Phloretin Inhibits Phorbol Ester–Induced Tumor Promotion and Expression of Cyclooxygenase-2 in Mouse Skin: Extracellular Signal-Regulated Kinase and Nuclear Factor- κ B as Potential Targets

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ABSTRACT The present study investigated the effect of phloretin [2',4',6'-trihydroxy-3-(4-hydroxyphenyl)-propiophenone] on 12-*O*-tetradecanoylphorbol 13-acetate (TPA)–induced cyclooxygenase-2 (COX-2) expression and tumor promotion in mouse skin and explored the underlying molecular mechanisms. Topical application of phloretin significantly inhibited 7,12-dimethylbenz[*a*]anthracene-initiated and TPA-promoted mouse skin carcinogenesis. Pretreatment with phloretin on the dorsal skin of mice inhibited TPA-induced COX-2 expression in a dose-dependent manner. To elucidate the molecular mechanism underlying COX-2 inhibition by phloretin, we examined its effect on TPA-induced activation of nuclear factor- κ B (NF- κ B), a ubiquitous transcription factor responsible for TPA-induced COX-2 expression in mouse skin. Topically applied phloretin decreased the TPA-induced DNA binding of NF- κ B. In addition, phloretin inhibited the phosphorylation as well as the catalytic activity of extracellular signal-regulated kinase (ERK), which was previously found to activate NF- κ B and induce COX-2 expression in TPA-treated mouse skin. Taken together, the inhibitory effects of phloretin on TPA-induced NF- κ B activation and COX-2 expression through the modulation of ERK signaling may partly account for its antitumor-promoting effect on mouse skin carcinogenesis.

KEY WORDS: • chemoprevention • cyclooxygenase-2 • mouse skin carcinogenesis • nuclear factor- κ B • phloretin

INTRODUCTION

CYCLOOXYGENASE-2 (COX-2), the key pro-inflammatory enzyme that catalyzes the rate-limiting step in prostaglandin biosynthesis, has been implicated in carcinogenesis.¹ An abnormally elevated COX-2 expression has been observed frequently in multiple malignancies, including those of the esophagus, stomach, breast, pancreas, lung, colon, skin, urinary bladder, and prostate.² In addition, mice genetically engineered to overexpress COX-2 are prone to develop tumors,³ whereas COX-2 knockout mice are less susceptible.⁴ Selective COX-2 inhibitors have also been reported to possess antitumor-promoting activities in different organ-specific cancer models.⁵ Thus, COX-2 is regarded as a critical molecular target of many chemopreventive as well as anti-inflammatory agents.⁶

The wide variety of anti-inflammatory phytochemicals present in fruits and vegetables are known to possess chemopreventive properties. Phloretin [2',4',6'-trihydroxy-3-(4-hydroxyphenyl)-propiophenone], a naturally occurring dihydrochalcone present in apple, pear, and other plants of the Rosaceae family, has been reported to elicit anticancer effects in HL-60 human leukemia cells,⁷ B16 mouse melanoma

cells,⁷ HT29 human colon cancer cells,⁸ and H-Ras-transformed MCF-10A human breast epithelial cells.⁹ Phloretin selectively inhibits 12-*O*-tetradecanoylphorbol 13-acetate (TPA)–induced calcium- and phospholipid-dependent protein kinase activity in mouse epidermis.¹⁰ Several phloretin derivatives have been reported to inhibit croton oil–induced mouse ear edema.¹¹ Moreover, phloretin inhibits TPA-induced transformation of BALB/3T3 cells.¹² However, the antitumor-promoting effects of phloretin and its underlying mechanisms have not been investigated yet.

In the present study, we examined whether phloretin could suppress TPA-induced tumor promotion and COX-2 expression in mouse skin. To elucidate the molecular mechanism of the COX-2-inhibitory activity of phloretin, we examined its effect on TPA-induced activation of nuclear factor- κ B (NF- κ B), a principal transcription factor known to play a key role in COX-2 expression in mouse skin. We also explored the effect of phloretin on the activation of extracellular signal-regulated kinase (ERK), which was previously found to activate NF- κ B and induce COX-2 expression in TPA-treated mouse skin.

MATERIALS AND METHODS

Chemicals and antibodies

Phloretin (purity $\geq 99\%$) and 7,12-dimethylbenz[*a*]anthracene (DMBA) were purchased from Sigma-Aldrich Co.

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(St. Louis, MO, USA). TPA was obtained from Alexis Biochemicals (San Diego, CA, USA). The antibody against COX-2 was from Cayman Chemical Co. (Ann Arbor, MI, USA). The antibodies against ERK and phospho-ERK (pERK) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA). Enhanced chemiluminescence detection reagent was purchased from GE Healthcare Life Sciences (Little Chalfont, United Kingdom). All other chemicals used were in the purest form available commercially.

Animal treatment

Female ICR and HR-1 hairless mice (6–7 weeks of age) were obtained from Central Lab. Animal Inc. (Seoul, Korea). The animals were housed in climate-controlled quarters ($24 \pm 1^\circ\text{C}$ at 50% humidity) with a 12-hour light/12-hour dark cycle. In the case of ICR mice, the dorsal side of the skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used in all experiments. Indicated doses of phloretin and TPA were dissolved in 200 μL of acetone and applied to the shaven area of the dorsal skin.

Two-stage mouse skin carcinogenesis

Female HR-1 hairless mice were randomly divided into four groups of 25 each. Mice from Groups II, III, and IV were treated with a single topical application of DMBA (0.2 μmol) dissolved in 0.2 mL of acetone/dimethyl sulfoxide (85:15, vol/vol), and animals in Group I were treated with only vehicle. One week after treatment with DMBA, animals in Groups II, III, and IV were treated topically with TPA (10 nmol) twice a week for 18 weeks. Thirty minutes before each TPA treatment, phloretin was applied topically at doses of 1 and 5 μmol to mice in Groups III and IV, until the end of the experiment at week 18. Animals in Group I were treated with vehicle alone, twice a week for 18 weeks. Tumors of at least 1 mm in diameter were counted every week until week 18.

Western blot analysis

Animals were sacrificed by cervical dislocation at indicated times. For isolation of protein from mouse skin, the dorsal skin was excised, the fat was removed on ice, and the skin was immediately placed in liquid nitrogen and pulverized with a mortar. The pulverized skin was homogenized on ice for 20 seconds with a Polytron tissue homogenizer and lysed in 1 mL of ice-cold lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tris-HCl [pH 7.4], 20 mM EGTA, 1 mM dithiothreitol, and 1 mM Na_3VO_4 [Cell Signaling Technology, Inc., Beverly, MA, USA]) for 10 minutes. Lysates were centrifuged at 14,800 g for 15 minutes, and supernatant containing 30 μg of protein was boiled in sodium dodecyl sulfate sample loading buffer for 10 minutes before electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel as described previously.¹³

Electrophoretic mobility shift assay for determining NF- κ B DNA binding

The nuclear extract from mouse skin was prepared as described previously.¹⁴ Electrophoretic mobility shift assay was performed using a DNA-protein binding detection kit (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's protocol. In brief, the oligonucleotide for NF- κ B (Promega, Madison, WI, USA) was labeled with [γ - ^{32}P]ATP (GE Healthcare Life Sciences) by T4 polynucleotide kinase and purified on a Nick column (GE Healthcare Life Sciences). The binding reaction was carried out in a total volume of 25 μL containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 4% (vol/vol) glycerol, 0.1 mg/mL sonicated salmon sperm DNA, 10 μg of nuclear extracts, and 100,000 cpm of the labeled probe. After a 50-minute incubation at room temperature, 2 μL of 0.1% bromophenol blue was added, and samples were electrophoresed through a 6% nondenaturing polyacrylamide gel at 150 V for 2 hours. Finally, the gel was dried and exposed to an X-ray film.

ERK activity assay (nonradioactive)

The kinase assay for determining the catalytic activity of ERK was carried out by using a nonradioactive ERK assay kit (Cell Signaling Technology, Inc.) as described by the protocol provided by the manufacturer. Collected tissues were lysed in 600 μL of lysis buffer per sample (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3VO_4 , and 1 g/mL leupeptin). The lysates were centrifuged, and the supernatant was incubated with specific immobilized pERK monoclonal antibodies with gentle rocking overnight at 4°C . The beads were washed twice each with 500 μL of lysis buffer and the same volume of kinase buffer (25 mM Tris-HCl [pH 7.5], 5 mM glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4 , and 10 mM MgCl_2). The kinase reaction was carried out in the presence of 100 μM ATP and 2 μg of Elk-1 (ERK substrate) at 30°C for 30 minutes. Phosphorylation of Elk-1 was selectively measured by immunoblotting with specific antibodies detecting phosphorylation of Elk-1 at Ser-383.

RESULTS AND DISCUSSION

In recent years, numerous dietary components have received considerable attention for cancer chemoprevention. However, chemopreventive effects of dihydrochalcone phloretin and its underlying molecular mechanisms have not been reported. We therefore attempted to investigate the effect of phloretin on chemically induced mouse skin tumor promotion. The onset of papillomagenesis in DMBA-initiated mouse skin occurred 6 weeks after TPA treatment, which resulted in an average of 23.35 tumors per mouse (Fig. 1A) and a 100% incidence at week 18 (Fig. 1B). Although phloretin did not affect the tumor incidence, it reduced the multiplicity of skin tumors by 26% and 40% at topical doses of 1 μmol and 5 μmol , respectively (Fig. 1).

FIG. 1. Inhibitory effects of phloretin on 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated and 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-promoted mouse skin papillomagenesis: (A) average numbers of papillomas per mouse in different treatment groups and (B) percentage of tumor-bearing mice. Female HR-1 hairless mice ($n=25$ per group) were first treated with DMBA ($0.2 \mu\text{mol}$) or acetone for the control group. At 1 week after DMBA treatment, animals were treated with vehicle or phloretin (1 or $5 \mu\text{mol}$) 30 minutes before each topical application of TPA (10 nmol) twice a week for 18 weeks. Treatment groups were vehicle only, DMBA plus TPA, DMBA plus phloretin ($1 \mu\text{mol}$) plus TPA, and DMBA plus phloretin ($5 \mu\text{mol}$) plus TPA.

Carcinogenesis can be caused by dysregulation of intracellular signal transduction pathways responsible for the normal growth of cells. Therefore, current chemopreventive as well as chemotherapeutic strategies target enzymes and transcription factors involved in these intracellular signaling cascades.¹⁵ The modulation of intracellular signaling pathways associated with aberrant COX-2 expression by dietary phytochemicals provided one of the rational approaches for molecular target-based cancer chemoprevention.^{5,15} In the present study, we found that topical application of phloretin 30 minutes prior to TPA treatment resulted in a dose-dependent decrease in the expression of COX-2 protein (Fig. 2).

FIG. 2. Inhibitory effect of phloretin on TPA-induced COX-2 expression. Female ICR mice were treated topically with phloretin at a dose of 1 or $5 \mu\text{mol}$ dissolved in 0.2 mL of acetone. After 30 minutes, mice were treated topically with 0.2 mL of acetone or 10 nmol of TPA and killed 4 hours later. Control animals were treated with acetone in lieu of TPA. Protein was analyzed for COX-2 by immunoblotting as described in Materials and Methods.

The murine COX-2 promoter contains binding sites for several transcription factors, including NF- κ B, activator protein-1, and CCAAT/enhancer-binding protein- β , that, either independently or in combination, play a role in the induction of COX-2.¹⁶⁻¹⁸ Because our previous studies demonstrated a consistent activation of NF- κ B in TPA-stimulated mouse skin, which was inhibited by diverse phytochemicals, we examined the effects of phloretin on activation of this ubiquitous eukaryotic transcription factor. Phloretin pretreatment attenuated TPA-induced DNA binding of NF- κ B in mouse skin (Fig. 3). Inactive NF- κ B is

FIG. 3. Effects of phloretin on the activation of NF- κ B in mouse skin treated with TPA. Female ICR mice were treated topically with phloretin at a dose of 1 or $5 \mu\text{mol}$ dissolved in 0.2 mL of acetone. After 30 minutes, mice were treated topically with acetone or 10 nmol of TPA and killed 1 hour later. The epidermal nuclear extracts were prepared and incubated with the radiolabeled oligonucleotide containing NF- κ B consensus sequence for analysis by electrophoretic mobility shift assay: lane 1, acetone control; lane 2, TPA alone; lane 3, phloretin ($1 \mu\text{mol}$) + TPA; and lane 4, phloretin ($5 \mu\text{mol}$) + TPA.

sequestered in the cytoplasm with the inhibitory protein κB - α ; NF- κB activation normally accompanies inhibitory protein κB - α phosphorylation and subsequent degradation. Topically applied TPA induced inhibitory protein κB - α phosphorylation in mouse skin, which was partly inhibited by a higher dose of phloretin treatment (data not shown).

Previous studies from this laboratory have revealed that topical application of a pharmacologic inhibitor of ERK abrogated the phosphorylation of inhibitory protein κB - α and expression of COX-2.¹⁹ Because TPA-induced COX-2 expression and NF- κB activation are regulated, at least in part, by ERK,¹⁹ we determined the effect of phloretin on TPA-induced activation of this upstream kinase. ERK activation via phosphorylation was reported to peak at 1 hour and was sustained up to 4 hours after topical application of TPA in mouse skin.¹⁹ Pretreatment with phloretin attenuated both TPA-induced phosphorylation and the kinase activity of ERK at 4 hours (Fig. 4). Our previous studies have shown that topical application of the pharmacologic inhibitor of

ERK attenuated NF- κB activation and COX-2 expression in mouse skin *in vivo*.¹⁹

In this study, we demonstrated that phloretin suppressed TPA-induced expression of COX-2 by inactivating NF- κB via blockade of upstream ERK signaling pathway, which provides a mechanistic basis of anti-inflammatory and antitumor-promoting activities of this phytochemical in mouse skin *in vivo*. Because TPA-induced COX-2 expression in mouse skin is mediated through diverse signaling pathways,²⁰ further work with phloretin to explore additional molecular mechanisms underlying its antitumor promoting activity is warranted.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Cao QJ, Einstein MH, Anderson PS, *et al.*: Expression of COX-2, Ki-67, cyclin D1, and P21 in endometrial endometrioid carcinomas. *Int J Gynecol Pathol* 2002;21:147–154.
2. Mohan S, Epstein JB: Carcinogenesis and cyclooxygenase: the potential role of COX-2 inhibition in upper aerodigestive tract cancer. *Oral Oncol* 2003;39:537–546.
3. Muller-Decker K, Neufang G, Berger I, *et al.*: Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. *Proc Natl Acad Sci USA* 2002;99:12483–12488.
4. Tiano HF, Loftin CD, Akunda J, *et al.*: Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis. *Cancer Res* 2002;62:3395–3401.
5. Surh YJ, Kundu JK: Signal transduction network leading to COX-2 induction: a road map in search of cancer chemopreventives. *Arch Pharm Res* 2005;28:1–15.
6. Surh YJ: Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. *Food Chem Toxicol* 2002;40:1091–1097.
7. Kobori M, Iwashita K, Shinmoto H, *et al.*: Phloretin-induced apoptosis in B16 melanoma 4A5 cells and HL60 human leukemia cells. *Biosci Biotechnol Biochem* 1999;63:719–725.
8. Park SY, Kim EJ, Shin HK, *et al.*: Induction of apoptosis in HT-29 colon cancer cells by phloretin. *J Med Food* 2007;10:581–586.
9. Kim MS, Kwon JY, Kang NJ, *et al.*: Phloretin induces apoptosis in H-Ras MCF10A human breast tumor cells through the activation of p53 via JNK and p38 mitogen-activated protein kinase signaling. *Ann NY Acad Sci* 2009;1171:479–483.
10. Gschwendt M, Horn F, Kittstein W, *et al.*: Calcium and phospholipid-dependent protein kinase activity in mouse epidermis cytosol. Stimulation by complete and incomplete tumor promoters and inhibition by various compounds. *Biochem Biophys Res Commun* 1984;124:63–68.
11. Blazso G, Gabor M: Effects of prostaglandin antagonist phloretin derivatives on mouse ear edema induced with different skin irritants. *Prostaglandins* 1995;50:161–168.

FIG. 4. Effects of phloretin on TPA-induced activation of extracellular signal-regulated kinase (ERK). Mice were given indicated doses of phloretin topically. After 30 minutes, animals were treated topically with acetone or 10 nmol of TPA and sacrificed 4 hours later. Total protein was isolated from dorsal skin and quantified. (A) Protein was analyzed for ERK1/2 and phospho-ERK (pERK) 1 and 2 by immunoblotting. (B) In another experiment, mice were sacrificed 1 hour after TPA treatment for the measurement of ERK catalytic activity. Cell lysates containing 200 μg of protein for the kinase assay were treated with specific immobilized phospho-ERK 1 or 2 kinase monoclonal antibody. The resulting immunoprecipitate was then incubated with Elk-1 fusion protein for the ERK kinase assay in the presence of 100 μM ATP. pElk-1, phospho-Elk-1.

12. Tanaka K, Ono T, Umeda M: Inhibition of biological actions of 12-*O*-tetradecanoylphorbol-13-acetate by inhibitors of protein kinase C. *Jpn J Cancer Res* 1986;77:1107–1113.
13. Kundu JK, Na HK, Chun KS, *et al.*: Inhibition of phorbol ester-induced COX-2 expression by epigallocatechin gallate in mouse skin and cultured human mammary epithelial cells. *J Nutr* 2003;133(11 Suppl 1):3805S–3810S.
14. Han SS, Keum YS, Seo HJ, *et al.*: Capsaicin suppresses phorbol ester-induced activation of NF- κ B/Rel and AP-1 transcription factors in mouse epidermis. *Cancer Lett* 2001;164:119–126.
15. Surh YJ: Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003;3:768–780.
16. Wadleigh DJ, Herschman HR: Transcriptional regulation of the cyclooxygenase-2 gene by diverse ligands in murine osteoblasts. *Biochem Biophys Res Commun* 1999;264:865–870.
17. Kim Y, Fischer SM: Transcriptional regulation of cyclooxygenase-2 in mouse skin carcinoma cells. Regulatory role of CCAAT/enhancer-binding proteins in the differential expression of cyclooxygenase-2 in normal and neoplastic tissues. *J Biol Chem* 1998;273:27686–27694.
18. Cieslik K, Zhu Y, Wu KK: Salicylate suppresses macrophage nitric-oxide synthase-2 and cyclo-oxygenase-2 expression by inhibiting CCAAT/enhancer-binding protein-beta binding via a common signaling pathway. *J Biol Chem* 2002;277:49304–49310.
19. Chun KS, Keum YS, Han SS, *et al.*: Curcumin inhibits phorbol ester-induced expression of cyclooxygenase-2 in mouse skin through suppression of extracellular signal-regulated kinase activity and NF- κ B activation. *Carcinogenesis* 2003;24:1515–1524.
20. Chun KS, Surh YJ: Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. *Biochem Pharmacol* 2004;68:1089–1100.