Journal of Pharmacological Sciences 131 (2016) 259–266

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

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

Full paper

Water-extracted *Perilla frutescens* increases endometrial receptivity though leukemia inhibitory factor-dependent expression of integrins

Eun-Yeong Kim [a], Hee-Jung Choi [a], Tae-Wook Chung [a], Jun-Yong Choi [a, b], Hyung Sik Kim [c], Yeon-Seop Jung [d], Syng-Ook Lee [d], Ki-Tae Ha [a, b, *]

[a] School of Korean Medicine and Healthy Aging Korean Medical Research Center, Pusan National University, Busandaehak-ro 49, Mulgeum-eup, Yangsan, Gyeongsangnam-do 50612, Republic of Korea
[b] National Clinical Research Center for Korean Medicine, Korean Medicine Hospital of Pusan National University, Geumo-ro 20, Mulgeum-eup, Yangsan, Gyeongsangnam-do 50612, Republic of Korea
[c] Laboratory of Molecular Toxicology, School of Pharmacy, SungKyunKwan University, Seobu-ro 2066, Jangan-gu, Suwon, Gyeonggi-do 16419, Republic of Korea
[d] Department of Food Science and Technology, Keimyung University, Dalgubeoldae-ro 1095, Dalseo-gu, Daegu 42601, Republic of Korea

ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form
29 June 2016
Accepted 11 July 2016
Available online 25 July 2016

Keywords:
Perilla frutescens
Infertility
Endometrial receptivity
LIF
Integrin

ABSTRACT

The leaves and stems of *Perilla frutescens* var. *acuta* Kudo (PF) have been used to prevent threatened abortion in traditional medicine in the East Asian countries. Because reduced receptivity of endometrium is a cause of abortion, we analyzed the action of PF on the endometrial receptivity. PF increased the level of leukemia inhibitory factor (LIF), a major cytokine regulating endometrial receptivity, and LIF receptor in human endometrial Ishikawa cells. The PF-induced LIF expression was mediated by c-jun N-terminal kinase (JNK) and p38 pathways. Adhesion between Ishikawa cells and trophoblastic JAr cells stimulated by PF treatment was abolished by knock down of LIF expression or antagonism of LIFR. In addition, the expressions of integrin β3 and β5 were increased by PF treatment in Ishikawa cells. The PF-induced expression of integrin β3 and β5 was reduced with an LIFR antagonist. Neutralization of both integrins successfully blocked PF-stimulated adhesion of JAr cells and Ishikawa cells. These results suggest that PF enhanced the adhesion between Ishikawa cells and JAr cells by increasing the expression of integrin β3 and β5 via an LIF-dependent pathway. Given the importance of endometrial receptivity in successful pregnancy, PF can be a novel and effective candidate for improving pregnancy rate.

1. Introduction

In spite of the development of diverse assisted reproduction technologies (ARTs), including *in vitro* fertilization, prevention of incomplete embryo implantation remains a major unmet need (1). To solve the problem of embryo implantation, it is crucial to enhance endometrial receptivity toward a properly developed embryo (2). It is well known that diverse biomolecules, such as cytokines and growth factors, play important roles in the process of developing a receptive endometrium (3). Among them, several previous studies have shown that leukemia inhibitory factor (LIF), a member of interleukin-6 family of cytokines, is one of the major factors that regulates endometrial receptivity (4). In addition, LIF expression defects have been shown to be involved in multiple implantations failures in patients with female infertility (5). Therefore, research has focused on developing novel candidates that stimulate embryo implantation rates by enhancing LIF expression, especially by using natural herbal medicines or traditional therapy, including acupuncture (6–8).

The leaves and stems of *Perilla frutescens* var. *acuta* Kudo (Labiatae family), a perennial herb, has being used in traditional medicine of East Asian countries for treating symptoms of the common cold, including shivering fits, fever, chest pains, and cough, and for preventing threatened abortion (9). In addition, previous pharmacological studies have shown that *P. frutescens* has anti-inflammatory and anti-tumor effects (10,11). However, there

* Corresponding author. School of Korean Medicine and Healthy Aging Korean Medical Research Center, Pusan National University, Yangsan, Gyeongsangnam-do 50612, Republic of Korea. Fax: +82 51 510 8420.
E-mail address: hagis@pusan.ac.kr (K.-T. Ha).
Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2016.07.004

are no reports available on the effects of *P. frutescens* on enhancing endometrial receptivity or pregnancy rate. Because reduced endometrial receptivity is a cause of abortion in early stage of pregnancy (12), we predicted that PF may be involved in the regulation of endometrial receptivity.

In this study, we investigated the effect of *P. frutescens* on the expression of LIF and adhesion between trophoblast and endometrium *in vitro*. The expression of adhesion molecules, including integrins involved in PF-induced endometrial receptivity was also examined.

2. Materials and methods

2.1. Materials

Antibodies against LIF, LIFR, phospho-extracellular signal-regulated kinases (ERK) 1/2, ERK2, p38, integrin αV, β3, and β5, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against phospho-c-Jun N-terminal kinases (JNK) and phospho-p38 were purchased from Cell signaling (Danver, MA, USA), and the antibody for JNK was purchased from Sigma–Aldrich (St. Louis, MO, USA). Specific inhibitors against signaling pathways, including U0126 (inhibitor for mitogen-extracellular signal-regulated kinase/extracellular signal-regulated kinase; MEK/ERK), SB203580 (inhibitor for p38), and SP600125 (inhibitor for JNK) were obtained from Merck Millipore (Billerica, MA, USA). Recombinant antagonists for human LIFR (hLA) expressed in *Escherichia coli* were prepared as described previously (13).

2.2. Plant material and extract preparation

The leaves and stems of *P. frutescens* were purchased from Omniherb Co. (Daegu, Korea). The plant was collected in Gyeongsangbuk-do Province, Republic of Korea in 2013, and identified by a botanical expert working at Omniherb Co. A boucher specimen (KMRC-DC-H21) was deposited at the Healthy Aging Korean Medicine Research Center, Pusan National University. The sample was extracted as previously described (14). Briefly, the leaves and stems of *P. frutescens* (100 g) were extracted with distilled water (1 L) for 2 h at 100 °C, and then centrifuged at 4000 rpm for 10 min. The supernatant was extracted with 70% ethanol for polysaccharide precipitation at 4 °C. After centrifugation at 4000 rpm for 10 min, the supernatant was evaporated and lyophilized by freeze-drier to give a powder (abbreviated as PF, 7.856 g). PF was freshly dissolved in dimethyl sulfoxide (Sigma–Aldrich) before experiments.

2.3. Fingerprinting high-performance liquid chromatography (HPLC) analysis

Phytochemical characteristics of PF were identified by HPLC analysis as previously described (9,15), with some modifications. HPLC analysis was performed using an Agilent 1200 series system (Agilent Technologies, Santa Clara, CA, USA) and LC solution software (version 1.24) was used for data analysis. AkzoNobel KR100-5C18 column (AkzoNobel, Amsterdam, Netherlands; 4.6 × 250 mm; pore size, 3.5 μm) was used as an analytical column. The mobile phases were solvent A [0.1% formic acid aqueous (v/v)] and solvent B (methanol). The gradient flow was as follows: (A)/(B) = 100/0 (5 min) → (A)/(B) = 50/50 (20 min) → (A)/(B) = 0/100 (30 min) → (A)/(B) = 100/0 (5 min). The column temperature was maintained at 35 °C. The analysis was carried out at a flow rate of 1 mL/min with detection at 254 nm. The column injection volume was 20 μL. A standard solution, containing protocatechuic acid, coumaric acid, ferulic acid, and rosmarinic acid was prepared by dissolving in distilled water (10 μM). The solution was filtered through a 0.45 μm membrane filter before HPLC analysis.

2.4. Cell culture

The human endometrial Ishikawa cell line was generously provided by Dr. Jacques Simard (CHUL Research Center, Quebec, Canada) and human trophoblastic JAr cells were provided from the Korean Cell Line Bank (Seoul, Korea). Ishikawa cells were cultured as monolayers at 37 °C in an atmosphere containing 5% CO_2/air and Dulbecco's Modified Eagle Medium (DMEM; Welgene, Daegu, Korea) with 10% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA). JAr cells were cultured as monolayers at 37 °C in an atmosphere containing 5% CO_2/air and Roswell Park Memorial Institute 1640 (RPMI1640; Welgene) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin.

2.5. Cell viability assay

Ishikawa cells were cultured in 24-well plates with the indicated concentrations of PF in serum free medium for 24 h. Next, the medium was replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (2 mg/mL) and incubated at 37 °C in a cell culture incubator for 3 h. The formazan crystals formed by MTT reduction were dissolved with DMSO and EtOH solution and measured at 540 nm with a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from Ishikawa cells using GeneJET RNA Purification Kit (Thermo Fischer Scientific, Waltham, MA, USA). Total RNA of each sample was subjected to reverse transcription with oligo-dT primers by using M-MLV reverse transcriptase (Enzynomics, Daejeon, Korea). The cDNA was amplified by PCR using DiaStarTM Taq DNA Polymerase (Solgent Co., Daejeon, Korea). The primers and PCR conditions used for amplifying *LIF, β-actin, ITGAV, ITGB3,* and *ITGB5* are shown in Table 1. The amplified DNA was separated by electrophoresis in 1% agarose gels containing ethidium bromide and visualized under ultraviolet (UV) light. The images were acquired with the GelDoc-It TS Imaging System (UVP, Upland, CA, USA).

2.7. Western blot analysis

Total proteins were isolated from Ishikawa cells. Equal amount of proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) and then incubated with each antibody for target proteins, including LIF, LIFR, phospho-ERK1/2, ERK2, phospho-p38, p38, phospho-JNK, JNK, integrin αV, integrin β3, integrin β5, GAPDH, and β-actin, overnight. Membranes were then washed in TBS and incubated with appropriate secondary antibodies conjugated with the horseradish peroxide. The signals were visualized by using the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

2.8. Adhesion assay

Ishikawa cells (1.5×10^6 cells) were seeded in 6 well plates overnight. Media were replaced and incubated in serum free-