Suppression of Prostaglandin E2-Mediated Cell Proliferation and Signal Transduction by Resveratrol in Human Colon Cancer Cells

Su-Hyun Song¹, Hye-Young Min¹, and Sang Kook Lee¹,²,*

College of Pharmacy, ¹Ewha Womans University, Seoul 120-750,
²Seoul National University, Seoul 151-742, Republic of Korea

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Abstract — Although the overproduction of prostaglandin E2 (PGE2) in intestinal epithelial cells has been considered to be highly correlated with the colorectal carcinogenesis, the precise mechanism of action remains poorly elucidated. Accumulating evidence suggests that the PGE receptor (EP)-mediated signal transduction pathway might play an important role in this process. In the present study, we investigated the mechanism of action underlying PGE2-mediated cell proliferation and the effect of resveratrol on the proliferation of human colon cancer cells in terms of the modulating PGE2-mediated signaling pathway. PGE2 stimulated the proliferation of several human colon cancer cells and activated growth-stimulatory signal transduction, including Akt and ERK. PGE2 also increased the phosphorylation of GSK-3β, the translocation of β-catenin into the nucleus, and the expressions of c-myc and cyclin D1. Resveratrol, a cancer chemopreventive phytochemical, however, inhibited PGE2-induced growth stimulation and also suppressed PGE2-mediated signal transduction, as well as β-catenin/T cell factor-mediated transcription in human colon cancer cells. These findings present an additional mechanism through which resveratrol affects the regulation of human colon cancer cell growth.

Keywords: β-catenin, Colon cancer cells, EP receptors, Prostaglandin E2, Resveratrol

INTRODUCTION

Elevated cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) levels have been associated with inflammation and carcinogenic processes. Increased expression of COX-2 was predominant in colorectal cancer tissues and chemically-induced tumors in animals (Eberhart et al., 1994; DuBois et al., 1996). PGE2 levels are also elevated in colorectal tumor tissues when compared to normal mucosa (Rigas et al., 1993; Pugh and Thomas, 1994). Indeed, PGE2 treatment was also shown to enhance the incidence of colorectal tumors in azoxymethane (AOM)-treated rats and to alleviate the effect of non-steroidal anti-inflammatory drug (NSAID)-mediated tumor regression in ApcMin/+ mice, through increasing intestinal epithelial cell proliferation and reducing apoptosis (Hansen-Petrik et al., 2002; Kawamori et al., 2003; Wang et al., 2005). Additionally, the reduction of tissue PGE2 levels by NSAID treatment led to more effective regression of human adenoma (Giardiello et al., 2002; Ulrich et al., 2006). PGE2 also increased cell survival, invasion, and migration of human colon cancer cells, suggesting the involvement of PGE2 in colorectal carcinogenesis (Sheng et al., 2001; Buchanan et al., 2003; Pai et al., 2003). Therefore, PGE2-mediated signal transduction might be an important target for the development of cancer chemopreventive agents.

It is known that the PGE2-mediated signal is transduced through four G-protein coupled receptors, designated as EP1, EP2, EP3, and EP4 within the family of prostaglandin E receptors (Hull et al., 2004; Sugimoto and Narumiya, 2007). EP1 is linked to Gq protein and thus activates PLC/IP3, leading to the increase of intracellular [Ca2+]²; EP2 and EP4 bind to Gs protein and elevate cAMP concentration; (Editor Note: Do you mean “activate” or “elevates?” EP3 is linked to Gi protein and thus decreases intracellular cAMP levels (Hull et al., 2004). Therefore, the effects of PGE2 on cancer cell growth seem to be associated with overall secondary responses in the cells mediated through EP receptors. EP receptors are distributed throughout several
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MATERIALS AND METHODS

Cell culture and reagents

Human colorectal cancer cells (HCT-15, HCT-116, and LS 174T) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and antibiotics - antimycotics (PSF; 100 units/ml penicillin G sodium, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B) at 37°C with 5% CO2. SW 480 colorectal carcinoma cells were grown in RPMI 1640 medium containing 25 mM HEPES, 10% FBS, and PSF at 37°C. Mouse monoclonal anti-cyclin D1, anti-PARP, and anti-GSK-3β antibodies were purchased from BD Biosciences (San Diego, CA, USA). Mouse monoclonal anti-c-myc, anti-ERK1/2, and anti-phospho-ERK (Tyr204) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against anti-phospho-β-catenin (Ser45), anti-phospho-β-catenin (Ser33/37, Thr41), anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), and anti-Akt were from Cell Signaling (Danvers, MA, USA). Rabbit polyclonal anti-EP1, EP2, EP3, and EP4 antibodies and PGE2 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Dual luciferase assay system was purchased from Promega (Madison, WI, USA). PGE2 was dissolved in ethanol and stored at −20°C until use. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) was obtained from Sigma (St. Louis, MO, USA), and dissolved in dimethyl sulfoxide (DMSO) (Fig. 1A).

Cell proliferation assay (SRB assay)

Cell proliferation was evaluated using the sulforhodamine B (SRB) assay as previously described (Nam et al., 2003). Cells were seeded into 96 well plates at a density of 5×10^3 cells/well and incubated for 24 h. The cells were serum starved for 24 h, and then treated with test sample for an additional 48 h. Cells were fixed with 10% trichloroacetic acid solution for 30 min at 4°C, washed 5 times with tap water, and dried in the air. Cells were stained with 0.4% SRB in 1% acetic acid solution for 30 min at room
temperature. After washing unbound dye and drying, stained cells were dissolved in 10 mM Tris (pH 10.0), and absorbance was measured at 515 nm. Cell proliferation was calculated by comparison with absorbance of a vehicle-treated control group.

**Western blot analysis**
Cultured cells were seeded into 100 mm dishes at a density of $8 \times 10^5$ cells/dish and incubated for 24 h. Cells were washed twice with PBS, and then incubated for 24 h without serum. The cells were washed with PBS, treated with test sample 30 min prior to the addition of PGE$_2$, and then incubated for the indicated time periods. After harvesting, cells were washed twice with PBS, suspended with boiling 2xsample loading buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol, 50 mM sodium fluoride, and 5 mM sodium orthovanadate) and further incubated for 5-20 min at 100°C for complete lysis. After cooling at room temperature, samples were stored at −20°C until the experiment.

Protein concentration of cell lysates was determined using the BCA method.

Equal amounts (30-50 μg) of protein samples were subjected to 8-11% SDS-PAGE. Separated proteins were electrically transferred onto PVDF membranes (Millipore, MA, USA). Membranes were blocked with blocking buffer (5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST)) for 1 h at room temperature. After washing 3 times with PBST, membranes were incubated with primary antibodies diluted in 3% non-fat dry milk in PBS (1:1,000-1:2,000) overnight at 4°C. Membranes were washed 3 times with PBST and incubated with corresponding secondary antibodies diluted in 1% non-fat dry milk in PBS (1:2,000-1:5,000) for 2-3 h at room temperature. Membranes were washed 3 times with PBST, and then exposed to enhanced chemiluminescence (ECL) detection kit (LabFrontier, Suwon, Korea). Blots were detected by LAS 3000 (Fuji Film Corp., Japan).

**Isolation of cytosolic and nuclear extracts**
LS 174T cells were seeded into 100 mm dishes at a density of $8 \times 10^5$ cells/dish and incubated for 24 h. Cells were treated with test sample diluted in the serum-free medium for 24 h. Harvested cells were washed with PBS, suspended with ice-cold lysis buffer (10 mM HEPES (pH 7.4), 250 mM sucrose, 1.5 mM MgCl$_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2% NP-40, 10 mM PMSF) on ice for 5 min. Following centrifugation at 2,500×rpm for 4 min at 4°C, supernatant was collected as the cytosolic fraction, and pellets were washed twice with ice-cold lysis buffer without NP-40. Pellets were resuspended in hypertonic nuclear extract buffer (20 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol, 50 mM sodium fluoride, and protease inhibitor cocktail) on ice for 10 min, and then centrifuged at 14,000×rpm for 15 min at 4°C. The supernatant containing nuclear extract was collected and stored in aliquots at −70°C. The protein content of cell lysates was determined using the Bradford assay.

**Reporter gene assay**
Transient transfection was performed using lipofectamine 2000 (Invitrogen, CA, USA). Briefly, LS 174T cells ($8 \times 10^4$ cells/ml) were seeded in a 48-well plate. After 24 h, cells were co-transfected for 24 h with the luciferase reporter constructs (500 ng of TOPflash (β-catenin/Tcf reporter plasmids) or FOFLASH (mutated β-catenin/Tcf reporter plasmids) (Millipore, MA, USA) and 25 ng of pRL-SV40 reporter plasmids (Promega, Madison, WI, USA)). After transfection, cells were incubated with PGE$_2$ and/or test sample for 24 h. Luciferase activity was measured using the dual-luciferase reporter gene assay system (Promega). Transfection efficiency was measured using the Renilla luciferase activity. The activity was expressed as a relative value compared to control.

**Statistics**
Data were presented as the means ± S.D. for the indicated number of independently performed experiments. Fig. data are shown as one representative of at least three independent experiments. Non-linear regression analysis for calculation of EC$_{50}$ values was performed by TableCurve program (ver. 1.0, AISN software). Statistical significance was analyzed by Student’s t-test (SigmaStat 3.1, Systat software Inc.). A difference was considered to be statistically significant when $p < 0.05$.

**RESULTS**

**Expression of EP receptor subtypes in human colon cancer cells**
It is well known that PGE$_2$-mediated signal transduction is associated with the G protein-coupled receptors designated subtypes of EP1, EP2, EP3 and EP4 [13]. Therefore, we primarily determined the protein expression profiles of EP receptor subtypes in several human colon cancer cells. As illustrated in Fig. 1B, Western blot showed that all EP receptors were expressed to a certain level in human colon cancer cells, and especially, EP4 expression was the most prominent in all cell lines tested. In LS 174T
cells, the expressions of EP2 and EP4 were more abundant compared to EP3, which is consistent with previous findings by Sheng et al. (2001).

**Effects of resveratrol on PGE2-mediated cell proliferation in human colon cancer cells**

To determine whether PGE2 enhances cell proliferation, we evaluated the growth of colon cancer cells treated with 0.5 μM of PGE2 in a serum-free condition for 48 h. PGE2 increased the growth of colon cancer cells >1.2-fold compared to control group (Fig. 2A). Based on this result, the effect of resveratrol on PGE2-enhanced cell proliferation was evaluated in cultured human colon cancer cells. To determine the non-cytotoxic concentration ranges of resveratrol in cancer cells, the growth inhibitory activity of resveratrol on LS 174T cells was measured using the SRB assay. The resultant IC50 value was 51.2 μM, and the survival rate was over 90% at 25 μM (Fig. 2B). Therefore, the subsequent experiments were performed with less than 25 μM resveratrol to exclude the possibility of unexpected effects due to its cytotoxicity. As shown in Fig. 2C, we found that resveratrol effectively inhibited the PGE2 stimulated growth of cancer cells in all tested human colon cancer cells.

**Suppression of PGE2-stimulated Akt and ERK activation by resveratrol**

Based on the PGE2-mediated enhancement of cell proliferation, the signaling molecules which are related to the activation of cell proliferation were monitored in colon cancer cells. Especially, Akt and ERK activation by treatment with PGE2, in the presence or absence of resveratrol, was

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**Fig. 2.** (A) Stimulation of cell proliferation in various colon cancer cell lines by treatment with 0.5 μM PGE2. Serum-starved cells were treated with 0.5 μM PGE2 for 48 h. Cell proliferation was determined by SRB assay. Fold-changes of cell proliferation in PGE2-treated cells were calculated by comparison of the absorbance of PGE2-treated cells with that of vehicle-treated control cells. (*p < 0.05). (B) Effect of resveratrol on the proliferation of LS 174T cells. Cells were exposed to the increasing concentrations of resveratrol for 48 h. Cell viability was determined by SRB assay. (C) Effect of resveratrol on PGE2-stimulated cell proliferation in various human colon cancer cells. Serum-starved cells were treated with PGE2 alone or in combination with various concentrations of resveratrol for 48 h. Cell proliferation was evaluated by SRB assay. Fold-changes were determined by comparing the absorbance of treated cells to that of vehicle-treated control cells. RES: resveratrol. *p < 0.05 compared to PGE2-treated control group.
determined. When monitored during a short-term course of 2 h in LS 174T cells, PGE2 (0.5 μM) increased Akt phosphorylation at an early time point, reached peak levels at 30 and 60 min, and then decreased. The pre-treatment with resveratrol (30 min prior to PGE2 treatment) alleviated the increased phosphorylation of Akt during the incubation as shown Fig. 3A. We next examined the activation of Akt and ERK with PGE2 for up to 24 h in LS 174T cells. As shown in Fig. 3B, the Akt activation by PGE2 was suppressed in cells treated with resveratrol during the incubation. However, the activation of ERK phosphorylation was induced at a relatively later time point after 2 h incubation and was predominant at 8 h. This activation was also alleviated by pre-treatment with resveratrol.

**Suppression of PGE2-stimulated GSK-3 phosphorylation and β-catenin phosphorylation by resveratrol**

It is reported that PGE2 increases the phosphorylation of GSK-3 in human embryonic kidney and neural cells that express prostanoid receptors (Fujino et al., 2002). The phosphorylation of GSK-3 inhibits its kinase activity, which is required for phosphorylation and degradation of β-catenin. To determine whether PGE2 increased the phosphorylation of GSK-3 in colon cancer cells, LS 174T cells were treated with PGE2 (0.5 μM). Treatment with PGE2 rapidly induced the increase of phosphorylation of GSK-3α/β at the early time point of 0.5 h. An increased level of β-catenin was also detected. Consequently, the phosphorylation of β-catenin was decreased after 2 h. When LS 174T cells were treated with resveratrol, the phosphorylation of GSK-3α/β was decreased at 0.5 and 1 h, and the total β-catenin was also decreased throughout a time period of 0.5 to 24 h incubation (Fig. 4).

**Resveratrol suppression of PGE2-stimulated c-myc and cyclin D1 expression**

Based on the influence of PGE2 treatment on β-catenin expression, the protein expressions of β-catenin/Tcf-target...
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Regulation of β-catenin translocation by resveratrol in PGE2-stimulated colon cancer cells

To further elucidate the underlying mechanism of action by which resveratrol suppresses c-myc and cyclin D1 expression, (which are known as β-catenin/Tcf-target genes), in LS 174T cells, the effect of resveratrol on the translocation of β-catenin into the nucleus as mediated by PGE2 was determined. As shown in Fig. 4, PGE2 treatment increased the phosphorylation of GSK-3α/β at 0.5 h and decreased the phosphorylated β-catenin level. This event might lead to the decrease of β-catenin in cytosol as well as the increase of β-catenin translocation into the nucleus. When treated with PGE2 (0.5 µM) for 24 h, the level of β-catenin in the nucleus was increased (Fig. 5A). However, nuclear translocation of β-catenin by PGE2 was definitely alleviated by treatment with resveratrol, as illustrated in Fig. 5B.

Suppression of PGE2-induced TCF-dependent transcription in reporter gene assay

Accumulated β-catenin binds and activates the transcriptional factor of the Tcf/Lef family, which in turn, up-regulates the transcription of target genes. To determine whether PGE2 was able to stimulate β-catenin/Tcf-mediated transcription, LS 174T cells were transfected with a reporter vector harboring the Tcf-binding site -TOPflash. Exposure to PGE2 increased the transcriptional activity of TOPflash. In contrast, in the reporter construct containing mutated TCF sites, FOPflash showed minimal transcriptional activity in PGE2-treated LS 174T cells, suggesting the specificity of the transcriptional activation by

![Fig. 5](image-url)
PGE2 stimulation. In this experimental condition, resveratrol markedly inhibited the TOPflash activity without affecting FOPflash activity (Fig. 5C).

**DISCUSSION**

Accumulating evidence suggests that chronic inflammation is highly associated with the carcinogenic process. Indeed, inflammatory mediators such as PGE2 and NO enhance the proliferation and metastasis of cancer cells, and also suppress the apoptotic death of cancer cells (Wiseman and Halliwell, 1996; Sheng et al., 2001; Torok et al., 2002). PGE2-mediated signal transduction is mediated by triggering the activation of EP receptors which are distributed in several tissues and play important roles in inflammation and maintenance of homeostasis in the body. Therefore, signal transduction activated by PGE2/EP receptors might be a candidate target for cancer chemoprevention. In the present study, we investigated the effects of resveratrol on PGE2-stimulated cell proliferation and the mechanisms of action regarding PGE2/EP receptors/Wnt signaling in colon cancer cells.

Primarily, we determined whether EP receptors are expressed in human colon cancer cells (LS 174T, HCT-15, HCT 116 and SW480). All these colon cancer cells express subtypes of EP receptors (EP1-4) to various extents. Especially, LS 174T cells which do not express endogenous COX-2, also show the expression of EP2 and EP4 receptors, which are major receptors of PGE2-mediated signal transduction. These results suggest that PGE2-mediated signal transduction might be linked with the relay of EP receptors in these colon cancer cells. Although PGE2 was known to enhance the proliferation of cells, we confirmed whether PGE2 stimulates the growth of colon cancer cells. PGE2 stimulated the proliferation of all the tested colon cancer cells, with ~1.2-fold induction at 0.5 μM PGE2 after a 3 day incubation (Fig. 2).

To further determine whether the enhancement of cell proliferation might be associated with the expression of cell proliferation-related biomarker proteins, Western blot analysis was performed in LS 174T cells. PGE2 effectively stimulated the expression of phosphorylated Akt and ERK in LS 174T cells, indicating the activation of growth-stimulatory signaling by PGE2 treatment (Fig. 3). Next, we investigated the effect of PGE2 on the Wnt signaling pathway linked with a downstream signaling of EP receptors. PGE2 also affected GSK-3α/β, which is associated with the Wnt signaling, and subsequently activates downstream oncogenic protein expression, including c-myc and cyclin D1 (Fig. 4).

Based on the information of PGE2-mediated signaling and activation of proliferation biomarkers, we examined, for the first time, whether a cancer chemopreventive agent (resveratrol) could modulate the PGE2-stimulated growth-promoting signaling pathway in colon cancer cells. Resveratrol, a natural stilbenoid, exhibits potential cancer chemopreventive activity, and is known to inhibit the COX-2 activity and induce apoptosis of cancer cells (Mutoh et al., 2000; Wolter et al., 2001; Bhat and Pezzuto, 2002; Joe et al., 2002; Liang et al., 2003). However, the cancer cells which do not express COX-2 were also growth inhibited by resveratrol, suggesting that resveratrol-mediated growth inhibition might involve both COX-2-dependent and -independent mechanisms. Because there is no report showing the regulatory effects of resveratrol in the PGE2-mediated signaling pathway, in the present study, we determined the effect of resveratrol on the stimulation of cell proliferation as mediated by PGE2 and its action mechanism in colon cancer cells. Resveratrol inhibited the PGE2-stimulated cell proliferation of colon cancer cells in a dose-dependent manner over a range of non-cytotoxic concentrations (Fig. 2). The extent of the decrease of cell proliferation by treatment with resveratrol appears to vary in each cell line, which might be due to different expression levels of each EP receptor subtype. Resveratrol exhibited the most profound suppression of cell proliferation in LS 174T cells, which express relatively higher levels of EP2 and 4 receptors than that of vehicle-treated control cells (the fold-change of cell viability was < 1.0). It is reported that activation of EP2 receptor by PGE2 results in promotion of colon cancer cell growth through the modulation of β-catenin-mediated signaling, a component of the Wnt signaling pathway (Castellone et al., 2005). Moreover, in previous studies, resveratrol triggered cell cycle arrest and apoptosis in human prostate and uterine cancer cells (Hsieh and Wu, 1999; Sexton et al., 2006). Since resveratrol modulated growth-promoting signal transduction, including β-catenin/Tcf-mediated transcription as discussed below, it is suggested that modulation of EP2-mediated signaling might partially explain the effectiveness of resveratrol on the proliferation of LS 174T cells. In addition, the down-regulation of cell proliferation at the highest concentration of resveratrol might be mediated by induction of cell cycle arrest and/or apoptosis. Additional studies should be conducted to prove these suggestions.

Next, we examined the effect of resveratrol on the signal transduction activated by PGE2 in LS 174T cells, which do
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not express endogenous COX-2. Again, resveratrol suppressed the phosphorylation of Akt and ERK as stimulated by PGE₂ (Fig. 3). In addition, resveratrol remarkably inhibited the expressions of oncoproteins c-myc and cyclin D1, which were known to be induced by Wnt signaling activation in PGE₂-stimulated colon cancer cells (Fig. 4). To further examine the inhibitory mechanism of resveratrol on the Wnt signaling activation by PGE₂ treatment, we next determined the translocation of β-catenin into the nucleus (Fig. 5B), and this event might lead to the inhibition of Tcf/β-catenin transcriptional activity in the reporter gene assay (Fig. 5C). These results again suggest that resveratrol might inhibit PGE₂-mediated cell proliferation through modulating growth-stimulatory signal transduction in LS 174T colon cancer cells.

In summary, PGE₂, which is known to be highly correlated with the carcinogenic process in colon, stimulates cell proliferation of colon cancer cells through activation of growth-promoting signal transduction, and a cancer chemopreventive agent (resveratrol) modulates the PGE₂-mediated signal transduction and thereby inhibits the cell proliferation stimulated by PGE₂ treatment in colon cancer cells. Therefore, these results suggest an additional mechanism of action for resveratrol in its cancer chemopreventive effect.

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