Honokiol, a phytochemical from *Magnolia spp.*, inhibits breast cancer cell migration by targeting nitric oxide and cyclooxygenase-2

TRIPTI SINGH² and SANTOSH K. KATIYAR¹,²,³,⁴

¹Birmingham Veterans Affairs Medical Center, Birmingham, AL; ²Department of Dermatology; ³Comprehensive Cancer Center; ⁴Nutrition Obesity Research Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Received October 13, 2010; Accepted November 22, 2010

DOI: 10.3892/ijo.2011.899

Abstract. In the present study, we report the effects of honokiol, a phytochemical from *Magnolia spp.*, on cancer cell migration capacity and the molecular mechanisms underlying these effects using breast cancer cell lines as an in vitro model. Using cell migration assays, we found that the treatment of human breast cancer cells (MCF-7) and murine mammary cancer cells (4T1) with honokiol resulted in a dose-dependent inhibition of migration of these cells, which was associated with a reduction in nitric oxide (NO) levels. The cell migration capacity was decreased in the presence of N⁰-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase. Honokiol reduced the elevated levels of cyclic guanosine monophosphate (cGMP) in the cells, while the treatment of 4T1 cells with guanylate cyclase (GC) inhibitor 1-H-[1,2,4]oxadiaxolo[4,3-a]quinolalin-1-one (ODQ) reduced the migration of cells and the levels of cGMP. The presence of 8-bromoguanosine 3'5'-cyclic monophosphate, an analogue of cGMP, enhanced the migration of these cells, suggesting a role for GC in the migration of 4T1 cells. Honokiol also inhibited the levels of cyclooxygenase-2 (COX-2) and prostaglandin (PG) E₂ in 4T1 cells. The transfection of 4T1 cells with COX-2 siRNA resulted in a reduction in cell migration. ODQ and L-NAME also decreased the levels of PGE₂ in 4T1 cells suggesting a role for COX-2/PGE₂ in cell migration. Moreover, honokiol inhibited the activation of nuclear factor κB (NF-κB), an upstream regulator of COX-2 and iNOS, in 4T1 cells. These results indicate that NO and COX-2 are the key targets of honokiol in the inhibition of breast cancer cell migration, an essential step in invasion and metastasis.

Introduction

It is expected that one in eight to thirteen out of one hundred women in the United States will develop breast cancer over the course of an entire lifetime. Breast cancer is the second most frequent cause of cancer-related death among women in the United States (1,2). The metastatic spread of cancer continues to be the greatest barrier to a cure. Understanding the molecular mechanisms of metastasis is crucial for the effective therapeutic strategies to combat metastasis. Cancer cell migration is considered to be a major event in invasion and the metastatic cascade. Although breast cancer can be treated by using several available modalities, such as surgery, hormone therapy and radiation therapy (3), all these modalities may not work effectively in the advanced stages, where cancer cells have already metastasized to different parts of the body and the median survival time for most breast cancer patients is not more than a few years (3).

The role of nitric oxide (NO) in tumor biology has been extensively studied, and many studies have suggested a positive association between NO and tumor progression (4,5). The production of NO at low levels is an important mediator of physiological functions, such as vasodilation, smooth muscle relaxation and the regulation of neurotransmission (6-8), while higher levels of NO can mediate antibacterial and antitumor functions. The chronic and sustained production of NO contributes to many pathological conditions, including inflammation-associated diseases and the development of cancers (8-10). Studies have shown that NO promotes tumor cell migration, and that tumor cell migration requires the activation of NO synthase. They have also shown a positive association between endothelial-specific NO synthase expression in tumor cells and the growth and metastasis of tumors (4). These cancer-promoting effects have made NO a potential target for cancer chemoprevention.

Many studies have indicated that iNOS, as well as inducible cyclooxygenase-2 (COX)-2, are constitutively expressed in many cancers, suggesting that the indirect effect of NO, through the regulation of COX activity, is present in...
COX-2 overexpressing cancers (13-16) in addition to its direct roles (11,12). Until now, a number of interactions have been suggested between NOS and COX during inflammatory response. There are, however, conflicting reports with respect to whether NO activates or inhibits prostaglandin (PG) synthesis. Certain studies have suggested that during inflammation, NO upregulates COX-2 expression and increases COX-2 and COX-1 activity directly or indirectly (17-20), while others have reported that NO mediates COX inhibition (21-23). However, there are limited reports regarding cancer cell migration, particularly breast cancer cells, under the combined influence of NO and COX-2. As the role of COX-2 and NO induction in carcinogenesis has become among the most intensive areas of study in the metastasis of cancer cells, we examined the role of these biomarkers in mammary cancer cell migration in our in vitro model system of chemoprevention.

Honokiol (C18H18O2) (Fig. 1) is an active constituent found in the bark and leaves of the Magnolia plant species. It is used in the traditional Japanese medicine, Saiboku-to, for the treatment of various ailments due to its anxiolytic, anti-thrombotic, anti-depressant and antibacterial properties (24). Honokiol has been shown to possess antibacterial, anti-inflammatory, anti-oxidative and anti-carcinogenic properties (24-30). In this study, we examined the chemotherapeutic effects of honokiol on the migration of breast cancer cells, as the migration of cancer cells is a major event in the metastatic cascade of cancers. For this purpose, human breast cancer cells (MCF-7) and mouse mammary carcinoma cells (4T1) were used as an in vitro cancer model. We investigated the role of NO and COX-2 and its metabolite PGE2 on the migration of cancer cells and ascertained whether honokiol inhibits the migration of breast cancer cells and whether it is mediated through its action on NO and COX-2 expressions. We present evidence that honokiol inhibits breast cancer cell migration and that it does so through: (i) A reduction in the levels of NO production, inhibiting COX-2 expression and the production of PGE2 by cancer cells, and that (ii) this involves the downregulation of cyclic guanosine monophosphate (cGMP) and nuclear factor κB (NF-κB), as shown in our in vitro cell culture model.

Materials and methods

Cells and cell culture conditions. The MCF-7 and 4T1 cells were obtained from the American Type Culture Collection (Rockville, Maryland) and cultured in monolayers in Dulbecco’s Modified Eagle’s Medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin from Invitrogen (Carlsbad, CA) and maintained in a humidified incubator at 37°C. Honokiol was dissolved in a small volume of ethanol, which was added to the complete cell culture medium [maximum concentration of ethanol, 0.02% (v/v), in medium] prior to the addition to the subconfluent cells (60-70% confluent). Cells treated with ethanol (0.02%, v/v) only served as the vehicle control.

Antibodies and reagents. Purified honokiol was procured from LKT Laboratories, Inc. (St. Paul, MN). Boyden chambers and polycarbonate membranes (8 μm pore size) for cell migration assays were obtained from Neuroprobe, Inc. (Gaithersburg, MD). The antibodies specific for COX-2, iNOS and their secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). The NO- nitro-L-arginine methyl ester (L-NAME), D-NAME (inactive enantiomer of L-NAME) and 1-H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of guanylate cyclase (GC), an enzyme immunoassay kit for PGE2 analysis and the cyclic GMP EIA Kit, were obtained from Cayman Chemical Co. (Ann Arbor, MI). 8-Bromoguanosine 3’5’-cyclic monophosphate (8-Br cGMP), a cGMP analogue, was purchased from Sigma-Aldrich (St. Louis, MO).

Cell migration assay. The in vitro migration capacity of breast cancer cells and the inhibitory or stimulatory effects of any agent were determined using Boyden chambers in which two chambers were separated with Millipore membranes (8-μm pore size), as described previously (31). Briefly, cancer cells (1x104 cells/100 μl serum-reduced medium) were placed in the upper chamber of a Boyden apparatus. Honokiol or any other test agents were added alone or in combination to the upper chamber which contained cells, while the lower chamber contained the medium alone (150 μl). Boyden chambers with cells were kept in an incubator at 37°C for 24 h. At the termination of the incubation period, cells from the upper surface of the Millipore membranes were completely removed with gentle swabbing and the migrating cells on the lower surface of the membranes were fixed and stained with crystal violet. The membranes were examined microscopically and cellular migration was determined by counting the number of cells on the membranes in at least 3-4 randomly selected fields using an Olympus BX41 microscope fitted with a Qcolor5 digital camera system. The cell migration experiment was repeated at least two times, and data are presented in terms of the number of migrating cells/microscopic field ± SD, at x10 magnification.

Measurement of NO in cell supernatants. In NO assay, nitrate is enzymatically converted into nitrite by the enzyme nitrate reductase and the nitrate is quantified using Griess reagent. Briefly, cell culture media were collected from cells treated identically to those used in the migration assays and stored at -20°C until the assay. The levels of NO in cell culture supernatants were measured by determining the levels of their stable degradation products, nitrate and nitrite, using a colorimetric Nitric Oxide Assay Kit (Oxford Biomedical Research, Inc., Oxford, MI) following the manufacturer’s instructions.
PGE$_2$ immunoassay for quantification of PGE$_2$. The levels of PGE$_2$ in cell homogenates were determined using the Cayman PGE$_2$ Enzyme Immunoassay Kit following the manufacturer's instructions. Briefly, cells were harvested at the desired time-point and homogenized in 100 mM phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid and 10 μM indomethacin (a COX inhibitor) using a homogenizer. PGE$_2$ concentration was determined in the supernatants according to the manufacturer's instructions.

COX-2-siRNA transfection of 4T1 cells. COX-2 siRNA was transfected into 4T1 cells using the siRNA Transfection Reagent Kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) according to the manufacturer's instructions. Briefly, 2x10$^5$ cells/well were seeded into a 6-well plate and allowed to grow to 70% confluency. The COX-2 siRNA mix with transfection reagents was overlaid on the cells for ~6 h at 37°C and transferred into 2X growth medium for ~18-20 h. At 24 h post-transfection, fresh medium was added to the cells, and the cells were incubated for an additional 48 h. The knockdown of COX-2 expression in the cells after transfection was confirmed by Western blot analysis. Thereafter, the cells were subjected to cell migration assay.

NF-$\kappa$B/p65 activity assay. Quantitative analysis of NF-$\kappa$B/p65 activity was performed using the NF-$\kappa$B TransAM Activity Assay Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. For this assay, the nuclear extracts from cells were prepared using the Nuclear Extraction Kit (Active Motif) according to the manufacturer's instructions. Absorbance was recorded at 450 nm using the absorbance at 650 nm as the reference. The results are expressed as the percentage of the optical density of the control (non-honokiol-treated) group.

Western blot analysis. Breast cancer cells were treated with or without honokiol or any other agent for the desired time period. Thereafter the cells were harvested and lysed with ice-cold lysis buffer supplemented with protease inhibitors, as described previously (32,33). Proteins were resolved on 10% Tris-Glycine gels and transferred onto a nitrocellulose membrane. After blocking the non-specific binding sites, the membrane was incubated with the primary antibody at 4°C overnight, washed and then incubated with the peroxidase-conjugated secondary antibody and the specific protein bands were detected using enhanced chemiluminescence reagents. To verify equal protein loading, the membrane was stripped and reprobed with anti-β-actin antibody.

Statistical analysis. For cell migration assays, the control, honokiol- or ODQ-treated groups were compared separately using one-way analysis of variance (ANOVA). All quantitative data are shown as the means ± SD. In each case a value of P<0.05 was considered to be statistically significant.

Results

Honokiol inhibits the migration of breast cancer cells by inhibiting the endogenous levels of NO. Before performing cell migration assay, preliminary experiments were performed to determine the effects of lower concentrations of honokiol on cell viability and cell death using trypan blue exclusion assays, as described previously (32,33). Only those in vitro concentrations of honokiol were selected which did not significantly inhibit cell viability or induce cell death as that could adversely affect the cancer cell migration. The molecular structure of honokiol is shown in Fig. 1. As shown in Fig. 2, relative to the untreated control cells, treatment of cells with honokiol for 24 h at the concentrations of 5, 10 and 20 μM reduced the cell migration capacity of MCF-7 and 4T1 cells in a concentration-dependent manner. The density of the migrating cells on the membrane after staining with crystal violet dye is shown in Fig. 2A, and the numbers of migrating cells/microscopic field are summarized in Fig. 2B. The cell migration was inhibited by 18-65% in MCF-7 cells and by 28-82% in 4T1 cells in a concentration-dependent manner after treatment with honokiol.

In order to determine whether the inhibitory effect on the migration of cancer cells was due to the inhibition of NO production by honokiol, the levels of NO in cell supernatants of the various treatment groups were determined. It was observed that the treatment of MCF-7 and 4T1 cells with honokiol (5-20 μM) resulted in a significant reduction in the levels of NO production in a dose-dependent manner compared to the non-honokiol-treated control cells (Fig. 2C). As the levels of NO decreased after honokiol treatment, we determined whether the levels of NOS were reduced in the honokiol-treated cells. Western blot analysis revealed that the treatment of MCF-7 and 4T1 cells with honokiol decreases the expression of iNOS in these cells in a dose-dependent manner (Fig. 2D). These results also reveal that the migrating capacity of murine mammary carcinoma cells (4T1) is greater than that of human breast cancer cells MCF-7. Also, 4T1 cells are considered to be highly metastasis-specific cancer cells. Therefore, further studies were conducted with only 4T1 cells to determine the mechanism of inhibition of cell migration by honokiol.

L-NAME, an inhibitor of NO, inhibits breast cancer cell migration. L-NAME is a well known inhibitor of NOS, and thus an inhibitor of NO production. We therefore examined the effect of L-NAME on the migrating capacity of 4T1 cells using cell migration assay. The treatment of 4T1 cells with L-NAME at concentrations of 250, 500 and 1000 μM for 24 h resulted in a significant inhibition of cell migration in a concentration-dependent manner (42±5-76±5%, P<0.01-0.001), compared to the non-L-NAME-treated control 4T1 cells. Resultant data on number of migrating cells/microscopic field from three independent experiments are summarized as the means ±SD (Fig. 3A). Treatment of cells with D-NAME (an inactive enantiomer of L-NAME) for 24 h did not result in the inhibition of cell migration and did not reduce the levels of NO in the cell supernatants (data not shown).

Honokiol decreases the basal levels of cGMP in 4T1 cells. NO stimulates the GC/cGMP pathway in a variety of cancer cells and thus affects cell migration (34). We determined whether honokiol has any effect on the levels of cGMP in mammary cancer cells. The treatment of 4T1 cells with honokiol (5, 10 and 20 μM) for 24 h resulted in a dose-
dependent decrease in the levels of cGMP (17±2-83±6%, Fig. 3B) compared to the non-honokiol-treated control. To determine the role of cGMP on cell migration, certain agents which are known to block cGMP formation (e.g. ODQ, a GC inhibitor) or mimic cGMP action (8-Br cGMP, a cell permeable cGMP analogue) were used. As shown in Fig. 3C, the treatment of 4T1 cells with ODQ for 24 h resulted in a significant reduction in the levels of cGMP (33±3-82±4%, P<0.01-0.001) in the cell supernatants similar to that observed with the treatment of the cells with honokiol (Fig. 3B). The treatment of 4T1 cells with ODQ (25, 50 and 100 μM) also resulted in a significant inhibition of cell migration in a dose-dependent manner (20-76%, P<0.01-0.001) (Fig. 3D). The treatment of cells with 8-Br cGMP (cGMP analogue) increased the numbers of migrating cells (Fig. 3E) under identical conditions, suggesting a role for cGMP in mammary cancer cell migration.

The inhibition of cell migration by honokiol is associated with the inhibition of endogenous COX-2 expression and PGE2 production. As the overexpression of COX-2 has been shown to stimulate invasion and metastasis in cancer cells, we determined whether the inhibitory effect of honokiol on the migration of the cells is associated with inhibition of COX-2 expression. We determined the levels of COX-2 expression in lysates of cells from the various treatment groups by Western blot analysis. As shown in Fig. 4A, treatment of 4T1 cells with honokiol reduced the levels of COX-2 expression in a concentration-dependent manner compared to the expression in the untreated controls. As the COX-2 metabolite, PGE2, has been implicated in COX-2-mediated effects, we determined the levels of PGE2 in the honokiol-treated cells. Immunoassay data on PGE2 revealed that treatment of 4T1 cells with honokiol for 24 h resulted in
significant inhibition of PGE\(_2\) production (18-68%, P<0.05-0.001) in a concentration-dependent manner (Fig. 4B), suggesting that the honokiol-induced reduction in PGE\(_2\) production is associated with an inhibitory effect on COX-2 activity by honokiol in these mammary cancer cells.

**siRNA knockdown of COX-2 leads to reduction of 4T1 cell migration.** We further verified the role of COX-2 in breast cancer cell migration through siRNA knockdown of COX-2 in the 4T1 cells and examined whether it could lead to the inhibition of cell migration in these cells. The transfection of 4T1 cells with COX-2 siRNA resulted in a significant reduction in cell migration in 4T1 cells (86%, P<0.001) after 24 h compared to the migration of the control siRNA-transfected 4T1 cells (Fig. 4C).

**ODQ and L-NAME reduced the levels of PGE\(_2\) in 4T1 cells.** In order to determine whether ODQ and L-NAME affect cell migration through their inhibitory effect on PGE\(_2\) production, in addition to their effect on NO, 4T1 cells were treated with ODQ or L-NAME for 24 h, and the levels of PGE\(_2\) were determined. As shown in Fig. 4D, the treatment of cells with ODQ significantly inhibited (P<0.001) the production of the PGE\(_2\) metabolite in a concentration-dependent manner. A similar inhibitory effect on PGE\(_2\) production was also found when the cells were treated with L-NAME (Fig. 4E). These data suggest that there is an interrelationship between NO and COX-2 expressions and that both have an effect on cancer cell migration.

**Honokiol reduces the activation of NF-κB/p65 in 4T1 cells:** NF-kB is an important regulator of COX-2 and iNOS. iNOS and COX-2 are downstream targets of NF-κB or NF-κB is a regulator of COX-2 and iNOS expression. Therefore we assessed whether honokiol affects the level of NF-kB in 4T1 cells. For this purpose, cells were treated with various concentrations of honokiol (0, 5, 10 and 20 μM) for 24 h, and thereafter the cells were harvested and nuclear fractions were subjected to Western blot analysis. The results of Western blot analysis revealed that the treatment of cells with honokiol downregulated the translocation of NF-kB/p65 from the cytosol to the nucleus in a dose-dependent manner (Fig. 5A). The activity of NF-kB was also significantly reduced (25-80%, P<0.05 and P<0.001) after the treatment of cells with honokiol (Fig. 5B). In order to examine whether NF-kB has a role in breast cancer cell migration, 4T1 cells were treated with
various concentrations of caffeic acid phenethyl ester (0, 5 and 10 μg/ml), a potent inhibitor of NF-κB, and cell migration was determined. As shown in Fig. 5C, the treatment of cells with caffeic acid phenethyl ester resulted in a significant reduction in cell migration (47 and 83%, P<0.001) relative to the untreated control cells, similar to that observed with the treatment of cells with honokiol (Fig. 2A). Cell migration data are summarized in Fig. 5D.

Discussion

Understanding the molecular mechanisms of cancer cell metastasis is crucial for the effective therapeutic strategies to combat metastasis and the successful treatment of breast cancer. Endogenous NO promotes tumor progression and metastasis through multiple pathways, which include the stimulation of tumor angiogenesis, invasiveness and tumor cell migration (34,35). Similarly, many human cancers express elevated levels of COX-2. The COX-2 overexpression and abundant production of PGE2 have been linked with tumor progression, invasion and metastasis (36). Due to their important roles in tumor invasion and metastasis, NO and COX-2 are considered to be promising targets for cancer therapy (36,37).

The significant findings in the present study are that the treatment of breast cancer cells with honokiol inhibits cell migration in a dose-dependent manner, and that this is associated with the inhibition of NO production, COX-2 overexpression and PGE2 synthesis. Our study reveals that the inhibition of cancer cell migration by honokiol is mediated through the reduction in the levels of endogenous NO in both MCF-7 and 4T1 cells. The reduction in NO levels by honokiol is due to the inhibition of NOS expression in cells. NO-mediated migration also was blocked by the use of L-NAME, validating the NO-mediated stimulation of tumor cell migration and demonstrating that the migration-inhibitory effects of honokiol are NO-specific. Studies have shown that NO activates soluble GC and elevates cGMP, which affects the biological responses of NO via the activation of cGMP-dependent protein kinase or by modulating the phosphodiesterase activity (38,39). We provide evidence that the NO-mediated stimulation of mammary cancer cell migration is cGMP-dependent, showing that: (i) The cGMP analogue, 8-Br cGMP, which mimicked the migration-
promoting role of NO, has migration inhibitory effects. (ii) A selective inhibitor of soluble GC, ODQ, inhibited 4T1 cell migration, attesting to the GC selectivity of ODQ in the migration-inhibitory response. Under identical experimental conditions, the treatment of 4T1 cells with honokiol reduced the basal levels of cGMP and these effects were similar to those of ODQ in this in vitro cell culture model. These observations support the evidence that the inhibition of cancer cell migration by honokiol requires the inhibition of cGMP.

Another important finding of this study is that the inhibition of 4T1 cell migration by honokiol is associated with the inhibition of COX-2 expression and PGE2 production. The mammary cancer cells overexpress COX-2, and the inhibition of COX-2 by honokiol can contribute to the inhibition of cell migration of these cells. This concept is supported by the evidence that the transfection of 4T1 cells with murine COX-2 siRNA resulted in a significant reduction in cell migration. Many of the carcinogenic effects of COX-2 are mediated through its metabolite, PGE2. Honokiol inhibits PGE2 production in 4T1 cells. These observations support the evidence that the inhibition of 4T1 cell migration by honokiol also requires the downregulation of COX-2 expression.

The interaction between NOS and COX-2 has been reported mainly in cells involved in inflammatory responses, such as activated macrophage-like cells (17,40). These studies have suggested that the COX-2 regulation by NO occurred through the modulating effects of transcription factors, such as NF-kB (41,42). We found that the treatment of 4T1 cells with ODQ or L-NAME decreased the levels of PGE2, while ODQ and L-NAME also reduced the migration of cells and decreased the basal levels of NO in these cells. These results suggest that cross-talk exists between COX-2 and NOS in breast cancer cell migration. These findings indicate that endogenous NO, which is produced by 4T1 cells, plays an important role in COX-2 upregulation and PGE2 production. Thus, it can be hypothesized that the continuous upregulation of COX-2 and NOS could facilitate cancer cell migration.

NF-kB is a regulator of COX-2 and iNOS. Certain studies have suggested that the COX-2 regulation by NO occurs through the transcription factor NF-kB. We therefore examined the effect of honokiol on the basal levels as well as the activity of NF-kB in 4T1 cells, and found that the treatment of 4T1 cells with honokiol downregulates the basal level as well as the activity of NF-kB in 4T1 cells, plays an important role in COX-2 upregulation and PGE2 production. Thus, it can be hypothesized that the continuous upregulation of COX-2 and NOS could facilitate cancer cell migration.

In summary, our study shows that honokiol inhibits the migration of breast cancer cells by targeting NO and COX-2, and successively downregulates the migration-promoting signals induced by NO and COX-2. More mechanism-based studies using in vivo models are required in order to develop honokiol as a pharmacologically safe agent alone, or in combination with other anti-metastatic drugs, for the treatment of metastatic breast cancer.
Acknowledgements

This study was supported by funds from the Veterans Administration Merit Review Award (S.K.K.).

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