Food and Chemical Toxicology 68 (2014) 218–225

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Melittin suppresses EGF-induced cell motility and invasion by inhibiting PI3K/Akt/mTOR signaling pathway in breast cancer cells

Yun-Jeong Jeong [a,1], Yongsoo Choi [b,1], Jae-Moon Shin [a,c], Hyun-Ji Cho [a], Jeong-Han Kang [a,2], Kwan-Kyu Park [a], Jung-Yoon Choe [a], Young-Seuk Bae [c], Sang-Mi Han [d], Cheorl-Ho Kim [e], Hyeun-Wook Chang [f], Young-Chae Chang [a,*]

[a] Research Institute of Biomedical Engineering and Department of Medicine, Catholic University of Daegu School of Medicine, Daegu 705-718, Republic of Korea
[b] National Medicine Center, Korea Institute of Science and Technology, Gangneung 210-340, Republic of Korea
[c] School of Life Sciences, KNU Creative BioResearch Group (BK21 plus program), Kyungpook National University, Daegu 702-701, Republic of Korea
[d] Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Suwon, Kyunggi-Do 441-100, Republic of Korea
[e] Department of Biological Science, Sungkyunkwan University, Suwon, Kyunggi-Do 440-746, Republic of Korea
[f] College of Pharmacy, Yeungnam University, Gyeongsan 701-947, Republic of Korea

ARTICLE INFO

Article history:
Received 4 November 2013
Accepted 17 March 2014
Available online 25 March 2014

Keywords:
Bee venom
Melittin
FAK
MMP
Cancer invasion

ABSTRACT

Bee venom is a natural compound produced by the honey bee (*Apis mellifera*), and has been reported as having the biological and pharmacological activities, including anti-bacterial, anti-viral and anti-inflammation. In the present study, the inhibitory effects of bee venom and its major peptide components on the tumor invasion were demonstrated. It was confirmed the inhibitory effects of bee venom, melittin, and apamin on the EGF-induced invasion of breast cancer cells. Transwell invasion and wound-healing assays showed that bee venom and melittin significantly inhibits the EGF-induced invasion and migration of breast cancer cells. Also, bee venom and melittin reduced the EGF-stimulated F-actin reorganization at the leading edge, but apamin did not affect. Particularly, melittin inhibited the EGF-induced MMP-9 expression via blocking the NF-κB and PI3K/Akt/mTOR pathway. In addition, melittin significantly suppressed the EGF-induced FAK phosphorylation through inhibition of mTOR/p70S6K/4E-BP1 pathway. These results suggest that inhibitory effects of melittin on breast cancer cell motility and migration may be related to the inhibition of mTOR pathway.

1. Introduction

The regulation of metastasis and invasion represent important therapeutic targets, as the inability to control metastasis and cancer invasion remains the most formidable obstacle to successful treatment. The biological processes of metastasis require the destruction of extracellular matrix (ECM) to include mesenchymal collagen and the endothelial basement membrane. Cell metastasis through the tissues is caused by highly integrated multistep cellular events regulated by various signaling molecules, including the MMPs, the Rho family small GTPases, and focal adhesion kinase (FAK) (Li et al., 2013; Mitra et al., 2005).

The main ECM degradation enzyme families are MMPs, which have essential roles in tissue development, remodeling and wound healing. Among the MMPs, MMP-9 is known to be involved in the degradation of type IV collagen, an important component of ECM (Nabeshima et al., 2002). FAK is also one of the critical genes for cell motility and metastasis, and is found in the cell membrane, where the cytoskeleton interacts with the proteins of the ECM (McLean et al., 2005). In addition, FAK has been shown to be overexpressed compared with its normal tissue counterparts in many human tumors, including in the breast, ovarian, and colon carcinomas (Ayaki et al., 2001; Sood et al., 2004).

Recent reports suggested that MMP-9 and FAK are associated with the epidermal growth factor receptor (EGFR) upon epidermal growth factor (EGF) induction, and promote tumor cell motility and invasion (Hauck et al., 2001). EGF/EGFR signaling has also been identified as an essential contributor to various tumor types including breast cancer (Rothhut et al., 2007). When EGF binds to 179-kDa EGFR, the receptor activation involves homo- and hetero-dimerization with other EGFR family members (Riese and

* Corresponding author. Address: Department of Cell Biology, Catholic University of Daegu School of Medicine, 3056-6, Daemyung-4-Dong, Nam-gu, Daegu 705-034, Republic of Korea. Tel.: +82 53 650 4848; fax: +82 53 650 4849.
E-mail address: ycchang@cu.ac.kr (Y.-C. Chang).
[1] These authors have contributed equally to this paper.
[2] Current address: Mayo Clinic, Stabile Building, Rochester, MN 55905, USA.

http://dx.doi.org/10.1016/j.fct.2014.03.022
0278-6915/

Stern, 1998), as well as the activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) and MAPK pathways which enhance tumor growth and invasion (Martin-Orozco et al., 2007; Wells, 1999). Furthermore, the EGFR/PI3K/Akt signaling pathways regulate the mammalian target of rapamycin (mTOR), which is known as a key controller of cancer cell proliferation. The inhibition of the mTOR signaling pathway suppresses F-actin reorganization and FAK phosphorylation, which is a functional indicator of cell migration (Berven et al., 2004).

Bee venom is a natural toxin produced by the honey bee (*Apis mellifera*), and has been used as a traditional medicine for treating various diseases, such as, arthritis, rheumatism, cancerous tumors, and various skin diseases (Billingham et al., 1973; Son et al., 2007). Bee venom contains a large number of biologically active peptides, including melittin, apamin, adolapin, and mast cell-degranulating peptide (MCCP) (Kwon et al., 2002). In particular, melittin constitutes approximately 50% of the dry weight of bee venom. Many previous studies have examined the biological and pharmacological activities of melittin such as the anti-bacterial, anti-viral, anti-inflammatory, and anti-cancer effects (Boman et al., 1989; Jo et al., 2012; Park et al., 2008). In addition, our previous study showed that melittin inhibits invasion in the CasKi cells (Park et al., 2010). The underlying molecular mechanisms of the anti-tumor motility effects of melittin, however, are still under investigation, especially the anti-metastatic potential of melittin in breast cancer cells.

In this study, the anti-metastatic effects of bee venom and its major peptides on EGF-stimulated breast cancer cells were investigated. It was found that bee venom and melittin suppress the EGF-induced motility and invasion of cancer cells by inhibiting FAK phosphorylation and MMP-9 expression, and that the inhibitory effects of melittin on EGF-induced FAK and MMP-9 are related to the target PI3K/Akt/mTOR signaling pathway.

2. Materials and methods

2.1. Cells and materials

MDA-MB-231, MCF-7 (breast carcinoma cancer) cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM or RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 5% CO_2 at 37 °C. Bee venom was obtained from the National Institute of Agricultural Science and Technology (NIAST), Suwon, Korea. All chemicals were obtained from Sigma (St. Louis, MO) including melittin (2 μg/ml) and apamin (2 μg/ml), unless otherwise indicated.

2.2. Cell viability assays

Cells were plated in 96-well culture plates at 1×10^4 cells/well in culture medium and allowed to attach for 24 h. Media were then discarded and replaced with 100 μl of new medium containing various concentrations of drugs and cultured for 24 h. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT) (Roche Molecular Biochemicals, Indianapolis, IN) was added to each well. The amount of formazan deposits was quantified according to the supplier's protocol after 4 h of incubation with MTT test solution in a 37 °C and 5% CO_2 incubator.

2.3. Transwell invasion assays

Matrigel-coated filter inserts (8 μm pore size) that fit into 24-well migration chambers were obtained from Becton–Dickinson (New Jersey, USA). Breast cancer cells were then plated on the upper chamber. The lower chamber was filled with culture media containing drugs. Cells in the chamber were incubated for 24 h at 37 °C and cells that invaded the lower membrane surface were fixed with methanol and stained with hematoxylin and eosin. The cells that passed through the matrigel and were located on the underside of the filter were counted. Random fields were counted by light microscopy under a high power field (400×).

2.4. Wound-healing assays

Cells were seeded at 5×10^5 cells/well in 6-well plates and incubated until they reached 80% confluence. Monolayers were scratched with a 200 μl pipette tip to create a wound, and cells were then washed twice with serum-free culture media to remove floating cells. Media were then replaced with fresh serum-free medium. Cells were subjected to the indicated treatment for 24 h, and cells migrating from the leading edge were photographed at 24 h.

2.5. F-actin staining

Serum-starved cells grown in 6-well-plates were pretreated with or without drugs for 2 h, followed by stimulation with or without EGF (20 ng/ml) for 1 h. Cells were then fixed with 4% ice cold formaldehyde in PBS for 20 min at 4 °C and washed with 0.2% Triton X-100 in PBS for 5 min. Cells were stained with FITC conjugated phalloidin (1 μg/ml, Sigma) for 30 min. Actin filaments (F-actin) were photographed with a Nikon TE300 digital inverted microscope.

2.6. Western blot analysis

Cells lysates, SDS–PAGE, transfer to an Immonobilon-p-membrane (Millipore, USA), and immunoblotting were performed as described previously (Jeong et al., 2010). To determine the nuclear translocation of p65, nuclear extracts of cells were subjected to describing as follows. Cells were suspended in tubes with 0.4 ml of lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin. Cells were then allowed to swell on ice for 15 min, and 25 μl of 10% Nonidet P-40 was added. Homogenates were centrifuged at 4 °C for 2 min at 13,000 rpm. The nuclear pellets were resuspended in 50 μl of ice-cold nuclear extraction buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin, and incubated on ice for 15 min with intermittent mixing. Nuclear extracts were then centrifuged at 4 °C for 5 min at 13,000 rpm and supernatants were either used immediately. Specific antibodies for MAPKs and phosphorylation form of MAPKs were purchased from Santa Cruz (Santa Cruz, CA).

2.7. Transient transfection with small interfering RNA

Breast cancer cells at 50% confluency were transfected with 50 nM negative control small interfering RNA (siRNA) or mTOR specific siRNA duplexes (Dharmacon Inc., Chicago, IL) using Trans IT-TKO (Mirus Bio Corp., Madison, WI), according to the manufacturer's instructions.

2.8. Statistical analysis

All in vitro-results presented here derived from at least three independent experiments performed in triplicate. The significances of differences between experimental and control values were calculated using analysis of variance with the Newman–Keuls multi-comparison test. p Values of <0.05 were deemed to be significantly different.

3. Results

3.1. Inhibitory effects of bee venom and its peptides on the EGF-induced migration and invasion of breast cancer cells

Before investigating the pharmacological potentiality of bee venom, melittin, and apamin on breast cancer cells, the cytotoxic effects of drugs were examined via MTT assay. Bee venom and melittin did not demonstrate any significant effect on the cell viability at concentrations of up to 2 μg/ml (Fig. 1A), and the apamin also did not affect in cell viability at 4 μg/ml. Based on these results, drugs at 2 μg/ml were used in subsequent experiments.

The inhibitory effects of bee venom, melittin, and apamin on the EGF-induced migration and invasion of the breast cancer cells, MDA-MB-231 and MCF-7cells, were investigated using wound-healing and cell transwell invasion assays. As illustrated in Fig. 1B, the migration of both the MDA-MB-231 and MCF-7 cells was increased by treatment with EGF (20 ng/ml). Bee venom and melittin inhibited EGF-induced cell migration, whereas apamin had a weak effect on cell migration. Furthermore, the transwell invasion assays indicated that the EGF-induced invasion was significantly inhibited by bee venom or melittin. The inhibitory effect of apamin on cells invasion are similar to the effect on migration (Fig. 1C).