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BIOMEDICAL SCIENCES

D-Pinitol Increases Insulin Secretion and Regulates Hepatic Lipid Metabolism in Msg-Obese Mice

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Abstract: D-pinitol is one of the major inositol found in plants and studies suggest its potential hypoglycemic and hypolipidemic actions in diabetic rodents. Here, we investigated the actions of D-pinitol on adiposity, and in lipid and glycemic homeostasis in monosodium glutamate (MSG)-obese mice. *Swiss* mice received daily subcutaneous injections of MSG [(4g/kg of body weight (BW)] or saline [1.25g/kg BW; control (CTL)] during their first five days of life. From 90-120 day-old, half of the MSG and CTL groups received 50 mg D-pinitol/kg BW/day (MPIN and CPIN groups) or vehicle (saline; MSG and CTL groups) by gavage. MSG mice displayed higher abdominal adiposity and hepatic triglycerides (TG) deposition, and increased hepatic expression of lipogenic genes (SREBP-1c, ACC-1 and FASN), but downregulation in AMPKα mRNA. MSG mice also exhibited hyperinsulinemia, islet hypersecretion and hypertrophy, glucose intolerance and insulin resistance. D-pinitol did not change adiposity, glucose intolerance, insulin resistance, but increased hepatic triglycerides (TG) content in MPIN mice, which was associated with increases in gene expressions of SREBP-1c and FASN, but reduction in AMPKα. Furthermore, D-pinitol enhanced insulin secretion in MPIN and CPIN groups. Therefore, D-pinitol enhanced glucose-induced insulin secretion, which may account to enhances hepatic lipogenesis and TG deposition in MPIN mice.

Key words: D-chiro-inositol, Hypothalamus, β-cell, obesity.

INTRODUCTION

Obesity is a major health problem that is increasing worldwide. This chronic non-communicable disease predisposes individuals to comorbidities such as cardiovascular diseases, glucose intolerance, type 2 diabetes (T2D) and non-alcoholic fat liver disease (NAFLD) (Berlanga et al. 2014, Jelenik et al. 2017). In addition, insulin resistance and T2D also induce NAFLD (Berlanga et al. 2014); and NAFLD can induce these obesity comorbidities (Brouwers et al. 2017). This disease is characterized by ectopic fat deposition in the liver and affects around 25% world people (Araujo et al. 2018). Such epidemiologic data are worrying, since the progression of this disease leads to the establishment of nonalcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma (Kawano & Cohen 2013). The ectopic accumulation of fat in the liver occurs due to an increased exogenous fatty acid supply resulting from a high-calorie diet, or enhanced adipose tissue lipolysis. Additionally, increases in the activation of the transcription factors and enzymes involved in *de novo* lipogenesis, or downregulation of the β-oxidation process, together or not, with

reduced hepatic secretion of very low-density lipoproteins (VLDLs) are key mechanisms involved in NAFLD physiopathology (Berlanga et al. 2014). The therapeutic possibilities for treating NAFLD are still limited due to its complexity and high variability between individuals. Weight loss, nutritional interventions and bioactive food compounds have been demonstrated to be good strategies for the management of NAFLD (Vilar-Gomez et al. 2015, Zelber-Sagi et al. 2017).

D-pinitol (3-O-methyl-D-chiro-inositol) is a naturally-occurring compound, found in pine trees, legumes, seeds, flowers and leaves, and it is actively converted into D-chiro-inositol in mammalians. Some studies have demonstrated that D-chiro-inositol metabolism is impaired in insulin-resistant experimental rodents (Larner 2002) and humans (Ostlund et al. 1993). As such, D-pinitol ingestion may correct D-chiro-inositol levels in insulin resistance. However, severe insulin-resistant but not diabetic leptin deficient obese mice treated with D-pinitol did not show any amelioration in glucose homeostasis (Bates et al. 2000). However, when type 1 diabetes (T1D) or T2D are established this inositol demonstrated hypoglycemic (Gao et al. 2015, Kang et al. 2006, Kim et al. 2007, Sivakumar et al. 2010b, Sivakumar & Subramanian 2009b) and hypolipidemic effects (Geethan & Prince 2008). As such, more investigations regarding the actions of D-pinitol on obesity are necessary to understand whether this molecule may represent a good bioactive compound against adiposity, insulin resistance and hepatic steatosis.

Early postnatal administration of monosodium glutamate (MSG) in rodents produces neuronal necrosis in the arcuate nuclei of the hypothalamus and median eminence, leading to the development of hypothalamic obesity (Olney et al. 1971). MSG–obese rodents are not diabetic, but display several features of impairments in glucose and lipid homeostasis characterizing they as pre-diabetic rodents, such as severe glucose intolerance (Araujo et al. 2019), insulin resistance (Hirata et al. 2003), and pancreatic β-cell (Lubaczeuski et al. 2015, Lucinei Balbo et al. 2000, Ribeiro et al. 2013) and α-cell hypersecretion (Araujo et al. 2019), and hepatic steatosis due to high hepatic *de novo* lipogenesis activation (Bonfleur et al. 2015). With regard to hypothalamic obesity treatment, weight loss is much more difficult due to the devastating neuroendocrine disruption that characterizes this disease; however, natural bioactive compounds may represent an important strategy for combating this disorder (Alarcon-Aguilar et al. 2007, Fortis-Barrera et al. 2017, Gao et al. 2018). Therefore, we aimed herein to investigate the actions of D-pinitol on obesity, and in lipid and glycemic homeostasis in MSG mice.

MATERIALS AND METHODS

MSG induction of obesity and D-pinitol treatment

All experiments were approved by the UFRJ's Animal Care and Use Committee (CEUA, license nº.: MAC021). Male newborn *Swiss* mice received a daily subcutaneous injection of MSG (4 g/kg body weight [BW]; MSG group) or hyperosmotic saline solution (1.25 g/kg BW/day, CTL group), during the first 5 days of life. All mice groups were weaned at 21 days of life. From 90 to 120 days of age, the mice were randomly distributed into the groups: CTL and MSG mice, which received 0.2 mL of vehicle daily (0.9% saline; CTL and MSG), or CTL and MSG mice that received 50 mg/kg BW/day D-pinitol (Sigma-Aldrich Chemical, St Louis, MO, USA) diluted in vehicle (CPIN and MPIN groups; representation of experimental groups and treatment can be see in Fig. 1a). The vehicle or D-pinitol treatments were performed daily at 10:00 am. The D-pinitol dose used here

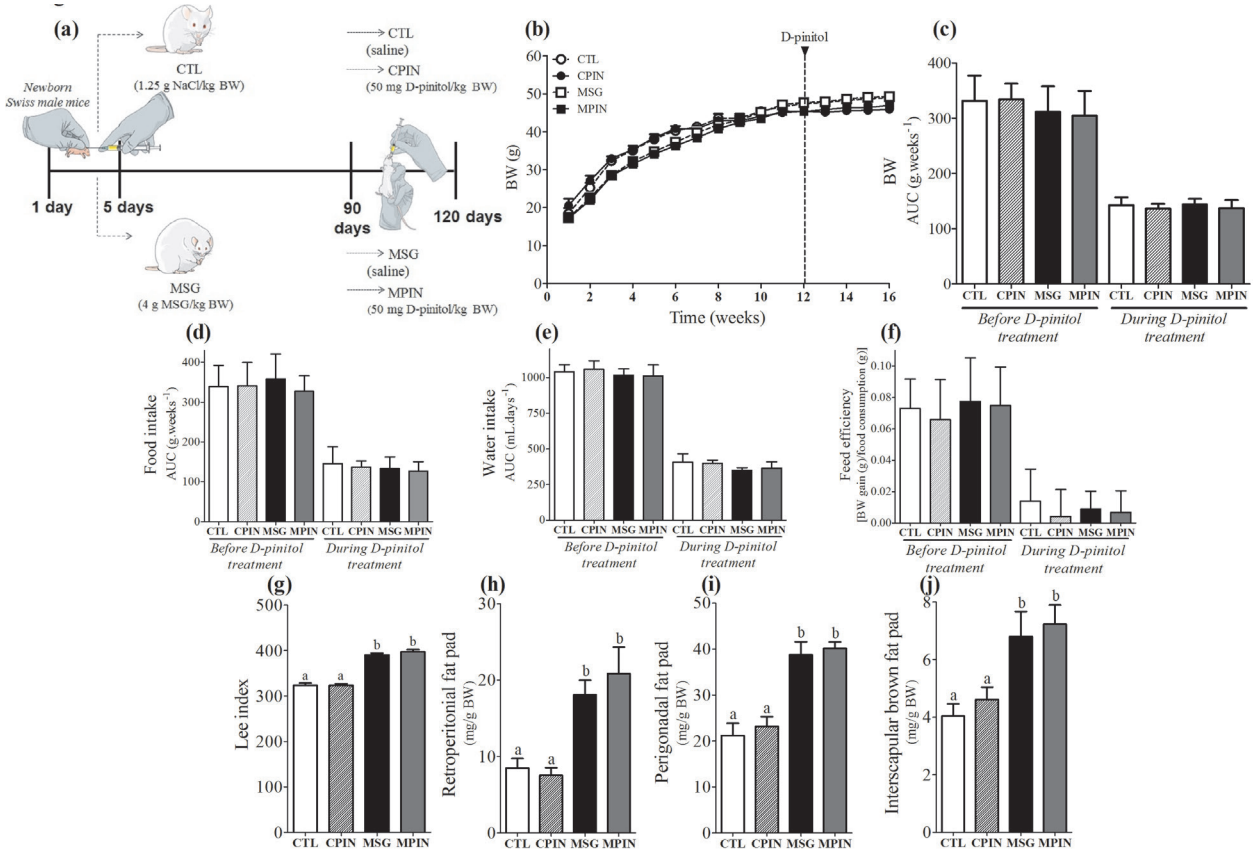

Figure 1. D-pinitol treatment did not change body weight or abdominal adiposity in MSG-obese mice. (a) Schematic representation of the experimental mice groups. From 90 to 120 days of age, CTL and MSG mice received a daily saline solution without (vehicle; CTL and MSG groups) or with 50 mg D-pinitol/kg BW via gavage (CPIN and MPIN groups). (b) Body weight (BW) of CTL (n = 26), CPIN (n = 28), MSG (n = 25) and MPIN (n = 27) mice, weekly registered before and during D-pinitol or vehicle treatments. Means ± SEM of total of BW (c), food (d) and water intake (e), and feed efficiency (f), expressed by the area under curve (AUC), registered before and during D-pinitol or vehicle treatments. Obesity parameters at the end of D-pinitol or vehicle treatments, expressed by the means ± SEM of Lee index (g), retroperitoneal (h) and perigonadal white fat pad (i), and interscapular brown fat pad weights (j). Different letters over the bars represent significant differences (Kruskal-Wallis followed by Dunns post-test, P < 0.05).

was based on that of previously reports showing that the daily administration of 50 mg D-pinitol/kg BW for 30 days via gavage reduced oxidative stress, had hypoglycemic and anti-inflammatory actions in diabetic rodents (Sivakumar et al. 2010a, b, Sivakumar & Subramanian 2009a, b). During the entire experimental period, the mice had free access to standard rodent chow (Nuvilab, Colombo, PR, BRA) and water and were housed in standard cages maintained on a 12 h light/dark cycle (lights on 08:00-20:00h) and at controlled temperature (21 ± 2ºC). All experimental procedure developed in vehicle or D-pinitol MSG and CTL groups of this study were performed after 30 days of the experimental treatment, and at least 20-21 hour of the last vehicle or D-pinitol gavage administration.

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

For ipGTT, glycemia was measured in blood samples obtained from the tip of the tails (time 0) of 12h-fasted mice, using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic®, Switzerland). Subsequently all mice received an i.p. injection of 2 g/kg BW glucose. Glycemia was measured again at 15, 30, 60, 120 and 180 min after glucose administration. For insulin sensitivity analysis, all mice were food deprived for 2h and glycemia was measured. Afterwards, the mice were i.p. injected with 1.25 IU/kg BW regular human insulin (Humulin®R, Eli Lilly and Company, Indianapolis, IN, USA). Glycemia was also measured at 4, 8, 12, 16 and 20 min after insulin administration. The values of glycemia were converted to natural logarithmic values to assess the decay rate constant (K_{ITT}). Using linear regression, the slope was calculated (time × Ln ^{glycemia}) and the glycemia decay rate constant (%/min) was obtained by multiplying the result by 100 (Araujo et al. 2019).

Food and water intake, obesity and biochemical parameters

All groups of mice were weighed weekly before and during the D-pinitol or vehicle treatments. Food consumption was measured once a week and water intake was measured every two days; results are expressed as the food or water ingestion per day. Feed efficiency was obtained by the ratio from the total BW gained, divided by the total food consumption before or during the treatment period (Araujo et al. 2019).

At the end of the D-pinitol or vehicle treatments, mice from both groups were weighed and the nasoanal lengths were measured to obtain the Lee index [BW $(g)^{1/3}$/ nasoanal length (cm) x 1000]. Fasted and fed mice were euthanized by decapitation, their blood was collected and plasma was used for measurements of triglycerides (TG) total cholesterol (CHOL), using colorimetric commercial kits, according to the manufacturer's instructions (Bioclin, Belo Horizonte, MG, BRA), insulin by radioimmunoassay (RIA). Glucose was measured in blood samples collected from the tip of the tail, using a glucose analyzer (as mentioned above).

Lipid content in the liver

Fragments of 100 mg of the livers of all mice groups were collected and lipids were extracted by FOLCH's method (Folch et al. 1957). The extract was evaporated and diluted in isopropanol for measurement of the TG and CHOL, as described above.

Real time quantitative RT-PCR in liver samples

Fragments of 50 mg of the livers of all mice groups were submitted to RNA extraction using TRIzol® and reverse transcription of mRNA was performed using the Superscript II kit (Invitrogen, Carlsbad, CA, USA). The relative mRNA contents were quantified using the Fast System 7500 & 7500 Real-Time PCR (Applied Biosystems, CA, USA). Primer sequences used for mice genes are provided in Table I. The relative mRNA levels were determined by the $2^{-\Delta\Delta Ct}$ method and normalized by the 36B4 gene.

Islet isolation and static insulin secretion

Pancreatic islets were isolated by collagenase digestion of the exocrine pancreas. For static incubations, four islets from each mice group were first incubated for 30 min at 37°C in Krebs–bicarbonate (KBR) buffer with the following composition; 115 mM NaCl, 5 mM KCl, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM $NaHCO_3$, 15 mM HEPES, supplemented with 5.6 mM glucose, 3 g of BSA/L (Sigma Chemical, St Louis, MO, USA), and gassed with a mixture of 95% O_2/5% CO_2, pH 7.4. This medium was discarded and replaced by fresh

Table I. Primer sequences for genes involved in lipid metabolism in the liver.

	Forward (5' to 3')	Reverse (5' to 3')
SREBP-1c	CCACTAGAGGTCGGCATGGT	TCCCTTGAGGACCTTTGTCATT
ACC-1	TTTGAGGAGGACCGCATTTATC	ACATGGGATGGCATAAGGT
FASN	GATATTGTCGTCTGAGGCTGTTG	GGAATGTTACACCTTGCTCCTTGC
AMPKα	CAAAGCCGACCCAATGAC	TCTTCCTTCGCACACGC
UCP-2	AGCAGTTCTACACCAAGGGC	TGGAAGCGGACCTTTACCAC
36B4	GAGGAATCAGATGAGGATATGGGA	AAGCAGGCTGACTTGGTTGC

SREBP-1c, sterol regulatory element-binding protein 1c; ACC-1, Acetyl-CoA carboxylase 1; FASN, fatty acid synthase; AMPKα, subunit alpha of adenosine monophosphate-activated protein kinase; UCP-2, uncoupling protein 2; 36B4, acidic ribosomal phosphoprotein PO.

KBR and the islets were incubated for a further 1h with 2.8, 11.1 and 22.2 mM glucose. In another series of experiments, islets were isolated from CTL mice for the evaluation of insulin release in response to 2.8, 11.1 and 22.2 mM glucose in association or not with 10 μM, 100 μM or 1 mM D-pinitol [these concentrations were based on those of a report that incubated *L6* myotubes with this inositol (Yap et al. 2007)]. At the end of the incubation period, the supernatant was collected and stored at –20°C for posterior insulin measurement by RIA using ^{125}I human insulin (Perkin Elmer, MA, USA).

Endocrine pancreas morphology

After euthanasia the pancreases were collected from all mice groups and weighted, followed fixation in sodium-phosphate buffer (PBS) pH 7.4 containing 4% paraformaldehyde for 24h. Afterwards, the pancreases were dehydrated in alcohol, permeabilized with xylene and then embedded in paraffin. Sections of 5 μm in thickness were stained with hematoxylin and eosin. All islets present in the sections were covered systematically by capturing images with a digital camera (Olympus DP71, Olympus Optical, São Paulo, SP, BRA) coupled to an optical microscope (Olympus BX51, Olympus Optical, São Paulo, SP, BRA). For islet morphometry, the areas of all islets per section and the area of the histological section analyzed were manually measured using the *straight tool* of the Image J software (http://ddsdx.uthscsa.edu/dig/itdesc.html). The classification of the percentage of the islets per section by size was made as previously reported (Freitas et al. 2019). The islet mass was calculated by multiplying the absolute pancreas weight by the ratio obtained from the sum of all islet areas in the pancreas divided by the area of respective pancreatic section analyzed (Ribeiro et al. 2012).

Statistical analysis

Results are presented as means ± SEM for the number of determinations (n) indicated. The area under the curve (AUC) was calculated by trapezoidal integration using GraphPad Prism® version 5.00 for Windows (San Diego, CA, USA). Data were first analyzed using the Komorogov-Smirnov test and then submitted to parametric (one-way ANOVA followed by Newman-Keuls post-test) or non-parametric (Kruskal-Wallis followed by Dunns multiple comparison post-test) variance analysis using GraphPad Prism® version 5.00. The level of significance was set at $P < 0.05$.

RESULTS

Obesity evaluation

As previously demonstrated (Araujo et al. 2019, Balbo et al. 2000, Bonfleur et al. 2015, Macho et al. 2000, Nardelli et al. 2011, Oida et al. 1984), from 30 to 120 days of age, MSG mice displayed similar BW (Fig. 1b and 1c), food (Fig. 1d) and water (Fig. 1e) intakes, and feed efficiency (Fig. 1f) to those observed for CTL mice. D-pinitol treatment did not change BW, or total food and water intake and feed efficiency in MPIN and CPIN mice, when compared with MSG and CTL groups, respectively (Fig. 1b-1f).

At the end of the experimental period, MSG mice displayed an increase of 21% in Lee index ($P < 0.0001$; Fig. 1g) and enhanced adiposity, since the weights of the retroperitoneal ($P < 0.0001$; Fig. 1h) and perigonadal ($P < 0.0001$; Fig. 1i) white fats, and interscapular brown fat pad ($P < 0.002$; Fig. 1j) were 113%, 83% and 79% higher, respectively, than those observed for CTL mice. D-pinitol treatment did not modify the Lee index or white and brown adiposity in MPIN mice, when compared with MSG mice (Fig. 1g-1j).

Plasma and hepatic lipids profile, and expression of genes involved in hepatic lipid metabolism

MSG mice exhibited higher triglyceridemia in the fasting (Fig. 2a) and fed (Fig. 2c) states, than CTL mice ($P < 0.05$), while fasting and fed cholesterolemia did not differ between MSG and CTL groups (Fig. 2b and 2d). In addition, MSG livers had 41% more TG deposition ($P < 0.05$; Fig. 2e), without modifications in hepatic cholesterol content (Fig. 2f), when compared with CTL liver. D-pinitol treatment in MPIN mice led to an increase of 37% in hepatic TG content (Fig. 2e), compared to the MSG group ($P < 0.05$). Furthermore, the TG content in the liver of the CPIN group was 46% higher than that observed for CTL ($P < 0.05$; Fig. 2e). Furthermore, liver weight was lower in the MSG mice (30.38 ± 2.23 mg/g BW), when compared with the CTL group (38.46 ± 1.35 mg/g BW). D-pinitol treatment did not change the liver weight in MPIN (33.58 ± 1.48 mg/g BW) or in CPIN mice (41.91 ± 1.26 mg/g BW), when compared with their respective controls.

The hepatic expression of the lipogenic genes, sterol regulatory element-binding protein (SREBP)-1c, acetyl-CoA carboxylase (ACC)-1 and fatty acid synthase (FASN), were 169%, 146% and 156%, respectively, higher in the MSG liver, than in the CTL liver ($P < 0.05$, $P < 0.01$ and $P < 0.0001$; Fig. 2g). Additionally, uncoupling protein (UCP)-2 mRNA was 71% higher in MSG liver, while the subunit alpha of adenosine monophosphate-activated protein kinase (AMPKα) mRNA expression was 38% reduced in the MSG, when compared with CTL ($P < 0.05$ and $P < 0.0001$; Fig. 2g). D-pinitol treatment increased the SREBP-1c and FASN gene expressions in MPIN livers by 52% and 41%, respectively, when compared with CTL livers ($P < 0.0001$; Fig. 2g). MPIN livers also exhibited a reduction of 33% in AMPKα mRNA expression, when compared to MSG ($P < 0.05$; Fig. 2g). Furthermore, D-pinitol also enhanced the gene expressions of SREBP-1c and FASN, but reduced the AMPKα mRNA amount in the liver of CPIN group ($P < 0.05$; Fig. 2g).

Glucose homeostasis

As previously reported (Bonfleur et al. 2015, Nardelli et al. 2011), under fasting and fed conditions, MSG-obese mice displayed normoglycemia (Fig. 3a and 3c), which was maintained by hyperinsulinemia (Fig. 3b and 3d), when compared with CTL mice ($P < 0.05$). In the ipGTT, glycemia reached maximal levels at 60 min after the glucose load in MSG-obese mice, but reached maximal levels at 15 min in the CTL group (Fig. 3e). In addition, MSG mice displayed hyperglycemia at 15, 30 and 60 min of

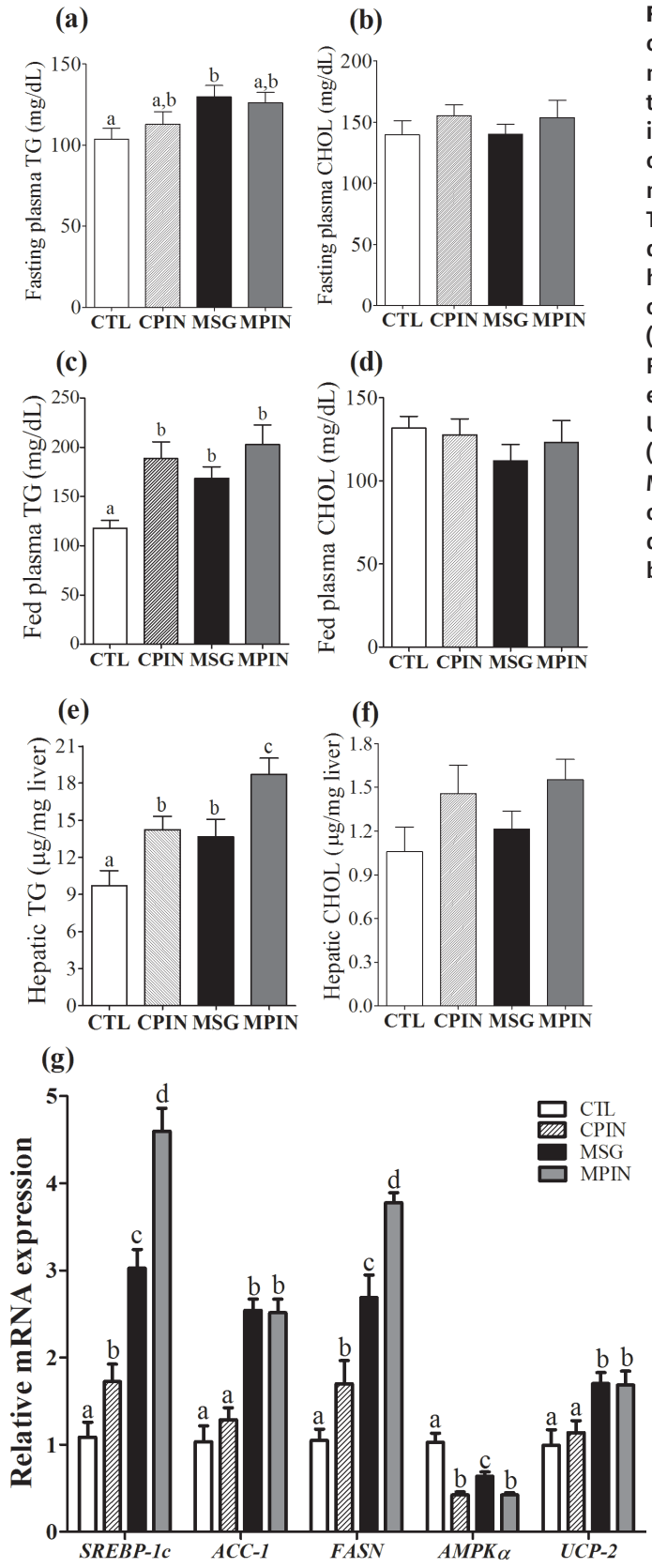

Figure 2. D-pinitol did not change circulating lipids in MSG-obese mice, but increased TG content in the liver of these rodents and also in CPIN mice, through regulation of genes involved in hepatic lipid metabolism. Means ± SEM of plasma TG (a and c) and total CHOL (b and d) plasma concentrations, and hepatic TG (e) and CHOL (f) contents of CTL (n = 15), CPIN (n = 15), MSG (n = 15) and MPIN (n = 12) mice. (g) Relative mRNA expressions of genes encoding SREBP-1c, ACC-1, FASN, UCP-2 and AMPKα in the liver of CTL (n = 7), CPIN (n = 7), MSG (n = 7) and MPIN (n = 7) mice. Different letters over the bars indicate significant difference (Kruskal-Wallis followed by Dunn's post-test, $P < 0.05$).

the test, when compared with the CTL group ($P < 0.05$; Fig. 3e). Total glycemia during the ipGTT, expressed as the area under the curve (AUC), was 80% higher in MSG mice, than that observed for CTL mice ($P < 0.0001$; Fig. 3f). MPIN mice exhibited glucose intolerance to levels similar of those observed for vehicle-treated MSG mice (Fig. 3e and 3f). Furthermore, in the ipITT, after insulin i.p. administration, MSG mice displayed a lower glycemia reduction, than that observed for CTL mice (Fig. 3g). Accordingly, the constant of plasma glucose disappearance (K_{ITT}) was 32% lower in MSG mice, than that observed for CTL mice ($P < 0.01$; Fig. 3h). D-pinitol treatment did not modify the action of insulin in MPIN mice, since these rodents displayed insulin resistance at similar levels to those of MSG mice (Fig. 3g and 3h).

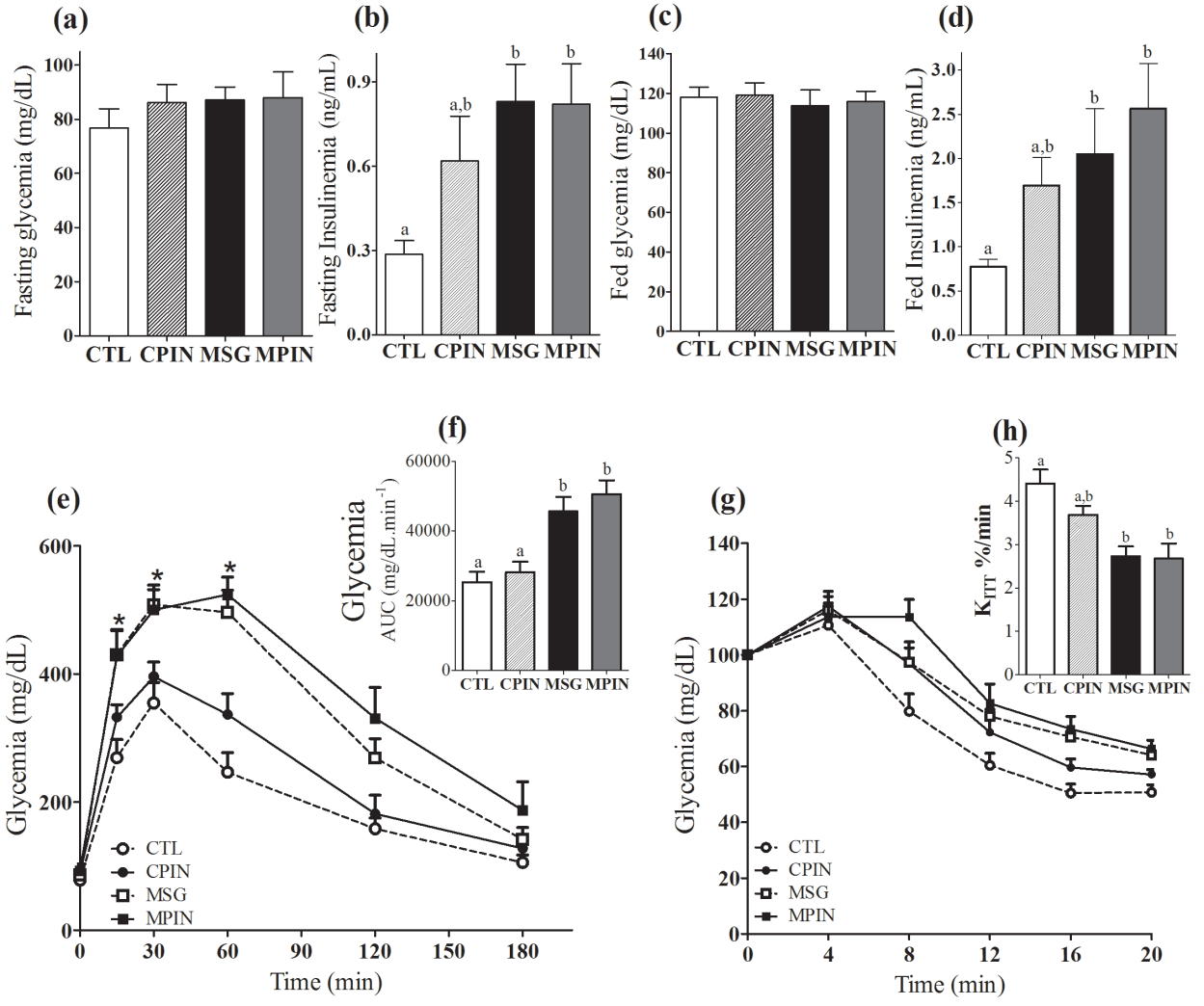

Figure 3. D-pinitol treatment did not change glucose homeostasis parameters in MSG-obese mice. Means ± SEM of fasting and fed glycemia (a and c), insulinemia (b and d) in CTL (n = 12), CPIN (n = 12), MSG (n = 12) and MPIN (n = 14) mice. Changes in glycemia during the ipGTT (e) or ipITT (g) in CTL (n = 11), CPIN (n = 11), MSG (n = 12) and MPIN (n = 11) mice. Means ± SEM of the total glycemia during ipGTT (f), expressed by the AUC; and insulin sensitivity expressed by the K_{ITT} (h). Different letters over the bars represent significant differences (Kruskal-Wallis followed by Dunns post-test, with exception of data in figures b and f, which were analyzed by one-way ANOVA followed by Newman-Keuls post-test, $P < 0.05$).

Insulin secretion and pancreatic islet morphology

As previously reported (Lucinei Balbo et al. 2000, Nardelli et al. 2011), islets isolated from MSG-obese mice secreted more insulin at 11.1 mM glucose than islets of CTL mice ($P < 0.0001$; Fig. 4a); while at basal (2.8 mM glucose) or supraphysiological (22.2 mM glucose) glucose concentrations, the amount of insulin secreted by MSG islets was similar to that of CTL islets (Fig. 4a). D-pinitol treatment increased insulin secretion by islets isolated from MPIN mice, in response to 11.1 mM glucose, when compared

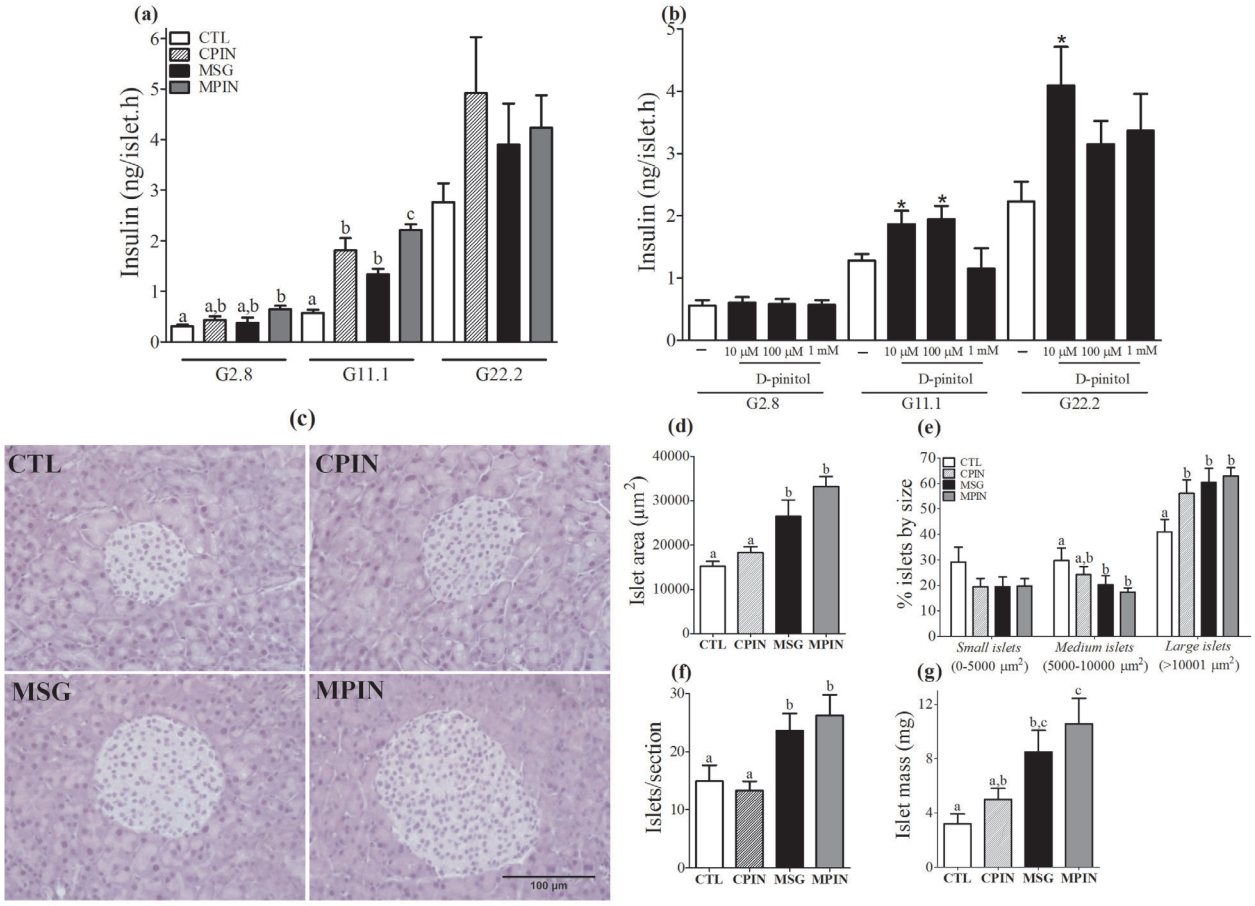

Figure 4. D-pinitol treatment increases glucose-induced insulin secretion in both, MSG and CTL mice, possibly due to a direct insulinotropic action on pancreatic β-cells. (a) Static insulin secretion in response to 2.8, 11.1 or 22.2 mM glucose (G) in islets isolated from CTL, CPIN, MSG and MPIN mice. (b) Insulin secretion of islets isolated from the CTL mice incubated with 2.8, 11.1 or 22.2 mM G without or with 10 µM, 100 µM or 1 mM D-pinitol. Groups of 4 islets of similar sizes were incubated for 1h under the different G or D-pinitol concentrations as indicated in figures a and b. Each bar represents means ± SEM of 10 groups of islets in three independent experiments from islets isolated from 3 mice for each experimental group. Different letters over the bars represent significant differences between the groups at the same G concentration evaluated (Kruskal-Wallis followed by Dunns post-test; $P < 0.05$). (c) Panels show representative images from 5-µm sections of pancreases, stained with hematoxylin and eosin, from CTL, CPIN, MSG and MPIN mice. Scale Bar = 100 µm. Means ± SEM of islet area (d), the percentage of distribution of islets by size (e), number of islets per pancreatic section (f) and islet mass (g) in the pancreases of CTL (n = 5), CPIN (n = 5), MSG (n = 5) and MPIN (n = 5) mice. Endocrine pancreatic morphological analyzes were performed in 3 sections from each pancreas collected. Different letters over the bars represent significant differences (Kruskal-Wallis followed by Dunns post-test, $P < 0.05$).

with MSG islets (P < 0.05; Fig. 4a). The treatment with this inositol also enhanced insulin secretion at 11.1 mM glucose in CPIN islets, in comparison with CTL islets (P < 0.01; Fig. 4a). Trying to investigate whether D-pinitol has direct insulinotropic actions on β-cells, we incubated CTL islets for 1h with 10 μM, 100 μM or 1 mM D-pinitol. We observed that this inositol, at the concentrations of 10 or 100 μM, enhanced insulin secretion by the CTL islets in response to 11.1 mM glucose, but at 22.2 mM glucose, only 10 μM D-pinitol increased insulin release (Fig. 4b).

Figure 4c-g shows representative images from pancreatic sections and morphologic analyses of the endocrine pancreas from MSG and CTL mice, treated or not with D-pinitol. As can be observed in Fig. 4c, MSG islets were hypertrophic, displaying higher islet area and enhanced percentage of medium and large islets, when compared with CTL islets (P < 0.01 and P M; Fig. 4d and 4e). Additionally, MSG pancreata had increased number of islets per pancreatic section evaluated (P < 0.05; Fig. 4f) and increased islet mass (P < 0.05; Fig. 4g), compared to CTL pancreas. D-pinitol treatment did not alter endocrine pancreatic morphology and mass in the MPIN group, when compared with MSG (Fig. 4c-4g). However, an increase in the percentage of large islets was observed in CPIN pancreata, compared to CTL (P < 0.05; Fig. 4e). In addition, the pancreas weight of MSG mice was lower (7.05 ± 0.35 mg/g BW), than that registered for CTL mice (9.33 ± 0.41 mg/g BW; P < 0.05). D-pinitol did not change pancreas weight in the MPIN (7.38 ± 0.50 mg/g BW) and CPIN (9.03 ± 0.62 mg/g BW) groups, when compared with their respective controls.

DISCUSSION

Investigations describing nutraceutical agents with properties that may combat the comorbidities of obesity, such as glucose intolerance, insulin resistance and ectopic fat deposition, have been increasing in the literature (Cicero et al. 2018, Vilar-Gomez et al. 2015, Zelber-Sagi et al. 2017). These agents may enhance weight loss, which represents the best treatment for NAFLD and obesity. D-pinitol has demonstrated hypoglycemic, hypolipolidemic and anti-inflammatory actions mainly in diabetic conditions (Gao et al. 2015, Geethan & Prince 2008, Lopez-Domenech et al. 2018, Sivakumar et al. 2010a, b, Sivakumar & Subramanian 2009a, b). However, whether this inositol has metabolic benefits on hypothalamic obesity is not yet known. Herein, we provide the first evidence that the administration for 30 days of D-pinitol in hypothalamic MSG-obese mice increases insulin secretion, but does not change glucose intolerance and insulin resistance. Furthermore, in contrast to observations that were provided with diet-induced obesity in rodents (Choi et al. 2009, Gao et al. 2015, Kim et al. 2014), herein D-pinitol treatment in hypothalamic obese mice increased TG content in the liver, the hepatic expressions of genes involved in *de novo* lipogenesis, and down-regulated the gene expressions of AMPKα in the liver of MPIN mice.

The hypoglycemic effects of D-pinitol were previously reported in experimental rodents and humans with T1D or T2D (Bates et al. 2000, Geethan & Prince 2008, Kang et al. 2006, Kim et al. 2007, Sivakumar et al. 2010b). The benefits of D-pinitol on body glucose control have been linked with a possible insulin-sensitizing action of this inositol, as D-chiro-inositol has been reported to be one of the compounds that form the phosphoglycan mediators of insulin which are produced after insulin receptor activation

(Larner et al. 1988). In addition, D-pinitol seems to regulate the expression of proteins involved in insulin signaling, since the administration of 60 mg D-pinitol/kg BW/day to obese and diabetic rats increased the hepatic protein content of phosphatidylinositol-3 kinase after 5 weeks, as well as, phosphorylated protein kinase B/Akt (Gao et al. 2015). However, in our study, D-pinitol did not result in any modification in glucose intolerance or insulin resistance in MPIN mice. It is possible that the glucose lowering action of D-pinitol may manifest only when DM is already established. Accordingly, obese subjects that ingested 20 mg D-pinitol/kg BW/day for 1 week did not display modifications in glucose tolerance, insulin sensitivity or in endogenous glucose production (Davis et al. 2000). Similar results were observed in elderly individuals that consumed 2 grams D-pinitol per day for 7 weeks (Campbell et al. 2004) and in leptin-deficient (*ob/ob*) severe obese mice, in which D-pinitol administration also did not improve glucose intolerance or insulin resistance (Bates et al. 2000).

Another novel finding obtained of our study is that D-pinitol treatment enhanced glucose-induced insulin secretion in both MPIN and CPIN mice. This effect may be linked to a direct insulinotropic action of this inositol, since CTL islets incubated with 10 or 100 μM D-pinitol displayed a significantly enhancement in glucose-stimulated insulin secretion. Previously, studies have only indicated a possible action of D-pinitol on β-cell morphofunction, with no evidence that this inositol regulates insulin secretion. For example, in leptin receptor-deficient (*db/db*) diabetic mice, 10 mg D-pinitol/kg BW/day treatment, for 8 weeks, increased insulinemia and the immunoreactivity of insulin in the pancreas (Huang et al. 2015). In streptozotocin-diabetic rats treated with 50 mg D-pinitol/kg BW/day for 4 weeks, an attenuation of β-cell loss through a reduction in oxidative stress in the pancreas has been reported (Sivakumar & Subramanian 2009b). In mice fed on a high-fat diet containing 0.15% D-pinitol for 12 weeks, an increased islet area, but reduced insulinemia were reported (Kim et al. 2014). In the present study, besides the insulinotropic action of D-pinitol, we found that (as mentioned above) this inositol, at least in the CPIN pancreata, might regulate islet-cells hyperplasia, since this group displayed enhanced percentage of large islets in their pancreas.

We also observed in MPIN group that D-pinitol administration did not change abdominal adiposity. Interestingly, a similar effect was previously demonstrated, in a study developed with obese women, in which the consumption of 2.29 g of an inositol mixture (2.00 g pinitol, 0.23 g myo-inositol plus D-chiro-inositol and 0.08 g other polyols) during 12 weeks did not modify BW or adiposity (Lopez-Domenech et al. 2018). Therefore, we speculate that the anti-adiposity action of this inositol could not manifest after obesity installation. However, D-pinitol treatment increased TG content in the liver of MPIN and CPIN mice. This hepatic effect may occur through enhancement in the mRNA amounts for the lipogenic transcription factor, SREBP-1c, which accounts for the enhancement in FASN mRNA gene expression and lipogenesis activation in these groups. In addition, the reduction in the gene expression of AMPKα in MPIN and CPIN livers may contribute to hepatic TG deposition in these groups, since AMPK is a well-recognized protein that inhibits lipogenesis via phosphorylation of ACC, increasing fatty acid flux to β-oxidation (Gruzman et al. 2009), and through the phosphorylation of SREBP-1c, AMPK suppress SREBP-1c cleavage and nuclear translocation, leading to reduced lipogenesis and lipid accumulation in the liver (Li et al. 2011). Therefore, we hypothesize that these

modifications in lipid metabolism in livers of MPIN and CPIN groups could have been resulted from both direct and indirect actions of D-pinitol. The indirect effect could be ascribed to the insulinotropic action of this inositol, in which the stimulus for expression of genes involved in *de novo* lipogenesis and the reduction in the expression of AMPKα mRNA, can be attributed to the enhanced insulin release by the islets from MPIN and CPIN groups.

In conclusion, this study demonstrated for the first time that D-pinitol treatment for 30 days in MSG-obese mice enhanced insulin secretion through a direct effect on glucose-induced insulin secretion. However, D-pinitol increase ectopic fat deposition in the liver of MPIN and CPIN mice by increasing hepatic expression of transcription factors and enzymes involved in *de novo* lipogenesis, and reducing AMPKα gene which might be protective against TG deposition in the liver.

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