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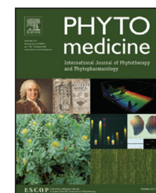

Vitexin reduces neutrophil migration to inflammatory focus by down-regulating pro-inflammatory mediators via inhibition of p38, ERK1/2 and JNK pathway

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ABSTRACT

Background: Vitexin is a flavonoid found in plants of different genus such as *Vitex* spp. and *Crataegus* spp. Despite being an important molecule present in phytomedicines and nutraceuticals, the mechanisms supporting its use as anti-inflammatory remains unclear.
Purpose: To investigate the cellular and molecular mechanisms involved in acute anti-inflammatory effect of vitexin with regard to neutrophil recruitment and macrophages activation.
Methods: Anti-inflammatory properties of vitexin were evaluated in four models of neutrophil recruitment. The regulation of inflammatory mediators release was assessed *in vivo* and *in vitro*. Vitexin (5, 15 and 30 mg/kg p.o) effects on leukocytes migration to peritoneal cavity induced by zymosan (ZY), carrageenan (CG), *n*-formyl-methionyl-leucyl-phenylalanine (*f*MLP) and lipopolysaccharide (LPS) were evaluated in Swiss-Webster mice and the effects on the levels of TNF-α, IL-1β and IL-10 cytokines, and NO concentration were in the LPS-peritonitis. RAW 264.7 macrophages viability were determined by Alamar Blue assay as well as the capacity of vitexin in directly reducing the concentrations of TNF-α, IL-1β, IL-10, NO and PGE_2. Additionally, vitexin effects upon the transcriptional factors p-p38, p-ERK1/2 and p-JNK were evaluated by western blotting in cells activated with LPS.
Results: Vitexin was not cytotoxic ($IC_{50} > 200$ μg/ml) in RAW 264.7 and at all doses tested it effectively reduced leukocyte migration *in vivo*, particularly neutrophils in the peritoneal lavage, independently of the inflammatory stimulus used. It also reduced TNF-α, IL-1β and NO releases in the peritoneal cavity of LPS-challenged mice. Vitexin had low cytotoxicity and was able to reduce the releases of TNF-α, IL-1β, NO, PGE_2 and increase in IL-10 release by LPS activated RAW 264.7 cells. Vitexin was also able to regulate transcriptional factors for pro-inflammatory mediators, reducing the expression of p-p38, p-ERK1/2 and p-JNK in LPS-elicited cells.
Conclusions: Vitexin presented no *in vitro* cytotoxicity. Inhibition of neutrophil migration and pro-inflammatory mediators release contributes to the anti-inflammatory activity of vitexin. These effects are associated with the inactivation of important signaling pathways such as p38, ERK1/2 and JNK, which act on transcription factors for eliciting induction of inflammatory response.
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Introduction

Flavonoids are one of the most important and diverse phenolic groups distributed in the plant kingdom. Structurally, they have a characteristic $C_6C_3C_6$ core biosynthesized from shikimic acid and acetic acid pathway. Changes to the central ring leads to the formation of subclasses like chalcones, flavanones, flavanonois, flavones, flavonols, isoflavones, flavan-3-ols and anthocyanidins (Cook and Samman 1996).

Among these compounds, apigenin glycoside flavone identified as vitexin (8-β-D-glucopyranosyl-apigenin), originally isolated from

Abbreviations: ZY, zymosan; CG, carrageenan; *f*MLP, *N*-formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor α; NF-kB, nuclear factor-kB; MAPKs, mitogen-activated protein kinase; p38, p38 MAP kinase; ERK1/2, extracellular signal-regulated protein kinase; JNK, c-Jun *N*-terminal protein kinase; DEXA, dexamethasone; PGE_2, prostaglandin E_2.

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Vitex lucens Kirk (formerly *V. littoralis*), stands out as an important secondary plants metabolites, such as *Crataegus* spp. (Edwards et al. 2012) and *Vitex agnus-castus* L. (Hajdu et al. 2007). Apart from its medicinal relevance, vitexin is also used as a marker of flavonoids contents in a variety of phytomedicines (Urbonavičiūtė et al. 2006), like *Passiflora* spp. based phyto-pharmaceuticals products, such as *P. incarnata* L. and *P. foetida* L., among others (Pongpan et al. 2007).

Evidences of vitexin uses in human health are being supported by clinical trials that evaluated prescription of phytomedicines containing such compound in the treatments of generalized anxiety disorder (NCT00794456), insomnia (NCT01100645), and also as a nutraceuticals (NCT01647984). Besides, in experimental models, vitexin has shown activity as anticonvulsant (Abbasi et al. 2012), antitumoral (Yang et al. 2013), antinociceptive (Borghi et al. 2013; Demir Özkay and Can 2013), neuroprotector (Abbasi et al. 2013), cardioprotector (Dong et al. 2011) and as new anti-inflammatory molecule (Prabhakar et al. 1981). However, despite such evidences, little is known about the cellular mechanisms involved in its anti-inflammatory action.

In regard to anti-inflammatory compounds, the discovery of new pharmacological strategies of immunomodulation is particularly important in diseases that involve multifactorial mechanisms of development, as observed in rheumatoid arthritis, Crohn's diseases, sepsis, intestinal inflammatory diseases, just to mention but few (Nathan 2002). Part of limitations in controlling inflammatory conditions by well-known pharmaceutical products in market, such as NSAIDs, is due to their serious side effects on the gastrointestinal tracts, especially in some groups of patients with bleeding risk (Scheiman and Hindley 2010). Moreover, other classes of drugs, such as immunosuppressives (e.g. glucocorticosteroids), long term use may result in serious adverse effects, which includes infections susceptibility. It is worthwhile to mention that curiously, the success observed with some classes of NSAIDs, were due to other mechanisms of action of such compounds, and not merely inhibition of COX-2. In fact, indomethacin may also reduce directly leukocyte chemotaxis, acetylsalicylic acid may induce lipoxins (anti-inflammatory endogenous molecule), contributing to its overall pharmacological effect (Barton et al. 2000).

In this context, new pharmacological strategies are being sought, like those that interfere with leukocyte recruitment and activation, pro-inflammatory mediators release (e.g. interleukin (IL)-1β, IL-10, tumor necrosis factor α (TNF-α) and nitric oxide (NO) and more recently by regulation of transcriptional factors, such as nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAPKs) pathways (Alessandri et al. 2013). In fact, the intracellular pathways have received special attention, because they regulate the expression of inflammatory genes that can be activated by different inflammatory stimuli. For instance, it was demonstrated that LPS and *f*MLP (derived from *Escherichia coli*) and CG (phlogistic agent derived from algae *Chondrus crispus*), are capable of increasing cytokines release and migration of neutrophils to inflammatory sites by up-regulating the expressions of p38, ERK1/2 and JNK (Azuma et al. 2007). Furthermore, ZY (derived from *Saccharomyces cerevisiae*), also used as phlogistic agent in experimental models, is known to induce the recruitment of neutrophils and cytokines release into the inflammatory sites by increasing expressions of p38 and ERK1/2 (Di Paola et al. 2010). More recently, compounds such as quercetin, curcumin, (-)-epigallocatechin gallate (EGCG) and resveratrol were shown to present immunomodulatory effects by acting on alternative pathways such as inhibition of 5-lipoxygenase, protein kinases (MAPKs, PKC, Akt) and transcriptional factors, such as NF-kB (Koeberle and Werz 2014).

Thus, this study investigated the effects of vitexin in acute inflammatory response after different phlogistic stimuli and the regulatory mechanisms upon pro-inflammatory mediators release and MAPK signaling pathways in macrophages.

Materials and methods

Drugs and reagents

ZY, CG, *f*MLP, LPS (*E. coli*, serotypes 055:B5 and 055:B8), dexamethasone acetate (DEXA), DMEM medium, RPMI-1640 phenol red free, fetal bovine serum, penicillin, streptomycin, doxorubicin, dimethyl sulfoxide, SB203580, PD98059, SP600125, NS-398 and Griess reagent were obtained from Sigma-Aldrich (USA). Vitexin (PubChem CID: 5280441), was obtained from Fluka (USA). Alamar Blue was obtained from Invitrogen (USA). All other chemicals were of reagent grade. Drugs were diluted in distilled water or 0.02% dimethyl sulfoxide as solubility same.

Animals

Male Swiss-Webster mice (25–30 g) from the Central Animal House of UFMT were used. The animals were maintained in propylene cages at 25 ± 1 °C with a 12 h light/dark cycle and had free access to standard pellet chow and water. Groups of six to eight animals were used for each experiment. The experimental protocol followed the International Principles for the Biomedical Research Involving Animal (CIOMS/OMS, 1985) and was approved by the Committee on the Use of Animal for Experimentation (CEUA/UFMT) with protocol no. 23108.028454/12-2.

Cell culture

The RAW 264.7 mouse macrophages cell line (ATCC TIB-71) was obtained from the Cell Bank of Rio de Janeiro. Cells were cultured in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37 °C in an atmosphere of 5% of CO_2 and 90% humidity.

In vivo *anti-inflammatory evaluation*

Peritonitis induced by different stimuli

In order to assess the possible effect of vitexin on leukocyte recruitment into the peritoneal cavity, the mice were orally (p.o) pretreated (0.1 ml/10 g) with vehicle (2% Tween 80 in distilled water), vitexin (5, 15 and 30 mg/kg) or DEXA (0.5 mg/kg). After 1 h, it was injected by intraperitoneal (i.p.) route 0.2 ml/cavity of ZY (1 mg), CG (300 μg), *f*MLP (10 μg) and LPS (250 ng) dissolved in sterile saline for induction of peritonitis. A sham group received p.o the same volume of vehicle and sterile saline i.p. Four or six hours after the injection of stimulus, mice were anesthetized with 180 mg/kg ketamine and 30 mg/kg xylazine by i.p. route and the cells in the peritoneal cavities were collected through injecting of 3 ml of cold PBS 1x (pH 7.4) containing EDTA (3 mM). The abdomens were gently massaged, and the blood-free cell suspension was carefully aspirated with a syringe. The peritoneal lavage collected was used for cellular counting in Neubauer chamber, while an aliquot of this lavage was used to make smear for differential cell counting. Aliquots of peritoneal washing were stored in a freezer at −80 °C for posterior dosage of cytokines (Da Silva et al. 2014).

Cytokine and nitrite quantification in the peritoneal lavage

The cytokines concentrations (pg/ml) of TNF-α, IL-1β and IL-10 in peritoneal fluid of mice with LPS induced peritonitis was measured with commercially available ELISA kits according to manufacturer's instructions (eBioscience, USA). Indirect estimation of NO through nitrite (NO_2^-) determination was performed by the colorimetric method based on the Griess reaction, employing the modified method of Ni et al. (2010). The lavage collected in LPS-induced peritonitis was incubated with an equal volume of Griess reagent complete at room temperature for 15 min and the absorbance (540 nm)

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