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RESEARCH PAPER

Honokiol inhibits gastric tumourigenesis by activation of 15-lipoxygenase-1 and consequent inhibition of peroxisome proliferator-activated receptor-γ and COX-2-dependent signals

Shing Hwa Liu[1], Chin Chang Shen[2,3], Yu Chiao Yi[4], Jaw Ji Tsai[5,6], Chih Chien Wang[6], Ju Ting Chueh[7], Keh Liang Lin[7], Tso Ching Lee[5], Hung Chuan Pan[5,6] and Meei Ling Sheu[5,6]

[1]*Institute of Toxicology, Taipei, Taiwan,* [2]*Department of Chemistry, National Taiwan University, Taipei, Taiwan,* [3]*Institute of Nuclear Energy Research, Atomic Energy Council, Longtan, Taoyuan, Taiwan,* [4]*Department of Obstetrics and Gynecology, Taichung Veterans General Hospital, Taichung, Taiwan,* [5]*Department of Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan,* [6]*Institute of Biomedical Sciences, National Chung Hsing University, Taichung, Taiwan and* [7]*School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*

Background and purpose: Peroxisome proliferator-activated receptor-γ (PPAR-γ), COX-2 and 15-lipoxygenase (LOX)-1 have been shown to be involved in tumour growth. However, the roles of PPAR-γ, COX-2 or 15-LOX-1 in gastric tumourigenesis remain unclear. Here, we investigate the role of 15-LOX-1 induction by honokiol, a small-molecular weight natural product, in PPAR-γ and COX-2 signalling during gastric tumourigenesis.
Experimental approach: Human gastric cancer cell lines (AGS, MKN45, N87 and SCM-1) were cultured with or without honokiol. Gene and protein expressions were analysed by RT–PCR and Western blotting respectively. Small interfering RNAs (siRNAs) for COX-2, PPAR-γ and 15-LOX-1 were used to interfere with the expressions of these genes. A xenograft gastric tumour model in mouse was used for *in vivo* study.
Key results: PPAR-γ and COX-2 proteins were highly expressed in gastric cancer cells. Inhibitors, or siRNA for COX-2 or PPAR-γ, significantly decreased cell viability. Honokiol markedly inhibited PPAR-γ and COX-2 expressions in gastric cancer cells and tumours of xenograft mice, and induced apoptosis and cell death. Honokiol markedly activated cellular 15-LOX-1 expression and 13-*S*-hydroxyoctadecadienoic acid (a primary product of 15-LOX-1 metabolism of linoleic acid) production. 15-LOX-1 siRNA could reverse the honokiol-induced down-regulation of PPAR-γ and COX-2, and cell apoptosis. 15-LOX-1 was markedly induced in tumours of xenograft mice treated with honokiol.
Conclusions and implications: These findings suggest that induction of 15-LOX-1-mediated down-regulation of a PPAR-γ and COX-2 pathway by honokiol may be a promising therapeutic strategy for gastric cancer.
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Keywords: gastric tumourigenesis; honokiol; calpain; 15-LOX-1; PPAR-γ; COX-2

Abbreviations: 13-*S*-HODE, 13-*S*-hydroxyoctadecadienoic acid; 15-LOX-1, 15-lipoxygenase-1; DTT, dithiothreitol; IHC, immunohistochemistry; MTS, 3,4-(5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; NSAIDs, non-steroidal anti-inflammatory drugs; PGE_2, prostaglandin E_2; PI, propidium iodide; PPAR-γ, peroxisome proliferator-activated receptor-γ; RT–PCR, reverse transcription–PCR; VEGF, vascular endothelial growth factor

Introduction

Gastric cancer is the second most common cause of death in the world (Leung *et al.*, 2008). Tumour angiogenesis is closely related to the development of growth and metastasis in

Correspondence: Meei-Ling Sheu, Institute of Biomedical Sciences, National Chung Hsing University, Taichung 40227, Taiwan. E-mail: mlsheu@nchu.edu.tw
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human gastric carcinomas, particularly on the basis of peritoneal disseminated metastasis (Fidler, 2003; Roukos and Kappas, 2005). Gastric cancer remains a difficult disease to cure in Western countries. In search of new strategies for the treatment of cancer, the peroxisome proliferator-activated receptor-γ (PPAR-γ), COX-2 and other related genes have attracted increasing attention (Hafner *et al*., 2005). Alteration of PPAR-γ or COX-2 expression or function involved in tumour growth and angiogenesis has been evolved into a promising target for cancer therapy (Panigrahy *et al*., 2005; Arber and Levin, 2008; Rouzer and Marnett, 2008). Several well-established drugs (e.g. PPAR-γ agonists and COX-2 inhibitors) have initially been developed in clinical applications, which exhibit anti-tumour activity, but many questions have arisen regarding their side effects (Badawi *et al*., 2004; Hau *et al*., 2007). Several angiogenesis inhibitors have been assessed in clinical trials, including vascular endothelial growth factor (VEGF) and prostaglandin (PG) E_2 inhibitors (Mohammed *et al*., 2002; Yao *et al*., 2003; Chu *et al*., 2004). Both COX-2/PGE_2 and VEGF signalling have been shown to play a role in human gastric tumourigenesis and angiogenesis (Uefuji *et al*., 2000; Oshima *et al*., 2006; Guo *et al*., 2008; Raica *et al*., 2008). However, the role of COX-2 and PPAR-γ, and the upstream signalling regulating their activity in gastric tumourigenesis remain unclear.

From the studies of the relationship between polyunsaturated fatty acid metabolism and carcinogenesis, some molecular targets for cancer chemoprevention research have been identified, including pro-carcinogenic lipoxygenases (LOXs), such as 5-, 8- and 12-LOX, and anti-carcinogenic LOXs, such as 15-LOX-1 (Shureiqi and Lippman, 2001). 15-LOX-1 is a major enzyme for metabolizing linoleic acid to 13-*S*-hydroxyoctadecadienoic acid (13-S-HODE), which has been demonstrated to link to colon tumourigenesis (Shureiqi *et al*., 1999). Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to increase 15-LOX enzymatic activity in normal human leucocytes (Vanderhoek and Bailey, 1984) and human colorectal cancer cells (Shureiqi *et al*., 2000). It has been further demonstrated that the NSAIDs-induced increase of 15-LOX-1 activity in colorectal cancer cells is associated with subsequent growth inhibition and apoptosis (Shureiqi *et al*., 2000). 15-LOX-1 has been suggested to exhibit anti-carcinogenic effects in colorectal cancer, dependent or independent of its metabolites, and is manifested through downstream pathways such as cGMP, PPAR, p53, p21 and NAG-1, which increase cancer cell apoptosis (Bhattacharya *et al*., 2009). 15-LOX-1 expression has been shown to induce apoptosis in colon cancer cells via modulating PPAR-β/δ suppression of PPAR-γ (Zuo *et al*., 2006). However, the relationship between 15 LOX-1, PPAR-γ and COX-2 in gastric tumourigenesis remains unclear.

Honokiol, a small-molecular weight natural product, is a main active compound of *Magnolia officinalis*. *Magnolia* bark is widely known to ameliorate microbial infection, inflammation and gastrointestinal disorder in traditional Asian medicinal systems such as traditional Chinese medicine and Kampo medicine in Japan. Recent studies have reported that honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells, and inhibits growth of PC-3 prostate cancer cell xenografts *in vivo* in association with apoptosis induction (Battle *et al*., 2005; Hahm *et al*., 2008). Garcia and colleagues have recently shown that honokiol suppresses survival signals mediated by Ras-dependent phospholipase D activity in several human cancer cell lines (Garcia *et al*., 2008). Glucose-regulated protein-94 signalling has been shown to be involved in the apoptosis of human gastric cancer cells induced by honokiol (Sheu *et al*., 2007). However, the precise molecular mechanism(s) underlying the inhibition of gastric tumourigenesis by honokiol still needs further investigation. In this study, we have investigated the role of PPAR-γ and COX-2 signalling in gastric tumourigenesis, and if 15-LOX-1 induction played a regulatory role in the PPAR-γ and COX-2 signalling pathway, and affected gastric tumourigenesis. We found that honokiol is an inducer of 15-LOX-1 signalling, which inhibited gastric tumourigenesis.

Methods

Cell culture

Human gastric cancer cell lines including Caucasian cell lines (AGS, a moderately/poorly (mixed) differentiated adenocarcinoma cells, and N87, well-differentiated carcinoma cells) and Asian cell lines (MKN45 and SCM-1, the undifferentiated adenocarcinoma cells) were obtained from the cell bank of Taipei Veterans General Hospital (Taiwan). Cells were maintained in RPMI1640 medium containing 10% FCS and streptomycin/penicillin in a humidified 5% CO_2 atmosphere.

Honokiol was obtained from Wako Chemical Company (Osaka, Japan), and its purity was determined to be a minimum of 99% by HPLC.

Animals

All animal care and experimental procedures were approved by the Committee of Animal Experiments, National Chung Hsing University, Taichung, Taiwan. The procedures we used were as humane as possible and complied with the guideline for animal care of National Chung Hsing University, Taichung, Taiwan. Four- to six-week-old male BALB/c nude mice were purchased from NLAC (Taipei, Taiwan). The mice were bred and maintained under specific pathogen-free conditions, provided with sterilized food and water *ad libitum* and housed in a barrier facility with 12 h light/dark cycles.

Xenograft tumour mouse model

In the tumour growth experiment, MKN45 cells were subcutaneously injected (4×10^6 cells·per mouse) into BALB/c nude mice (4- to 6-week-old male mice). After the tumours have established (~50 mm^3), the tumours in mice were injected with vehicle, 1.5 and 5 $mg \cdot kg^{-1}$ honokiol twice per week. The body weights and the volumes of the implanted tumours in the dorsal side of the mice were recorded twice per week. The tumour sizes were measured by vernier caliper and calculated using the formula $V = (LW^2)\pi/6$ [V, volume (mm^3); L, biggest diameter (mm); W, smallest diameter (mm)]. Twenty-eight and 49 days after treatment with honokiol, the mice were killed with a lethal intraperitoneal injection of pentobarbital.

Flank tumours were excised, cut into blocks and placed in 10% formalin for paraffin blocks or snap-frozen in liquid nitrogen.

Reverse transcription–PCR (RT–PCR) for mRNA expression
The expressions of PPAR-γ, COX-2 and 15-LOX-1 mRNA were determined by the RT–PCR analysis technique. In brief, approximately 5×10^5 cells were homogenized with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA was isolated according to the manufacturer's protocols. The first-strand cDNA was synthesized by the extension of (dT) primers with 200 units of SuperScript II reverse transcriptase (Invitrogen) in a mixture containing 1 μg of total RNA digested by RNase-free DNase (2 units·μg^{-1} of RNA) for 15 min at 37°C. Then, the cDNA served as a template in a PCR using the PerkinElmer DNA Thermal Cycler (model 480, Waltham, MA, USA). The primers (5′ to 3′) used for 15-LOX-1 (500 bp) were sense primer, 5′-GCTGCGGCTCTGGGAAATCATCT-3′; antisense primer 5′-GGGCCCGAAAAATACTCCTCCTCA-3′. PPARγ (473 bp) sense primer 5′-TCTCTCCGTAATGGAAG ACC-3′; antisense primer 5′-GCATTATGAGACATCCCCAC-3′. COX-2 (304 bp) sense primer 5′-TTCAAATGAGATTGTGGG AAAAT-3′; antisense primer 5′-AGATCATCTCTGCCTGAGTA TCTT-3′; β-actin (358 bp) sense primer 5-GATGATGATA TCGCCGCGCT-3′ antisense primer 5-TGGGTCATCTTCT CGCGGTT-3′. The amplification cycles included 94°C for 60 s, 55°C for 60 s and 72°C for 90 s. Then, the PCR products were subjected to electrophoresis on a 1.8% agarose gel after 30 cycles. The electrophoresis products were visualized by ethidium bromide staining. The mRNA of β-actin served as control for the sample integrity and loading.

Western blotting
Protein levels of PPAR-γ, COX-2 and 15-LOX-1 were analysed by Western blot. Cells were washed with PBS and lysed with RIPA buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg·mL^{-1} aprotinin and 1 μg·mL^{-1} leupeptin]. Proteins (50–100 μg) were separated by pre-cast 8–20% SDS–polyacrylamide gel electrophoresis, and then electrophoretically transferred from the gel onto polyvinylidene difluoride membranes. After blocking, blots were incubated with anti-COX-2 (New England BioLabs, Ipswich, MA, USA), anti-15-LOX-1 (Abcam, Cambridge, MA, USA) and anti-PPAR-γ (dilution: 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies in PBS with 0.1% Tween 20 for 1 h followed by three 10 min washes in PBS with 0.1% Tween 20. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 60 min. Detection was performed with Western blotting reagent ECL, and chemiluminescence was exposed by the filters of Kodak X-Omat films (Rochester, NY, USA).

Cell viability [3,4-(5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay]
Cell viability was measured using a non-radioactive cell proliferation assay kit (CellTiter 96 AQueous; Promega, Madison, WI, USA). The assay was composed of a solution of tetrazolium compound (MTS) and an electron coupling reagent (phenazine methosulphate). The assay was based on the cellular conversion of the colorimetric reagent MTS into soluble formazan by dehydrogenase enzymes found only in metabolically active cells. All assays were at least repeated threes times in duplicate.

Preparation of nuclear extracts
At the end of the culture, approximately 1×10^6 cells were harvested and suspended in hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT) and 0.5 mM PMSF] for 5 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 12 000× *g* for 2 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was suspended in a buffer [20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol and 0.42 M NaCl] for 30 min on ice. The supernatants containing nuclear proteins were collected by centrifugation at 18 000× *g* for 30 min and stored at −70°C.

Immunohistochemistry
The expressions of PPAR-γ and COX-2 proteins in mouse solid tumours were examined by immunohistochemistry. All tumours were fixed in 10% buffered formalin immediately after death. After pressure cooker pretreatment in citrate buffer (pH 6.0) for 30 min, 5 μm sections of the tumours were incubated at room temperature for 1 h with a monoclonal antibody (dilution: 1:250; Santa Cruz Biotechnology). After incubation with an appropriate primary antibody enhancer, the slides were incubated with HRP polymer (Laboratory Vision Corporation, Fremont, CA, USA). Reaction products were visualized by immersing the slides in peroxidase-compatible chromogen. Finally, the slides were counterstained with hemalaun.

Annexin-V FITC staining
The annexin V/propidium iodide (PI) assay (Clontech, Mountain View, CA, USA) was used to quantify numbers of apoptotic cells as described previously (Sheu *et al*., 2007). Cells were washed twice with PBS and stained with annexin V and PI for 20 min at room temperature. The level of apoptosis was determined by measuring the fluorescence of the cells by flow cytometer (Becton Dickinson, Rockville, MD, USA). Data acquisition and analysis were performed by the CellQuest program (Becton Dickinson).

13-S-HODE production
The level of 13-*S*-HODE in cell culture supernatants was determined using the EIA kit (Assay-Designs, Ann Arbor, MI, USA).

Small interfering RNA (siRNA) and transfection assays
The siRNA duplexes specific for the inhibition of 15-LOX-1, COX-2 and PPAR-γ expression in human cells were obtained

from Dharmacon (Lafayette, CO, USA) (15-LOX-1, L-003808-00; COX-2, L-004557-00) and MWG Biotech (Ebersberg, Germany) (PPAR-γ). The control siRNA (Santa-Cruz, sc-37007) is a non-targeting 20–25 nt siRNA designed as a negative control. The siRNAs were used at a concentration of 100–200 nM for transient transfection of cells with Lipofectin (Invitrogen) per well in a six-well plate with fresh medium.

Statistical analyses

The values given in this study are presented as mean ± SEM. All analyses were performed by ANOVA followed by a Fisher's least significant difference test. *P* value of less than 0.05 was viewed as statistically significant.

Results

Honokiol suppresses PPAR-γ and COX-2 expressions in gastric cancer cells and tumours, and inhibits tumour growth

Initially, we examined mRNA and protein expressions of PPAR-γ and COX-2 in four human gastric carcinoma cell lines (AGS, MKN45, N87 and SCM-1). PPAR-γ and COX-2 expression was high in MKN45 and SCM-1 cells, but relatively low levels were found in AGS and N87 cells [Figure 1A,B (cell lysates and nuclear extract)]. Moreover, COX-2 inhibitor NS398 (20 and 50 μM) and transfection of siRNA targeting for COX-2 or PPAR-γ significantly decreased the cell viability in MKN45 and SCM-1 cells (Figure 1C). These siRNAs for COX-2 and PPAR-γ specifically blocked the expression of the corresponding proteins, COX-2 and PPAR-γ (Figure 1D).

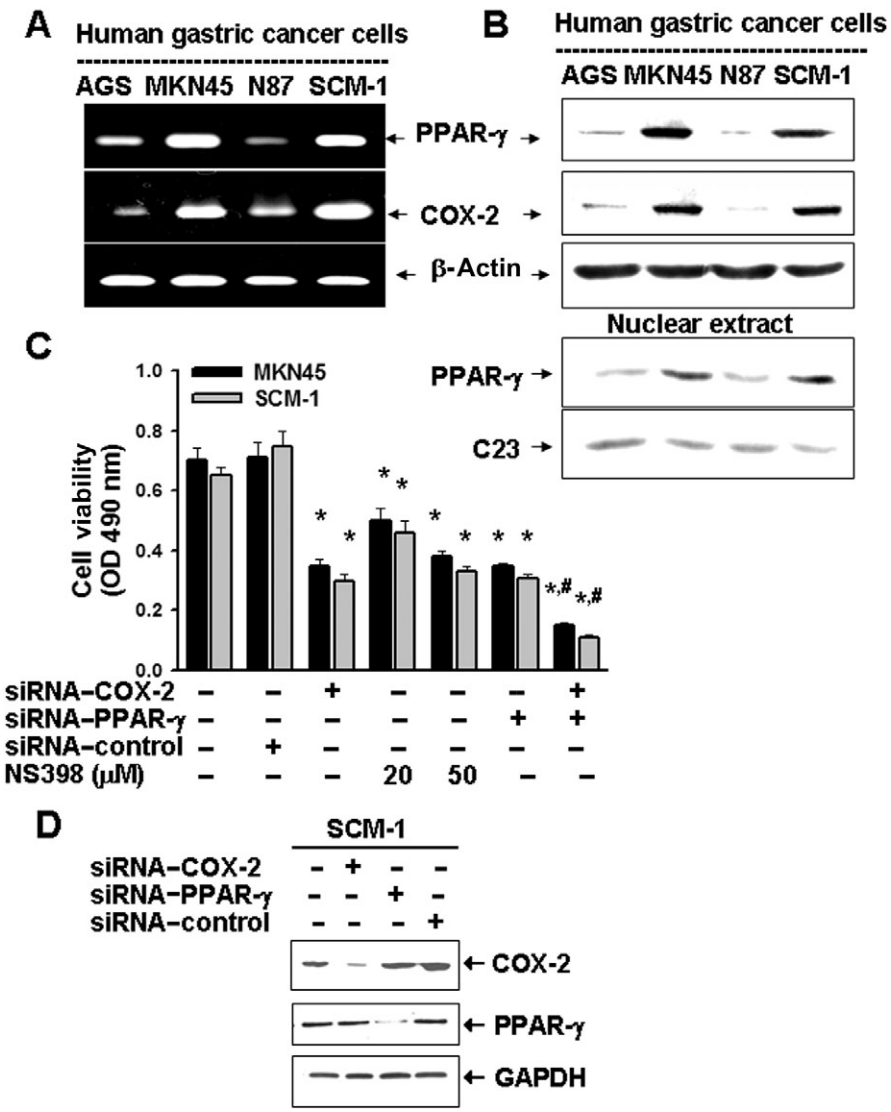

Figure 1 PPAR-γ and COX-2 expression, and cell viability in human gastric cancer cells. Four human gastric cancer cell lines (AGS, MKN45, N87 and SCM-1) were examined. The mRNA and protein levels of PPAR-γ and COX-2 were analysed using RT–PCR (A) and Western blotting (B). Results shown are representative of four independent experiments. In C, the effects of NS398 (20 and 50 μM) or transfection of siRNA–COX-2 or siRNA–PPAR-γ on cell viability in MKN45 and SCM-1 cells are shown. Cells were cultured for 24 h, and cell viabilities were determined by MTS assay. Data are presented as mean ± SEM. **P* < 0.05 versus control. # *P* < 0.05 versus siRNA–COX-2 or siRNA–PPAR-γ alone. In D, the protein expression of COX-2 and PPAR-γ in siRNA–COX-2 or siRNA–PPAR-γ or siRNA–control-transfected SCM-1 cells is shown. Results shown are representative of three independent experiments.

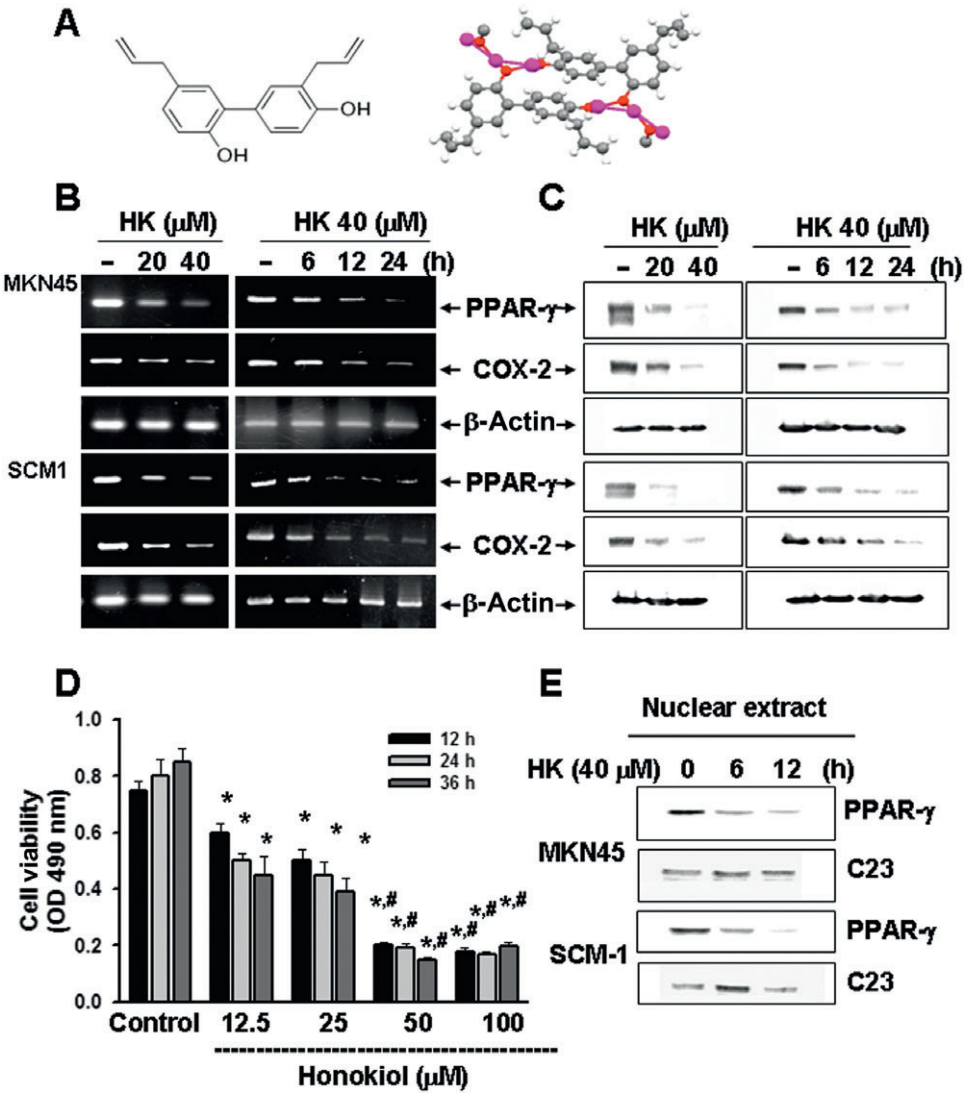

Figure 2 Effects of honokiol on PPAR-γ and COX-2 expressions, and cell viability in human gastric cancer cells. (A) Structure of honokiol. The left panel shows a stereoscopic illustration of the unit cell packing in the structure of honokiol. (B–D) Human gastric cancer cell lines (MKN45 and SCM-1) were cultured with or without honokiol (HK, 20 and 40 μM) over time, and then the mRNA and protein levels of PPAR-γ and COX-2 were analysed using RT–PCR (B) and Western blotting (C). Results shown are representative of four independent experiments. In D, the effects of honokiol on the cell viability in MKN45 cells in a time- and concentration-dependent manner are shown. Data are presented as mean ± SEM. *$P < 0.05$ versus control. In E, the protein expression of PPAR-γ in nuclear extracts of MKN45 and SCM-1 cells. Results shown are representative of three independent experiments.

Honokiol is a small-molecular weight natural product with the formula $C_{18}H_{18}O_2$. Its chemical structure is shown in Figure 2A. Honokiol effectively suppressed PPAR-γ and COX-2 mRNA (Figure 2B) and protein (Figure 2C) expressions in MKN45 and SCM-1 cells in a dose- and time-dependent manner. Honokiol could also effectively reduce the cell viability in MKN45 cells (Figure 2D). The PPAR-γ protein expression in nuclear extracts of MKN45 and SCM-1 cells was also reduced by honokiol (Figure 2E).

We next investigated whether honokiol administration inhibits gastric tumour growth. Nude mice were inoculated with MKN45 cells and treated with honokiol 1.5 and 5 $mg \cdot kg^{-1}$ or vehicle when tumour became evident. Honokiol effectively ameliorated tumour growth displayed in gross morphology, tumour weight and tumour volume (Figure 3A–C). Honokiol could also significantly reverse the decrease in body weight of the tumour xenograft-bearing mice (Figure 3D). Immunohistochemical staining showed that both PPAR-γ and COX-2 were over-expressed in tumours of xenograft mice (Figure 3E-a and -c), and both were reversed by honokiol treatment (Figure 3E-b and -d) (data of densitometric analysis of staining score were: PPAR-γ: control, 164 ± 16.3, HK treatment, 57.6 ± 12.7; COX-2: control, 176.2 ± 14.4, HK treatment, 51.1 ± 9.8 arbitrary units; $n = 5$, $P < 0.05$). These results indicate that the suppression of PPAR-γ and COX-2 over-expression may contribute to the honokiol-induced inhibition in xenografts of gastric cancer cells.

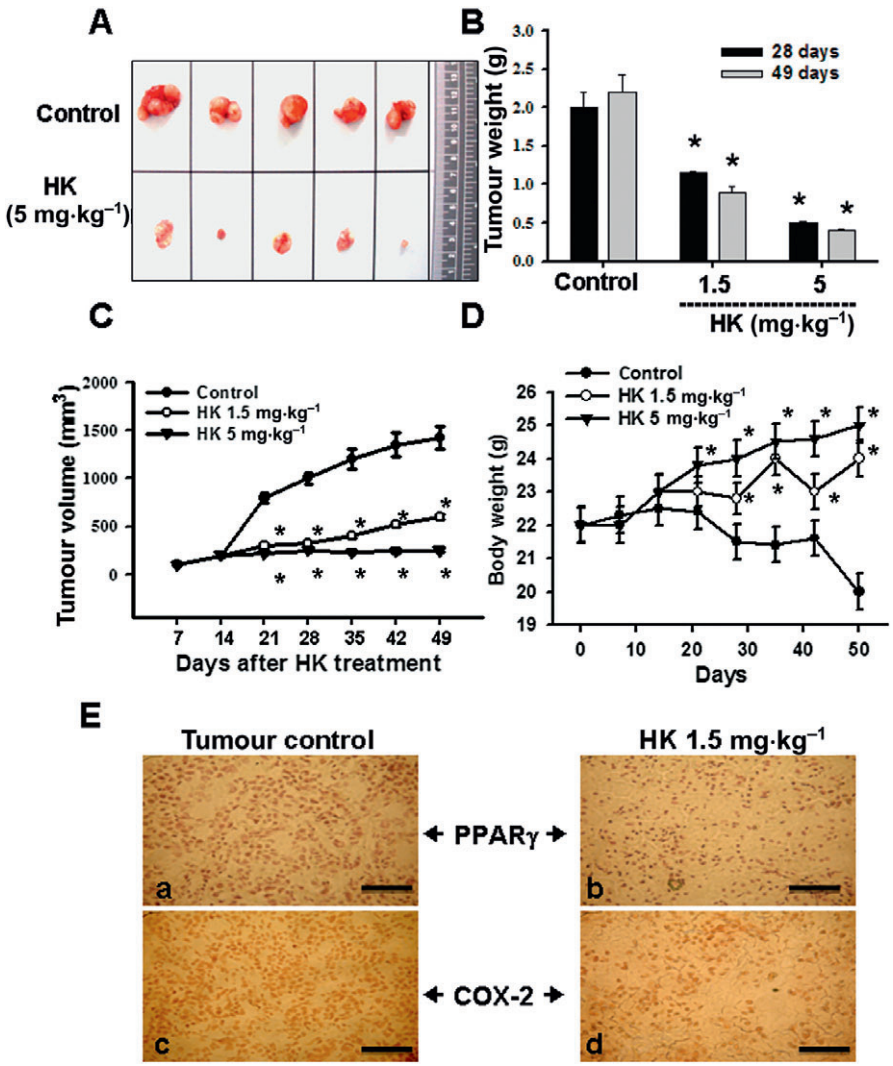

Figure 3 Inhibitory effects of honokiol on xenograft tumour growth and expression of PPAR-γ and COX-2 in tumours. MKN45 cells were inoculated 5×10^6 cells per mouse into nude mice. After the tumours had established (~50 mm^3), the mice were injected with or without honokiol (HK, 1.5 and 5 $mg \cdot kg^{-1}$) twice a week. Twenty-eight and 49 days after treatment with honokiol, the mice were killed, the gross morphology of the tumours was recorded and samples of tumour were taken for immunohistochemistry. The observations on gross morphology (A), tumour weight (B), tumour volume (C) and body weight (D) in honokiol-treated groups were compared with control groups. In E, immunohistochemistry for PPAR-γ and COX-2 expressions in tumours of xenograft mice with or without honokiol treatment was observed. Honokiol treatment (b,d) effectively decreased the immunohistochemical staining compared with control groups (a,c); scale bar = 50 μm. In A and E, results shown are representative of 10 animals per group. In B–D, data are presented as mean ± SEM ($n = 10$). *$P < 0.05$ versus control.

The role of 15-LOX-1 in honokiol-suppressed PPAR-γ and COX-2 expressions

We further investigated the role of 15-LOX-1 in honokiol-induced suppression of PPAR-γ and COX-2 expression in human gastric cancer cells. Honokiol markedly induced 15-LOX-1 mRNA (Figure 4A) and protein expression (Figure 4B) in SCM-1 cells in a dose-dependent manner. Honokiol also markedly increased the production of 13-*S*-HODE, a primary product of 15-LOX-1 metabolism of linoleic acid, in gastric cancer cells, which could be reversed by silencing of 15-LOX-1 by siRNA (Figure 4C). Furthermore, silencing of 15-LOX-1 by siRNA effectively reversed the down-regulation of PPAR-γ and COX-2 expressions by honokiol (Figure 5A) and honokiol-induced apoptosis (Figure 5B) in human gastric cancer cells. The decreased cell viability induced by honokiol could also be reversed in siRNA–15-LOX-1-transfected cells (Figure 6A). Co-treatment of honokiol (40 μM) and 13-*S*-HODE (20 μM) induced a further decrease in SCM-1 cell viability (Figure 6A), and PPAR-γ and COX-2 protein expression (Figure 6B) than honokiol or 13-*S*-HODE treatment alone. Moreover, immunohistochemical staining showed that 15-LOX-1 was up-regulated in tumours of xenograft mice treated with honokiol (Figure 7A). Honokiol treatment could also up-regulate 15-LOX-1 protein expression, but down-regulated the PPAR-γ and COX-2 protein expressions in tumours (Figure 7B). These results indicate that 15-LOX-1 is involved in the honokiol-induced down-regulation of PPAR-γ and COX-2 in human gastric cancer cells.

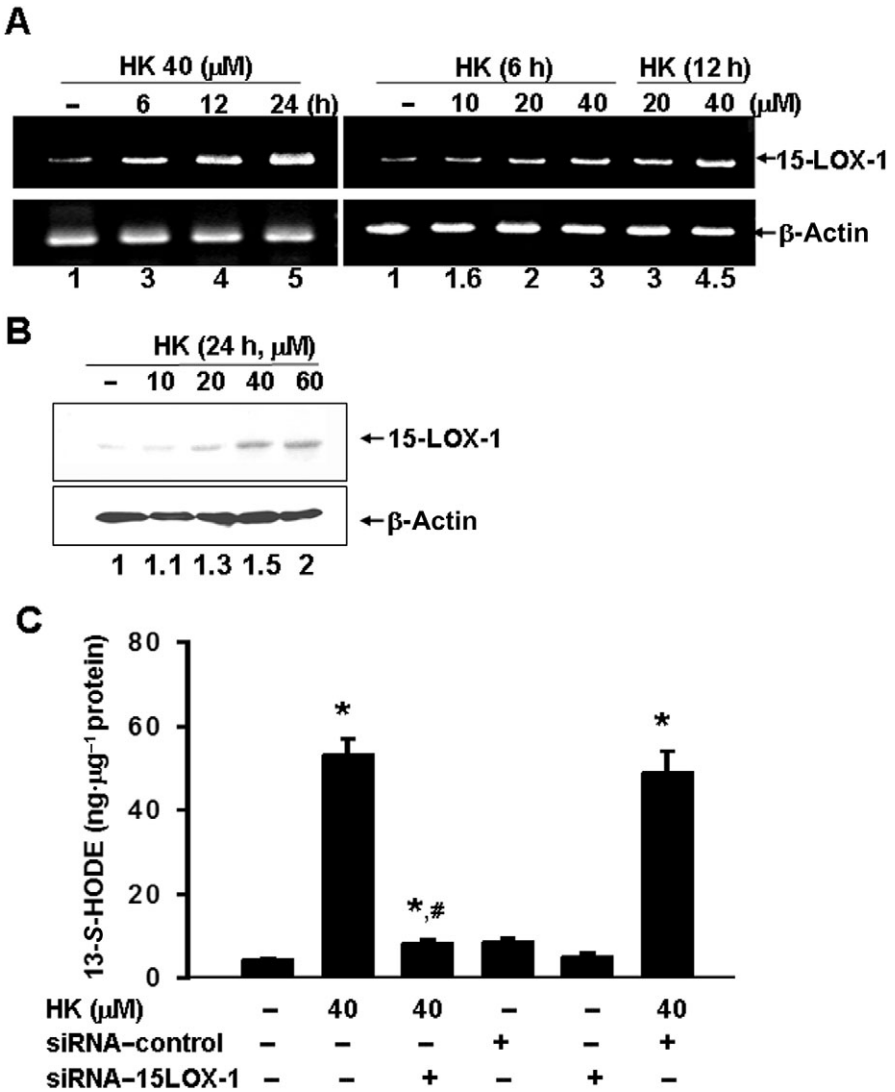

Figure 4 Honokiol up-regulates 15-LOX-1 expression and 13-*S*-HODE production, and affects cell viability in human gastric cancer cells. SCM-1 cells were cultured with or without honokiol (HK, 10–40 μM) for 6–24 h, and then the 15-LOX-1 mRNA (A) or protein (B) levels were analysed. Results shown are representative of four independent experiments. The numbers below represent the fold increase in proteins relative to the untreated group after normalization to the loading control. In C, cells were cultured with or without honokiol (40 μM) for 24 h in the presence or absence of silencing of 15-LOX-1 by siRNA, and then the culture media were collected and the level of 13-*S*-HODE were assayed using EIA kit. Data are presented as mean ± SEM ($n = 5$). *$P < 0.05$ versus control. # $P < 0.05$ versus honokiol alone.

Discussion

Both inhibition of COX-2 and activation of PPAR-γ have been shown to synergistically inhibit proliferation and induce apoptosis of pancreatic carcinoma cells (Sun *et al*., 2009), breast cancer cells (Mustafa and Kruger, 2008) and mammary gland cancer cells (Badawi *et al*., 2004). Conversely, several reports have shown that PPAR-γ possesses tumour-promoting properties (Krishnan *et al*., 2007; Thompson, 2007; Eibl, 2008). Over-expression of PPAR-γ was observed in many human tumours including colon, lung, breast, bladder and prostate (Lehrke and Lazar, 2005; Han and Roman, 2007). A recent study has indicated that both COX-2 and PPAR-γ immunostaining were significantly increased in melanomas, and metastatic melanomas were more likely to have a higher number of PPAR-γ-immunopositive cells (Lee *et al*., 2008). Therefore, the biological significance of PPAR-γ in cancer development and progression is still controversial.

The role of COX-2 and PPAR-γ, and their up-stream regulation in gastric tumourigenesis remain unclear. In the present work, we found that PPAR-γ and COX-2 expressions were highly expressed in human gastric cancer cells MKN45 and SCM-1 (the undifferentiated adenocarcinoma cells), and tumours of xenograft mice. COX-2 inhibitor and transfection of siRNA targeting for COX-2 or PPAR-γ significantly decreased the cell viability in human gastric cancer cells. These results suggest that over-expression of PPAR-γ and COX-2 may participate in the induction of gastric tumourigenesis. Moreover, we also found that honokiol, a natural biphenolic compound, effectively suppressed PPAR-γ and COX-2 expressions in human gastric cancer cells and tumours of xenograft mice. Honokiol also effectively reduced cancer

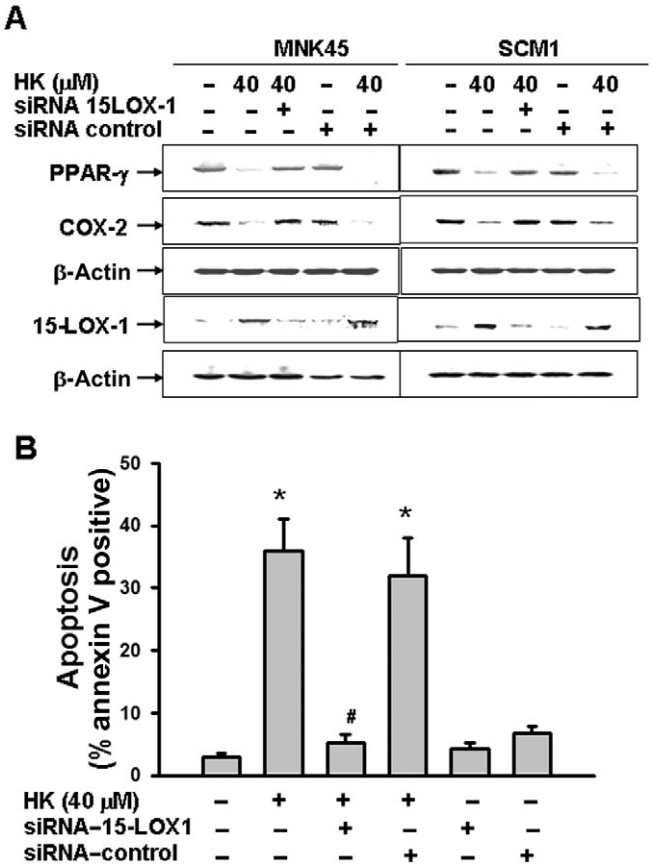

Figure 5 Honokiol suppresses PPAR-γ and COX-2 expression, and enhances cell apoptosis through up-regulation of 15-LOX-1 expression in human gastric cancer cells. SCM-1 and MKN45 cells were cultured with or without honokiol (HK, 40 μM) for 24 h, and then protein levels were analysed using Western blotting (A). In some experiments, cells were transfected with 15-LOX-1–siRNA. In B, apoptosis in 15-LOX-1–siRNA-transfected MKN45 cells with or without honokiol (40 μM) treatment was analysed by annexin V staining. Data are presented as mean ± SEM ($n = 5$). *$P < 0.05$ versus control. #$P < 0.05$ versus honokiol alone.

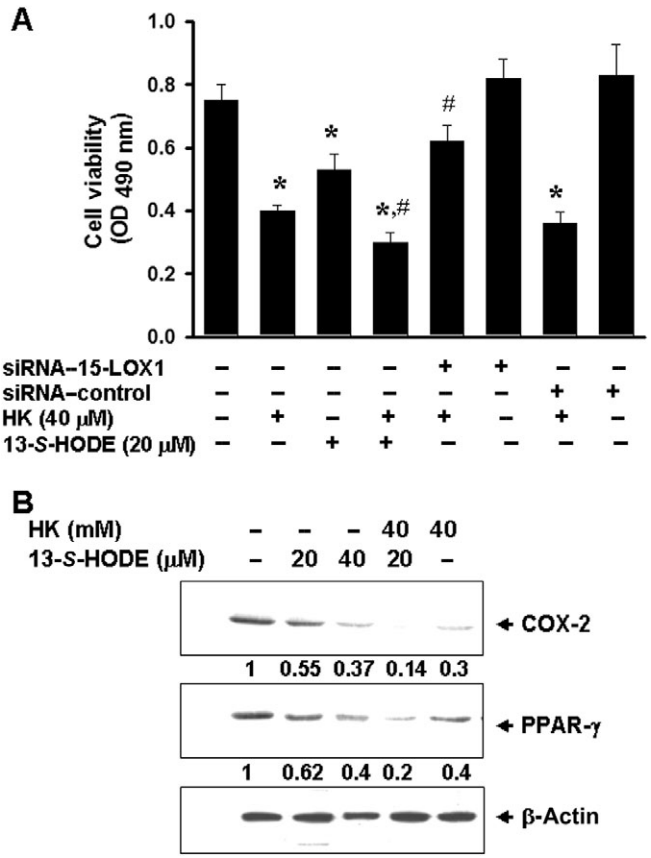

Figure 6 Effects of honokiol and 13-*S*-HODE on the cell viability and the protein expression of PPAR-γ and COX-2 in SCM-1 cells. Cells were treated with 13-*S*-HODE (20 μM) and honokiol (40 μM) or both for 24 h with or without siRNA–15-LOX-1 transfection. The cell viability (A), and PPAR-γ and COX-2 protein expressions (B) were detected. In A, data are presented as mean ± SEM ($n = 5$). *$P < 0.05$ versus control. #$P < 0.05$ versus honokiol alone. In B, results shown are representative of three independent experiments.

cell viability. The NSAID-induced growth inhibition and apoptosis of colorectal cancer cells were associated with increased 15-LOX-1 activity (Shureiqi *et al*., 2000). Here, we found that honokiol triggered not only the COX-2 inhibition, but also the PPAR-γ inhibition; the combination of COX-2 and PPAR-γ inhibition possessed more effective decrease in cell viability than COX-2 inhibition alone. We also found that honokiol did not affect the expression of COX-1 protein in human gastric cancer cells (data not shown), implying that honokiol may possess less side effect than some NSAIDs; however, this issue may need further confirmation in the future.

Shureiqi *et al*. (2000) have identified that human colon cancers are associated with a down-regulation in 15-LOX-1 expression and a reduction in 13-*S*-HODE levels, and suggested that 15-LOX-1 is a novel molecular target of NSAIDs for inducing apoptosis in colorectal carcinogenesis (Shureiqi *et al*., 2000). They further found that down-regulation of PPAR-δ by 15-LOX-1 through 13-*S*-HODE is an apoptotic signalling pathway in colorectal cancer cells, which is activated by NSAIDs (Shureiqi *et al*., 2003). A recent study has also shown that 15-LOX-1-related 13-*S*-HODE formation promotes PPAR-γ activity via down-regulating PPAR-β/δ and induces apoptosis in colon cancer cells (Zuo *et al*., 2006). In gastric cancer cells, it has been found that SC-236, a COX-2 inhibitor, induced cell apoptosis via up-regulation of 15-LOX-1 and 13-*S*-HODE, but did not affect the expression of COX-1 and COX-2 (Wu *et al*., 2003). Thus, the role of 15-LOX-1 and its relationship with signalling by COX-2 or PPAR-γ in gastric tumourigenesis need further clarification.

In the present work, honokiol also up-regulated 15-LOX-1 expression and 13-*S*-HODE production in cultured human gastric cancer cells. The expression of 15-LOX-1 in tumours of xenograft mice was increased by honokiol treatment. The siRNA of 15-LOX-1 significantly inhibited honokiol-induced apoptosis and suppression of PPAR-γ and COX-2 expressions. Our unpublished data also showed that combined treatment with 13-*S*-HODE and the COX-2 inhibitor NS398 exerted a synergistic effect on cell death in human gastric cancer cells. Thus, these results suggest that 15-LOX-1-regulated PPAR-γ and COX-2 signalling pathway may be a potential therapeutic

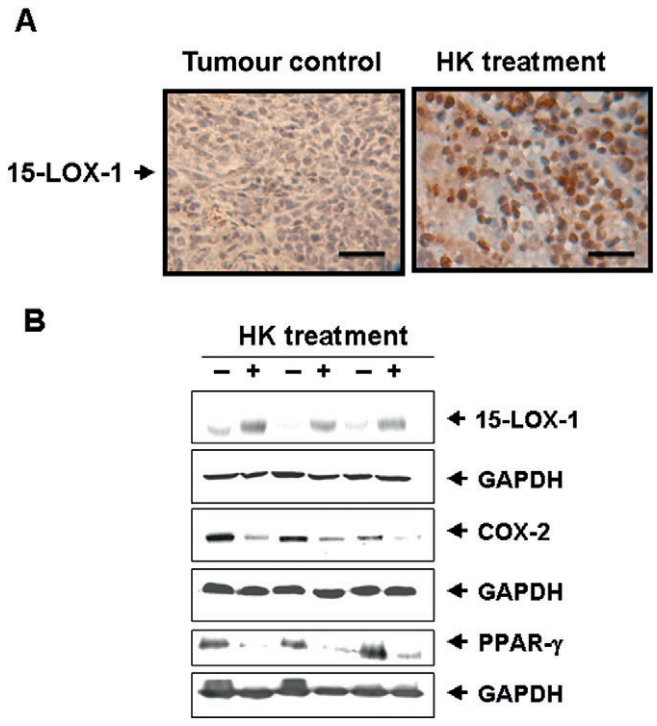

Figure 7 Honokiol up-regulates 15-LOX-1 expression in tumours. MKN45 cells were inoculated (5 × 106 cells per mouse) into nude mice. After the tumours had established (~50 mm^3), the mice were subcutaneously injected with or without honokiol (HK 5 $mg \cdot kg^{-1}$) twice a week. After 30 days, the mice were killed and tumours were removed for immunohistochemistry and Western blot analysis. In A, immunohistochemistry for 15-LOX-1 expression in tumours of xenograft mice with or without honokiol treatment is presented. In B, 15-LOX-1, PPAR-γ and COX-2 protein expression was detected by Western blotting. Results shown are representative of 10 animals per group; scale bar = 50 μm.

target for the treatment of gastric cancer. Moreover, honokiol has been shown to decrease 5-LOX activity (Hamasaki *et al*., 1996). Inhibitors of 5-LOX have pre-clinical anti-carcinogenic activity and are being developed for clinical chemoprevention study (Shureiqi and Lippman, 2001). Therefore, the possibility that honokiol could induce gastric cancer cell death through the inhibition of 5-LOX cannot be ruled out.

Honokiol-containing natural products have been widely used in traditional Chinese and Japanese medicine for several thousand years. Natural products are one of the important sources of potential cancer chemotherapeutic and chemopreventive agents (Pezzuto, 1997). The results of the present study provide *in vitro* and *in vivo* evidence for the antagonistic effect of honokiol on gastric tumourigenesis, and a molecular basis for its effect. We found that honokiol-induced activation of 15-LOX-1 signalling contributes to the down-regulation of PPAR-γ and COX-2 expression in human gastric cancer cells, which may in turn trigger cell apoptosis and inhibition of gastric tumourigenesis. These findings also suggest that the induction of the down-regulation of a PPAR-γ and COX-2 signalling pathway by 15-LOX-1 may be a promising therapeutic strategy for gastric cancer and provide insight for the design of potential clinical testing of honokiol in gastric cancer.

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Conflict of interest

None.

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