Article

D-Pinitol from *Ceratonia siliqua* is an orally active natural inositol that reduces pancreas insulin secretion and increases circulating ghrelin levels in Wistar rats

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Abstract: To characterize the metabolic actions of D-Pinitol, a dietary inositol, in male Wistar rats, we analysed its oral pharmacokinetics and its effects on a) the secretion of hormones regulating metabolism (insulin, glucagon, IGF-1, ghrelin, leptin and adiponectin), b) insulin signaling in the liver and c) the expression of glycolytic and neoglucogenesis enzymes. Oral D-Pinitol administration (100 or 500 mg/Kg) resulted in its rapid absorption and distribution to plasma and liver compartments. Its administration reduced insulinemia and HOMA-IR, while maintaining glycaemia thanks to increased glucagon activity. In the liver, D-Pinitol reduced the key glycolytic enzyme pyruvate kinase and decreased the phosphorylation of the enzymes AKT and GSK-3. These observations were associate with an increase in ghrelin concentrations, a known inhibitor of insulin secretion. The profile of D-Pinitol suggests its potential use as a pancreatic protector decreasing insulin secretion through ghrelin upregulation while sustaining glycaemia through liver-based mechanisms of glycolysis control.

Keywords: AKT; D-Pinitol; ghrelin; insulin; insulin resistance; liver; phosphorylation

1. Introduction

Insulin resistance refers to a poor response of insulin receptors through the phosphatidylinositol-3-kinase (PI3K) pathway [1]. This state demands greater secretion of insulin by the pancreas but without effective control on blood glucose levels. Inefficient insulin signaling can occur by several causes [1]. Usually, insulin resistance is associated with high sugar or -fat diets, obesity, hyperglycemia and hyperinsulinemia. This is the prelude to a type 2 diabetes mellitus (T2DM), a condition in which cells cannot respond properly to insulin [1].

Alterations in inositol metabolism are evident in situations of insulin-resistance leading to T2DM. Indeed, insulin resistance is related with changes in levels of two inositol stereoisomers such as D-Chiro-inositol (DCI) and Myo-inositol (MI) that have been proposed to act as second messengers in insulin receptor cell signaling [2]. MI can be produced from D-glucose and is converted to DCI by the action of a specific isomerase so that each tissue has its own MI/DCI ratio. DCI is especially crucial for glycogen synthesis through promoting the dephosphorylation of glycogen synthase (GS) enzyme [3,4]. In agreement with these observations, insulin resistance is associated with i) low levels of DCI in plasma, urine, and insulin-target tissues; ii) excess urine excretion of MI; iii) intracellular deficiency in MI in insulin target tissues. Moreover, administration of DCI or MI restores normal insulin sensitivity in some situations of insulin resistance [5]. But, despite all these evidences, the mechanisms through which MI and DCI exert their effects on insulin signaling are not well understood.

DCI can be synthetized endogenously in small quantities, but in humans most DCI can be obtained from its methylated derivative D-Pinitol (3-O-methyl-chiro-inositol) which pertains to the family of inositols. It is a natural compound found in high concentrations in legumes and soy foods and can be isolated from Bougainvillea spectabilis leaves and carob tree pods [6]. D-Pinitol exhibits important pharmacological properties, among which are its insulinomimetic effects [7,8], its beneficial effects against oxidative stress [9] and its impact on the attenuation of the effects of some pro-inflammatory cytokines [10,11]. Single dose of D-Pinitol was described to lower the plasma levels of glucose in healthy subjects and in patients with T2DM [12,13] and long term treatment with D-Pinitol decreased hyperglycemia and insulin levels in patients with insulin resistance [10,14,15]. Likewise, the chronic administration of D-Pinitol increased the hepatic expression of the PI3K as well as the phosphorylation of the downstream target protein kinase B/AKT, both components of the insulin receptor-signaling pathway [16]. Moreover, treating myocytes with a PI3K inhibitor prevented the increased glucose uptake mediated by D-Pinitol [7]. Therefore, all these observations suggest that D-Pinitol participates directly in the intracellular insulin-signaling pathway. Consequently, and because it can be easily incorporated to the organism from dietary, D-Pinitol was considered a beneficial dietary supplement to prevent T2DM. However, notwithstanding all the aforementioned, other studies showed no effect of acute or chronic D-Pinitol supplementation on glycemic control [17-19], thus indicating that the effects and mechanisms of action of D-Pinitol on glucose metabolism remain inconclusive.

To date, most data about the effects of D-Pinitol are limited to its role in attenuating the hyperglycemia in experimental diabetic scenarios and/or in postprandial period. No pharmacokinetics of D-Pinitol has been published. The present study aimed to investigate the acute effects of D-Pinitol on the insulin levels as also on several hepatic components of the insulin-mediated cell signaling in fasted Wistar rats. We focused also on hepatic glycogenesis and gluconeogenesis pathways as well as on the secretion profile of those cytokines involved in the control of the insulin-glucose metabolism [1,20-22].

Our hypothesis is that D-Pinitol is rapidly absorbed and distributed to the plasma and liver compartment, modulating insulin actions and contributing to glucose handling.

2. Materials and Methods

2.1. Ethics statement

Animal experimental procedures were carried out in accordance with the European Communities directive 2010/63/EU and Spanish legislation (Real Decreto 53/2013, BOE 34/11370–11421, 2013) and approved by Bioethics Committee for Animal Experiments of the University of Malaga, Spain, and in accordance with the ARRIVE guidelines [23]. Accordingly, all efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Animals

The experiments were performed with 4 to 5 week-old male Wistar rats (Crl:WI(Han)) weighting 400±20 g Charles River Laboratories, Barcelona, Spain). The animals were kept under a standard conditions (light regimen of 12/12 hs, day/night) and under temperature and humidity control. The rats were fed on standard pellet diet (STD) (3.02 Kcal/g with 30 Kcal% protein, 55 Kcal% carbohydrates and 15 Kcal% fat; purchased from Harlam (Tecklad, Madison WI). Water and food were available ad libitum. Animals were anaesthetized with intraperitoneal (ip) sodium pentobarbital (50 mg/Kg body weight) before sacrificed by decapitation.

2.3. Drug preparation and experimental design

Caromax®-D-Pinitol (3 O methyl d chiro-inositol, 98% purity) was generously provided by Euronutra (https://www.euronutra.com/, Málaga, Spain), in the form of crystalline fine powder (lot: PPN-M0201). D-Pinitol was dissolved in water to be administered by gavage (orally) at 100 mg/Kg or 500 mg/Kg concentrations at a volume of 1 mL/Kg. After overnight fasting, the rats were orally administered with a single corresponding dose of D-Pinitol. The rats receiving an oral dose of 100 mg/Kg D-Pinitol, were sacrificed in groups (n=5) at different times: 10, 20, 30, 60, 120, 240 and 360 min after D-Pinitol load. For the oral dose of 500 mg/Kg, animals were sacrificed at times: 60, 120 and 240 min after D-Pinitol administration (n=8 per group). Water was administered by gavage to control group (n=8) in a volume of 1 mL/Kg body weight.

2.4. Sample collection

Blood and liver samples were immediately collected. Blood was centrifuged (2100 g for 8 min, 4 °C) and the plasma was kept at -80° C for biochemical analysis. Liver samples were flash frozen in liquid nitrogen, then stored at −80°C until analysis.

2.5. Plasma Pinitol Concentration

Plasma Pinitol concentrations were monitored by Medina Foundation (Parque Tecnológico de Las Ciencias de la Salud, Granada 18016, Spain) using a specific liquid chromatography-mass spectrometry method. Detection of analytes and internal standards were carried out in multiple reaction monitoring mode (MRM) with electrospray positive ionization. Detection limits were 333 to 20000 ng/mL D-Pinitol. Calculations were performed using non compartmental analysis of plasma data after extravascular input by means of the computer program PK Solver 2.0 [24].

2.6. Measurement of metabolites and hepatic enzymes in plasma

The following plasma metabolites were measured: glucose, urea, uric acid, creatinine, bilirubin, and the hepatic enzymes glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT) and gamma-glutamyl transferase (GGT). These metabolites were analyzed using commercial kits according to the manufacturer's instructions and a Hitachi 737 Automatic Analyser (Hitachi Ltd, Tokyo, Japan). The plasma levels of cytokines were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) method using commercial kits: leptin, adiponectin, insulin and ghrelin ELISA kits (EMD Millipore Corporation, Billerica, MA, USA, cat. number:

#EZRL-83K, #EZRADP-62K, #EZRMI-13K and #EZRGRT-91K, respectively); glucagon EIA kit (Sigma-Aldrich, Saint Louis, MO, USA, cat. number: RAB0202-1KT); and IGF 1 ELISA kit (Thermo Scientific, Waltham, MA; USA, cat. number: ERIGF1). All serum samples were assayed in duplicate within one assay, and results were expressed in terms of the particular standard hormone. The homeostasis model assessment-β (HOMA-β) was calculated following the formula HOMA-β = (20 × FINS) / (FBG − 3.5); FINS = fasting serum insulin, FGB = fasting blood glucose.

2.7. Glucose tolerance tests (GTT)

Before acute treatment, rats (n=8) were food deprived for 18 h and given a dose of 100 mg/Kg of D-Pinitol (via gavage) 240 and 30 min before an ip injection of 2 g D-glucose/Kg. Blood samples were collected from the tail vein at 0 (basal level), 5, 10, 15, 30, 45, 60 and 120 min after D-glucose injection and glucose concentrations were measured with a commercially available glucometer (AccuCheck, Roche, Germany).

2.8. Insulin tolerance tests (ITT)

Before acute treatment, overnight fasting rats (n=8) were given a dose of 100 mg/Kg of D-Pinitol (via gavage) 1 and 2 hours before an ip injection of 0.75 insulin units/Kg. Blood samples were collected from the tail vein at 0 (basal level), 5, 10, 15, 30, 45, 60 and 120 min after insulin injection and glucose concentrations were measured with a commercially available glucometer (AccuCheck, Roche, Germany).

2.9. RNA isolation and cDNA synthesis

Total RNA was extracted from tissue sections of liver (50–80 mg) using the Trizol® method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, EE.UU.). Total RNA was quantified using a spectrophotometer Nanodrop TM ND-1000 (Thermo Fisher Scientific Waltham, MA, EE.UU.) to ensure A260/280 ratios of 1.8 to 2.0. Reverse transcription was carried out from 1 µg of RNA using the Transcriptor Reverse Transcriptase kit and random hexamer primers (Transcriptor RT, Roche Applied Science, Mannheim, Germany). Negative controls included reverse transcription reactions that omitted the reverse transcriptase.

2.10. Real-time qPCR and Gene Expression Analysis

Real-time qPCR was performed following the criteria of the MIQE guidelines [25]. Real-time qPCR reactions were carried out in a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) as previously reported (Decara et al., 2018). The gene-specific probes for the target rat genes: *Fbp1* (Fructose 1,6 Bisphosphatase 1), *G6pc* (Glucose-6-Phosphatase Catalytic Subunit), *Pc* (Pyruvate Carboxylase), *Pck1* (Phosphoenolpyruvate Carboxykinase 1), *Pklr* (Pyruvate Kinase Liver/RBC), *Actb* (beta Actin), and *Gapdh* (Glyceraldehyde-3-Phosphate Dehydrogenase) are shown in Supplementary Table 1S. All probes were obtained based on TaqMan® Gene Expression Assays and the FAM™ dye label format (Life Technologies). For all reference and target gene studies, two independent biologic samples of each experimental condition were evaluated by qPCR which, in turn, was conducted in duplicate reactions as previously described [26]. The raw fluorescence data were submitted to the Miner algorithm available on line (http://www.miner.ewindup.info/) for calculation of respective quantification cycle (Cq) and efficiency values [27]. The target and reference gene sequence amplifications were verified to show comparable efficiencies. Repeatability between replicates was accepted when Cq values differed ≤0.7. For the relative quantification, the mean of duplicates was used. Expression of both *Actb* and *Gapdh* genes was unaffected during all experimental treatments. *Actb* gene was chosen as reference gene and the Cq values were normalized in relation to the Cq *Actb* (ΔCq). Relative quantification was calculated using the ΔΔCq

method and normalized with respect to the control group. Fold gene expression values were determined using the 2 ΔΔCq method [28].

2.11. Protein extraction and western blot analysis

Total protein from 15-25 mg of liver samples was extracted using ice-cold cell lysis buffer for 30 min as previously described [29]. Fifty micrograms of protein were resolved on a 4 12% (Bis-Tris) Criterion XT Precast Gels (Bio-Rad Laboratories, Inc., cat. number: 3450124) and electroblotted onto nitrocellulose membranes (BioRad). For specific proteins detection the membrane was incubated 1 h in TBS-T containing 2% BSA and the corresponding primary antibody. Phosphorylated form of proteins was determined using the corresponding rabbit anti-phospho-AKT, phospho-GSK3β, phospho-Glycogen Synthase, phospho-mTOR (Cell Signaling Technology Inc. MA.). The total protein was detected by using rabbit anti-AKT, anti-GSK3β, anti-Glycogen Synthase, anti-mTOR, respectively (Cell Signaling Technology Inc. MA.). Adaptin γ was detected using mouse anti-Adaptin γ (Becton, Dickinson and Company (BD), New Jersey, USA). Primary antibodies were detected using anti-rabbit or an anti-mouse HRP-conjugated antibody as appropriate (Promega, Madison, WI, USA, respectively). Specific proteins were revealed using ECL™ Prime Western Blotting System (GE Healthcare, Chicago, IL, USA), in accordance with the manufacturer's instructions. Images were visualized in ChemiDoc MP Imaging System (Bio Rad, Hercules, CA, USA). After measuring phosphorylation proteins, the specific antibodies were removed from membrane by incubation with stripping buffer (2% SDS, 62.5 mM Tris HCL pH 6.8, 0.8% ß-mercaptoethanol) 30 min at 50 °C. Membranes were extensively washed in ultrapure water and then re-incubated with the corresponding antibody specific for total protein. Quantification of results was performed using ImageJ software (http://imagej.nih.gov/ij). The specific signal level for total proteins was normalized to signal level of the corresponding Adaptin γ band of each sample and in the same blot. The phosphorylation stage of a protein was expressed as the ratio of the signal obtained with the phospho- specific antibody relative to the appropriate total protein antibody. The amounts of the protein of interest in control samples were arbitrarily set as 1.

2.12. Cell culture and in vitro experimental design

INS-1E β-cells were cultured in complete medium containing RPMI 1640 (GIBCO) supplemented with 5% FBS (GIBCO), 1mM Sodium Pyruvate (GIBCO), 2mM glutamine (GIBCO), 50 μM 2-mercaptoethanol (GIBCO), 10mM HEPES (Lonza), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma) at 37 °C in a humidified atmosphere containing 5% CO_2 [30]. For experiments the cells were seeded at a density of 2.5x105 cells/well in 12-well plates in 2 mL of complete medium until 80% of confluence. Then, medium was changed and cells were maintained in 1 mL of complete medium and stimulated with 3 mM or 11 mM glucose (D-(+)-Glucose Solution (Sigma) for 15 hs. Subsequently, cells were washed twice with glucose free complete medium and incubated for 2 hs in this medium. Cell cultures were washed twice with glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH; 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 10 mM HEPES and BSA 0.1 %, pH 7.4). Next, cells were incubated in 500 μL of KRBH and stimulated 30 min with different concentrations of D-Pinitol (1 μM, 10 μM, 100 μM and 1000 μM). Control cell samples were maintained in glucose free KHRB, or in glucose 3 mM KHRB, or in glucose 11 mM KHRB. Incubation was stopped placing the plates on ice. Supernatants were collected and their insulin content was measured by ELISA kit (EMD Millipore Corporation, Billerica, MA, USA, cat. number: #EZRMI-13K).

2.13. Statistical analysis

Graph-Pad Prism 6.0 software was used to analyse the data. Values are represented as mean ± standard error of the mean (SEM) of 4-10 determinations for each *in vivo* experimental group

according to the assay. The significance of differences within and between groups was evaluated by one-way analysis of variance (ANOVA) followed by post-hoc test for multiple comparisons. Alternatively, for comparisons between two groups, a Student t-test was also used. A P value ≤ 0.05 was considered statistically significant. (*= P<0.05; †= P<0.01; ‡= P<0.001).

2.14. Data availability

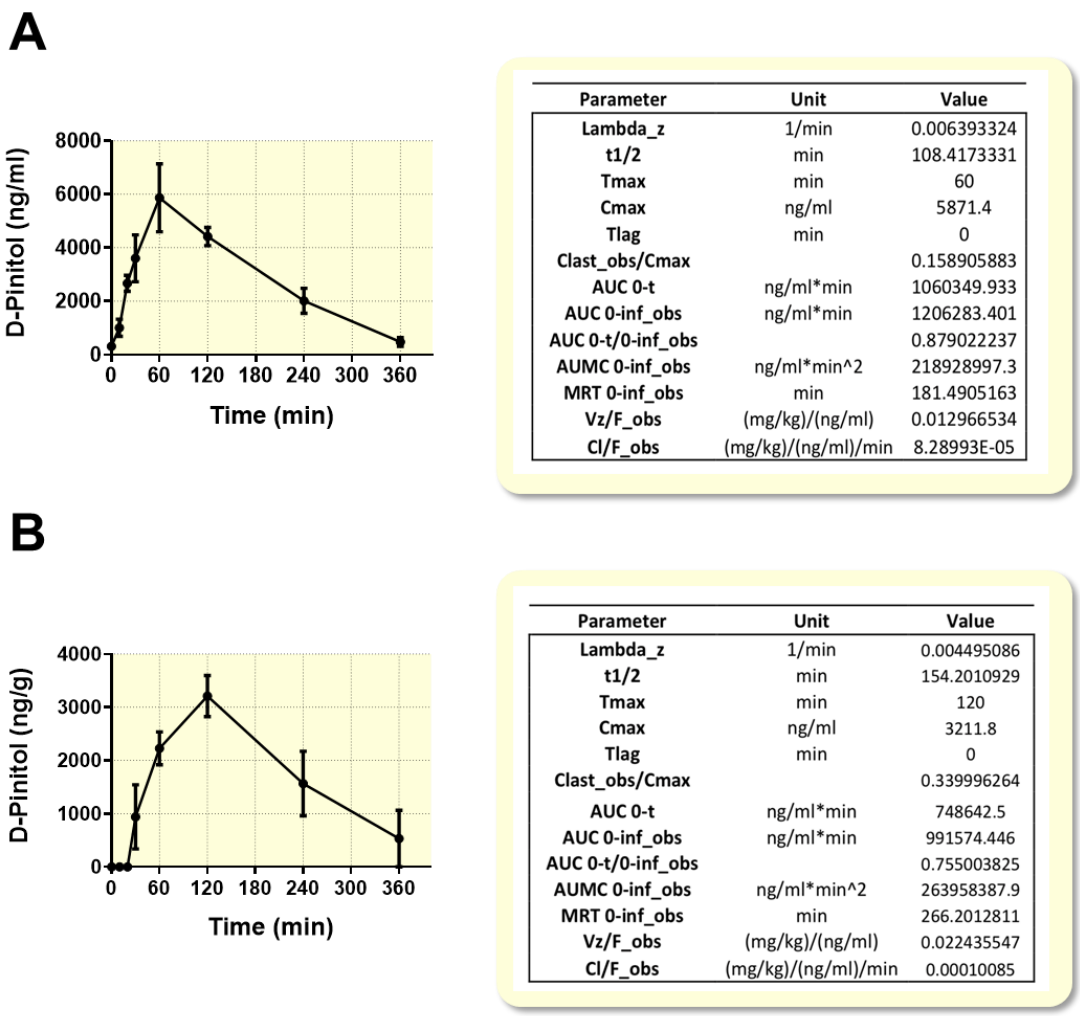
All data generated or analysed during this study are available from the corresponding author on reasonable request.

3. Results

3.1. Pharmacokinetics analysis shows a rapid absorption and detection of D-Pinitol in plasma and liver of Wistar rats after acute oral load

We performed preliminary studies in plasma and liver to examine the absorption and clearance of D-Pinitol orally administrated to Wistar rats. Pharmacokinetics analysis of oral administration of D-Pinitol was obtained by monitoring plasma concentration of D-Pinitol at 0, 10, 20, 30, 60, 120, 240 and 360 min after the oral administration of a 100 mg/Kg in 18 hours food-deprived male Wistar rats. As shown in Figure 1A, fasting plasma D-Pinitol concentrations were below the level of detection (minute 0). After supplementation, plasma D-Pinitol became detectable as soon as 10 min, and peaked at 60 min (Tmax) showing a rapid absorption and clearance with a half-life (t1/2) of 100 min. Liver concentration of D-Pinitol was below detectable levels at 0, 10 and 20 min and became detectable at 30 min after oral administration. A peak of liver concentration of D-Pinitol was observed at 120 min (Figure 1B), thus 1 h later with respect to plasma Tmax. Of note is that no accumulation was detected in liver showing a half-life (t1/2) of 154 min, slightly greater than plasma clearance (Figure 1B), suggesting either, a rapid metabolism or clearance from hepatic tissue.

When tested higher oral dose of D-Pinitol (500 mg/Kg) it peaked at 120 min in plasma and liver, with plasma Cmax of 54.42 ± 12.73 μg/mL and liver Cmax of 9.87 ± 1.07 μg/mL but still showing a rapid clearance.



Parameter	Unit	Value
Lambda_z	1/min	0.006393324
t1/2	min	108.4173331
Tmax	min	60
Cmax	ng/ml	5871.4
Tlag	min	0
Clast_obs/Cmax		0.158905883
AUC 0-t	ng/ml*min	1060349.933
AUC 0-inf_obs	ng/ml*min	1206283.401
AUC 0-t/0-inf_obs		0.879022237
AUMC 0-inf_obs	ng/ml*min^2	218928997.3
MRT 0-inf_obs	min	181.4905163
Vz/F_obs	(mg/kg)/(ng/ml)	0.012966534
Cl/F_obs	(mg/kg)/(ng/ml)/min	8.28993E-05

Parameter	Unit	Value
Lambda_z	1/min	0.004495086
t1/2	min	154.2010929
Tmax	min	120
Cmax	ng/ml	3211.8
Tlag	min	0
Clast_obs/Cmax		0.339996264
AUC 0-t	ng/ml*min	748642.5
AUC 0-inf_obs	ng/ml*min	991574.446
AUC 0-t/0-inf_obs		0.755003825
AUMC 0-inf_obs	ng/ml*min^2	263958387.9
MRT 0-inf_obs	min	266.2012811
Vz/F_obs	(mg/kg)/(ng/ml)	0.022435547
Cl/F_obs	(mg/kg)/(ng/ml)/min	0.00010085

Figure 1: Concentration of D-Pinitol in **A)** plasma (ng/ml) and **B)** liver tissue (ng/g), after oral load (dose: 100 mg/kg) at different times. The values are means ± SEM (5 animals per treated group, Wistar male rats). Lambda_z: first order rate constant associated with the terminal (log-linear) portion of the curve. Estimated by linear regression of time vs. log concentration. t1/2: half-life. Tmax: time of maximum observed concentration. For non-steady-state data, the entire curve is considered. For steady-state data, Tmax correspond to points collected during a dosing interval. If the maximum observed concentration is not unique, then the first maximum is used. Cmax: maximum observed concentration, occurring at Tmax. If not unique, then the first maximum is used. Tlag: extravascular input (model 200) only. Tlag is the time prior to the first measurable (non-zero) concentration. Cl: clearance. Clast_obs: total body clearance for extravascular administration. AUC: area under the curve. AUMC 0-inf_obs: area under the first moment curve (AUMC) extrapolated to infinity, based on the last observed concentration. MRT: mean residence time. Vz: Volume of distribution.

3.2. *Acute administration of D-Pinitol showed no signs of liver and kidney toxicities*

The effects of a single dose of D-Pinitol in fasted rats were also investigated on plasma biomarkers revealing the state of the kidney or liver function [31,32]. Kidney function analysis included the study of creatinine, urea, and uric acid. After D-Pinitol (100 mg/Kg) administration at 60, 120 and 240 min, no changes in the plasma concentrations of uric acid, creatinine, and urea were detected regarding the concentrations found in untreated control group (0 min), therefore revealing no symptoms of renal malfunction at any time after D-Pinitol administration (Table 2S). Likewise,

liver function parameters included the analysis of the plasma concentration of bilirubin and the concentration of the hepatic enzymes aspartate aminotransaminase/glutamic oxaloacetic transaminase (AST/GOT), and alanine aminotransaminase/glutamic pyruvic transaminase (ALT/GPT). After 60, 120 and 240 min of D-Pinitol (100 mg/Kg) administration, no changes in the basal levels of these hepatic enzymes and bilirubin were detected with respect to the levels found at 0 min (Table 2S).

The same set of analyses was performed in rats treated acutely with 500 mg/Kg dose of D-Pinitol. At this dose, the plasma levels of creatinine found between 60 to 240 min after D-Pinitol load were reduced ($P<0.01$) in comparison with levels found at 0 min. Also, urea levels in plasma were reduced ($P<0.01$) after 60 min (Table 3S). Likewise, D-Pinitol 500 mg/Kg dose reduced ($P<0.05$) the levels of the hepatic transaminase AST/GOT at 120 min while increased ($P<0.01$) the levels of ALT/GPT at 240 min (Table 3S). Despite this last observation, the global analysis of these results indicated that D-Pinitol can be considered safe for liver and kidney at a single oral administration at the amounts of 100 and 500 mg/Kg.

3.3. *Acute D-Pinitol oral administration reduced both plasma insulin levels and insulin resistance index in fasted rats*

D-Pinitol is reported to facilitate the glucose uptake by the skeletal muscle in mouse model acting as insulin mimetic [33], thus its oral administration in the short-term-fasted Wistar rats was expected to reduce the glucose levels below its basal levels in plasma. Surprisingly, D-Pinitol administration did not affect glycaemia but clearly reduced plasma insulin levels. Oral D-Pinitol intake at either 100 (Figure 2A) or 500 (Figure 2B) mg/Kg dose decreased insulin concentration in plasma, showing levels below baseline at all times tested ($P<0.01$ and $P<0.001$). Unlike for insulin, the levels of glucagon were slightly increased after 60 min of 100 mg/Kg of oral dose of D-Pinitol (Figure 2A), and significantly raised ($P<0.05$) after 120 min of 500 mg/Kg of D-Pinitol oral load (Figure 2B). Accordingly, the analysis of the glucagon/insulin ratio in all groups tested showed a trend to increase with respect to the corresponding ratio observed at 0 min; this increase was statistically significant after 60 min ($P<0.05$) of 100 mg/Kg D-Pinitol (Figure 2A) dose and after 120 min ($P<0.05$) of 500 mg/Kg dose of D-Pinitol (Figure 2B).

The hormone glucagon leads the processes of glycogenolysis and gluconeogenesis to compensate for the decrease of plasma glucose during fasting. According to the above exposed, it would be expected that the levels of circulating glucose would increase due to the combined effect of glucagon and the decrease in insulin levels after D-Pinitol intake. However, we found that baseline glucose levels remained unchanged within the period of 0 to 240 min after acute administration of 100 or 500 mg/Kg of D-Pinitol (Figure 2A & 2B). We reasoned that the decrease in insulin levels could be accompanied by an increase in insulin sensitivity by D-Pinitol treatment, therefore counteracting the effect of glucagon and thus keeping baseline plasma glucose levels constant. Thus, the homeostatic model assessment of insulin resistance (HOMA IR) was evaluated as the insulin-sensitive range. As shown in Figure 2A & Figure 2B, acute intake of D-Pinitol at either dose of 100 or 500 mg/Kg, reduced significantly the HOMA IR, observing this effect at 60, 120 and 240 min post-D-Pinitol oral load. Hence, this indicated that D-Pinitol could exert a clearly beneficial effect by increasing insulin sensitivity and probably relieving the pancreas from more insulin secretion.

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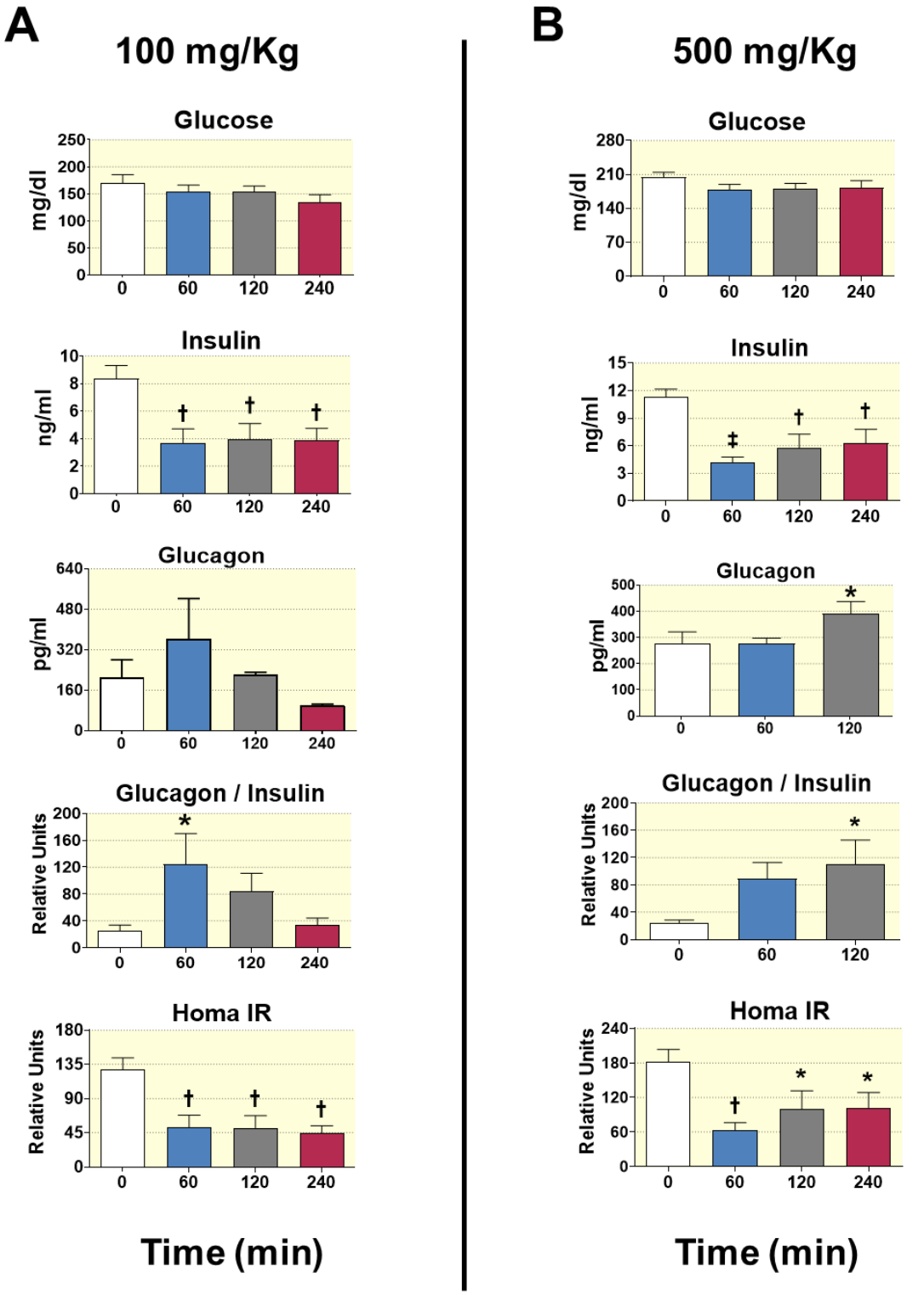

Figure 2: Effect of D-Pinitol on glucose, insulin and glucagon levels; on glucagon/insulin ratio and on insulin resistance index (Homa IR) at different times after administration. **A)** Dose: 100 mg/Kg, **B)** Dose: 500 mg/Kg. Values measured in plasma of Wistar male rats. The values are means ± SEM, 4-5 animals per group. Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0.05, † P<0.01, ‡ P<0.001 vs 0 min group.

3.4. D-Pinitol reduces insulin secretion in INS-1 cells

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For further verification of the inhibitory action of D-Pinitol on insulin secretion, we performed a set of in vitro experiments in rat insulinoma INS 1 cells under basal conditions (3 mM glucose) or stimulated by high glucose (11 mM) glucose to insulin secretion in the absence or presence of different concentrations of D-Pinitol (1, 10, 100 and 1000 μM). The results obtained indicated that D-Pinitol partially inhibited glucose-stimulated insulin secretion in vitro, (Figure 3). Therefore, these results are in agreement with the suggestion that D-Pinitol reduces insulin plasma levels in Wistar rats by inhibiting insulin secretion in pancreas.

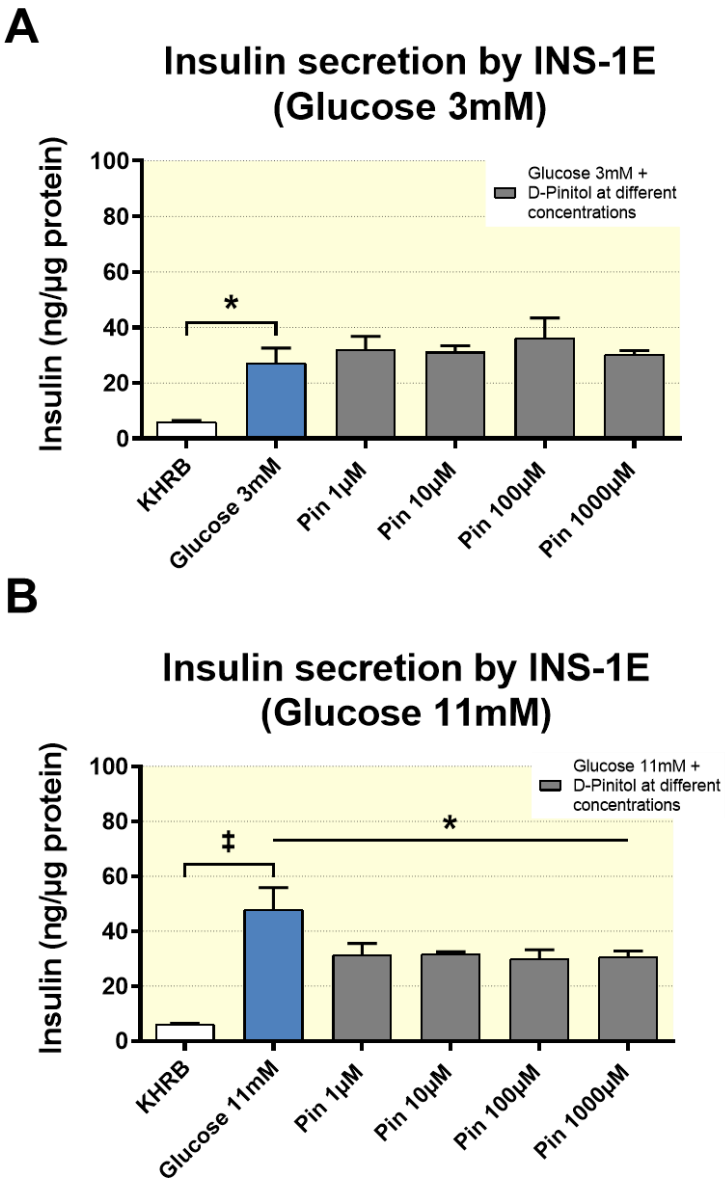

Figure 3: Effect of increasing concentrations of D-Pinitol (1 μM, 10 μM, 100 μM and 1000 μM) on insulin secretion by INS-1 cells under two glucose concentration conditions: **A)** 3mM glucose medium, **B)** 11mM glucose medium. Control groups shown in both conditions are represented by cell cultures treated with glucose-free Krebs-Ringer Bicarbonate HEPES medium (KRBH). The values are means ± SEM, 3 samples per treated group. Differences between groups were evaluated using one-way Anova + Fisher's LSD test: **A)**: * P<0.05 vs KHRB medium group, **B)**: * P<0.05 vs 11mM glucose medium group, ‡ P<0.001 vs KHRB medium group.

3.5. Acute D-Pinitol oral administration induced transient intolerance to glucose and insulin

We next studied alterations in glucose tolerance in rats receiving D-Pinitol. To this purpose, the glucose tolerance test (GTT) was assessed in fasted Wistar rats orally loaded with 100 mg/Kg of D-Pinitol 30 min (DP-30 group) or 240 min (DP-240 group) before glucose (2 g/Kg) injection. A control group received only glucose injection. Values for the blood glucose at 0, 5, 10, 15, 30, 45, 60 and 120 min after glucose load were measured and the area under the curve (AUC) was calculated for each group. As shown in Figure 4A, DP 30 group did not improve the glucose tolerance but rather showed a delay in glucose lowering being the blood glucose levels statistically higher than those of the control group at 30 min after the exogenous glucose load. Indeed, the AUC in the DP-30 group was significantly higher than the AUC of the control group (Figure 4A) thus suggesting glucose intolerance in this group, although the magnitude was small (less than 20% of increase). Nevertheless, it is relevant to indicate that glucose lowering efficiency in DP-30 group was similar to control group from 45 min to 120 min thus indicating that the glucose intolerance observed at 30 min post glucose load was transient (Figure 4A). Indeed, no major differences were observed between the DP-240 min group and the control group regarding the glucose kinetics in blood after exogenous glucose load (Figure 4A).

Insulin tolerance test (ITT) was performed to determine whether the administration of D-Pinitol (100 mg/Kg) affected the response to exogenous insulin. The ITT was assessed 1 h (DP-1h) or 2 hs (DP-2hs) after D-Pinitol administration and the blood glucose concentration were measured before and after (5, 10, 15, 30, 45, 60 and 120 min) insulin ip administration. We found that there was a diminished response ($P<0.01$ and $P<0.05$) to insulin in group DP-1h at 45, 60 and 120 min regarding the control group (Figure 4B). This effect was no longer observed in group DP-2hs, thus suggesting that the effect of D-Pinitol on insulin intolerance was transient. Nevertheless, no statistical difference was observed between the AUC of control group and the AUC from D-Pinitol treated groups (Figure 4B).

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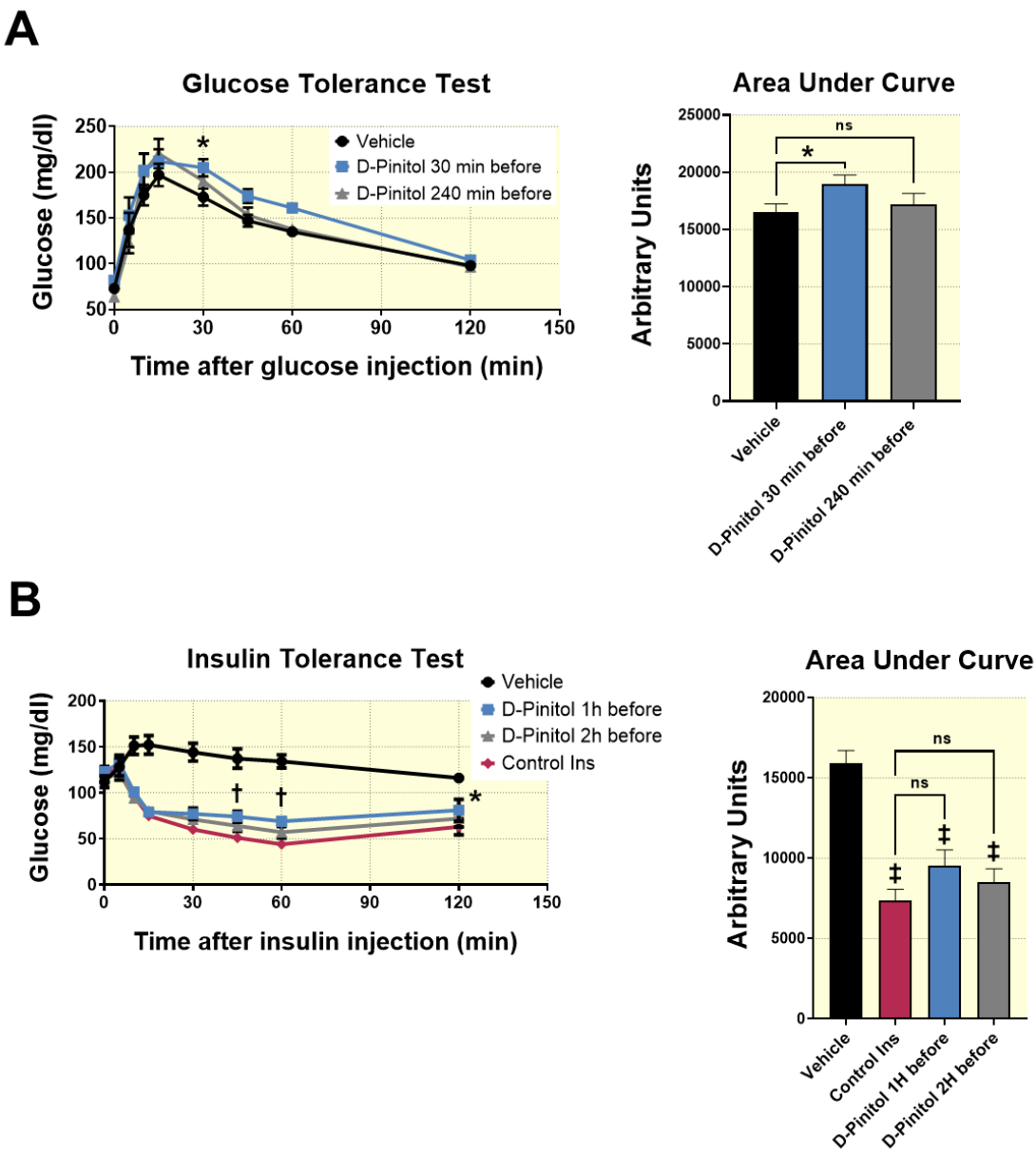

Figure 4: Changes in Wistar male rats blood glucose (mg/dl) during: **A)** glucose tolerant test (GTT). Animals were fasted for 18 h before they received an i.p. injection of glucose (2 g/Kg i.p.). Blood glucose concentrations were measured in blood drawn from the tail vein using a glucometer (AccuCheck, Roche, Germany) at 0 (basal level), 5, 10, 15, 30, 45, 60 and 120 min after the glucose injection. The GTT was done at the end of 30 or 240 min of D-Pinitol (100 mg/Kg p.o.) administration. A control group (vehicle) received only glucose injection. The area under the curve (AUC) was calculated for each group. The values are means ± SEM, 8 animals per group. Data were analysed using two-way Anova + Fisher's LSD test: * P<0.05 D-Pinitol 30 min group vs vehicle group, ns = not significant. **B)** insulin tolerance test (ITT). Animals were fasted for 18 h before they received an i.p. insulin injection (0,75 insulin units/Kg). Blood glucose concentrations were measured in blood drawn from the tail vein using a glucometer (AccuCheck, Roche, Germany) at 0 (basal level), 5, 10, 15, 30, 45, 60 and 120 min after the insulin injection. The ITT was done at the end of 1 or 2 h of D-Pinitol (100 mg/Kg p.o.) administration. Vehicle group received only a saline solution injection; Control Ins group received only insulin injection. The area under the curve (AUC) was calculated for each group. The values are means ± SEM, 8 animals per group. Data were analysed using two-way Anova + Fisher's LSD test: * P<0.05 D Pinitol 1h

group vs Control Ins group, † P<0.01 D-Pinitol 1h group vs Control Ins group, ‡ P<0.001 vs Vehicle group, ns = not significant.

3.6. *Acute oral administration of D-Pinitol inhibits the gene expression of the glycolytic enzyme Pyruvate Kinase in liver*

We next investigated the effect of D-Pinitol oral intake on the gene expression of key enzymes controlling glycolysis and gluconeogenesis in the liver of Wistar rats. The gene expression of the hepatic enzymes such as pyruvate kinase (*Pklr*), glucose-6-phosphatase catalytic subunit (*G6pc*), fructose-1,6-bisphosphatase (*Fbp1*), and pyruvate-carboxykinase (*Pck1*), is normally under dietary and hormonal control. Their expression in liver was evaluated at 0, 20, 30 and 60 min after D-Pinitol load in fasted rats by PCR analysis. The results revealed that the expression of the three-gluconeogenic enzymes: *Fbp1*, *G6pc*, and *Pck1* was not altered in all groups of rats that received D-Pinitol at the times tested as compared with the control group (0 min) (Figure 1S). In contrast, the analysis of the gene expression of the glycolytic enzyme *Pklr* showed a clear and sustained inhibition of transcription as soon as from 10 min (P<0.001) after D-Pinitol load, with a trend to recover normal levels from 60 min (P<0.05) post D-Pinitol administration (Figure 5). This last observation might indicate that oral D-Pinitol treatment under fasting conditions reduced the hepatic glycolysis, probably diverting liver metabolism to an active release of glucose. This was further analyzed in the following set of experiments.

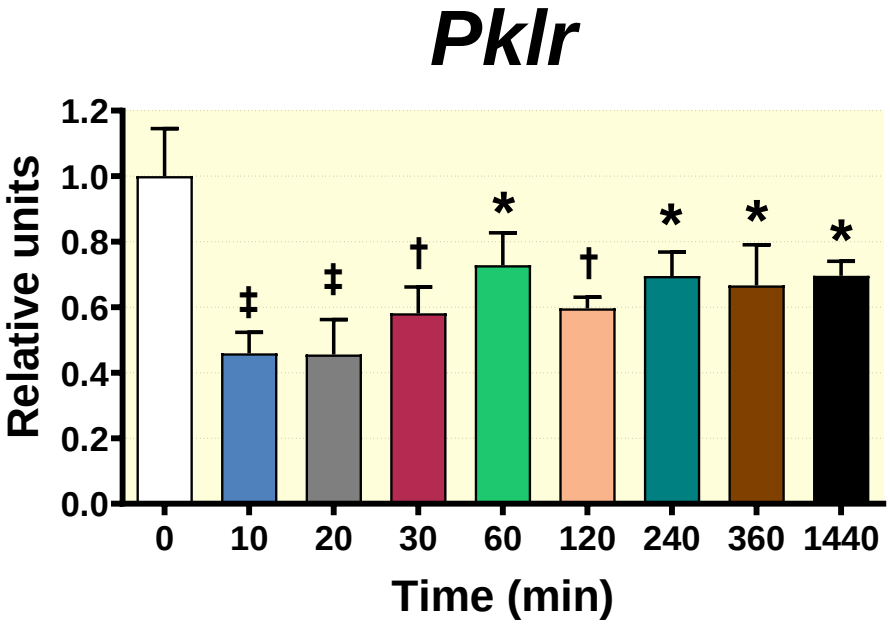

Figure 5: qPCR analysis of *Pklr* gene expression in liver tissue of Wistar male rats measured at different times (0, 10, 20, 30, 60, 120, 240, 360 and 1440 min) after D-Pinitol treatment (100 mg/Kg p.o.). The values are means ± SEM, 4-5 animals per group. Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0.05, † P<0.01, ‡ P<0.001 vs 0 min group.

3.7. *Acute D-Pinitol oral load increases ghrelin levels in plasma*

The effects of D-Pinitol on peripheral hormones modulating both glucose metabolism and insulin response, (adiponectin, leptin, IGF 1 and ghrelin) were also evaluated in plasma after D-Pinitol administration. Neither adiponectin nor leptin, nor IGF 1 plasma concentrations were affected after acute D-Pinitol load at 60, 120 and 240 min as compared with 0 min control group

(Table 4S). In contrast, examination of the plasma levels of the hormone ghrelin, which is known to inhibit the insulin secretion in pancreatic ß-cells *in vivo* [34], showed that 100 mg/Kg dose of D-Pinitol was able to increase significantly the levels of ghrelin in plasma as soon as 10 min (P<0.01) after D-Pinitol load with a clear trend to maintain the levels of ghrelin above the baseline levels in plasma (Figure 6). Likewise, the higher dose of 500 mg/Kg of D-Pinitol showed a significant (P<0.001) increase of this hormone in plasma 60 min and (P<0.05) 240 min after D-Pinitol administration (Figure 2S). Together, these results indicated that the effect of D-Pinitol on ghrelin levels are specific for this hormone.

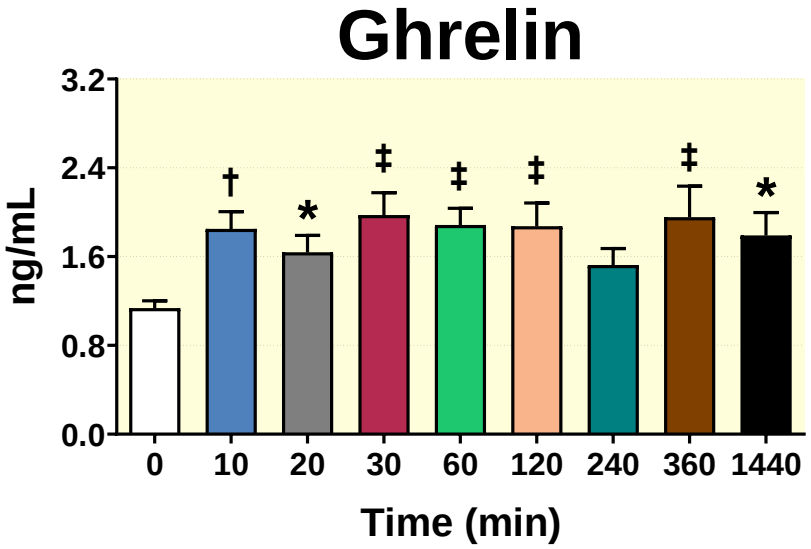

Figure 6: Effect of D-Pinitol (100 mg/Kg p.o.) on ghrelin levels (ng/ml) in plasma of Wistar male rats at 0, 10, 20, 30, 60, 120, 240, 360 and 1440 min after administration. Values measured using a commercial ELISA kit. The values are means ± SEM, 3-5 animals per group. Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0.05, † P<0.01, ‡ P<0.001 vs 0 min group.

3.7. Acute D-Pinitol administration attenuates insulin signaling in liver

Next, we sought to examine the consequences of the reduced insulin circulating levels in the liver tissue of fasted rats receiving oral administration of D-Pinitol. To this aim, we examined several key proteins of the insulin mediating signaling in the liver of rats treated with 100 or 500 mg/Kg of D-Pinitol. Figure 7A displays the western blot analysis of the AKT phosphorylation stage (p-AKT) showing its clearly reduction after 60 min of D-Pinitol (500 mg/Kg) administration, while the levels of total AKT protein remained unchanged. The analysis also indicated that this effect was transient since the amounts of basal p AKT were recovered after 120 min post-D-Pinitol intake. According with a decreased activity of AKT, the phosphorylation of its substrate GSK-3ß (p-GSK3ß) was also temporarily reduced at 60 min after D-Pinitol intake (Figure 7B), thus rendering this GSK-3ß more active. When active, GSK 3ß phosphorylates and inhibits the enzyme glycogen synthase (GS). However, not only was no increase in GS phosphorylation but even a significant reduction of GS phosphorylation was observed 60 min after the administration of the inositol (Figure 3SA). Although no significant, the phosphorylation status of the mTOR protein, a downstream substrate of AKT, was slightly reduced at 60 min of acute D-Pinitol intake as compared with control groups (Figure 3SB). All these observations in the insulin-AKT pathway were compatible with the reduced insulin secretion levels observed after D-Pinitol administration.

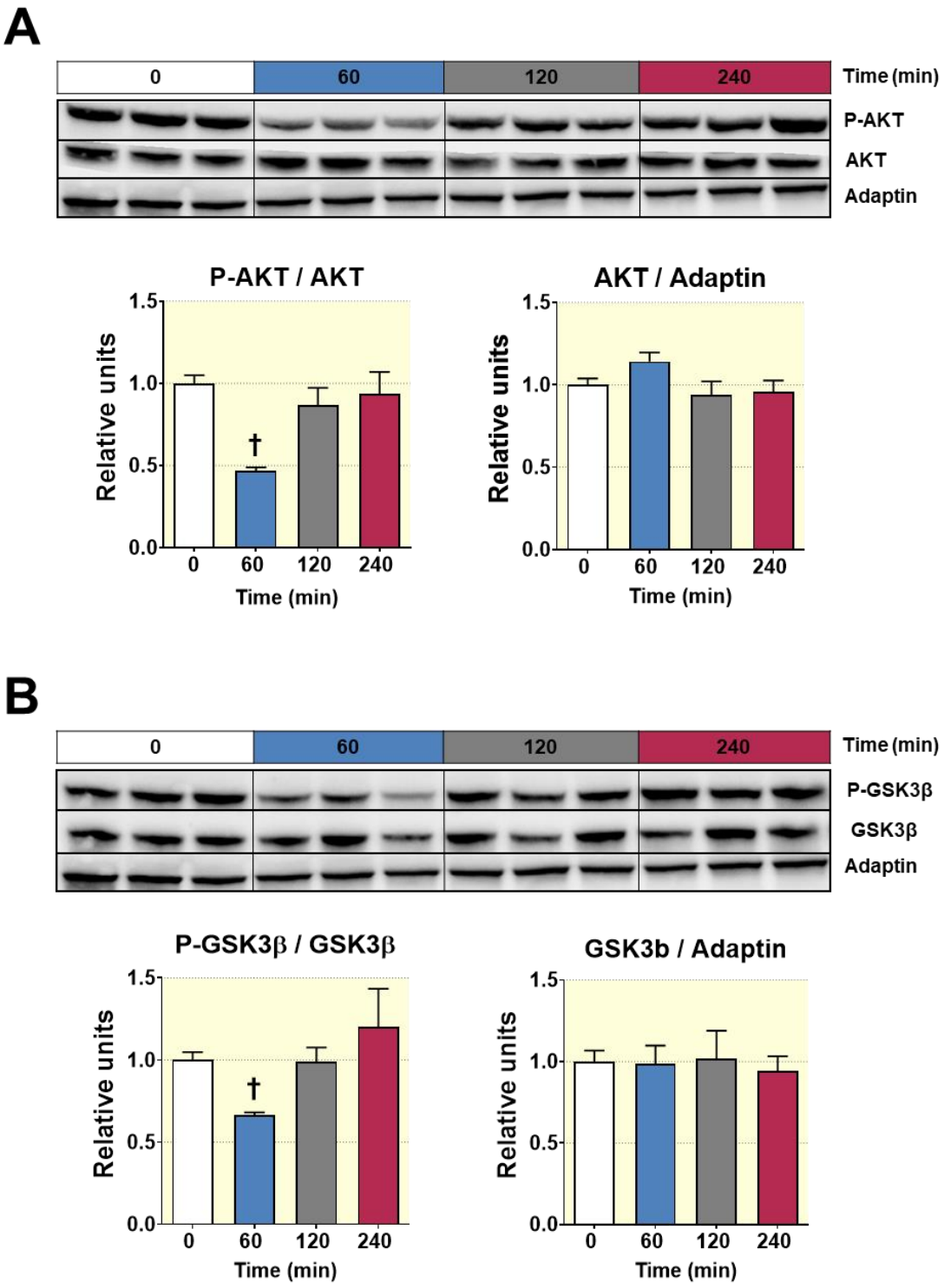

Figure 7: Western blot analysis of the phosphorylation status of the enzymes AKT and GSK3β from liver lysates of Wistar rats treated with 500 mg/Kg of D-Pinitol (p.o.) for 0, 60, 120 and 240 min. **A)** Representative western blot analysis for AKT (upper panels) and p-AKT/AKT ratio and AKT/adaptin ratio (bottom panels) from liver samples of Wistar rats treated with D-Pinitol for times indicated in figure. The blot shows analysis from three independent samples from each treatment group. The corresponding expression of adaptin is shown as a loading control per lane. **B)** Representative western blot analysis for GSK3β (upper panels) and p-GSK3β/GSK3β ratio and GSK3β/adaptin ratio (bottom panels) of liver samples from Wistar rats treated with D-Pinitol at times indicated in figure. The blot shows analysis from three independent samples from each treatment group. The

corresponding expression of adaptin is shown as a loading control per lane. All samples shown in the figure were derived at the same time and processed in parallel in the corresponding blot. The adjustment to digital images did not alter the information contained therein. Differences between groups were evaluated using one-way Anova + Fisher's LSD test: † P<0,01 vs 0 min group.

4. Discussion

The search for natural ingredients that might optimize human glucose metabolism is becoming a priority because of the current obesity pandemics. In this regard, natural inositols, as insulinomimetics, have gained attention as potential functional foods that might help to prevent the development of insulin resistance and diabetes type 2. Our current study provides new perspectives about the metabolic effects of D-Pinitol, a natural inositol from carob fruit, under fasting conditions, when circulating glucose is expected to be at baseline levels. A first innovative approach was to study the oral absorption of this natural compound, under these dietary conditions. The pharmacokinetics analysis showed that the oral D-Pinitol intake is easily absorbed and quickly detected in plasma and in liver in a few minutes, reaching a peak 60 min after its oral ingestion, and suffering a quick clearance from plasma there on, practically disappearing 6 h after its administration. Liver pharmacokinetics indicates an accumulation of this inositol in the liver, shifted in time 60 min, and with a similar rate of clearance. Thus, half-life for plasma was 108 min and 154 min for liver. This pharmacokinetics parallels the transient effects observed on different metabolic aspects here studied and discussed below. It also accounts for the possibility of repeated administration (i.e. twice a day) in case this compound has to be used as an endocrine pancreas protector as it is deduced from its endocrinological profile, discussed below. Another important consequence of these findings is the fact that despite the potential conversion of D-Pinitol into DCI in the acid media of the stomach, a substantial amount of D-Pinitol is incorporated to the blood stream, being able of acting as an active nutritional ingredient.

The analysis of plasma standard markers of renal and liver dysfunction revealed that doses of 100 and 500 mg/Kg of D-Pinitol, are not toxic, being a safe compound. It should be noted that when high doses of D-Pinitol (500 mg/Kg) were tested, the levels of the transaminase GOT were transiently reduced at 120 min while the GPT levels were increased significantly at 240 min. In this sense, repeated administration of D-Pinitol for 9 weeks did not affect transaminase profile (data not shown).

Because D-Pinitol administration seems to ameliorate the hyperglycemia in some models of diabetes, it has been considered as an insulin mimetic, as other inositols such as myoinositol and DCI [10,12-15]. In our model in fasted rats, the main effect of D-Pinitol administration was a significant reduction of circulating insulin resulting in an increased glucagon/insulin ratio. Since plasma glucose did not vary along the experiment, the net result is a saving of insulin, since the secretion of this hormone was reduced up to a 50% for more than 4 h. Of note is that our *in vivo* experiments were carried out under fasting conditions in which glucagon is supposed to play a crucial role in maintaining basal glucose blood levels through promotion of the gluconeogenesis and glycogenolysis in liver. Therefore, after oral D-Pinitol intake, a favorable effect of glucagon on the circulating glucose levels should be expected. But as mentioned above, D-Pinitol did not alter basal plasma glucose concentrations over 240 min in fasted Wistar rats. Other reports showing the ability of D-Pinitol in reducing plasma glucose levels in fasting conditions can be attributed to the use of different *in vivo* models that exhibit hyperglycemia and with different regimen of D-Pinitol treatment. These models are usually models of animals affected by diabetes [7,14,16,35] and diabetic patients [13,15], or studies conducted after food or glucose oral uptake that demonstrate the ability of D-Pinitol in reducing the postprandial blood glucose level and stimulating GLUT4 translocation in the skeletal muscle [33]. In our model, we found that decreased insulin levels by a single D-Pinitol intake was directly related with the HOMA-IR index thus strengthening the sensibility to insulin. In this line, studies carried out in T2DM patients that took D-Pinitol three times a day and chronically showed also reduction in the HOMA-IR index, but in these patients the D-Pinitol treatment reduced

the fasting plasma glucose while no changes in insulin or peptide C was observed [15]. In our study, the inhibitory effect of D-Pinitol on insulin secretion in the rat insulinoma INS 1 strongly suggests that D-Pinitol exerts a direct effect on the pancreatic beta cells. These in vitro observations are in agreement with the negative effect of D-Pinitol on the insulin basal levels found in fasted Wistar rats, which in turn is in line with the ITT results showing a reduced ability of exogenous insulin to lower blood glucose in rats previously treated with D-Pinitol. Even though, as we will discuss later, other hormonal inputs modulating insulin secretion was also affected by this inositol.

Increase in glucagon activity stimulates glycogenolysis and gluconeogenesis via the cAMP/protein kinase A (PKA) activation pathway. PKA activation phosphorylates the CREB (cAMP-responsive element -binding protein) factor [36]. Among its target genes are *Fbp1*, *PCk1* and *G6pc*, all involved in gluconeogenesis. In our experiments, no changes in the transcription of these genes were observed after D-Pinitol administration therefore suggesting that this inositol derivative did not affect the gluconeogenic via, at least at the transcription level of these genes. In contrast, rats that took D-Pinitol presented a decreased expression of the *Pklr* gene which enzyme product (Pyruvate kinase enzyme, L-PK) is closely related with the glycolysis. The L-PK is a rate-controlling glycolytic enzyme and glucagon is known to inhibit both hepatic activity and the *Pklr* gene transcription [37,38] while insulin activates L-PK stimulating its dephosphorylation [39,40,41]. This finding indicates that D-Pinitol leads reduction of the glycolysis pathway, probably as a consequence of the lowering insulin levels. Likewise, the attenuation in the phosphorylation of the hepatic AKT points to a lessening action of insulin in liver which is compatible with the lower insulin levels detected in plasma.

This study also shows that acute D-Pinitol administration did not affect the levels of certain cytokines controlling the energy balance like IGF-1, adiponectin, or leptin (Table 3S). But, interestingly, here we show for the first time the positive effects of single D-Pinitol intake on ghrelin levels and this result is specific for this hormone. It is known that ghrelin inhibits the secretion of insulin in pancreatic β cells [34,42]. Further investigation is needed to decipher the mechanism of action of D-Pinitol on beta cells to inhibit insulin secretion as shown in our experiments with INS-1, but it is also plausible that the increase in ghrelin levels under oral D-Pinitol contributes for the transient decrease in insulin levels detected in the plasma of rats receiving a single dose of D-Pinitol.

In conclusion, our findings confirmed the hypothesis that D-Pinitol is an active ingredient that is rapidly incorporated into the blood stream upon its oral ingestion. The data pointed to D-Pinitol as an active nutrient with very specific actions beyond its known incorporation to glycans acting as insulin signaling mediators. Both, direct and ghrelin-mediated reduction of insulin secretion, while maintaining glycaemia, might help to protect the endocrine pancreas by alleviating pancreatic islets from the high insulin demand that occurs in insulin resistance conditions. This property makes of D-Pinitol a dietary supplement of potential utility in a pro-diabetes scenario (i.e. obesity, aging, etc.) in which pancreas becomes exhausted due to an overproduction of an inefficient insulin. Moreover, the ability of D-Pinitol to induce a transient increase in ghrelin levels opens new perspectives on the actions and use of this inositol in pathologic situations of weight loss such as cachexia and anorexia.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,

Supplementary Table 1 (Table 1S). Primer references for TaqMan® Gene Expression Assays (Applied Biosystems).

Gene description	Assay ID	Nº accession GenBank	Amplicon Length
Target genes			
Fbp1	Rn00561189_m1	NM_012558.3	77
G6pc	Rn00689876_m1	NM_013098.2	64
Pc	Rn00562534_m1	NM_012744.2	97
Pck1	Rn01529014_m1	NC_005102.4	87
Pklr	Rn01455286_m1	NC_005101.4	58
Reference genes			
Gapdh	Rn01775763_g1	NC_005103.4	174
Actb	Rn00667869_m1	NM_031144.2	91

Supplementary Table 2 (Table 2S). Effect of D-Pinitol (100 mg/Kg p.o.) on different metabolites measured in plasma of Wistar male rats at 60, 120 and 240 min post-administration. The values are means ± SEM, (4-5 animals per treated group). Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0.05 vs 0 min group.

Plasma metabolic parameters	0 min	60 min	120 min	240 min
Uric Acid (mg/dl)	1.8 ± 0.132	1.32 ± 0.139	1.88 ± 0.443	1.24 ± 0.068
Creatinine (mg/dl)	0.525 ± 0.025	0.54 ± 0.024	0.44 ± 0.04	0.54 ± 0.024
Urea (mg/dl)	32.25 ± 2.358	37.4 ± 2.42	41.6 ± 4.155	36.2 ± 3.76
Bilirubin (mg/dl)	0.175 ± 0.025	0.1 ± 0.000 * ⇩	0.14 ± 0.024	0.14 ± 0.024
GOT (I.U.)	104.5 ± 12.939	119.0 ± 14.765	127.4 ± 13.923	117.4 ± 12.504
GPT (I.U.)	45.5 ± 8.49	45.8 ± 0.969	59.6 ± 6.608	44.6 ± 4.106

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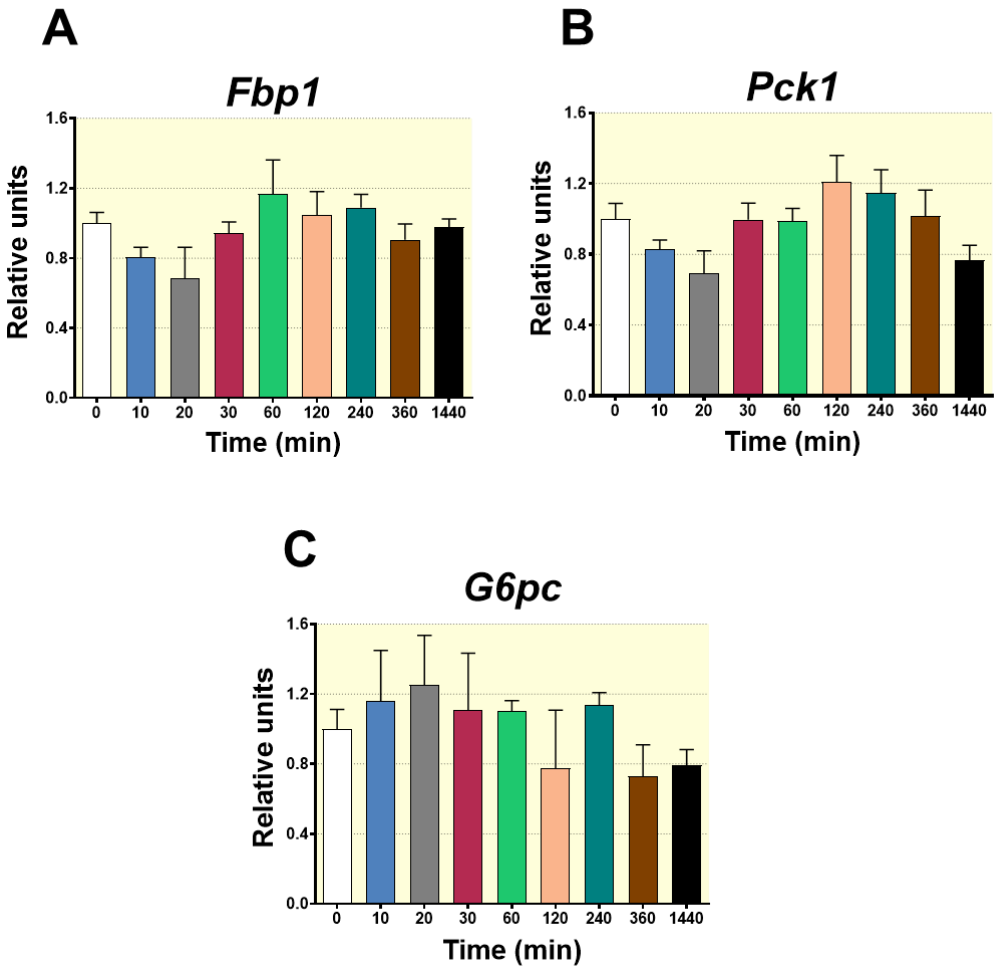
Supplementary Table 3 (Table 3S). Effect of D-Pinitol (500 mg/Kg p.o.) on different metabolites measured in plasma of Wistar male rats at 60, 120 and 240 min post-administration. The values are means ± SEM, (5 animals per treated group). Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0.05, † P<0.01 vs 0 min group.

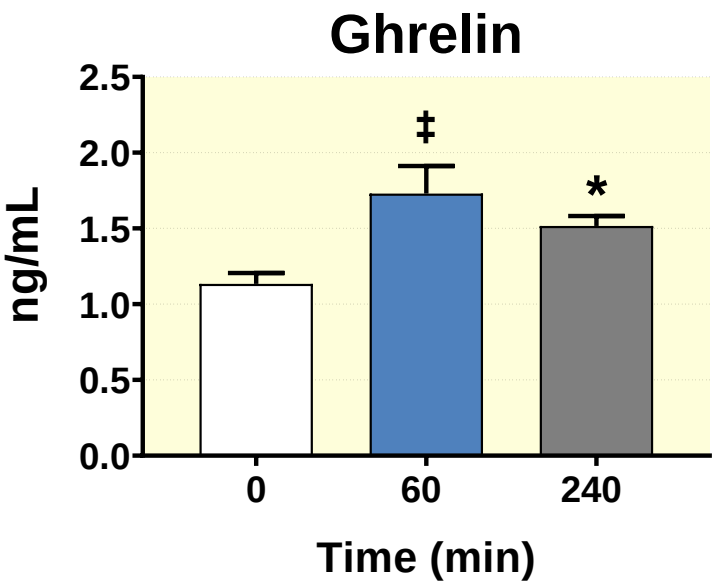
Plasma metabolic parameters	0 min	60 min	120 min	240 min
Uric Acid (mg/dl)	1.738 ± 0.107	1.786 ± 0.222	2.025 ± 0.314	1.2 ± 0.195
Creatinine (mg/dl)	0.688 ± 0.061	0.486 ± 0.023 † ⇩	0.475 ± 0.065 † ⇩	0.45 ± 0.027 † ⇩
Urea (mg/dl)	44.143 ± 2.340	34 ± 3.576 † ⇩	41.5 ± 1.476	44.25 ± 1.264
Bilirubin (mg/dl)	0.178 ± 0.043	0.1 ± 0.027	0.163 ± 0.038	0.1 ± 0.000
GOT (I.U.)	219.5 ± 23.291	191.625 ± 28.869	133.143 ± 6.859 * ⇩	220.125 ± 35.228
GPT (I.U.)	129.875 ± 17.513	103.375 ± 17.644	74.625 ± 7.653	220.125 ± 35.228 † ⇧

Supplementary Table 4 (Table 4S). Effect of D-Pinitol (100 mg/Kg p.o.) on different serum mediators in plasma of Wistar male rats at different times after administration (60, 120 and 240 min). ELISA kits used for this test. Nd: Not done. The values are means ± SEM, (4-5 animals per treated group). Differences between groups were evaluated using one-way Anova + Fisher's LSD test.

Serum mediators	0 min	60 min	120 min	240 min
Adiponectin (ng/ml)	1316.7 ± 62.127	1173.236 ± 87.018	1202.99 ± 76.144	1419.682 ± 75.994
Leptin (ng/ml)	4.669 ± 0.888	3.579 ± 0.631	5.91 ± 1.309	4.231 ± 1.201
IGF-1 (pg/ml)	1420.29 ± 704.41	1187.02 ± 350.534	Nd	920.56 ± 191.92



Supplementary Figure 1 (Figure 1S): qPCR analysis of: **A)** *Fbp1*, **B)** *Pck1* and **C)** *G6pc* gene expression in liver tissue of Wistar male rats measured at different times (0, 10, 20, 30, 60, 120, 240, 360 and 1440 min) after D-Pinitol treatment (100 mg/Kg p.o.). The values are means ± SEM, 4-5 animals per group. Differences between groups were evaluated using one-way Anova + Fisher's LSD test.



Supplementary Figure 2 (Figure 2S): Effect of D-Pinitol (500 mg/Kg p.o.) on ghrelin levels (ng/ml) in plasma of Wistar male rats at 0, 60 and 240 min after administration. Values were measured using a commercial ELISA kit. The values are means ± SEM, (5 animals per treated group). Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0.05, ‡ P<0.001 vs 0 min group.

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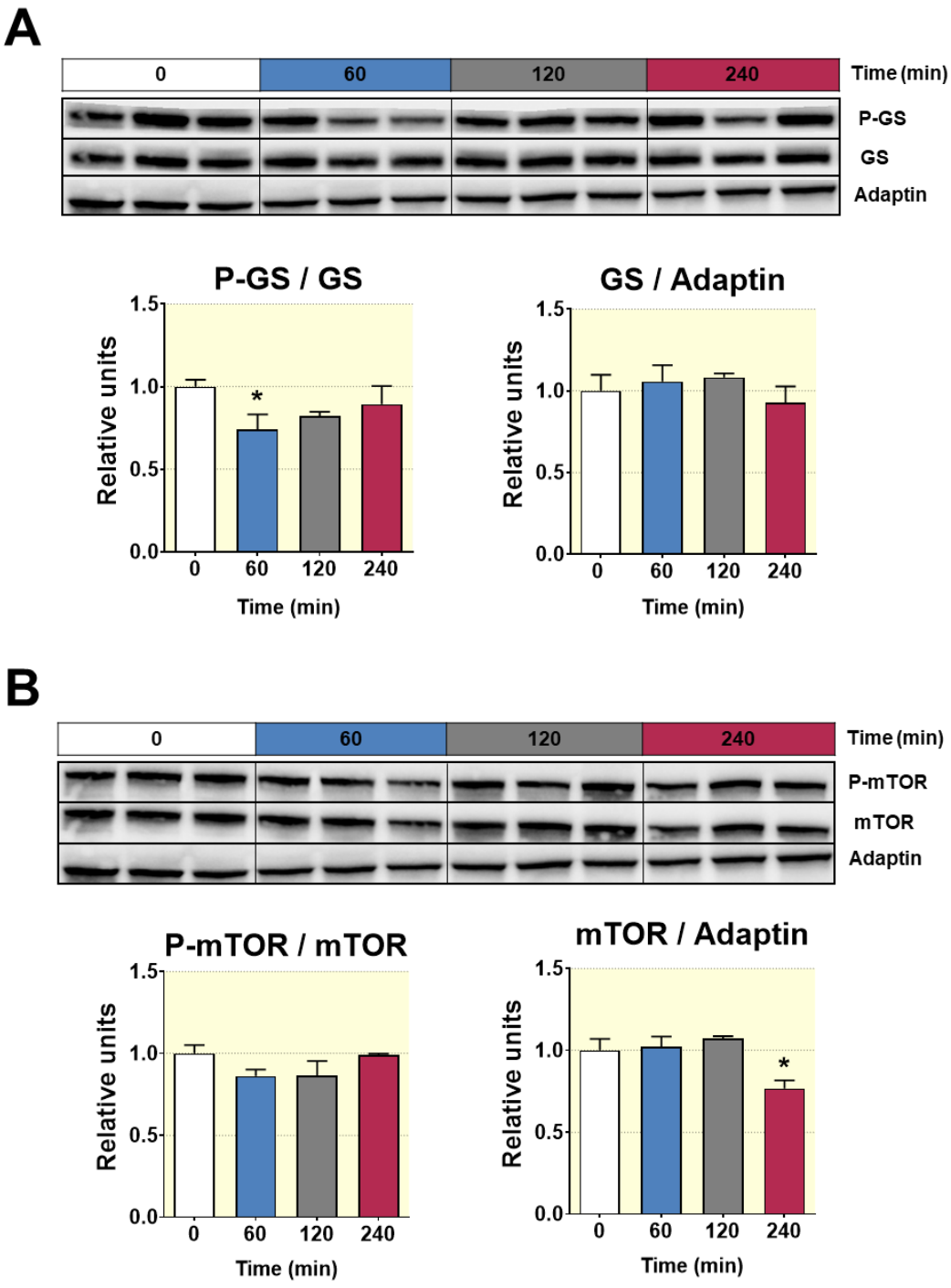

Supplementary Figure 3 (Figure 3S): Western blot analysis of the phosphorylation status of the enzymes GS and mTOR from liver lysates of Wistar rats treated with 500 mg/Kg of D-Pinitol (p.o.) for 0, 60, 120 and 240 min. **A)** Representative western blot analysis for GS (upper panels) and p-GS/GS ratio and GS/adaptin ratio (bottom panels) from liver samples of Wistar rats treated with D-Pinitol for times indicated in figure. The blot shows analysis from three independent samples from each treatment group. The corresponding expression of adaptin is shown as a loading control per lane. **B)** Representative western blot analysis for mTOR (upper panels) and p-mTOR/mTOR ratio and mTOR/adaptin ratio (bottom panels) of liver samples from Wistar rats treated with D-Pinitol at times indicated

in figure. The blot shows analysis from three independent samples from each treatment group. The corresponding expression of adaptin is shown as a loading control per lane. All samples shown in the figure were derived at the same time and processed in parallel in the corresponding blot. The adjustment to digital images did not alter the information contained therein. Differences between groups were evaluated using one-way Anova + Fisher's LSD test: * P<0,05 vs 0 min group.

Author Contributions: Conceptualization, Alfonso Gutierrez-Adan, Carlos Sanjuan, Elena Baixeras and Fernando Rodriguez De Fonseca; Data curation, Juan Antonio Navarro, Juan Decara, Dina Medina-Vera and Juan Suarez; Formal analysis, Juan Antonio Navarro, Juan Decara, Dina Medina-Vera, Javier Pavon, Antonia Serrano, Carlos Sanjuan, Elena Baixeras and Fernando Rodriguez De Fonseca; Funding acquisition, Alfonso Gutierrez-Adan, Carlos Sanjuan and Fernando Rodriguez De Fonseca; Investigation, Juan Antonio Navarro, Juan Decara, Dina Medina-Vera, Ruben Tovar, Juan Suarez, Javier Pavon, Antonia Serrano, Margarita Vida, Carlos Sanjuan and Elena Baixeras; Methodology, Juan Antonio Navarro, Juan Decara, Dina Medina-Vera, Ruben Tovar, Juan Suarez, Javier Pavon, Antonia Serrano, Margarita Vida, Carlos Sanjuan and Elena Baixeras; Project administration, Fernando Rodriguez De Fonseca; Resources, Alfonso Gutierrez-Adan; Supervision, Juan Suarez, Javier Pavon, Antonia Serrano, Alfonso Gutierrez-Adan, Elena Baixeras and Fernando Rodriguez De Fonseca; Writing – original draft, Juan Antonio Navarro, Juan Decara, Elena Baixeras and Fernando Rodriguez De Fonseca; Writing – review & editing, Juan Suarez, Javier Pavon, Antonia Serrano, Alfonso Gutierrez-Adan, Carlos Sanjuan and Fernando Rodriguez De Fonseca.

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Conflicts of Interest: Carlos San Juan declares he receives salary and has share from Euronutra company. The remaining 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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