men [5, 6].

Protein malnutrition during pregnancy is a frequent cause of low birth weight. Rodent models of dietary protein restriction are useful for understanding metabolic adaptations that may occur during this period and during adult life. Adult offspring from low-protein dams showed normal blood glucose during oral glucose tolerance test despite lower insulinemia. The higher glucose tolerance is explained by increased insulin sensitivity [7, 8]. However, this effect seems to be transitory, since, rats from malnourished mothers, at 15 months of age present impaired adipocyte glucose uptake and become diabetic [9].

Taurine (TAU), is a sulfur-containing amino acid found in high concentrations in the plasma and tissues of mammals [10]. This amino acid demonstrates several physiological properties and, amongst these, its antidiabetic activity is the object of intense research. TAU supplementation has been shown to improve insulin sensitivity and normalized blood glucose, plasma insulin, hypertension, dyslipidemia, and β-cell function in prediabetic and diabetic rodents [11–13]. In addition, TAU concentration is reduced in plasma of diabetic subjects [14, 15]. Finally, TAU-supplemented malnourished rats have showed enhanced nutrient-induced insulin secretion and recovered glucose tolerance [16, 17].

The aim of this study was to evaluate the progression of obesity and body glucose control in mice fed either a normal or a low-protein diet for 6 weeks, followed by a high-fat diet (HFD) for additional 8 weeks. In addition, the preventive effect of TAU supplementation upon the metabolic damages induced by HFD in normal and low-protein diet mice was studied.

2 Materials and methods

2.1 Animals and diets

All experiments were approved by the ethics committee at UNICAMP (protocol number: 1942–1). Male C57Bl/6J mice were obtained from the breeding colony at UNICAMP and maintained at 22 ± 1°C, on a 12-h light–dark cycle, with free access to food and water.

Weaned 30-day-old mice were randomly distributed in the following groups: mice that received a normal-protein diet (14% protein) without (Control group: C, $n = 21$) or with 5% TAU in their drinking water; or mice submitted to a protein-restricted diet (6% protein) without (Restricted group: R, $n = 19$) or with 5% TAU in their drinking water [13, 16, 18, 19]. After 6 weeks, C and R groups were subdivided and received, or not, an HFD (35% fat) for 8 weeks (CH and RH, $n = 21$) [20]. All TAU-treated mice were kept on the supplementation protocol and were also fed an HFD for 8 weeks (CHT, $n = 20$; and RHT, $n = 18$). All experimental procedures listed below were developed at the end of diet and TAU treatment (14 weeks). Diet compositions are described in Table 1.

Table 1. Composition of the diets

Ingredient (g/kg)	Control	Low protein	High fat
Casein	140	71.5	140
Cornstarch	465.7	502.5	208.7
Dextrinized cornstarch	155	166.5	100
Sucrose	100	121	100
L-cystine	1.8	1	1.8
Fiber	50	50	50
Soybean oil	40	40	40
Mineral mix (AIN-93M)[a)]	35	35	35
Vitamin mix (AIN-93M)[a)]	10	10	10
Choline chlorhydrate	2.5	2.5	2.5
Lard	-	-	312
Energy (kcal/kg)	3.88	3.88	5.44

a) For details, see Reeves et al. [61].

2.2 General nutritional parameters

Body weight (BW) was measured weekly throughout the experimental period. During the last week of HFD treatment all mice groups were placed in metabolic cages and had their food intake monitored, as previously reported [21]. At the end of the experimental period (14 weeks), fasted mice were euthanized in a CO_2 chamber followed by decapitation. Their blood was collected in heparinized tubes (5000 IU diluted 1:1000), centrifuged at 10 600 × *g* and the obtained plasma was stored at -20°C until use. A colorimetric kit was used according to the manufacturer's instructions for quantification of total plasma proteins (Laborlab, Guarulhos, SP, Brazil). Enzymatic kits were used for quantification of nonesterified fatty acids (Wako®; Richmond, USA), cholesterol (CHOL), and triglycerides (Roche/Hitachi®; Indianopolis, USA). ELISA kits were used for plasma leptin (Crystalchem; Downers Grove, USA) and adiponectin (Millipore; Billerica, USA). Plasma glucose was measured using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic, Switzerland), insulin was measured by radioimmunoassay as previously reported [22].

2.3 Plasma amino acid profile

Plasma-free amino acids were extracted using 80% ethanol containing 0.1 M HCl. The mixture was sonicated for 10 min and further homogenized for 1 h, followed by centrifugation at 10 600 × *g* for 15 min. The supernatant was filtered through a 0.22 μm membrane, 40 μL of the samples were derivatized with phenylisothiocyanate [waters pico-tag for free amino acids (WAT0 10954 Ver4)] and 20 μL of the phenylisothiocyanate derivatives were separated by chromatography using

a Luna C-18 5μ, 250 × 4.6 mm column (00G-4252-EQ; Phenomenex, Torrance, CA, USA), at 50°C, in an HPLC system (SCL-10avp, CTO10avp, SPDm10avp; Shimadzu Scientific Instruments, Columbia, MD, USA) with CLASS-VP 6.12 software. The solvent A was ACN 60% and solvent B was sodium acetate 0.58 M + 5% of ACN. An amino acid standard solution was derivatized and analyzed together with the samples, and methionine sulfone was used as an internal control.

2.4 Liver glycogen quantification

Fasting glycogen content in the liver were measured in sample pieces (weighing 15–20 mg from the two major lobules) collected from 8 h fasted mice. Glycogen was measured by the phenolsulfuric method [23] after KOH digestion and ethanol precipitation of glycogen. The glycogen content in the liver was calculated using D-glucose as standard curve.

2.5 Intraperitoneal glucose (ipGTT), insulin (ipITT), glucagon (ipGlTT), and pyruvate (ipPTT) tolerance tests

For ipGTT, overnight fasted mice (10 h) were injected with a 2 g/kg BW of a 20% glucose solution. Blood glucose was determined before (time 0) and 15, 30, 60, and 120 min after glucose injection using a glucose analyzer (Accu-Chek Advantage, Roche Diagnostic®) and blood sample was taken from the tip of the tail. For ipITT, fed mice were injected with 1.5 U/kg BW of human insulin (Biohulin®, Biobrás, Brazil). Blood glucose was determined before (time 0) and 10, 15, 30, 45, and 60 min after insulin injection. For ipGlTT, overnight fasted mice (8 h) were injected with 100 μg/kg BW of human glucagon (Glucagen®, Novo Nordisk, Denmark). Blood glucose was determined before (time 0) and 5, 10, 20, 40, and 60 min after glucagon injection. For ipPTT, overnight fasted mice (14 h) were injected with 2 g/kg BW of sodium pyruvate (Merck; Darmstadt, Germany). Blood glucose was determined before (time 0) and 15, 30, and 60 min after pyruvate injection.

2.6 Western blot

For the evaluation of Akt phosphorylation (p-Akt), 12 h food-deprived mice were anesthetized with a mixture of ketamine (50 mg/kg, i.p.,Vetbrands®, Paulínia, SP, Brazil) and xylazine (16 mg/kg, i.p., Rompun, Bayer®, São Paulo, SP, Brazil), and subsequently received an ip injection of insulin (100 μL; 1×10^{-6} M). After 5 min, fragments of the liver were excised and immediately frozen in liquid nitrogen. Phosphorylated AMPK (p-AMPK)/AMPK ratio was evaluated in liver samples from 12 h fasted mice that received ip 0.9% saline (100 μL). Liver fragments were homogenized using a Polytron PT 1200 C homogenizer (Brinkmann Instruments, NY, USA) in buffer containing: 100 mmol/L Tris pH 7.5, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L sodium vanadate, 2 mmol/L PMSF, and 1% Triton X-100. The extracts were then centrifuged at 15 300 × *g* at 4°C for 40 min to remove insoluble material. The protein concentration in the supernatants was assayed using the Bradford dye method [24], using BSA as a standard curve and Bradford reagent (Bio-Agency Lab, São Paulo, SP, Brazil). For SDS gel electrophoresis and Western blot analysis, the samples were treated with a Laemmli sample buffer containing DTT. After heating samples at 95°C for 5 min, the proteins were separated by electrophoresis (70 μg protein/lane, 10% gels). Following electrophoresis, proteins were transferred to nitrocellulose membranes. The nitrocellulose filters were treated overnight with a blocking buffer (5% nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20) and were subsequently incubated with a polyclonal antibody against p-Akt (1:1000, cat. sc-7985R, Santa Cruz Biotechnology), Akt (1:1000, cat. sc-8313, Santa Cruz Biotechnology), p-AMPK (1:1000, cat # 2535, Cell Signaling), and AMPK (1:1000, cat # 2532, Cell Signaling). Detection was performed after 2 h incubation with a horseradish peroxidase-conjugated secondary antibody (1:10 000, Invitrogen, São Paulo, SP, Brazil). The band intensities were quantified by optical densitometry using the free software, Image Tool (http://ddsdx.uthscsa.edu/dig/itdesc.html). Densitometry values obtained from phosphorylated proteins (p-Akt and p-AMPK) were normalized by total protein expression (Akt and AMPK) and expressed as % of C group, as previously described [16, 19].

2.7 Statistical analysis

Results are presented as means ± SEM for the number of determinations (*n*) indicated. The statistical analyses were carried out using a one-way analysis of variance (ANOVA) and two-way ANOVA with repeated measures (for glucose, insulin, glucagon, and pyruvate tolerance experiments) followed by Duncan post-test ($p \leq 0.05$) with the Statistica 5.0 software (Statsoft, Tulsa, OK, USA). Prior to ANOVA analysis a Gaussian distribution of the samples was assumed based on the Kolmogorov–Smirnov normality test using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Effects of diet and TAU treatment upon mice features

BW from normal protein and protein-restricted groups was measured weekly as illustrated in Fig. 1A and B, respectively. At the end of the experiment, the final BW of R mice was lower

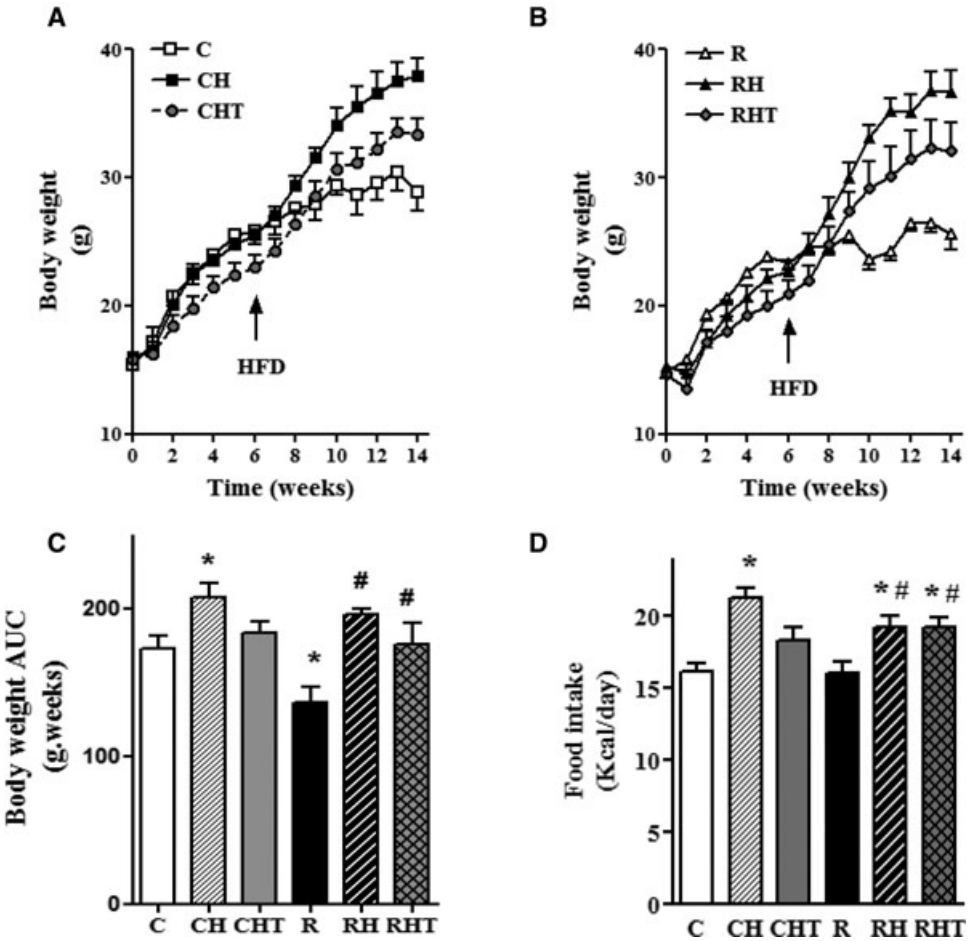

Figure 1. Taurine supplementation prevents obesity and hyperphagia in normal protein obese mice. Body weight of (A) C, CH, CHT and (B) R, RH, RHT mice registered for 14 weeks ($n = 5$). Arrows indicate the beginning of the HFD treatment. (C) Total body weight expressed by the area under growth curve. (D) Calorie intake registered during the last week of experimental period ($n = 5$–7). Data are means ± SEM; *different from C ($p < 0.05$); # different from R ($p < 0.05$).

than C (24 ± 2 versus 29 ± 1 g, respectively; $p < 0.05$). The final BW from CH (38 ± 1 g) and RH (37 ± 2 g) groups was increased when compared to controls ($p < 0.01$ and $p < 0.001$, respectively). The BW from CHT mice (34 ± 1 g) was similar to C, whereas RHT had final BW (32 ± 2 g) higher than R mice ($p < 0.01$). In accordance, total BW, assessed by the area under curve (AUC), was reduced in R compared to C mice ($p < 0.05$; Fig. 1C). HFD increased BW AUC in both CH and RH, when compared to C and R mice ($p < 0.05$ and $p < 0.01$, respectively). TAU supplementation reduced total BW in CHT mice, however RHT also showed a persistent higher total BW compared with R ($p < 0.05$). The final BW ratio between CH/C and RH/R mice, at the end of the experiment, was 1.3 and 1.5, respectively, indicating a catch up growth pattern in malnourished mice fed an HFD. CH and RH mice also showed increased adiposity, as demonstrated by a 2.8- and 1.9-fold increase in periepididymal ($p < 0.001$ and $p < 0.05$), and a 2.7- and 2.0-fold increase in retroperitoneal ($p < 0.001$) fat pads, compared to C and R mice, respectively (Fig. 2A and B). The increase in fat depots was accompanied by increased plasma leptin in CH and RH groups ($p < 0.01$ and $p < 0.05$, respectively; Fig. 2C). Despite increased plasma leptin levels, CH and RH mice showed a higher calorie intake when compared with C and R mice ($p < 0.001$ and $p < 0.05$, respectively; Fig. 1D). TAU supplementation impaired the development of obesity and normalized calorie intake only in normal protein mice, because the CHT group showed similar BW, calorie intake, and plasma leptin levels to those of the C group (Figs. 1C, D, and 2C), and a 20% reduction in fat depots, compared to the CH group ($p < 0.05$; Fig. 2A and B).

Mice submitted to low-protein diet developed malnutrition, as indicated by a reduction in the total plasma proteins in the R, compared with the C mice ($p < 0.01$; Table 2). Plasma nonesterified fatty acids levels were higher in RH when compared with the R group ($p < 0.05$). Plasma CHOL was increased in CH and RH groups, compared with their respective controls ($p < 0.001$ and $p < 0.05$, respectively). TAU supplementation lowered plasma CHOL in CHT mice with a 15% reduction, compared with the CH group ($p < 0.05$).

3.2 Glucose homeostasis

Blood glucose and plasma insulin levels were similar between fasted R and C mice (Fig. 3A and B). CH, but not RH mice,

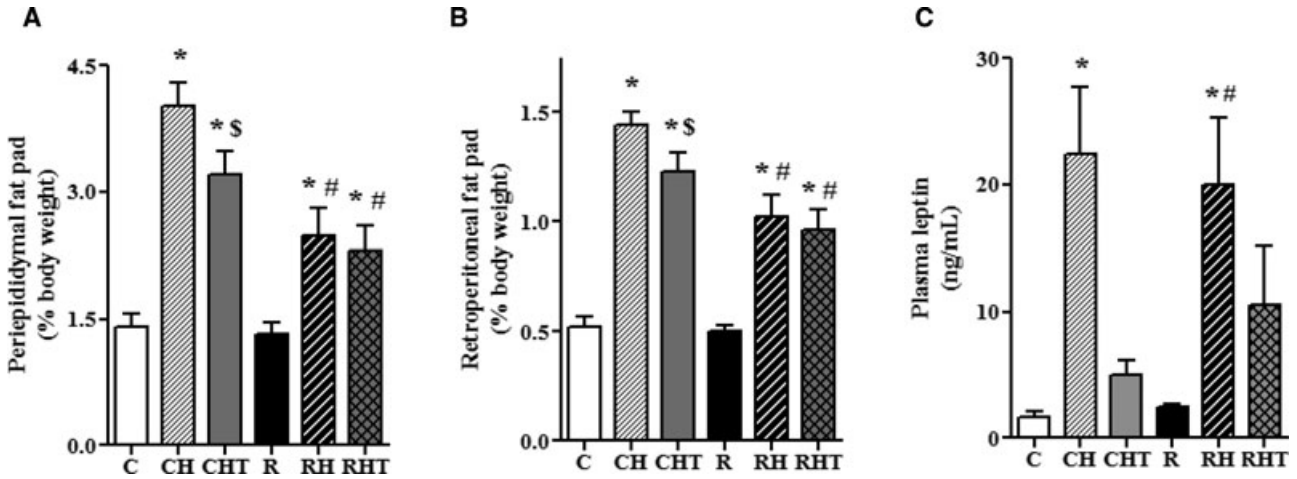

Figure 2. Taurine supplementation reduces adiposity and hyperleptinemia in normal protein obese mice. (A) Periepididymal and (B) retroperitoneal fat pads in C, CH, CHT, R, RH, and RHT mice ($n = 10$–12). (C) Plasma leptin in fasted mice ($n = 5$–7). Data are means ± SEM; *different from C ($p < 0.05$); # different from R ($p < 0.05$); $ different from CH ($p < 0.05$).

were hyperglycemic and hyperinsulinemic ($p < 0.05$; Fig. 3A and B). TAU supplementation normalized blood glucose and plasma insulin levels in CHT mice (Fig. 3A and B).

During the ipGTT, blood glucose reached maximal values at 30 min in all groups (Fig. 3C and D). CH mice showed increased blood glucose at 60 min of the test ($p < 0.001$; Fig. 3C). In RH group, hyperglycemia started at 30 min and persisted until 120 min of the test ($p < 0.001$; Fig. 3D). TAU improved glucose tolerance in CHT mice as judged by similar glucose levels to those observed in C group (Fig. 3C). However, RHT had higher blood glucose at 30 and 60 min of the test compared with R mice ($p < 0.001$ and $p < 0.01$; Fig. 3D, respectively). In addition, total plasma glucose levels (AUC) during ipGTT in CH and RH were higher than their respective controls ($p < 0.05$ and $p < 0.01$, respectively; Fig. 3E). TAU normalized glucose tolerance in CHT, but not in RHT mice (Fig. 3E).

During ipITT, R mice showed lower blood glucose compared with C mice (Fig. 3F and G), indicating increased insulin sensitivity, as demonstrated by the lower AUC in the R group ($p < 0.05$; Fig. 3H). HFD-induced insulin resistance since the total plasma glucose levels in all HFD fed mice were higher than their respective controls (Fig. 3H). TAU supplementation did not alter the insulin resistance induced by HFD.

In addition, liver glucose output was evaluated using ipGlTT and ipPTT (Fig. 4). After an ip glucagon administration, blood glucose in all groups, except for CH, reached the zenith after 10 min (Fig. 4A and B). In the CH group, the highest blood glucose concentration was observed at 20 min. The AUC during ipGlTT showed a lower liver glucose output in R compared to C mice ($p < 0.007$; Fig. 4C). In addition, hepatic glucose mobilization was increased in CH and RH mice, compared with their controls ($p < 0.05$ and $p < 0.01$; Fig. 4C). TAU supplementation normalized liver glucose output, induced by glucagon, in CHT but not in RHT mice. R and CH mice showed increased liver glycogen content ($p < 0.05$ and $p < 0.001$, respectively; Fig. 4G). TAU supplementation decreased liver glycogen content in both the CHT and RHT groups (Fig. 4G).

Figure 4D and E show an ipPTT performed in 14 h fasted mice. The AUC during ipPTT was similar between R and C mice, whereas CH and RH groups presented an increased glucose output as observed by the higher AUC compared to controls ($p < 0.01$; Fig. 4F). TAU supplementation improved liver glucose production after pyruvate administration only

Table 2. Total plasma proteins, nonesterified fatty acids (NEFA), cholesterol (CHOL), and triglyceride (TG) from fasted C, CH, CHT, R, RH, and RHT mice

	C	CH	CHT	R	RH	RHT
Total proteins (g/Dl)	5.21 ± 0.3	5.63 ± 0.2	5.44 ± 0.1	3.77 ± 0.5*	5.58 ± 0.1**	5.59 ± 0.3**
NEFA (mmol/L)	1.04 ± 0.06	0.80 ± 0.06	0.91 ± 0.03	0.82 ± 0.08	1.09 ± 0.06**	0.80 ± 0.07
Plasma CHOL (mg/dL)	96.12 ± 7.3	145.5 ± 6.7*	123.3 ± 7.7*,***	100.0 ± 6.8	134.5 ± 9.2**	129.4 ± 6.3**
Plasma TG (mg/dL)	69.20 ± 5.8	77.26 ± 3.9	77.46 ± 8.1	74.80 ± 11.9	71.57 ± 6.6	86.05 ± 5.1

Data are means ± SEM ($n = 4$–16).
*$p < 0.05$ versus C; **$p < 0.05$ versus R; ***$p < 0.05$ versus CH.

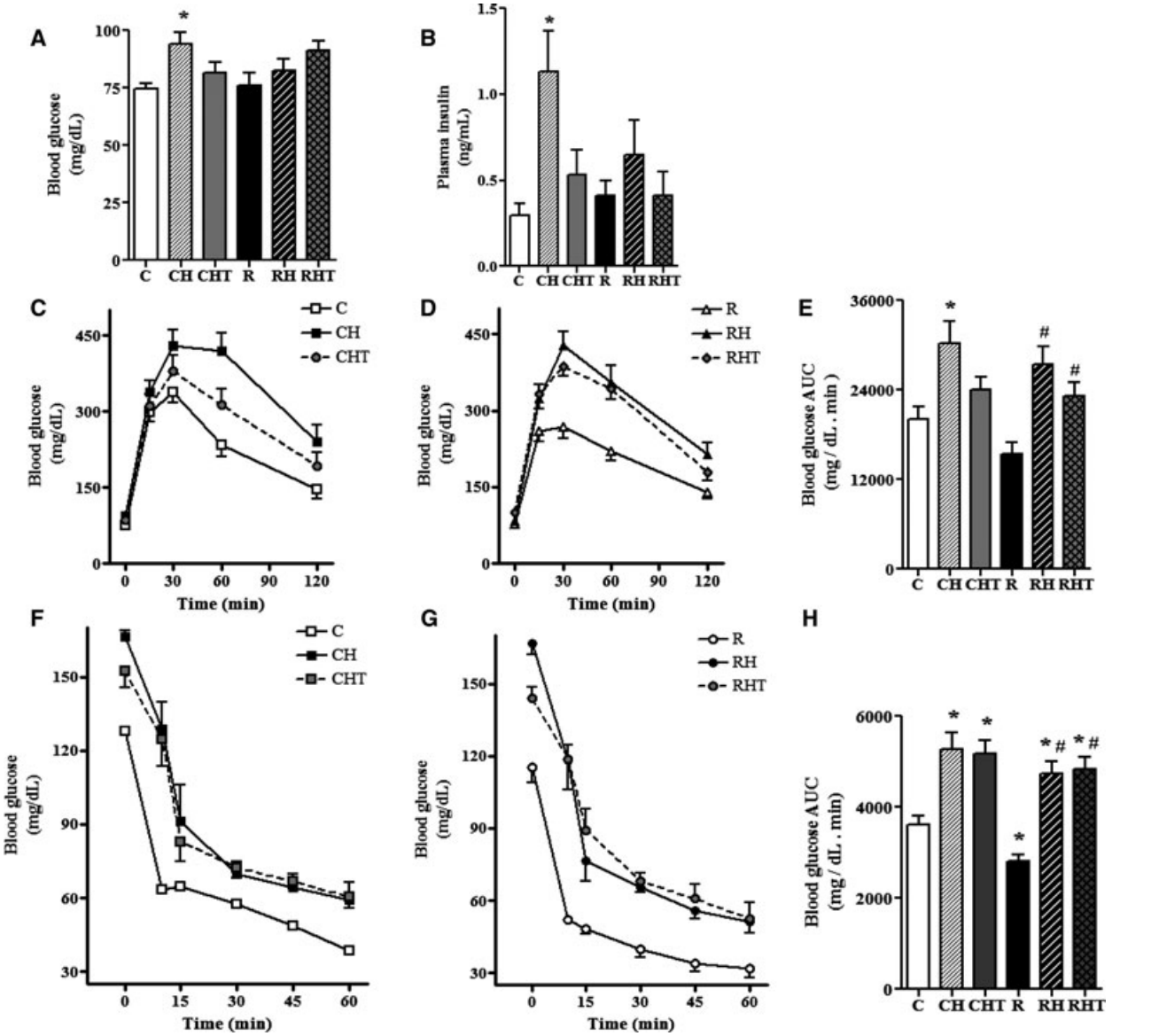

Figure 3. Taurine supplementation improves glucose but not insulin tolerance in normal protein obese mice. (A) Plasma glucose ($n = 8$–11) and (B) insulin ($n = 6$–10) levels in fasted C, CH, CHT, R, RH, and RHT mice. Changes in blood glucose during (C, D) ipGTT ($n = 8$–11) and (F, G) ipITT ($n = 7$–8). Total plasma glucose concentrations during (E) ipGTT and (H) ipITT expressed by the AUC. Data are means ± SEM; * different from C ($p < 0.05$); # different from R ($p < 0.05$).

in RHT. Despite the disruption in glucose homeostasis in all groups, no differences on liver CHOL and triglyceride content were observed (data not shown).

3.3 Akt and AMPK phosphorylation in the liver

Figure 5A shows p-Akt/Akt ratio in liver samples of fasted mice after an ip insulin administration. The R and C groups showed a similar liver p-Akt content (Fig. 5A). In addition, Akt activation was similar in obese controls and protein-restricted mice, but significantly increased in CHT, compared with all groups (Fig. 5A).

p-AMPK was significantly increased in the liver of fasted R mice in comparison with C ($p < 0.05$; Fig. 5B). HFD significantly reduced p-AMPK content in fasted RH mice ($p < 0.01$). TAU supplementation preserved the enhanced AMPK activation in RHT mice (Fig. 5B).

In addition, plasma adiponectin was higher in fasted R than C mice ($p < 0.001$; Fig. 5C). HFD lowered plasma adiponectin in RH and RHT, compared with R mice ($p < 0.001$).

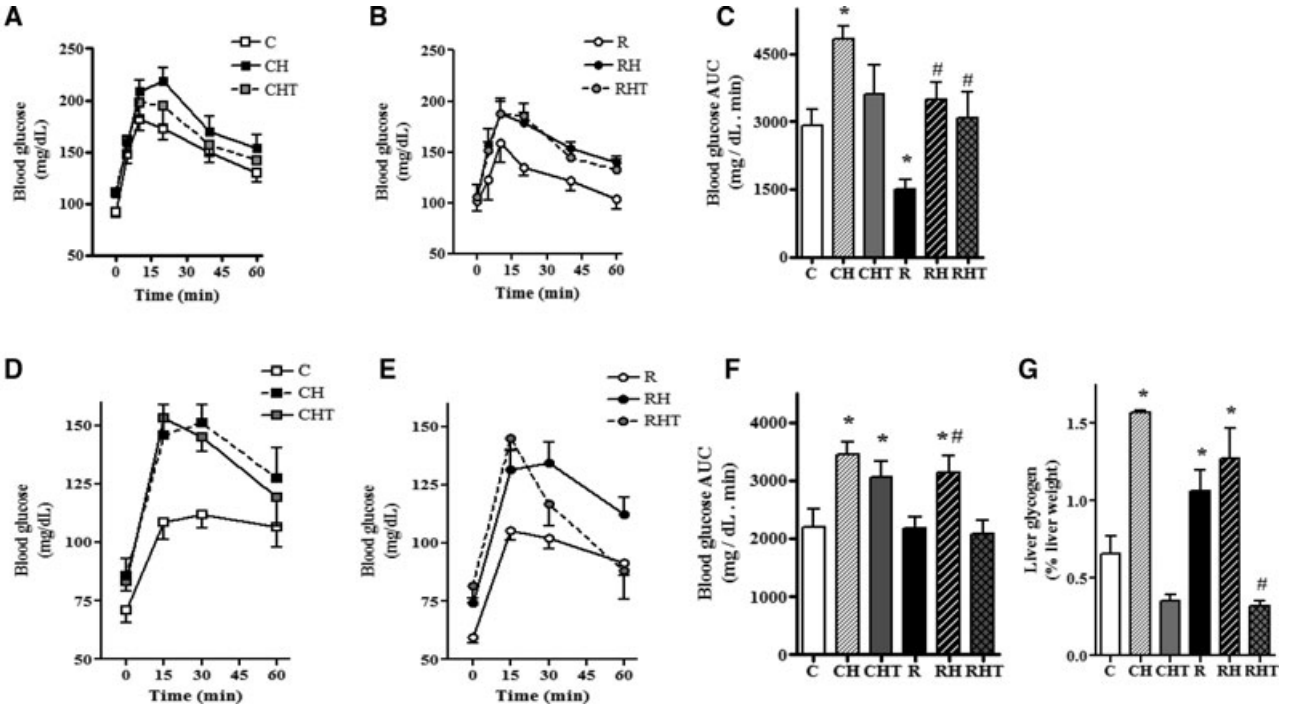

Figure 4. Taurine supplementation improves glucagon and pyruvate-induced liver glucose output in obese mice. Changes in plasma glucose levels during (A, B) ipGITT ($n = 5$–7) and (D, E) ipPTT ($n = 7$–12) in C, CH, CHT, R, RH, and RHT mice. Total plasma glucose concentrations during (C) ipGITT and (F) ipPTT expressed by the AUC. (G) Liver glycogen content ($n = 4$). Data are means ± SEM; * different from C ($p < 0.05$); # different from R ($p < 0.05$).

3.4 Plasma amino acid profile

Plasma TAU, arginine, leucine, valine, and isoleucine levels did not differ between R and C groups (Fig. 6). In addition, HFD treatment increased plasma leucine and valine levels in CH mice, compared with C ($p < 0.05$ and $p < 0.01$; Fig. 6C and D). TAU administration efficiently increased plasma TAU levels in CHT and RHT, in comparison with CH and RH mice, respectively ($p < 0.007$; Fig. 6A). Plasma arginine levels were 2.2 and 2.7 higher in CHT and RHT mice compared with

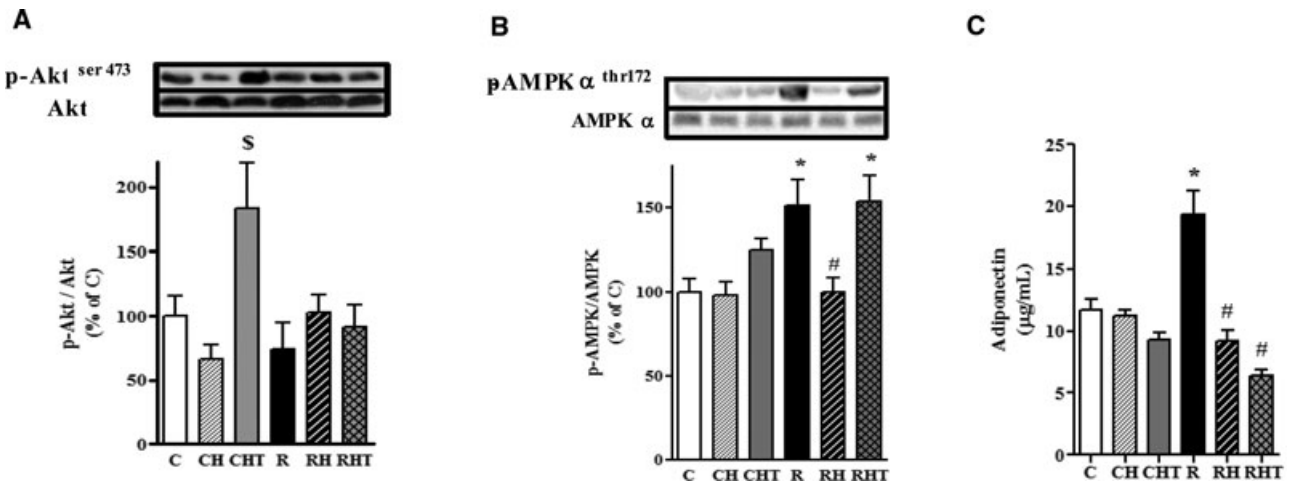

Figure 5. Taurine supplementation enhances insulin signaling in normal protein and AMPK phosphorylation in malnourished obese mice. (A) p-Akt^{ser473}/Akt protein expression in the liver from insulin-stimulated (100 µL; 10^{-6} M) C, CH, CHT, R, RH, and RHT mice ($n = 4$–6). (B) p-AMPK^{thr172}/AMPK protein expression in the liver from fasted C, CH, CHT, R, RH, and RHT mice ($n = 9$–10). (C) Plasma adiponectin ($n = 7$–8). Data are means ± SEM; * different from C ($p < 0.05$); # different from R ($p < 0.05$); $ different from CH ($p < 0.05$).

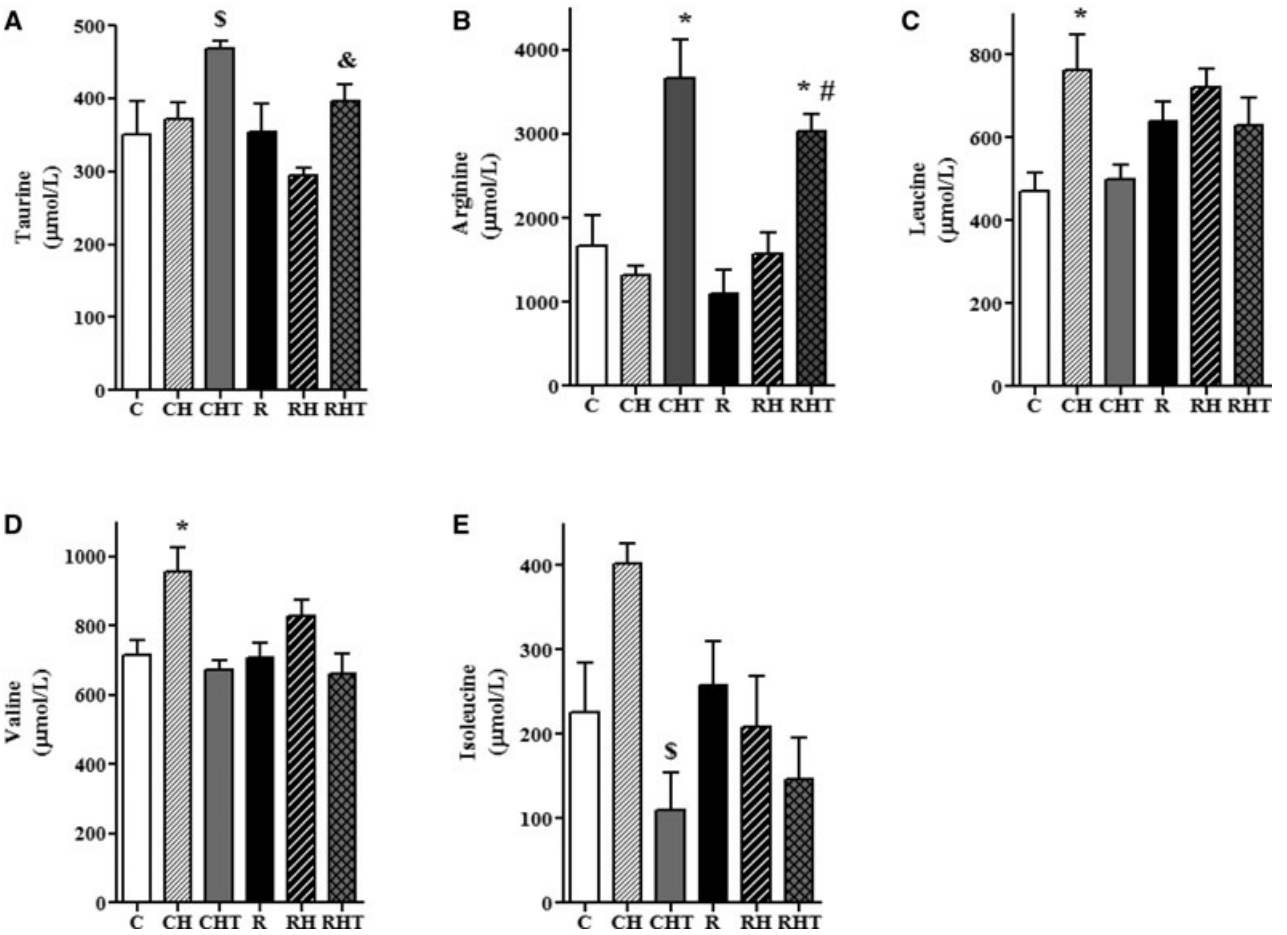

Figure 6. Taurine supplementation increases arginine plasma levels. Plasma (A) TAU, (B) arginine, (C) leucine, (D) valine, and (E) isoleucine in fasted C, CH, CHT, R, RH, and RHT mice ($n = 4$). Data are means ± SEM; * different from C ($p < 0.05$); # different from R ($p < 0.05$); $ different from CH ($p < 0.05$); & $p < 0.05$ versus RH.

their controls ($p < 0.001$ and $p < 0.01$; Fig. 6B). TAU supplementation also lowered plasma isoleucine in CHT, compared with the CH group ($p < 0.05$; Fig. 6E) and normalized plasma leucine and valine in CHT mice.

4 Discussion

Malnutrition during pregnancy leads to low birth weight, which may predispose to chronic diseases such as hypertension, glucose intolerance, and T2DM [2–4]. Rodents submitted to protein restriction during gestation and lactation showed catch up growth after receiving normal or hypercaloric diets, which was critical for the development of obesity and glucose intolerance [25–27]. Here, we assessed whether a similar susceptibility was present in HFD mice fed on a low-protein diet after weaning. Our results show that catch up growth was present in malnourished obese mice, since the BW ratio between RH/R was increased in comparison to CH/C mice. In addition, hyperleptinemia, hyperphagia, hypercholesterolemia, and insulin resistance were present in RH mice at a similar extent to those observed in CH. These RH mice features are in accordance with previous reports indicating that, after 8 months of hypercaloric diet, protein malnourished mice were overweight and expressed higher amounts of leptin in the adipose tissue, compared with the normal protein group [26]. In addition, isolated adipocytes from 15-month-old malnourished rats presented lower glucose uptake, associated with decreased PI3K and Akt activities [9].

In our study, although RH mice presented glucose intolerance and insulin resistance they did not develop hyperinsulinemia as observed in CH mice (Fig. 3). In fact, impaired islet function in protein restricted rodents during gestation, lactation, and after weaning were reported [7, 16, 28, 29]. However, for the first time, we showed that malnutrition after weaning may disrupt the β-cell capacity to compensate peripheral insulin resistance and may lead to the early development of T2DM in R mice submitted to an HFD.

In our study, TAU supplementation prevented excessive weight gain and hyperphagia (Fig. 1), lowered body fat depots,

plasma leptin (Fig. 2), CHOL (Table 2), glucose and insulin levels (Fig. 3), and improved glucose tolerance (Fig. 3) in control, but not in malnourished mice fed an HFD.

Improved plasma lipid profile and enhanced glucose tolerance after TAU supplementation have been previously reported [13, 30–32]. The better glucose control in CHT mice could be, at least in part, explained by enhanced Akt activation in the liver (Fig. 5A). TAU has been reported to increase p-Akt content in different cellular types [19, 33–37]. It is known that Akt activation diminishes liver glucose output through inhibition of glycogen synthase kinase-3 and forkhead box protein-1 enzymes [38, 39]. The mechanisms by which TAU enhanced Akt activation are still unclear and may be due to a direct interaction of this amino acid with the insulin receptor [40, 41].

Although TAU supplementation did not prevent obesity and glucose intolerance in RHT mice (Figs. 1–3), the pyruvate-induced glucose production was normalized in that group (Fig. 4). However, these effects were not associated with alterations in p-Akt content in the liver of RHT mice (Fig. 5) and may be related to the improvement of liver AMPK activation (Fig. 5B).

In the liver, AMPK suppresses lipid synthesis through inhibition of acetyl-CoA carboxylase [42]. This kinase also regulates liver glucose output, since the AMPK activator: 5-aminoimidazole-4-carboxamide ribonucleoside reduces transcriptional activity of the phosphoenolpyruvate carboxykinase gene promoter after phosphorylation and inhibition of the transducer of regulated CREB protein 2 and glycogen synthase kinase-3β [43–45]. In addition, adiponectin exerts insulin-sensitizing effects promoting nuclear export and cytosolic stabilization of the AMPK upstream activator kinase LKB1 [46, 47].

The enhanced insulin sensitivity in R mice (Fig. 3F–H) may be a consequence of increased plasma adiponectin levels, which promoted a higher AMPK activation in the liver (Fig. 5B and C). On the other hand, HFD decreased adiponectin levels and liver p-AMPK content in RH mice (Fig. 5B and C), which may contribute to defective insulin signaling in this group. Despite the lower plasma adiponectin concentrations in RHT (Fig. 5C), this group presented a persistent increase in AMPK phosphorylation in the liver (Fig. 5B). These observations suggest that TAU supplementation enhanced p-AMPK content by a different and yet unknown pathway.

Of note, plasma arginine concentrations were increased twofold in TAU-supplemented groups (Fig. 6B). Higher plasma arginine levels were reported in guinea pigs after a single ip injection of TAU [48]; however, the mechanisms by which these amino acids interact and regulate their own concentrations are not yet clear.

Arginine is a physiological precursor for nitric oxide (NO) generated by the reaction catalyzed by NO synthase [49]. Previous studies reported that AMPK activation is regulated by NO and its derivatives. Incubation of L6 myotubes with a NO donor, S-nitroso-N-penicillamine, has been found to increase GLUT4 mRNA and AMPK phosphorylation at the Thr^{172} residue. In addition, 5-aminoimidazole-4-carboxamide ribonucleoside-induced AMPK activation was prevented by incubation with L-NAME, an NO synthase inhibitor [50]. Furthermore, the antidiabetic drug metformin increased AMPK activation in endothelial and aortic cells via production of peroxynitrite ($ONOO^-$), a reactive molecule formed by binding of the superoxide radical ($O_2^{\bullet-}$) to NO [51, 52]. Although $ONOO^-$, at pathologic levels, may contribute to insulin resistance through protein nitration, physiological levels of this molecule are important for AMPK activation [53]. As such, we propose that TAU supplementation, by increasing plasma arginine levels, created an alternative pathway for AMPK activation in the liver of RHT mice, in turn, improving hepatic glucose control.

In addition, in our study CH mice showed higher plasma concentrations of the ketogenic amino acids: leucine and valine (Fig. 6C and D). This finding may be related to the impaired glucose control and insulin action in this group (Figs. 3 and 4). Increased branched-chain amino acids (BCAA) levels were reported in prediabetic and T2DM states [54–57]. Excessive amounts of BCAAs, especially leucine, can lead to hyperactivation of the mammalian target of rapamycin, an amino acid sensor, which abrogates insulin signaling through phosphorylation of several serine residues of the insulin receptor substrate 1 [58, 59]. On the other hand, insulin regulates BCAAs metabolism and insulin resistant subjects showed increased plasma BCAAs levels during euglycemic-hyperinsulinaemic clamp [54]. In leptin deficient (*ob/ob*) obese mice and Zucker fatty (*fa/fa*) diabetic rats a decrease in protein expression of the mitochondrial BCAA aminotransferase (BCATm) and branched-chain α-keto acid dehydrogenase complex in adipose tissue was reported [55]. Also, after bariatric surgery lower plasma BCAAs were present together with enhanced BCATm expression in white adipose tissues of human subjects [55]. Thus, lower oxidation of BCAAs may favor their accumulation in plasma of insulin resistant and diabetic subjects.

Here, TAU normalized BCAA levels in CHT mice. These observations support the improved glucose control in this group that was preserved by supplementation. The TAU action upon BCAAs metabolism is unclear. In a work from our laboratory pancreatic islets from TAU-treated mice presented enhanced leucine-induced insulin release associated with a better amino acid oxidation [60]. In this way, further studies are required to fully address this action of TAU upon the regulation of plasma BCAAs, but these evidences indicate that TAU may contribute to body nutrient control not only via insulin sensitizing effects but also through regulation of key metabolic enzymes.

In conclusion, we show that TAU supplementation, during early life and adulthood prevents obesity and improves glucose homeostasis in C mice fed an HFD. The later effect is probably due to increased p-Akt expression in the liver. In addition, for the first time, we provide evidence that an HFD treatment after a prolonged period of protein malnutrition,

after weaning, leads to the same metabolic damages such as: obesity, hyperphagia, and glucose intolerance seen in normal protein mice but they were not restored or prevented by TAU supplementation. The higher adiponectin levels in malnourished mice possibly enhance AMPK activation in the liver and contribute to normoglycemia in this group. Finally, TAU regulates plasma arginine levels, which may preserve AMPK activation and normal hepatic glucose control in RHT mice.

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