Inhibition of macrophage activation and lipopolysaccharide-induced death by seco-steroids purified from *Physalis angulata* L.

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**Abstract**

*Physalis angulata* L. is an annual herb widely used in popular medicine for the treatment of a variety of pathologies. Here, we tested immunomodulatory activities of physalins, seco-steroids purified from *P. angulata* extracts. Addition of physalins B, F or G, but not D, caused a reduction in nitric oxide production by macrophages stimulated with lipopolysaccharide and interferon-γ. In the presence of physalin B, macrophages stimulated with lipopolysaccharide, alone or in combination with interferon-γ, produced lower levels of tumour necrosis factor (TNF)-α, interleukin-6 and interleukin-12. The inhibitory activity of physalin B, unlike that of dexamethasone, was not reversed by RU486 [(4-dimethylamino) phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one], an antiglucocorticoid. Physalin B-treated mice had lower levels of serum TNF-α than control mice after lipopolysaccharide challenge. More importantly, mice injected with physalins B, F or G survived after a lethal lipopolysaccharide challenge. These results demonstrate that seco-steroids from *P. angulata* are potent immunomodulatory substances and act through a mechanism distinct from that of dexamethasone.

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**Keywords:** Steroid; Immunomodulation; (*Physalis angulata* L.); Endotoxic shock

1. **Introduction**

Glucocorticoids are potent modulators of inflammatory responses, exerting their effects in a variety of cell types, including macrophages and lymphocytes. They cause the suppression of immune–inflammatory responses and act by suppressing cell activation events, as well as modulating the production of several mediators, including arachidonic acid metabolites such as prostaglandins and leukotrienes, acute-phase reactants and several cytokines (Schimmer and Parker, 2001). The activities of glucocorticoids are mediated by the glucocorticoid receptor, which is a member of a superfamily of ligand-inducible transcription regulators for steroids and thyroid hormones (Beato and Klug, 2000; Duarte et al., 2002; Mangelsdorf et al., 1995). Upon binding to its ligands, the glucocorticoid receptor acts as a transactivator of responsive genes, as well as negative regulator of gene transcription (Beato and Klug, 2000).

Macrophage activation by lipopolysaccharide, the major component of gram-negative bacteria cell wall, results in the release of several inflammatory mediators such as nitric oxide (NO) and the proinflammatory cytokines tumour necrosis factor (TNF)-α, interleukin-6 and interleukin-12. The exacerbated production of these mediators by activated macrophages has deleterious consequences in septicemia or endotoxemia, and may lead to hypotension, disseminated intravascular coagulation, neutrophil extravasation, tissue hypoxia and death (Schimmer and Parker, 2001). Glucocorticoids and other steroid hormones inhibit the production of proinflammatory cytokines and NO by activated macrophages and protect against lipopolysaccharide-induced death. Administration of the synthetic glucocorticoid dexamethasone decreases while treatment with RU486 [(4-dimethylamino) phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one], a glucocorticoid receptor antagonist, increases the lipopolysaccharide-induced mortality in mice (Lazar et al., 1992).
Physalis angulata belongs to the Solanaceae family and is widely distributed throughout tropical and subtropical regions of the world. Extracts or infusions from this plant have been used in various countries in popular medicine as a treatment for a variety of illnesses, such as malaria, asthma, hepatitis, dermatitis and rheumatism (Lin et al., 1992; Chiang et al., 1992b). Several compounds from P. angulata have been isolated and chemically characterized. A group of steroids, known as physalins, are found in P. angulata stems and leaves (Lin et al., 1992). Antitumoral activities of purified physalins have been described (Chiang et al., 1992a,b). In this report, we have analyzed the immunomodulatory activity of physalins purified from P. angulata in activation-induced macrophage cytokine and nitric oxide production and in a murine model of endotoxic shock.

2. Materials and methods

2.1. Mice

BALB/c mice (male and female), 6–10 weeks old, were used to obtain cells from peritoneal cell exudate. Male, 6-week-old BALB/c mice were used in lipopolysaccharide-induced shock experiments. All mice were raised and maintained at the animal facilities at the Gonçalo Moniz Research Center-FIOCRUZ, and provided with rodent diet and water ad libitum. Animal numbers in experimental groups were the minimum necessary to allow statistical analysis. All animals were sacrificed under anaesthesia.

2.2. Physalin purification

P. angulata L. stems ethanolic extracts (15 g), obtained from dried plant material (collected at Belém do Pará, Brazil), were dissolved in 300 ml of methanol and mixed for 2 h with a lead acetate solution (25 g in 200 ml of distilled hot water). Twenty grams of activated charcoal (Merck Darmstadt, Germany) were added to the mixture and stirred up. The solution was then filtered and poured into a separatory funnel and extracted three times with 200 ml of chloroform. The chloroform layers (600 ml) were evaporated under reduced pressure giving 700 mg of a solid material (pool of physalins). This pool was chromatographed using a liquid medium pressure chromatographic column (MPLC). The elution was carried out with a gradient solvent system (hexane–ethyl acetate 7:3; thru 100% ethyl acetate). The collected fractions, after evaporation, were assayed in thin layer chromatography (TLC), against standard physalins. The substances, after recrystallization in ethyl acetate, gave the following results: 15 mg of physalin B (96% of purity), 75 mg of physalin D (95.6% of purity), 56 mg of physalin F (97.8% of purity) and 51 mg of physalin G (95% of purity). The chemical structures of physalins are described elsewhere (Matsuura et al., 1970; Mulchandani et al., 1979; Row et al., 1978; Row et al., 1980).

2.3. Macrophage cultures

Peritoneal exudate cells were obtained by washing with cold Hank’s balanced salt solution (HBSS; Life Technologies, GIBCO-BRL, Gaithersburg, MD) and stored in the peritoneal cavity of mice 5 days after injection of 3% thioglycollate in saline (1.5 ml per mouse). Cells were washed twice with HBSS and resuspended in RPMI medium (GIBCO-BRL) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 1-glutamine (2 mM), vitamins, sodium pyruvate (1 mM), HEPES (10 mM), 2-mercaptoethanol (50 μM) and gentamycin (50 μg/ml) (Sigma, St. Louis, MO). For TNF-α and interleukin-6 determinations, 5 × 10⁶ cells/well were plated in 24-well plates. For interleukin-12 and nitric oxide determinations, 2 × 10⁶ cells/well were plated in 96-well plates. After 1 h of incubation at 37 °C, nonadherent cells were removed by washing with complete RPMI. Cultures were then stimulated with lipopolysaccharide (from Escherichia coli serotype 0111:B4, Sigma) alone or in combination with interferon-γ (PharMingen, San Diego, CA) and treated with various concentrations of physalins or dexamethasone (Sigma), as described in figure legends. The glucocorticoid receptor antagonist RU486 (Sigma) was added in some cultures at a final concentration of 10 μM. Cell-free supernatants were collected at 6 h for determination of TNF-α and interleukin-6 concentrations and at 24 h for interleukin-12 and NO concentrations.

2.4. Endotoxic shock

Groups of mice were intraperitoneally (i.p.) injected with a dose of 600 μg of lipopolysaccharide in saline (previously determined as LD 100) alone or in combination with 0.5 or 1 mg of physalins. Mice were monitored daily for 4 days. To determine serum TNF-α levels, mice were sacrificed 90 min after injection of 400 μg of lipopolysaccharide alone or in combination with 0.5 mg of physalin B and bled by axilar vein puncture.

2.5. Cytokine and NO determinations

TNF-α, interleukin-6 and interleukin-12 (p40) concentrations in serum samples or in supernatants from macrophage cultures were determined by enzyme-linked immunosorbent assay (ELISA) using antibody pairs from PharMingen, according to the manufacturer’s instructions. After incubation with streptavidin-peroxidase conjugate (Sigma), the reaction was developed using 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (Kinkergaard & Perry Laboratories, Gaithersburg, MD) and read at 450 nm. Nitric oxide production was estimated by measuring nitrite content in supernatants using the Griess method, as described previously (Ding et al., 1988).
2.6. Statistical analyses

Data were analyzed using Student’s t-test, one-way analysis of variance (ANOVA) or Newman–Keuls multiple comparison test. Differences were considered significant when \( P < 0.05 \).

3. Results

3.1. Physalins B, F or G, but not D, inhibit nitric oxide production by activated macrophages

Addition of physalins B, F, or G to macrophage cultures stimulated with lipopolysaccharide and interferon-\( \gamma \) caused a dose-dependent inhibition in nitric oxide (NO) production by macrophage cultures stimulated with lipopolysaccharide and interferon-\( \gamma \), as indicated by the nitrite concentrations in supernatants (Fig. 1A). In contrast, addition of physalin D did not cause any inhibition in NO production (Fig. 1A). Physalin F was significantly more active than physalins B and G at 0.2 \( \mu \)g/ml (\( P > 0.01 \)). At a dose of 2 \( \mu \)g/ml (0.5 \( \mu \)M), physalin B reduced by 90% the NO production, whereas 1 \( \mu \)M of dexamethasone reduced only about 30% (Fig. 1B).

The effects of physalins on NO production by activated macrophages were not due to toxicity, as macrophages cultured in the presence of physalin concentrations below 2.5 \( \mu \)g/ml showed no difference in viability when compared to untreated controls (data not shown).

3.2. Physalin B modulates cytokine production by activated macrophages

We then evaluated the effects of physalin B on cytokine production by activated macrophages. Addition of physalin B to lipopolysaccharide-stimulated peritoneal macrophage cultures induced a decrease of TNF-\( \alpha \) and interleukin-6 production (Fig. 2A and B). Physalin B also caused a dose dependent inhibition in interleukin-12 production stimulated by lipopolysaccharide and interferon-\( \gamma \) (Fig. 2C). The inhibition of interleukin-12 production by physalin B were about 90% at 2 \( \mu \)g/ml, whereas in interleukin-6 and TNF-\( \alpha \) production, the same dose of physalin B inhibited about 60% and 30%, respectively (Fig. 2).
3.3. Physalins B, F or G protect mice against a lethal lipopolysaccharide challenge

To investigate the effects of physalins in endotoxic shock, groups of mice were challenged with a lethal dose of lipopolysaccharide and simultaneously treated with doses of 0.5 or 1 mg of physalins B, F or G. All mice in control group showed signs of endotoxemia such as piloerection, shivering and lethargy, and died after 24–36 h. Physalin-treated mice also exhibited endotoxic shock signs, although these were less pronounced than in control mice. More importantly, all mice treated with 1 mg of physalins B, F or G survived the lethal challenge (Fig. 3). At a dose of 0.5 mg/ml, physalins B and F protected 60% and 40% of mice, respectively, whereas treatment with 0.5 mg/ml of physalin G had no effect. TNF-α produced by lipopolysaccharide stimulation is a pivotal mediator in various events during endotoxic shock (Remick et al., 1990). As macrophages are a major source of TNF-α and other proinflammatory cytokines during endotoxic shock, we tested whether physalin B affects lipopolysaccharide-induced TNF-α production in vivo. Treatment of lipopolysaccharide-challenged mice with physalin B caused a reduction in serum TNF-α levels (Fig. 4). Physalin B-treated mice produced three times less TNF-α than control mice ($P < 0.02$).

![Fig. 3. Treatment of lipopolysaccharide-challenged mice with different physalins. Groups of 10–23 mice were simultaneously injected with 600 µg/ml of lipopolysaccharide (LD 100) and treated with doses of 0.5 mg (squares), 1 mg (triangles) of physalins B, F or G, or saline (circles) intraperitoneally. Lethality was monitored over 4 days.](image)

![Fig. 4. Reduction of TNF-α production in vivo by physalin B. BALB/c mice were simultaneously injected with 400 µg/ml of lipopolysaccharide and treated with 0.5 mg of physalin B or the same volume of saline. Blood samples from individual mice were collected 90 min later for TNF-α detection by ELISA. Data represent the means ± S.D. of five mice per group. *$P < 0.05$ (Student’s t-test).](image)

![Fig. 5. The glucocorticoid antagonist RU486 does not block the effects of physalin B. Peritoneal exudate macrophages were stimulated with lipopolysaccharide (500 ng/ml) and interferon-γ (5 ng/ml) and treated with physalin B (2 µg/ml) or dexamethasone (1 µM) in the absence or presence of RU486 (10 µM). Cell-free supernatants were collected 24 h later and tested for interleukin-12 p40 (A) or NO (B) production by ELISA or by the Griess assay, respectively. Data represent the means ± S.D. of four (A) and five (B) experiments. Statistical analyses were carried out using Newman–Keuls multiple comparison test.](image)
3.4. The glucocorticoid receptor antagonist RU486 does not block the in vitro effects of physalin B

Dexamethasone modulates cytokine and NO production, and protects mice against endotoxic shock by interaction with the glucocorticoid receptor, which can be blocked by the receptor antagonist RU486 (Lazar et al., 1992). We tested the ability of RU486 to reverse the action of physalin B in activated macrophage. The addition of RU486 did not block the inhibitory effects of physalin B in interleukin-12 and NO production by stimulated macrophages at of 10 μM (Fig. 5) or at concentrations 10- to 100-fold higher (not shown). RU486 also failed to reverse the inhibitory effects of physalins F and G in macrophage cultures (data not shown). Addition of RU486 (10 μM) to dexamethasone-treated, lipopolysaccharide- and interferon-γ-stimulated cultures abolished the inhibition of interleukin-12 production (Fig. 5A).

4. Discussion

Despite its use in folk medicine, little is known about the pharmacological properties of P. angulata and its compounds. In this report, we showed that physalins B, F and G have potent immunosuppressive activities in macrophages and in lipopolysaccharide-induced shock. These seco-steroids may therefore partially account for the antiinflammatory properties of P. angulata extracts empirically observed.

Lipopolysaccharide and interferon-γ act synergistically in the induction of NO synthesis by macrophages (Lorsbach et al., 1993). Physalins B, F and G showed a potent inhibitory action of NO production by macrophages activated by lipopolysaccharide and interferon-γ. The inhibition of NO production by the physalins was greater than that of dexamethasone, the gold standard of glucocorticoids. The active physalins reduced by 90–100% the NO production by macrophages activated with lipopolysaccharide and interferon-γ, whereas dexamethasone reduced NO production by 30%. Physalin B also reduced by 100% the production of NO by peritoneal exudate macrophages with lipopolysaccharide or interferon-γ alone (not shown).

TNF-α is a key mediator in septic or endotoxic shock. Administration of TNF-α induces shock, whereas treatment of mice with neutralizing monoclonal antibodies anti-TNF-α prevents the mortality caused by lipopolysaccharide challenge (Kettellhut et al., 1987; Remick et al., 1990). In this work we have showed that physalins inhibit lipopolysaccharide-induced TNF-α production both in vitro and in vivo. However, the protective effect of physalins in endotoxic shock may be due not only to the inhibitory action on TNF-α production, but also to the suppression of other cytokines such as interleukin-6 and interleukin-12, as well as of NO production. Excessive NO production under pathological conditions, such as endotoxemia, can lead to circulatory failure, hypotension, and septic shock (Thiiermann, 1997; Vincent et al., 2000). Moreover, interleukin-12 is a potent inducer of interferon-γ (Chan et al., 1991), another cytokine related to lethality in endotoxic shock (Heinzel, 1990). Thus, it is likely that a combination of effects may contribute to protection induced by the physalins in lipopolysaccharide-induced shock.

The effects of physalins on cytokine and NO production were not affected by the presence of the glucocorticoid receptor antagonist RU486. This demonstrates that physalins and dexamethasone suppress macophagy responses through different mechanisms. It was recently demonstrated that RU486 does not block the inhibitory effects of progesterone on NO synthesis by macrophages (Kohmura et al., 2000). The effects of progesterone, but not of dexamethasone, were blocked by another synthetic antagonist, ZK299 [11,-(4-dimethyl-aminopropyl)-17-hydroxy-17,-(3-hydroxypropyl)-13a-methyl-4,9gonadien-3-one] (Kohmura et al., 2000). As macrophages do not express progesterone receptor mRNA, and binding of progesterone to glucocorticoid receptor has been shown, it is possible that progesterone acts by activation of glucocorticoid receptor through a distinct pathway (Miller et al., 1996). Thus, the active physalins described here may act by the same mechanism used by progesterone. Studies aimed at the investigation of the intracellular mechanisms affected by the physalins are currently being carried out.

Glucocorticoids are among the most widely employed classes of drugs used in a variety of inflammatory and autoimmune diseases. However, the therapeutic use of glucocorticoids is strongly limited by major side effects caused by their wide range of actions, especially during long-term treatment. The search for new natural steroid compounds with similar beneficial activities to the glucocorticoids, but lower deleterious effects, is a promising field of research. This may be achieved by dissociating the transactivation and transrepression intracellular activities of glucocorticoid receptors (Vanden Berghe et al., 1999; Vayssiere et al., 1997). The potential toxicities of physalins remain still to be determined.

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