SHORT COMMUNICATION

Effects of a pinitol-rich Glycyrrhiza glabra L. leaf extract on insulin and inflammatory signaling pathways in palmitate-induced hypertrophic adipocytes

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ABSTRACT

Glycyrrhiza glabra roots have been well studied for their pharmacological activities, whereas less research has been conducted on liquorice aerial parts. Leaves represent a good source of D-pinitol, useful in the treatment of insulin resistance-related pathologies. Herein, we analyzed the in vitro effects of a D-pinitol-rich methanolic extract from Glycyrrhiza glabra leaves (GGLME) against lipotoxicity-related hypertrophy, inflammation, and insulin resistance in 3T3-L1 adipocytes exposed to palmitic acid (PA), comparing its activity with D-pinitol. GGLME pretreatment decreased lipid deposition, PPAR-γ, and NF-κB pathway induced by PA, similarly to D-pinitol, and improved insulin sensitivity, in presence or not of PA, increasing PI3K, pAkt, and GLUT1 levels. This study confirms that liquorice leaves, considered a waste of resource, could potentially be reused, and support further in vivo studies on animal and human models. In conclusion, liquorice leaves extract represents a potential candidate for prevention of metabolically induced inflammation, frequently leading to metabolic disorders.

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1. Introduction

Obesity is considered a major health problem since it is associated to the increasing incidence of its related comorbidities promoting a wide range of metabolic disorders, like insulin resistance, dyslipidemia, and hypertension (da Silva et al. 2020). Adipose tissue expansion, observed in obese people, is due to increased size of already present adipocytes (hypertrophy) and/or recruitment of other adipocytes (hyperplasia) contributing to the development of low-grade inflammation that represents a key risk factor for the onset of type 2 diabetes (Longo et al. 2019). In particular, IL-1β and TNF-α, released by infiltrated macrophages, induce IκB kinase (IKK) and c-Jun N-terminal kinase (JNK), which in turn activate proinflammatory gene transcription machinery and affect insulin signaling through the involvement of inhibitory serine–threonine phosphorylation of IRS-1, thus decreasing PI3K/Akt axis and glucose transporters (GLUTs) protein expression (Gustafson et al. 2007; Appari et al. 2018).

The rising attention about metabolic medicine addressed to main diseases like obesity and diabetes, favored research of dietary compounds, such as polyols and associated carbohydrates. Particularly, the effects of inositols in insulin resistance have been examined in glucose homeostasis disorders, as they may constitute a complementary and synergistic mechanism in metabolic diseases (Formoso et al. 2018).

Recently, leaves of Glycyrrhiza glabra L. belonging to Leguminosae family (generally known as liquorice) have been reported for their high content in the cyclic polyol D-pinitol (Biondi et al. 2005; Siracusa et al. 2018). The roots are the plant part generally used in traditional medicine for gastric and duodenal ulcers, and allergenic reactions (El-Saber Batiha et al. 2020). Indeed, leaves, which are the aerial portions of liquorice, are not regularly used, resulting in a waste of resources. In previous research, we analyzed the chemical profile of a methanolic extract from Glycyrrhiza glabra leaves, exhibiting high D-pinitol content, together with lower amounts of flavonones, dihydrostilbenes, and flavones (Siracusa et al. 2020). Plants containing D-pinitol or its derivatives have being traditionally employed as an empirical remedy for diabetes and inflammation (Sánchez-Hidalgo et al. 2021). Pinitol has shown insulin-sensitizing effects and antidiabetic properties, as well as anti-inflammatory activity (Sánchez-Hidalgo et al. 2021). However, to date, the underlying mechanism remains unclear. For this reason, the present study aimed to evaluate the in vitro activity of a D-pinitol rich methanolic extract from Glycyrrhiza glabra leaves (GGLME) on fully differentiated adipocytes against lipotoxicity-related hypertrophy, inflammation, and insulin resistance, focussing on the major molecular pathways implicated in the protective effects.

2. Results and discussion

Glycyrrhiza glabra L. roots and rhizomes have been extensively reported for their pharmacological activity, whereas less research has been conducted on aerial parts, considered a waste of resources of liquorice. In particular, Glycyrrhiza glabra leaves represent a good source of D-pinitol, a well-known cyclitol known for its insulin sensitizing properties (Sánchez-Hidalgo et al. 2021). In a previous study, we showed that a methanolic extract of liquorice prevented and/or reversed insulin resistance induced by free fatty acids (FFAs) in human vessel endothelial cells (Siracusa et al. 2020).
However, since little is known about the beneficial effects of pinitol in human obesity, the current study was therefore focused on the effects of GGLME on adipose dysfunctional tissue.

2.1. Antihypertrophic activity of GGLME

Excessive fat storage into the adipose tissue is the leading cause of adipose dysfunction (Hammarstedt et al. 2018). A positive energy balance due to excessive nutrient supply induces both the growth of preexisting adipose tissue as well as the development of other adipocytes (Gonzalez-Muniesa et al. 2017).

In order to verify adipocytes hypertrophy induced by PA, the levels of the transcription factor PPAR-γ was then evaluated (Figure S1). PPAR-γ is, indeed, the main transcriptional regulator of adipogenesis and the main responsible of lipid deposition in the adipogenic process. Results demonstrate that PA exposure induces a rise in PPAR-γ levels, compared to control cells. Instead, pretreatment with GGLME extract, dose-dependently decreased PA effect determining a reduction in PPAR-γ levels. In cells not exposed to PA, the higher dose of GGLME extract pretreatment significantly reduced PPAR-γ levels with respect to control cells.

The activity of GGLME on PA-stimulated lipid deposition in adipocytes was also confirmed through Oil Red O staining histological analysis (Figure S2). The activity of GGLME (40–80 μg/mL) was compared to that of D-pinitol (20–40 μM), as GGLME 40 μg/mL contains about 38 μM D-pinitol. Treatment with 1 mM PA for 24 h induced a size increase of lipid deposits compared to controls, whereas they were dose-dependently reduced by GGLME, or D-pinitol, pretreatment.

These data demonstrated that GGLME dose-dependently improves adipocytes hypertrophy, since it reduced intracellular lipid accumulation probably associated to reduced PA-triggered expression of PPAR-γ, considered the major regulator of adipogenesis. Interestingly, D-pinitol modulated lipid accumulation and PPAR-γ expression at the same extent of the extract. Accordingly, we can assume that the observed GGLME effects were in most part due to its D-pinitol content. In addition, GGLME and D-pinitol reduced adipocytes lipid accumulation also in absence of PA exposure and this effect was associated to reduced PPARγ, since its activation induces storage of fatty acids in mature adipocytes (Medina-Gomez et al. 2007).

2.2. GGLME inhibition of NF-κB activation triggered by PA

Obesity is characterized by excessive storage of fatty acids into adipose tissue, so leading to hypertrophic and dysfunctional adipocytes secreting proinflammatory adipokines, such as IL-6, IL-8, and TNF-α, and consequently to a condition of chronic low-grade inflammation (Longo et al. 2019). NF-κB is the main transcriptional factor implicated in the inflammatory process (Hayden and Ghosh 2014).

Results obtained show that PA induces nuclear translocation of NF-κB, as shown by higher p65 nuclear levels compared to controls, whereas pretreatment with GGLME, as well as with D-pinitol dose-dependently prevented PA-induced p65 nuclear translocation (Figure S3 A). Furthermore, PA exposure induced the activation (phosphorylation)
of IKK α/β, whereas pretreatment with GGLME and D-pinitol dose-dependently inhibited PA-induced IKK activation, reaching values similar to the control, and thus leading to the decrease in p65 nuclear levels shown above (Figure S3 B). Results of both p65 and IKK also showed that GGLME 40 μg/mL has a slightly lower activity than D-pinitol 40 μM (Figures 3A and 3B).

Moreover, IL6 gene expression was analyzed to evidence the transcriptional activity of NF-κB. Results confirmed that PA is able to induce a significant overexpression of IL6 compared with control, whereas pretreatment with GGLME and D-pinitol dose-dependently reduced the mRNA levels of this pro-inflammatory cytokine (Figure S3 C). In this case, the effect of GGLME 40 μg/mL on NF-κB transcriptional activity is comparable to that of D-pinitol 40 μM.

These data confirmed a proinflammatory state activated by PA exposure via NF-κB pathway in murine adipocytes, in agreement with a previous report (Muscarà et al. 2019), whereas, similarly to D-pinitol, GGLME dose-dependently reduced NF-κB nuclear translocation through IKK inhibition and IL-6 mRNA expression. Also in this case, data confirmed that the GGLME protective effect against inflammation induced by PA in adipocytes was probably due to the D-pinitol present in this extract. The anti-inflammatory effects of D-pinitol are supported by a previous in vivo study on obese patients who received a pinitol-enriched beverage for 12 weeks with a significant reduction in systemic inflammatory cytokines such as IL-6 and TNF-α (López-Domènech et al. 2018).

2.3. GGLME enhances insulin sensitivity altered by PA

Obesity-associated inflammation supports the onset of insulin-resistance and comes before the progression of type 2 diabetes mellitus (Martins et al. 2014). At a molecular level, insulin binding to its receptor induces autophosphorylation of the receptor followed by a phosphorylation cascade activating IRS and PI3K, leading to Akt phosphorylation. Akt has a crucial role in the metabolic activity of insulin, in particular regulating the translocation of glucose carriers (GLUTs) (Beg et al. 2017). FFAs can affect insulin-signal transduction, through phosphorylation of IRS-1 residue via IKK and JNK serine kinases (Rotter et al. 2003; Fratantonio et al. 2017).

PA reduced PI3K (Figure 1B) and pAkt (Figure 1C) phosphorylation compared to controls exposed only to insulin, whereas pretreatment with GGLME or D-pinitol

Figure 1. Effect of GGLME and D-pinitol on PI3K (p85) (A, B) and pAkt (Ser473) (A, C) phosphorylation, and on GLUT-1 (A, D) protein expression modulated by PA. Adipocytes were cultured with GGLME (40–80 μg/mL) or D-pinitol (20–40 μM) for 24 h, treated with PA (1 mM) for 24 h and finally incubated with 100 nM insulin for 15 min. Control cells (CTR) were treated with vehicles only and then incubated with insulin (Ins). A representative image of Western blot is shown for the graph. Densitometric analysis reports data as fold change against CTR (mean ± SD of three independent experiments). PI3K (p85), pAkt (Ser473), and GLUT-1 bands were normalized to β-actin. *p < 0.05 vs Ins; **p < 0.05 vs D-pinitol 20 μM; ***p < 0.05 vs GGLME 40 μg/mL; ^p < 0.05 vs respective dose of D-pinitol without PA; a p < 0.05 vs respective dose of GGLME without PA; b p < 0.05 vs PA; c p < 0.05 vs GGLME 40 μg/mL + PA; d p < 0.05 vs D-pinitol 20 μM + PA; e p < 0.05 vs D-pinitol 40 μM + PA.
restored the PI3K/Akt pathway affected by PA, with higher levels of PI3K and Akt phosphorylation with respect to cells exposed only to PA and insulin (Figure 1). The effect of GGLME 40 μg/mL on PI3K/Akt signaling pathway is comparable to that of D-pinitol 40 μM.

Finally, the decrease of GLUT-1 levels confirmed that PA causes insulin-resistance in 3T3-L1 (Figure 1D), whereas the higher levels of GLUT-1 demonstrate as well that both GGLME and D-pinitol dose-dependently increased adipocytes insulin-sensitivity, with a comparable activity of GGLME 40 μg/mL and D-pinitol 40 μM (Figure 1D). Moreover, all these data show an insulin-sensitizing activity of both GGLME and D-pinitol in cells not exposed to PA (Figure 1).

These results confirmed insulin resistance in PA-induced hypertrophic adipocytes reducing PI3K and pAkt levels. Furthermore, GLUT1 expression was also inhibited by PA exposure. Adipocytes pretreatment with GGLME or D-pinitol improved insulin sensitivity restoring PI3K/Akt axis altered by PA. Interestingly, GGLME and D-pinitol induced GLUT-1 expression, as well as PI3K and pAkt expression, either in presence or not of PA, thus confirming the insulin sensitizing properties of D-pinitol and supporting its role in the effects induced by the liquorice extract. Similar effects were also previously reported in endothelial cells, where GGLME and D-pinitol induced the insulin-PI3K/Akt/eNOS pathway in a dose-dependent way (Siracusa et al. 2020).

3. Conclusions

In conclusion, this study confirms previous findings reporting that liquorice leaves, considered a waste of resource, have the potential to be reused mainly thanks to its high D-pinitol content. The GGLME protective effects observed against adipocytes hypertrophy is very likely through the insulin sensitizing and anti-inflammatory properties of its main constituent D-pinitol, and support further in vivo studies.

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