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Parkinsonia aculeata (Caesalpineaceae) improves high-fat diet-induced insulin resistance in mice through the enhancement of insulin signaling and mitochondrial biogenesis

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ABSTRACT

Ethnopharmacological relevance: The search for natural agents that minimize obesity-associated disorders is receiving special attention. *Parkinsonia aculeata* L. (Caesalpineaceae) has long been used in Brazil as a hypoglycaemic herbal medicine, without any scientific basis.
Aims of the study: In this context, we aimed to use molecular and physiological methods to study the effect of a hydroethanolic extract partitioned with ethyl acetate from the aerial parts of *Parkinsonia aculeata* (HEPa/EtOAc) on insulin resistance in a mouse model of diet-induced obesity (DIO).
Material and methods: Firstly, C57BL/6J mice were fed either with standard rodent chow diet or a high-fat diet (HFD) for 12 consecutive weeks. Then, the animals were treated with HEPa/EtOAc at two doses (125 and 250 mg/kg/day) or metformin (200 mg/kg/day) for 16 days. At the end of the experiment, body weight, fat pad weight, fasting serum glucose (FSG), insulin (FSI) and leptin were measured. Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) was also calculated. Glucose, insulin and pyruvate tolerance tests were performed. The expression and phosphorylation of IRβ^{tyr}, Akt^{ser473}, AMPKα and PGC1α in liver, muscle and adipose tissue were determined by Western blot analyses.
Results: Herein we demonstrate for the first time an improvement in insulin resistance following HEPa/EtOAc administration in obese mice, as shown by increased glucose, insulin and pyruvate tolerance, as well as an improvement in FSG, FSI, HOMA-IR and circulating leptin levels, which together are in part due to enhancement of the insulin signaling pathway in its main target tissues. Surprisingly, the increase in activation of the AMPKα-PGC1-α axis by HEPa/EtOAc was similar to that produced by metformin treatment in the liver and muscle tissues.
Conclusion: In conclusion, *P. aculeata* appears to be a source of therapeutic agent against obesity-related complications.

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1. Introduction

Obesity is a disease with epidemic proportions around the world, especially in developed countries like the United States, as well as in developing countries similar to Brazil (Alberti and Zimmet, 2013; Atkinson, 2014; Ferreira and Magalhães, 2006; WHO, 2012). In a prospective study of 9 million people conducted from 1980 to 2008, it was shown that the average body mass index (BMI) had increased by 0.4–0.5 kg/m^2 per decade (Finucane et al., 2011). This alarming increase in the percentage of fat, which is associated with a reduction in insulin action (insulin resistance), is related to a wide range of health problems, including the increased risk of developing type 2 diabetes (T2D), several endocrine diseases, dyslipidemia, cardiovascular diseases, neurodegenerative diseases and a variety of cancers (Harcourt et al., 2013; Hotamisligil, 2006).

Insulin resistance (IR) is defined as a state of reduced metabolic response to circulating insulin levels (Harcourt et al., 2013; McArdle et al., 2013). It is directly related to abnormalities in peripheral tissues (muscle, liver and adipose tissue), in the central nervous system (hypothalamic neurons involved in the control of

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food intake) and pancreatic beta-cells (Gregor and Hotamisligil, 2011; Kahn et al., 2014; McArdle et al., 2013). Insulin resistance is often associated with a complex network of signaling pathways, including impairment of insulin signaling by changing the docking and downstream signaling of the insulin pathway in peripheral tissues such as the liver, muscle and adipose tissue (Boura-Halfon and Zick, 2009; Gregor and Hotamisligil, 2011). Among the causative mechanisms involved in the above-mentioned signaling impairment are defects in mitochondrial biogenesis associated with excess reactive oxygen species and the subsequent decrease in energy expenditure, which contributes to the metabolic dysfunctions observed in insulin-resistant states (Canto and Auwerx, 2009). In this context, AMP-activated protein kinase (AMPK) has a mitochondrial biogenesis function and it has been shown that peroxisome proliferator-activated receptor c coactivator 1alfa (PGC-1α) activity is necessary for AMPK-mediated mitochondrial activation (Fernandez-Marcos and Auwerx, 2011). Thus the AMPK-PGC-1α axis could be a possible therapeutic target for the treatment of IR.

The development of new pharmacological approaches to prevent or treat this complex metabolic disorder has become an overriding priority. The most commonly used drugs have many side effects and high doses are required in order to obtain any improvement in IR (Kahn et al., 2014). However, medicinal herbs, which have long been used in alternative and complementary medicine and are often considered safe, low-cost alternatives to conventional medicine with multiple targeting actions, are a potent source of IR drugs (Chang et al., 2013; Singh et al., 2013).

Parkinsonia aculeata L. (Caesalpineaceae) is a small, spiny deciduous tree found in the Northeast of Brazil. It has long been used as an ethnomedicine by the local population and traditional healers for the empiric treatment of hyperglycemia, without any scientific basis (de Almeida et al., 2005). In previous studies, we demonstrated the hypoglycaemic activity of different extracts of *P. aculeata* in a type 1 diabetes (T1D) model, i.e. induced by alloxan (Leite et al., 2007, 2011). Based on this evidence and the need for further studies of both the validity of their effects and their mechanisms of action, we set out to conduct a detailed study of the effects of the *P. aculeata* extract in a more comprehensive and complex model, the model of obesity, which has also received attention from the scientific community. In the present paper, we use physiological and molecular methods to study the effect of *P. aculeata* extract on insulin resistance mechanisms in a mouse model of diet-induced obesity (DIO), which closely mimics the metabolic syndrome disorder and initial T2D associated with an unhealthy lifestyle.

2. Materials and methods

2.1. Plant material

Aerial parts of *P. aculeata* were collected from the Xingó region (Sergipe, Brazil) (the geographical coordinates given by GPS – latitude: −37.8396; longitude: −9.619; altitude: 121 m), during February 2012. The plant was identified by Professor H. P. Bautista (INCRA-BA) and a voucher specimen was deposited (no. 500) in the Xingó Herbarium (Canindédo São Francisco, Sergipe, Northeast region, Brazil).

2.2. Preparation of plant extract

Dehydrated and powdered aerial parts of *P. aculeata* were macerated with EtOH:H_2O (1:1; v/v) at the ratio of 100 ml of ethanol to 10 g of dried plant. The suspension was subjected to mechanical agitation for two 24-h cycles at room temperature and at the end of each cycle the material was filtered and placed in a Soxhlet apparatus. After complete removal of the ethanol, the resulting extract was partitioned with ethyl acetate P.A. at a ratio of 1:1 (v/v). The biphasic suspension was subjected to separation in a decanting funnel and the polar phase was collected. Finally, the material was lyophilized and stored at −20 °C until use. The final yield of the hydroethanolic extract partitioned with ethyl acetate (HEPa/EtOAc) was 5.2 g/50 g of the dried powdered aerial parts. Based on two previous studies by our group (Leite et al., 2007, 2011), for pharmacological assays, a fresh dilution of lyophilized HEPa/EtOAc extract in vehicle (distilled water) was prepared on the day of treatment and administered by gavage at doses of 125 and 250 mg/kg b.w. in a fixed volume of 0.2 mL.

2.3. Animals

Male C57BL/6J mice (6-wk-old; obtained from the State University of Campinas Central Breeding Center) were housed under a 12/12 h light/dark cycle in a controlled environment (room temperature: 22 ± 3 °C, humidity: 55 ± 5%) and were randomly allocated into two dietary regimens of either the standard rodent chow diet Nuvilab CR-1 (Nuvital, Colombo, Paraná, Brazil) (8% fat, 26% protein, 54% carbohydrate, as a percentage of total kcal) and water ad libitum or a high-fat diet (HFD) (55% fat, 16% protein, 29% carbohydrate, as a percentage of total kcal) and water ad libitum, i.e. with the aim of developing obesity status, for a period of 12 weeks. Details regarding the composition and preparation of HFD were as described previously (Ropelle et al., 2006; Tobar et al., 2011). All animal protocols were approved by the Animal Care and Use Committee at the State University of Campinas (no. 1962-1) and were in accordance with the guidelines for the Care and Use of Laboratory Animals.

2.4. Evaluation of the subchronic oral treatment

After 12 weeks under the two different dietary regimens (i.e chow diet and HFD), the mice were randomly divided into five groups, comprising six animals each. The control group (CTL) and the diet-induced obesity group (DIO) received the vehicle, once daily, orally for 16 days. The other groups of obese mice received HEPa/EtOAc by gavage, once daily, at doses of 125 mg/kg (PA125) or 250 mg/kg (PA250) for 16 days. Another group of obese mice was treated with metformin (MET), once daily, at a dose of 200 mg/kg, orally for 16 days and was adopted as the positive treatment group. Metformin hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO) and for oral administration was suspended in a vehicle (distilled water). All groups of mice except the CTL group were on a HFD throughout the treatment period.

2.5. Biochemical assays

At the end of the treatments mice were fasted overnight, and blood samples were withdrawn from the retrobulbar intraorbital capillary plexus. The serum was separated by centrifugation (2500 × *g*, 5 min). Glucose values were measured from the tail venous blood using a glucose monitor (glucometer; Bayer Diagnostics, New York, NY). Readouts of fasting serum insulin (EZRMI-13K) and leptin levels (EZRL-83K) were determined by ELISA (Millipore, Bedford, MA). The homeostasis model of assessment (HOMA-IR) score, as a surrogate measurement of insulin resistance, was calculated as: (fasting insulin (μU/mL) × fasting glucose (mmol/l)/22.5), to determine the degree of insulin resistance (Mather, 2009; Matthews et al., 1985).

2.6. Intraperitoneal glucose tolerance test (ipGTT)

An ipGTT was performed after a 6 h fast. After collection of an unchallenged sample (time 0), a bolus of 1.0 g/kg body weight of glucose was administered into the peritoneal cavity, and the blood samples were collected from the tail vein at different time points up to 120 min to determine blood glucose levels, as previously described (Araujo et al., 2012; Caricilli et al., 2011).

2.7. Intraperitoneal insulin tolerance test (ipITT)

After fasting for 6 h, systemic insulin sensitivity was analyzed using the ipITT. Briefly, tail blood samples were withdrawn before (0 min) and at 5, 10, 15, 20, 25 and 30 min after an intraperitoneal injection of 1.5 IU/kg of regular insulin (Humulin-R, Eli-Lilly, Brazil). Glucose concentrations were measured using a glucometer and these values were used to calculate the constant rate of blood glucose disappearance (Kitt), which is based on the linear regression of the naperian logarithm of glucose concentrations obtained from 0 to 30 min of the test. Kitt was calculated using the formula $0.693/t_{1/2}$ (the plasma glucose $t_{1/2}$, was calculated from the slope of the least-square analysis of the plasma glucose concentrations during the linear decay phase) (Bonora et al., 1989).

2.8. Intraperitoneal pyruvate tolerance test (ipPTT)

The pyruvate tolerance test was performed in mice that were fasted for 15 h before being injected intraperitoneally with sodium pyruvate (2 g/kg; Sigma-Aldrich, St. Louis, MO) dissolved in saline. Blood was collected from the tail tip prior to and at various times after injection (Miyake et al., 2002).

2.9. Tissue extraction and immunoblotting

After overnight fasting, mice were anesthetized (anesthesia was ensured by the loss of pedal and corneal reflexes). The abdominal cavity was opened, the portal vein was exposed, and 0.1 ml of normal saline was injected with or without insulin (10^{-6} mol/l). At 30 and 90 s after insulin injection, the liver, gastrocnemius muscle and epididymal adipose tissue were removed, minced coarsely, and homogenized immediately in extraction buffer, as previously described (Araujo et al., 2012; Caricilli et al., 2011; Vecina et al., 2014). In direct immunoblot experiments, protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted, as previously described (Araujo et al., 2012; Caricilli et al., 2011; Vecina et al., 2014), with anti-IRβ, anti-phospho-IRβ^{tyr}, anti-Akt, anti-phospho-Akt^{ser473}, anti-AMPKα, anti-phospho-AMPKα and anti-PGC1α. The homogeneity of gel loading was evaluated by blotting the membranes with antibodies against IR, Akt, AMPK and β-actin as appropriate. All antibodies were from Santa Cruz Technology (Santa Cruz, CA), except anti-Akt, anti-phospho-Akt, anti-AMPKα and anti-phospho-AMPKα, which were obtained from Cell Signaling Technology (Beverly, MA).

2.10. Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM). The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (UN SCAN IT gel®, Silk Scientific Inc., Orem, UT, USA). Multiple comparisons were tested by one-way ANOVA, followed by Tukey's *post hoc* test, with the significance level set at $P < 0.05$ using SPSS software (SPSS for Windows, version 16.0, Chicago, IL, USA).

3. Results

3.1. Physiologic and metabolic characterization of studied animals

Fig. 1 displays comparative data regarding all studied groups, i.e. control group (CTL), diet-induced obesity group (DIO), obese animals treated with HEPa/EtOAc at 125 mg/kg (PA125), obese mice treated with HEPa/EtOAc at 250 mg/kg (PA250), and obese animals that received metformin at 200 mg/kg (MET). As mentioned in the methods section all animals were treated daily for 16 days. As expected, all animals fed on the HFD, regardless of treatment, had a similar body weight; however their weights were higher when compared to the CTL group (Fig. 1A). In terms of white adipose tissue (WAT) weight, all obese animals, treated or not, had higher weights compared to the CTL group and only the MET group showed a significant reduction compared to the DIO group (Fig. 1B). Regarding fasting serum glucose, the DIO, PA125 and PA250 groups showed significantly higher levels when compared to the CTL group, and all treatments reduced fasting glucose levels when compare to non-treated obese mice (Fig. 1C). With respect to fasting serum insulin, all treatments reduced this parameter compared to the DIO group (Fig. 1D). We then analyzed the HOMA-IR to assess insulin resistance, and the results showed a remarkable reduction in the HOMA-IR of all treated groups compared to non-treated obese mice (Fig. 1E). Leptin circulating levels were higher in obese mice compared to lean mice, and all treatments attenuated the deleterious effect of the HFD (Fig. 1F).

3.2. Effects of HEPa/EtOAc treatment on tolerance of glucose, insulin, and pyruvate

To estimate glucose tolerance, an ipGTT was conducted. Throughout the test, the DIO group showed higher glucose levels when compared to those obtained in the CTL group. However, treatment with HEPa/EtOAc for 16 days improved glucose tolerance in obese mice to the same extent as in the positive control group, i.e. the MET group (Fig. 2A). We then performed an insulin challenge, which showed that both concentrations of HEPa/EtOAc, as well as MET, were able to completely restore insulin tolerance in diet-induced obese mice, as demonstrated by the glucose disappearance rate (Kitt) (Fig. 2B and C). To study hepatic glucose production after a pyruvate challenge, we infused pyruvate, intraperitoneally, and evaluated blood glucose for 150 min. The results showed a marked increase in blood glucose levels throughout the pyruvate test in the DIO group when compared to the CTL group, whereas all treatments attenuated blood glucose levels (Fig. 2D), suggesting that HEPa/EtOAc administration is an efficient way to suppress the hepatic glucose output.

3.3. HEPa/EtOAc treatment improves insulin signaling pathway in liver, muscle and adipose tissue of diet-induced obese mice

We next assessed insulin signaling in the liver, muscle and adipose tissue of lean and obese mice. As expected, when comparing obese non-treated mice to lean mice, we observed a marked attenuation in insulin-induced tyrosine phosphorylation levels of IRβ^{tyr} along with lower levels of Akt^{ser473} phosphorylation in liver, muscle and adipose tissue (Fig. 3A–C), thus confirming the development of our insulin resistance model. However, the results showed that HEPa/EtOAc treatment, regardless of its concentration, was able to improve the insulin signaling pathway, as demonstrated by the increased insulin-stimulated IRβ^{tyr} and Akt^{ser473} phosphorylation in all studied tissues (Fig. 3A–C). It is important to mention that such extent of insulin signaling recovery was similar to that obtained in the metformin treatment (Fig. 3A–C).

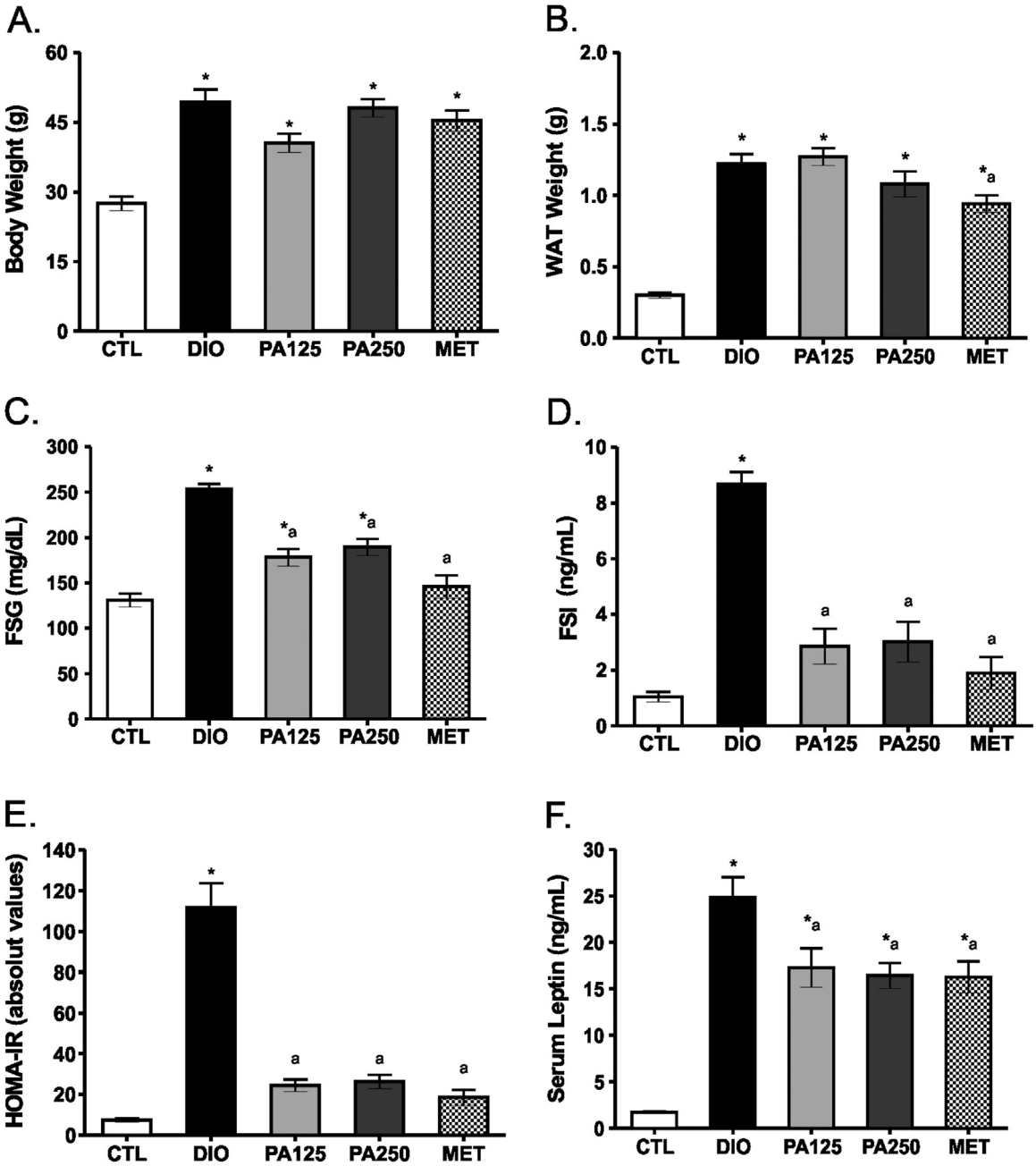

Fig. 1. Physiological and metabolic parameters in lean mice (CTL), non-treated obese mice (DIO), obese mice treated with HEPa/EtOAc at doses of 125 mg/kg (PA125) or 250 mg/kg (PA250), and obese mice treated with metformin at dose of 200 mg/kg (MET). In all groups, the treatments were performed during 16 days. The CTL and DIO groups received vehicle only. All groups except CTL group received the high-fat diet throughout the study. Panel (A), Body weight; panel (B), Epididymal fat pad weight; panel (C), Fasting Serum Glucose; panel (D), Fasting Serum Insulin; panel (E), HOMA-IR; panel (F), Fasting serum leptin. The values represent the mean ± SEM (n=6). One-way ANOVA with Tukey's *post hoc* test was applied. *$P < 0.05$ vs. CTL, $^{a}P < 0.05$ vs. DIO.

3.4. Effects of HEPa/EtOAc on mitochondrial biogenesis (AMPKα-PGC1-α axis)

Regarding AMPKα, the data showed that the high-fat diet induced a reduction in AMPKα phosphorylation in liver and adipose tissue compared with the chow diet (Fig. 4A and C). Surprisingly, treatment with HEPa/EtOAc at both doses resulted in higher phosphorylation levels of AMPKα in all studied tissues. It is worth mentioning that this effect on AMPKα activity following HEPa/EtOAc administration was similar to that obtained by metformin treatment (Fig. 4A and B). In the same way, when we analyzed PGC-1α expression in liver, muscle and adipose tissue, we observed a similar pattern to that of AMPKα activity (Fig. 4A and B). However, unlike AMPKα activity, HEPa/EtOAc treatment was not able to enhance PGC-1α expression in adipose.

4. Discussion

Current trends in obesity and diabetes management involve a significant number of medicines, and although these demonstrate some efficacy, they also have adverse effects. As a result, a much safer therapeutic agent is necessary. In this regard, there has been much interest from the scientific community in the search for alternative therapies, such as herbs used as a source of new chemical compounds. Against this background and based on previous

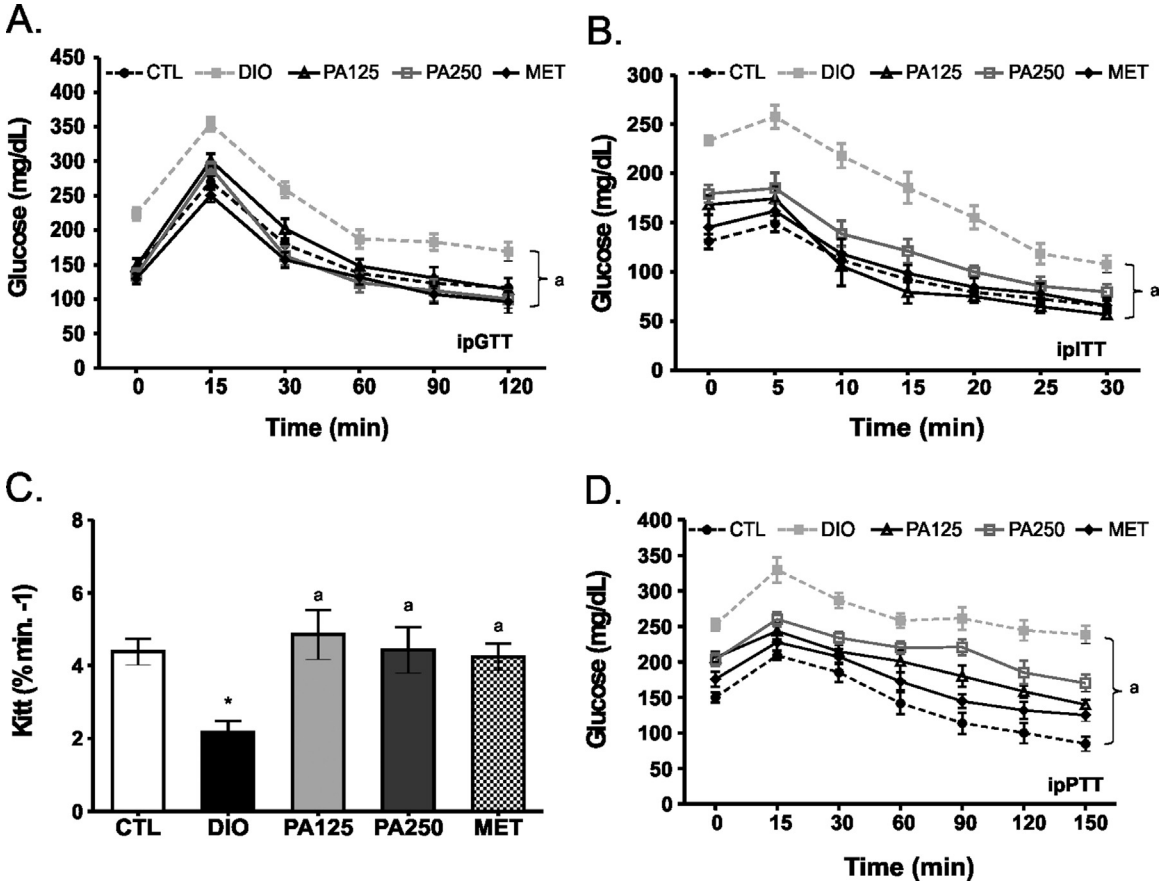

Fig. 2. Intraperitoneal challenge tests of glucose (ipGTT) (A), insulin (ipITT) (B) accompanied by the constant rate for blood glucose disappearance (Kitt) (C), and pyruvate (ipPTT) (D) in mice treated with HEPa/EtOAc or Metformin for 16 days. CTL: lean (vehicle); DIO: non-treated obese (vehicle); PA125: obese treated with HEPa/EtOAc (125 mg/kg); PA250: obese treated with HEPa/EtOAc (250 mg/kg); MET: obese treated with metformin (200 mg/kg). All groups except CTL group received the high-fat diet throughout the study. Data represent the means ± SEM (n=6). Statistical analysis was performed, $^{*}P < 0.05$ vs. CTL and $^{a}P < 0.05$ vs. DIO.

studies (Leite et al., 2007, 2011), *Parkinsonia aculeata* is an alternative therapy for the treatment of obesity-related complications. In this paper, we have demonstrated for the first time that HEPa/EtOAc administration improves high-fat diet-induced insulin resistance in obese mice, as shown by increased glucose, insulin and pyruvate tolerance, as well as an improvement in FSG, FSI, HOMA-IR and circulating leptin levels, along with increased phosphorylation levels of proteins such as $IR\beta^{tyr}$ and Akt^{ser473} in liver, muscle and adipose tissue. Moreover, we also observed that HEPa/EtOAc treatment was able to improve mitochondrial biogenesis in obese mice, thus presenting an additional mechanism of action and therapeutic effect.

Changes in insulin sensitivity and its secretion are metabolic events that can be identified in individuals who will develop diabetes years before the disease becomes evident. The insulin-resistant state is characterized by a reduction in the ability of insulin to activate its signaling pathway in the main target tissues such as muscle, fat and liver (Pessin and Saltiel, 2000; Saad et al., 1992). Insulin plays a crucial role in metabolism, mainly by increasing glucose uptake in both muscle and adipose tissue, and inhibiting glucose output in the liver. The main deleterious effects of IR on metabolic processes are the impaired suppression of hepatic glucose production, decreased glucose uptake in the main target tissue and a reduction in the beta cell compensatory response, which together contribute to hyperglycemia and hyperinsulinemia in T2D (DeFronzo et al., 2015). In this context, our results demonstrate that HEPa/EtOAc treatment for 16 days at 125 and 250 mg/kg reduced FSG and FSI levels, and improved the HOMA-IR index in obese mice. Furthermore, it is worth noting that the values obtained at both dosages were close to those of our positive control, i.e. metformin. This well-known drug is based on a biguanide compound from a herb (*French lilac)* and is now a first-line drug for T2D therapy (Chang et al., 2013). In terms of the metabolic effects of HEPa/EtOAc treatment, it should be noted that total body weight and adipose tissue weight did not differ significantly between the obese treated group and non-treated group, and therefore it is clear that the observed effects of HEPa/EtOAc treatment were elicited by physiological and molecular mechanisms and not by more common features, i.e. weight loss.

Leptin is secreted from the enlarged adipose tissues and plays a critical role in metabolism by controlling food intake and enhancing energy expenditure (Considine et al., 1996). Due to its importance, we decided to measure the circulating levels of leptin, and observed that treatment with HEPa/EtOAc, at both doses, was able to reduce leptin to levels very similar to those obtained with metformin treatment. One way to measure the uptake of glucose, insulin sensitivity, and hepatic glucose production is through well-established tolerance tests, such as ipGTT, ipITT and ipPTT, respectively. Our results demonstrated that HEPa/EtOAc treatment improves glucose, insulin and pyruvate tolerance in obese animals, with values close to those observed in the MET group. Based on these set of physiological parameters, we now have a greater insight into the effects of HEPa/EtOAc treatment on glucose homeostasis and can be sure of its ability to improve insulin resistance. The data presented herein reinforce the results of a previous study performed by our group, in which, using a model of type 1 diabetes, we also observed a reduction in blood glucose levels and increased glucose tolerance in animals treated with HEPa/EtOAc

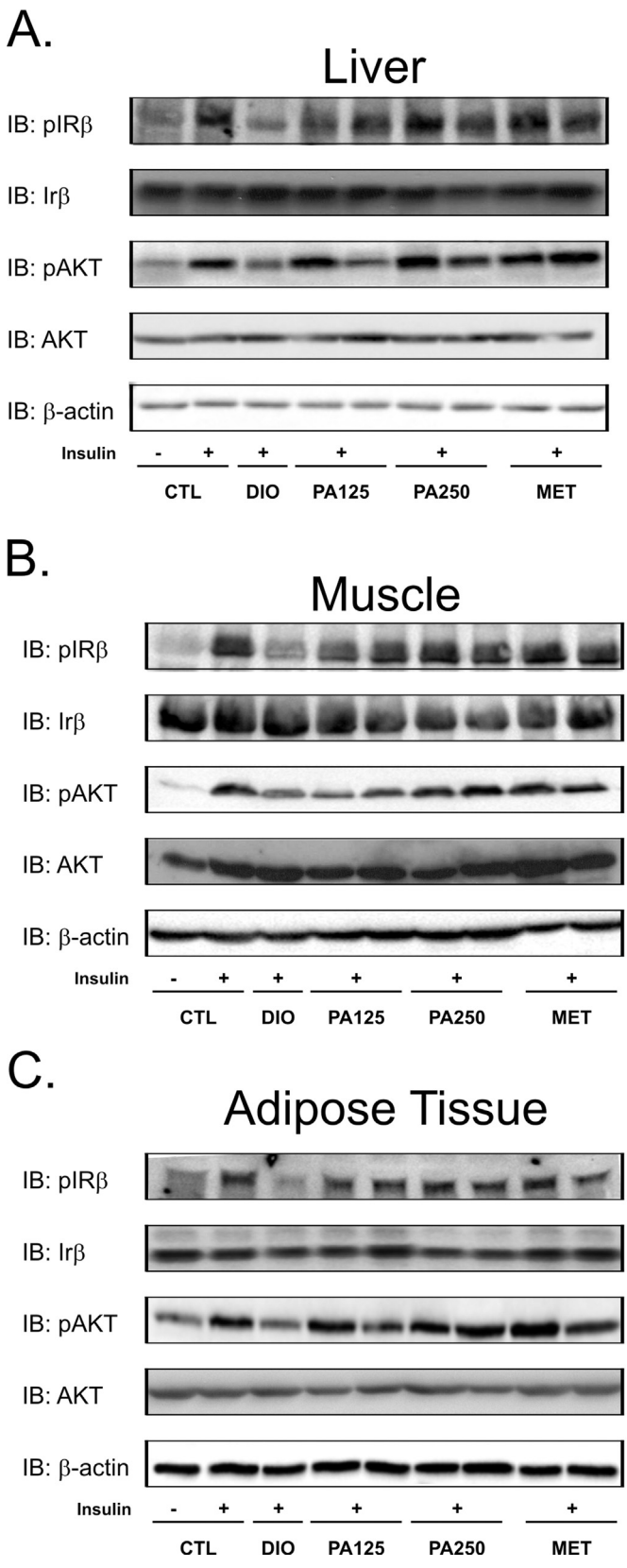

Fig. 3. HEPa/EtOAc treatment promotes the improvement on insulin signaling from obese mice. Representative blottings show tyrosine phosphorylation of IRβ, total protein expression of IRβ, serine phosphorylation of Akt and total protein expression of Akt in liver (A), muscle (B) and adipose tissue (C) from CTL, DIO, PA125, PA250 and MET groups. β-actin was used as a normalization housekeeping protein. IB, immunoblot.

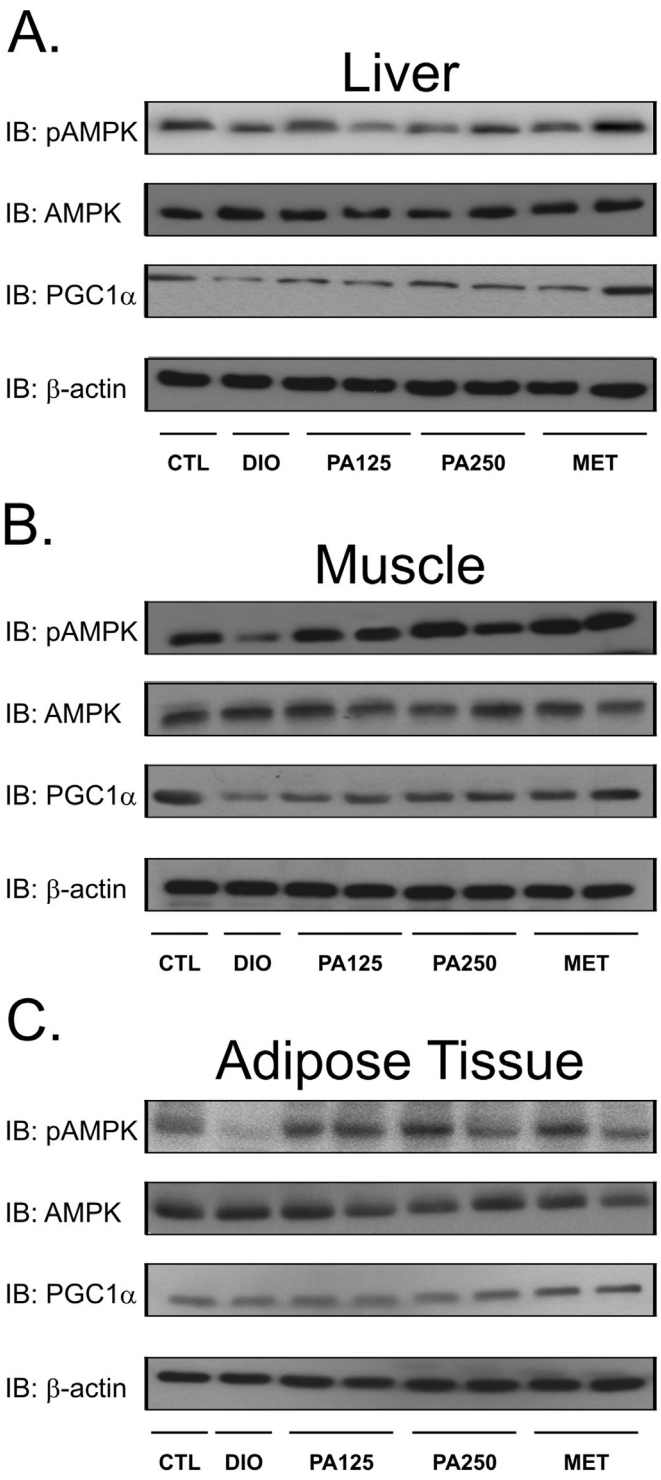

Fig. 4. HEPa/EtOAc treatment improves the mitochondrial biogenesis. Representative blottings show tyrosine phosphorylation of AMPK-α, total protein expression of AMPK-α, and expression of PGC1-α in liver (A), muscle (B) and adipose tissue (C) from CTL, DIO, PA125, PA250 and MET groups. β-actin was used as a normalization housekeeping protein. IB, immunoblot.

(Leite et al., 2011). In addition, it is worth mentioning that this previous study also confirmed the toxicological safety of HEPa/EtOAc, as oral administration of a maximum dose of 5 g/kg of HEPa/EtOAc did not result in any lethality or observable behavioral changes (Leite et al., 2011).

Notably, the current data that demonstrate the capacity of

HEPa/EtOAc to modulate the insulin signaling cascade are novel. Under physiological conditions, at the molecular level, insulin initiates its biological activity by binding to its receptor (IRβ) located in the plasma membrane of cells, resulting in its autophosphorylation. Activated IRβ results in the tyrosine phosphorylation of insulin receptor substrates (IRS-1 and IRS-2), leading to a signaling cascade by activating the pathway of phosphatidylinositol-3 kinase (PI3K) and consequently serine phosphorylation of Akt and GLUT-4 translocation (Boura-Halfon and Zick, 2009; Pessin and Saltiel, 2000). Conversely, under pathological conditions (insulin resistance), there is a reduction in phosphorylation levels of IRβ and Akt in the main target tissues (Boura-Halfon and Zick, 2009; Pessin and Saltiel, 2000). Interestingly, similarly to the effects of metformin, HEPa/EtOAc administration at both doses promoted an increase in the phosphorylation levels of IRβ and Akt in the liver, skeletal muscle and adipose tissue in obese animals, thus improving the state of insulin resistance as a whole.

It is known that the state of chronic low-grade inflammation and mitochondrial dysfunction are the main disruptors of insulin signaling in obesity (DeFronzo et al., 2015). It is also well known that AMPKα and PGC-1α are the main regulators of mitochondrial biogenesis (Pagel-Langenickel et al., 2008). Accordingly, AMPKα is a crucial sensor of the energy status of the cell, and has been reported to stimulate glucose uptake and lipid oxidation (Fei et al., 2012). AMPKα needs PGC-1α to modulate the expression of several key players in mitochondrial and glucose metabolism (Canto and Auwerx, 2009; Jager et al., 2007). Supporting this hypothesis, AMPKα activation leads to increased PGC-1α expression (Canto and Auwerx, 2009), thus emphasizing the importance of the AMPKα-PGC-1α axis in the metabolic process. In this regard, we decided to investigate the effects of HEPa/EtOAc on mitochondrial biogenesis by measuring the activity and expression levels of important proteins (AMPKα-PGC-1α axis). Surprisingly, we observed that HEPa/EtOAc treatment, mainly at the 250 mg/kg dose, induced a significant increase in the phosphorylation levels of AMPKα along with increased PGC-1α protein expression in the liver and muscle of obese mice. However, in the adipose tissue from these same animals we noted a slight increase in phosphorylation levels of AMPKα and no change in PGC-1α expression levels. Following this train of thought, the polyphenol resveratrol, physical exercise and metformin act via the activation of AMPKα and PGC-1α and the improvement of mitochondrial biogenesis, thereby mitigating high-fat diet-induced insulin resistance (Canto and Auwerx, 2009; Fei et al., 2012; Jager et al., 2007). Therefore, we can conclude that treatment with HEPa/EtOAc seems to mimics the effects of these three important therapies for T2D. For these reasons, it seems logical that the effects of HEPa/EtOAc on mitochondrial biogenesis may hold promise as a therapeutic strategy to reduce the burden of insulin resistance.

In conclusion, our findings expand our knowledge of the hypoglycaemic role of *P. aculeata*. Our results demonstrate that HEPa/EtOAc treatment improves high-fat diet-induced insulin resistance in obese mice. In detail, HEPa/EtOAc therapy significantly increases insulin sensitivity and normalizes fasting serum glucose, insulin and leptin levels, through multiple targeting actions, without significantly affecting body weight. At the molecular level, there was an improvement in mitochondrial biogenesis together with a recovery of insulin signaling by activating the AMPKα-PGC-1α axis. Thus, regarding glycemic control, our data suggest that HEPa/EtOAc exerts its effects similar to conventional insulin sensitizers, e.g. metformin, and based on its safety and multiple effects, *P. aculeata* may be a therapeutic alternative in the treatment of obesity-related complications.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.02.048.

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