Journal of Ethnopharmacology 175 (2015) 456–462

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Osthole, a coumadin analog from *Cnidium monnieri* (*L.*) *Cusson*, stimulates corticosterone secretion by increasing steroidogenic enzyme expression in mouse Y1 adrenocortical tumor cells

Zhiqiang Pan*, Zhaoqin Fang, Wenli Lu, Xiaomei Liu, Yuanyuan Zhang

Basic Medical School of Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

ARTICLE INFO

Article history:
Received 9 March 2015
Received in revised form
30 August 2015
Accepted 2 October 2015
Available online 9 October 2015

Keywords:
Osthole
Corticosterone
StAR
CYP11B1
Y1 adrenocortical cell

ABSTRACT

Ethnopharmacological relevance: Osthole is an O-methylated coumadin, which was isolated and purified from the seeds of *Cnidium monnieri* (*L.*) *Cusson*. Osthole is a commonly used traditional Chinese medicine to treat patients with Kidney-Yang deficiency patients, who exhibit clinical signs similar to those of glucocorticoid withdrawal. However, the mechanism of action of osthole is not fully understood.

Objective: This study was designed to reveal the effects of osthole on corticosterone production in mouse Y1 cell.

Materials and methods: Mouse Y1 adrenocortical cells were used to evaluate corticosterone production, which was quantified by enzyme-linked immunosorbent assay (ELISA) kits. Cell viability was tested using the MTT assay, and the mRNA and protein expression of genes encoding steroidogenic enzymes and transcription factors was monitored by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and western blotting, respectively.

Results: Osthole stimulated corticosterone secretion from mouse Y1 cells in a dose- and time-dependent manner, and osthole enhanced the effect of dibutyryl-cAMP (Bu_2cAMP) on corticosterone production. Further, osthole also increased StAR and CYP11B1 mRNA expression in a dose-dependent manner and enhanced the expression of transcription factors such as HSD3B1, FDX1, POR and RXRα as well as immediate early genes such as NR4A1. Moreover, osthole significantly increased SCARB1(SRB1) mRNA and StAR protein expression in the presence or absence of Bu_2cAMP; these proteins are an important for the transport of the corticosteroid precursor cholesterol transport into mitochondria.

Conclusions: Our results show that the promotion of corticosterone biosynthesis and secretion is a novel effect of osthole, suggesting that this agent can be utilized for the prevention and treatment of Kidney-Yang deficiency syndrome.

1. Introduction

Cnidium monnieri (*L.*) *Cusson* is a commonly used traditional Chinese medicine to treat Kidney-Yang deficiency patients, who exhibit symptoms suggestive of glucocorticoid deficiency (Zhao et al., 2013). Kidney-Yang deficiency syndrome is a typical condition in Chinese medicine, shares similar clinical signs of the glucocorticoid withdrawal syndrome. However, Kidney-Qi deficiency degree is less than Kidney-Yang deficiency. Additionally, Qi function is weak in Kidney-Qi deficiency syndrome, whereas both Qi function and Yang function are weak or poor in Kidney-Yang deficiency syndrome. Osthole is identified as the main bioactive component isolated from the seeds of *C. monnieri* (*L.*) *Cusson*, that is known to ameliorate Kindey-Yang deficiency syndrome. Osthole has been shown to have a wide array of biological effects, including anti-inflammatory (Liu et al., 2005), hepatoprotective (Zhang et al., 2011), anti-proliferative (Jarzab et al., 2014), anti-tumor (Kao et al., 2012), anti-allergic (Chen et al., 1988), anti-osteoporotic (Zhang et al., 2007a) and estrogen-like activities (Hsieh et al., 2004). Additionally, a pharmacological report showed that osthole improved the function of the pituitary–adrenocortical axis in a Kidney-Yang deficiency rat model induced by continuous administration of hydrocortisone acetate (Qin et al., 1997). However, effects of osthole on steroid-producing cells remain unclear. Latter-day traditional Chinese medicine theory holds that Kidney-Yang deficiency syndrome is related to a hypofunctioning of the hypothalamus–pituitary–adrenal axis (Shen et al., 2007).

Corticosterone, the major glucocorticoid secreted by adrenocortical cells, is important for the regulation of glucose, lipid and protein biosynthesis and metabolism. Corticosterone production is

* Corresponding author.
E-mail address: pzq527@163.com (Z. Pan).

http://dx.doi.org/10.1016/j.jep.2015.10.009
0378-8741/

under the control of circulating adrenocorticotropic hormone (ACTH) secreted by the pituitary, which also plays an important role in stress. It has been reported that components of Chinese herbs exhibit steroid hormone effects. Therefore, it is necessary to discover the effect of osthole on adrenocortical cells. In this study we demonstrate that osthole increased corticosterone secretion from Y1 adrenocortical cells, suggesting that osthole plays an important role in steroidogenesis.

2. Materials and methods

2.1. Materials

Osthole (with the chemical structure illustrated in Fig. 1) was obtained from the Shanghai Institute for Food and Drug Control (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1% in all osthole groups and had no effect on cell viability. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium-F12 (DMEM-F12) were purchased from Hyclone Thermo Fisher Scientific (Waltham, MA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Bu_2cAMP and β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). StAR, CYP11A1 and SRB1 antibodies were purchased from Abcam (Cambridge, MA, USA). Mouse Corticosterone EIA kits were obtained from Cayman Chemical Company (Ann Arbor, Michigan, USA).

2.2. Cell culture and treatment

The mouse adrenocortical tumor Y1 cells were grown in DMEM-F12 medium containing 1% penicillin–streptomycin and 10% FBS. For analysis of responses to Bu_2cAMP and/or osthole, cells were sub-cultured in 6-well plates to approximately 80% confluence. One day before the experiment, the medium was replaced with a low-serum experimental medium (DMEM-F12 medium supplemented with 1% FBS). The next morning, cells were treated with 0.1% DMSO or 25 μM osthole in the presence and absence of 1 mM Bu_2cAMP in fresh low-serum experimental medium. For the dose response experiments, after Y1 cells treated for 24 h with 10 μM, 25 μM, 50 μM osthole, the cell culture supernatants were collected and used for hormone analysis, and cells were harvested for genes or proteins expression assay. The vehicle DMSO (0.1%) treated group was used as the control group in all experiments. For the time course experiments, the cell supernatants were collected and cells were harvested after treatment with 10 μM osthole for 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 12 h as indicated.

2.3. MTT assay

Cell viability was tested using the MTT assay according to the supplier's instructions. Y1 cells were seeded into 96-well plates at a density of 1×10^4 per well for 24 h. Following treatment with osthole for 24–72 h, medium was removed and cells were washed with PBS. MTT (0.5 mg/mL) was then added to each well and the mixture was incubated for 4 h at 37 °C. MTT reagent was then replaced with DMSO (150 μL per well) to dissolve formazan crystals. After the mixture was shaken at room temperature for 10 min, absorbance was determined at 570 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

2.4. Measurement of steroid hormone production

Y1 cells were incubated for 16–20 h in low-serum DMEM-F12 medium containing 1% FBS. The cells were then treated with and without Bu_2cAMP and/or osthole for the times indicated. The supernatants were collected and stored frozen at −20 °C until corticosterone was assayed using the mouse corticosterone EIA kit according to the manufacturer's instructions.

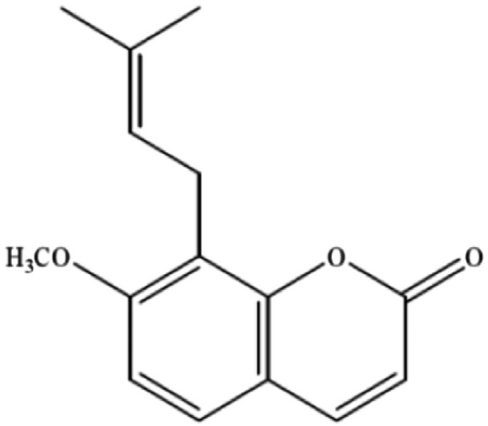

Fig. 1. Chemical structure of osthole.

2.5. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen) according to protocols from the manufacturers. The purity and integrity of the RNA were checked spectroscopically using a NanoDrop 2000/c spectrophotometer (Thermo). Then, for each sample, 2 μg RNA was reverse transcribed to obtain the cDNA template using PrimeScript®RT reagent Kit (TaKaRa). Each cDNA sample was diluted 5 times for qRT-PCR amplification; qRT-PCR was performed using the fluorescent dye SYBR® Premix Ex Taq™(Tli RNaseH Plus)II (TaKaRa) with a 7500 Fast Real-Time PCR System. Amplification was performed with the following fast time course: 95 °C 30 s, 95 °C 5 s, 65 °C for 30 s for 40 cycles. Relative mRNA expression values were determined by the $2^{-\Delta\Delta Ct}$ method using mouse cyclophilin (PPIA) and β-actin as the normalization control (Livak and Schmittgen, 2001).

2.6. Western blotting

Y1 cells were cultured in 6-well plates and were lysed with RIPA lysis buffer (Beyotime, Haimen, China) supplemented with PMSF. The cell lysates were centrifuged and the supernatants were mixed with 5 × Sample buffer (Beyotime, Haimen, China). The proteins were separated through 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes. The membranes were then blocked by incubating in the blocking buffer (5% nonfat dry milk, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5) for 1 h and then incubated with blocking buffer containing CYP11A1 antibody (1:1000), StAR antibody (1:1000), SRB1 antibody (1:2000) or β-actin antibody (1:20,000) overnight at 4 °C. Following extensive washing in Tris-buffered saline containing 0.1% Tween-20 (TBST) buffer, the transfer membranes were further incubated for 1.5 h in 5% nonfat dry milk blotting buffer that contained secondary antibodies (anti-rabbit IgG HRP-linked antibody or anti-mouse IgG HRP-linked antibody). Then, the membranes were washed three times with TBST and were finally developed using an alpha fluorchem E system detection kit (Protein-Simple, USA).

2.7. Statistical analysis

Data were expressed as the means ± SD. Significant differences were accepted at the 0.05 level of probability and were statistically determined with ANOVA followed by a Newman–Keuls post-hoc test using GraphPad Prism4 (San Diego, CA).