Steroids 117 (2017) 11–15

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Flow-cytometric analysis of reactive oxygen species in cancer cells under treatment with brassinosteroids

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ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 14 June 2016
Accepted 20 June 2016
Available online 23 June 2016

Keywords:
Brassinosteroids
28-Homocastasterone
Cancer cell line A549
Anticancer
Reactive oxygen species

ABSTRACT

To explore the underlying mechanism of cancer cell growth inhibition by brassinosteroids (BS), reactive oxygen species (ROS) generation under treatment with 28-homocastasterone and its synthetic derivatives (22*S*,23*S*)-28-homocastasterone was measured in A549 human lung adenocarcinoma cells. BS induced ROS generation in A549 cells and their growth in a time and dose-dependent manner. The maximal effect was observed for (22*S*,23*S*)-28-homocastasterone which at 30 μM concentration showed a 6-fold increase of ROS generation in comparison with the control.

1. Introduction

Reactive oxygen species (ROS) are well established to play an important role in a wide variety of physiological and pathological processes both in plant and animal cells [1]. At the early stages of studies, ROS were mainly characterized in terms of their harmful effects [2–4]. Gradually, an understanding was reached that the ROS adverse effects were overestimated and the concept of "oxidative signaling" was developed [5]. In plants, the ROS signaling cascade is involved in cell differentiation [6], cell cycle, programmed cell death, hormone signaling, growth, and development [7]. In addition, ROS generation was found to be a common signal triggering downstream response to biotic and abiotic stresses [8,9]. The corresponding plant defence system is governed by a crosstalk between phytohormones which produce ROS as second messengers. The latter convey the hormonal information to mediate a wide range of adoptive responses [10].

Brassinosteroids (BS) have been recognized to be an integral part of the plant hormonal system [11–13]. Among various biological activities of BS, the most significant one is the ability to increase plant resistance to unfavourable biotic and abiotic environmental factors. Therefore, it was quite natural and expectable that interplay between this relatively new group of phytohormones and ROS was studied also. The first experiments studied the effect of epibrassinolide (EBl) on the accumulation of H_2O_2 in cucumber [14]. The EBl treatment was shown to increase H_2O_2 level in the apoplast of mesophyll cells, and this was accompanied by an enhanced tolerance to oxidative stress. It is worth mentioning that the foliar treatment resulted in accumulation of H_2O_2 not only in locally treated but also in the non-treated upper and lower leaves [15]. A transient increase of the superoxide anion radical generation was observed in wheat coleoptiles treated with BS [16,17].

Although BS have so far been found in plant sources only, they were shown to exhibit also various biological effects outside the plant kingdom [18]. In animal systems BS revealed antiviral, antifungal, antibacterial, neuroprotective, immunomodulatory, and other activities that makes them promising candidates for a variety of medicinal applications [19]. Of particular interest are numerous reports describing the anticancer properties of BS [20–31]. However, very little is known about the mechanism by which BS exert their cytotoxic effects. Knowledge of the influence of these

Abbreviations: BS, brassinosteroids; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; EBl, 24-epibrassinolide; Et-Br, ethidium bromide; FBS, fetal bovine serum; 28-homocastasterone, 28-homoCS; (22*S*,23*S*)-28-homocastasterone, (22*S*,23*S*)-28-homoCS; MTT, 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; RFU, relative fluorescence units; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.steroids.2016.06.010
0039-128X/

compounds on the redox status of animal cells is likely to contribute to the explanation of the observed effects.

In this paper, we provide the first experimental characterization of BS impact on the ROS level in cancer cell line A549 (lung adenocarcinoma). The experiments were carried out using the natural phytohormone 28-homocastasterone and its synthetic derivative (22*S*,23*S*)-28-homocastasterone (Fig. 1).

2. Experimental

2.1. Materials

28-Homocastasterone (28-homoCS), (22*S*,23*S*)-28-homocastasterone ((22*S*,23*S*)-28-homoCS), and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), respectively, were synthesized as previously described [32–34]. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin were purchased from Sigma and 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Serva Electrophoresis (Heidelberg, Germany).

2.2. Cell culture

Human lung adenocarcinoma cell line A549 was purchased from the Russian Cell Culture Collection. A549 cells were cultivated in DMEM medium. The medium was supplemented with 10% FBS, L-glutamine (250 mg/L), penicillin (100 U/mL), streptomycin (100 mg/L). A549 cells were kept under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Cells were subcultured twice per week following standard trypsinization protocols.

2.3. MTT cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to determine IC_{50} concentrations of the studied agents [35]. In these assays 1.0×10^4 A549 cells in medium were added per well to 96-well plates, and grown for 24 h. The cells (70–80% confluent) were treated with BS in concentrations of 1–200 μM for 24 h in cell culture medium. Cells used as control were incubated solely with the maximum used amount of the diluent DMSO. After incubation for 24 h, MTT solution (5 mg/mL) was added and the cells were incubated for 4 h. The concentration leading to 50% inhibition of viability (IC_{50}) after 24 h was determined by measuring absorbance at 570 nm, using a microplate reader, as an indicator used to measure of MTT reductase activity. The viability of treated cells was expressed as a percentage relative to the viability of control vehicle-treated cells. Each experiment was performed in triplicate and independently repeated at least four times.

2.4. Measurement of intracellular ROS generation

ROS generation was analyzed by flow cytometry using DCFH-DA [34]. For this assay A549 cells were grown in 6-well plates, and grown for 24 h. At 70–80% confluence cells were treated with BS in concentrations of 1–100 μM and 10 μM DCFH-DA and incubated for 2 h in 5 mM PBS buffer. Cells used as control were incubated solely with 10 μM DCFH-DA and the maximum used amount of the diluent DMSO. Fluorescence generation due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by non-specific cellular esterases and the subsequent oxidation of DCFH by peroxides was measured by means of flow cytometry (Beckman Coulter Cytomics FC 500 (λ_{ex} = 495 nm and λ_{em} = 520 nm)).

2.5. Cell cycle determination

For this purpose the cells were seeded at densities of 4.0×10^4 - cells/cm^2 in DMEM culture media in T-25 cm^2 culture flasks. After 24 h incubation, the cells were treated with effective concentrations as determined by the intracellular ROS formation assay described above. Cells treated with DMSO alone were used as controls. After 24 h incubation, the cells were washed twice with PBS (pH = 7.4), trypsinized and washed from trypsin by centrifugation at 360*g* for 5 min. Then supernatant was removed and OptiLyse C (Beckman Coulter) was added for 10 min. Afer adding lysis buffer cells were washed twice with centrifugation and fixed in chilled ethanol (70%; v/v). To determine their DNA contents, the cells were stained with Et-Br and analyzed using a Beckman Coulter Cytomics FC 500 flow cytometer. The obtained results were processed using Multicycle AV Software (Phoenix Flow Systems, USA).

2.6. Determination of dead cells

The percentage of dead cells was determined using ethidium bromide (Et-Br) staining protocol [36]. Et-Br only stains cells that have lost their membrane integrity. Briefly, after incubation for 2 h with DCF-DA and BS in appropriate concentrations, cells were detached using 0.25% trypsin-EDTA solution, counted and treated with 10 μl of Et-Br for 10 min in the dark. The amount of dead cells was determined with the help of flow cytometry. The obtained results were processed using CXP Software (Beckman Coulter, USA).

2.7. Statistical analysis

The data were expressed as mean ± S.D. All statistics were calculated using the STATISTICA program (StatSoft, USA). A p-value of <0.05 was considered as significant.

3. Results and discussion

The cytotoxicity of BS was evaluated by the MTT assay [34]. As shown in Fig. 2, both compounds reduced the proliferation of A549

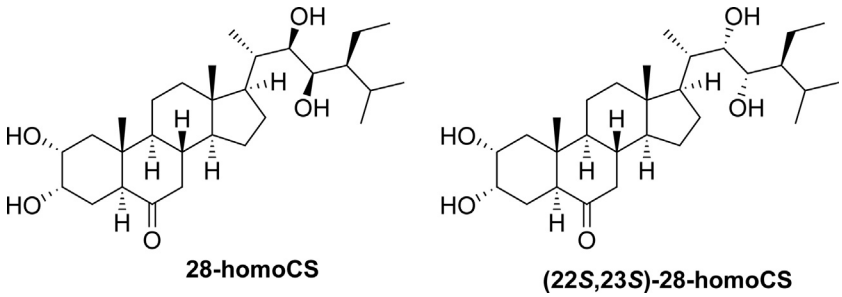

Fig. 1. Chemical structure of 28-homocastasterone and (22*S*,23*S*)-28-homocastasterone.