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Lack of interference of common phytoecdysteroids with production of nitric oxide by immune-activated mammalian macrophages

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ABSTRACT

Effects of selected common phytoecdysteroids on immunobiological responses triggered by lipopolysaccharide and interferon-γ (IFN-γ) were tested under *in vitro* conditions using murine resident peritoneal macrophages. Namely, production of nitric oxide was investigated. The series of test agents encompassed ecdysteroids occurring often as major components of the ecdysteroid fraction in numerous plant extracts: 20-hydroxyecdysone (20E), polypodine B, ajugasterone C, ponasterone A and inokosterone. Their structural variability concerns only variation in the number and position of hydroxyls. Two additional side-chain modified ecdysteroids: makisterone A (with a methyl substituent at position 24) and carthamosterone (with a cyclic side-chain lactone), and three ecdysteroid analogs: poststerone, rubrosterone and dihydrorubrosterone (devoid of side chains) were included into the test series. All test compounds, except of ponasterone A, represent natural substances isolated from the medicinal plant *Leuzea carthamoides* and are supposed to be significant for the often reported pharmacological activities of preparations derived from this species. However, the tested ecdysteroids did not interfere with the immunobiological activity of the immunocompetent cells. Our results thus differ from the so far reported information.

1. Introduction

Ecdysteroids represent a large family of polyhydroxylated steroid compounds with specific structural features [1] and with wide variety of biological functions and activities [2]. The principal function of the first and main identified ecdysteroid, ecdysone, was formulated by Karlson [3] as an insect molting hormone that controls the insect reproduction and development. The mode of action may be explained as a receptor-mediated control of gene activity, namely gene expression, inducing finally the targeted proteosynthesis. Majority of the so far known ecdysone analogues (structurally related ecdysteroids) induce such genomic action, and not only in insects, but also in other invertebrate groups (so far proven in athropods). However, already at the beginning of the ecdysteroid research, also other physiological and pharmacological activities, obviously not related to genomic action, were observed [4]. Moreover, they were observed not only in invertebrates, but also in mammals including humans [5].

The discovery of ecdysteroids in plants (phytoecdysteroids) made them available in large amounts and thereby allowed pharmacological studies and even a kind of pharmaceuti-

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cal (mostly nutraceutical) use. List of commercially available preparations with links to their producers are summarized in the review of Lafont and Dinan [6]. The list is saved and regularly updated in the products catalogue as well as in attached links of the Ecdybase [1]. The function of ecdysteroids in plants is still insufficiently explored [7,8]. Their effects on mammals including humans were extensively investigated and frequently reported in last three decades, however, not always with fully evident arguments. Several critical reviews [6–9] afforded opportunity to select the valuable facts out of the occasionally unconvincing information content.

Broad spectrum of reported biological activities of ecdysteroids includes also immunomodulatory effects [6]. However, the immunoactive properties of ecdysteroids were so far studied and reported only marginally [10–13]. It is rather difficult to compare the reported conclusions, as the relevant data resulted from different, unrelated methods. Our well-established bioassay [14] is based on evaluating effects of compounds on immunobiological responses triggered by lipopolysaccharide and interferon-γ (IFN-γ) tested under *in vitro* conditions using murine resident peritoneal macrophages. Namely, the production of nitric oxide is recorded and assessed. To learn more on immunological properties of ecdysteroids, we selected and tested a series of phytoecdysteroids, frequently occurring as major components of the ecdysteroid fraction in plant extracts, such as 20-hydroxyecdysone (20E), polypodine B, ajugasterone C and congeners.

2. Experimental

2.1. Chemicals

Phytoecdysteroids, 20-hydroxyecdysone, polypodine B, ajugasterone C, inokosterone, makisterone A, carthamosterone, poststerone, rubrosterone and dihydrorubrosterone (Fig. 1) were isolated and purified (each above 98% HPLC purity) from roots of *Leuzea carthamoides* [15–17], ponasterone A (99% HPLC purity) was isolated from mushroom *Tapinella panuoides* [18], but for our repeated experiments were prepared from 20-hydroxyecdysone by a four-step chemical transformation procedure [19]. The structural differences between the four major phytoecdysteroids and ponasterone A consist only in the number and positions of the hydroxyl groups (see Fig. 1). Ajugasterone C is isomeric to 20E with a missing hydroxyl in position 25 and with additional one in position 11 (in α configuration). Polypodine B is a derivative of 20E with one additional hydroxyl in position 5 (in β configuration). Ponasterone A is a 25-deoxy derivative of 20E. The minors, makisterone A and inokosterone are structurally also closely related to the 20E; makisterone A with an additional methyl group in position 24 and inokosterone with a hydroxyl at position 26 instead of 25. Carthamosterone, specific only to *L. carthamoides*, is a side-chain lactone analogue formed from an oxidized 24-ethyl precursor. Poststerone, rubrosterone and dihydrorubrosterone do not contain any side chain. For characteristic structural and HPLC analytical data, as well as for biological properties of these compounds, see [1,15–19]. The commercially available corticosterone was obtained from Aldrich.

The test compounds were dissolved in ethanol. Its final concentration for highest (i.e. 100 μM) concentration of compounds was 0.2%. This amount was devoid of any effects on production of NO (data not shown).

2.2. Animals; isolation and cultivation of macrophages

Female mice of the inbred strain C57BL/6, 10–12 weeks old, were obtained from Charles River Deutschland (Sulzfeld, Germany). They were kept in Independent Environmental Air Flow Cabinet (ESI Flufrance, Wissous, France). Lighting was set at 06:00–18:00 h, and temperature at 22 °C. All experimental protocols were approved by the institutional ethics committee. Animals, killed by cervical dislocation, were i.p. injected with 8 ml of sterile saline. Pooled peritoneal cells collected from mice ($n = 4$–6 in individual experiments) were washed, re-suspended in culture medium, and seeded into 96-well round-bottom microplates (Costar) in 100 μl volumes, 2×10^5 cells/well. Adherent cells (macrophages) were isolated by incubating the cells for 2 h at 37 °C, 5% CO_2, and then vigorously shaking the plate and washing the wells three times to remove non-adherent cells. Cultures were maintained at 37 °C, 5% CO_2 in humidified Heraeus incubator.

Complete RPMI-1640 culture medium (Sigma–Aldrich) contained 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 μg/ml gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all Sigma).

2.3. Nitric oxide (NO) bioassay

Peritoneal macrophages were cultured 24 h in presence of test compounds, applied either alone or in the presence of NO-priming immune stimuli, i.e. murine recombinant interferon-γ (5000 pg/ml; R&D Systems, Minneapolis, MN) or lipopolysaccharide (LPS from *E. coli* 0111:B4, 100 pg/ml; Sigma), or both. The concentration of nitrites in supernatants of cells was taken as a measure of NO production [20]. It was detected in individual, cell-free samples (50 μl) incubated 5 min at ambient temperature with an aliquot of a Griess reagent (1% sulphanilamide/0.1% naphtylendiamine/2.5% H_3PO_4). The absorbance at 540 nm was recorded using a microplate spectrophotometer (Tecan, Austria). A nitrite calibration curve was used to convert absorbance to μM nitrite.

2.4. Statistical analysis of data

Analysis of variance (ANOVA) with subsequent Tukey's multiple comparison test, and graphical presentation of data were done using the Prism program (GraphPad Software, San Diego, CA).

3. Results and discussion

The biological effects of all ten phytoecdysteroids in the concentration range of 0.1–100 μM, demonstrated by the NO production, were not significantly different from the controls (see Figs. 2 and 3). The observed inefficacy of the tested ecdysteroids in this bioassay is remarkable, especially when