# Antitumor Effect of Green Tea Polyphenol Epigallocatechin-3-Gallate in Ovarian Carcinoma Cells: Evidence for the Endothelin-1 as a Potential Target

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The green tea polyphenol, epigallocatechin-3-gallate (EGCG), has been shown to prevent cancer; however, a precise mechanism responsible for tumor growth inhibition has not yet been clearly described. The endothelin (ET) A receptor ($ET_AR$)/ET-1 autocrine pathway is overexpressed in ovarian carcinoma and triggers tumor growth, neoangiogenesis, and invasion. These latter tumor-promoting effects are mediated through the activation of cyclooxygenase (COX)-1– and COX-2–dependent pathways by ET-1. In the present study, pretreatment of HEY and OVCA 433 ovarian carcinoma cell lines with green tea and EGCG inhibited ET-1/$ET_AR$ expression, $ET_AR$-mediated COX-1/2 mRNA expression, and COX-2 promoter activity. These effects were associated with a significant reduction in the COX-1/2–derived prostaglandin $E_2$ ($PGE_2$) production. These results provide a novel insight into the mechanism by which EGCG, by affecting $ET_AR$-dependent COX-1/2 pathways may inhibit ovarian tumors suggesting that EGCG may be useful in preventing and treating ovarian carcinoma in which activation of $ET_AR$ by ET-1 plays a critical role in tumor growth and progression. Exp Biol Med 231:1123–1127, 2006

Key words: endothelin-1; $ET_A$ receptor; EGCG; green tea; ovarian carcinoma

## Introduction

Ovarian cancer represents the leading cause of death among gynecologic malignancies. Approximately 26,250 women are diagnosed yearly, with an overall 5-year survival rate of only 47%. Despite recent advances in surgery and chemotherapy, improvement in long-term survival of these patients has been slight (1). Development of new treatment options strongly relies on improved knowledge of the molecular mechanisms underlying ovarian carcinoma initiation and progression. Primary and metastatic ovarian carcinoma express high levels of endothelin (ET)-1 and $ET_A$ receptor ($ET_AR$) compared with normal ovarian tissue (2). Engagement of $ET_AR$ by ET-1 promotes tumor cell proliferation (2, 3), apoptosis protection (4), invasiveness (5), and neovascularization by stimulating vascular endothelial growth factor (VEGF) secretion (6–9). The proangiogenic and invasive properties of ET-1 are amplified by the capacity of ET-1 to induce cyclooxygenase (COX)-1 and COX-2 enzymes and prostaglandin (PG) $E_2$ production (10, 11). The COX-1 and COX-2 enzymes catalyze the rate-limiting step in the conversion of arachidonic acid to PGs (10), and are involved in tumor progression. We found that ET-1–induced $PGE_2$ participates in these complex mechanisms by stimulating VEGF secretion cell migration and matrix metalloproteinase (MMP) activation. $ET_AR$ blockade by the selective receptor antagonist, ABT-627, inhibits the growth of ovarian carcinoma xenografts concomitantly with a reduction of microvessel density, MMP-2, VEGF, and COX-2 expression, indicating that the blocking of the ET-1/$ET_AR$ axis may represent a potential therapeutic target in ovarian carcinoma management (11, 12).

Several studies on human and animal models have demonstrated that green tea may be effective in preventing and treating cancer of the breast, prostate, esophagus, pancreas, and colon. The anticancer property of green tea

polyphenol, (−)-epigallocatechin gallate (EGCG), is likely the result of blocking biochemical pathways involved in ovarian tumor growth (13).

Understanding the molecular mechanism of green tea polyphenol–mediated inhibition of ovarian tumor growth is essential in devising preventive and therapeutic approaches. In the present study, we show that green tea polyphenol induces inhibition of both ET-1 and $ET_AR$ expression, which, in turn, leads to the blockade of the COX-1/2–mediated signaling cascade. These results provide evidence that the ET-1/$ET_AR$ pathway is a critical target for green tea and EGCG in ovarian cancer growth and identify a potential role of green tea polyphenol in the prevention and treatment of this malignancy.

## Materials and Methods

**Cells and Cell Culture Conditions.** Human ovarian carcinoma cell lines, HEY and OVCA 433, previously characterized for ET-1 receptor expression and for ET-1 production (2, 6), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin and streptomycin at 37°C in 5% $CO_2$/95% air. The cells were serum-starved by incubation for 24 hrs in serum-free DMEM. All culture reagents were from Invitrogen (Paisley, Scotland, UK). ET-1 (Peninsula Laboratories, Belmont, CA), was used at 100 n$M$ and incubated with the cells for the indicated times. Green tea infusion was prepared from commercially available (China or Sri Lanka) green tea leaves steeped in 1 liter (12.4 g/l) of boiled distilled water for 1–2 mins. Pretreatment of cells with 1 μ$M$ BQ 123 (Peninsula Laboratories), or with the indicated concentrations of EGCG (Sigma Chemical Co., St. Louis, MO) or green tea infusion was performed for 30 mins before the addition of ET-1.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR).** Total RNA from HEY and OVCA 433 cells was extracted using TRIzol (Invitrogen). RT-PCR was performed using the AccessQuick RT-PCR System (Promega, Madison, WI) according to the manufacturer's instructions. The primer sets were as follows: ET-1, 5′-TGCTCCTGCTCGTCCCTGATGGATAAAGAG-3′ and 5′-GGTCACATAACGCTCTCTGGAGGGCTT-3′; $ET_AR$, 5′-CACTGGTTGGATGTGTAATC-3′ and 5′-GGAGATCAATGACCACATAG-3′; COX-1, 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ and 5′-GTGCATCAACACAGGCGCCTCTTC-3′; COX-2, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. Thirty-five cycles of amplification were performed under the following conditions: melting at 95°C for 30 secs; annealing at 58°C for 30 secs; and extension at 72°C for 60 secs. The PCR products were analyzed by electrophoresis on a 2% agarose gel, and the relative intensities of signals were quantified using NIH Image (Scion Corporation, Frederick, MD).

**Enzyme-linked Immunosorbent Assay (ELISA).** Subconfluent HEY cells were serum-starved for 24 hrs and incubated for the indicated times. The conditioned media were collected, centrifuged, and stored in aliquots at −20°C. Levels of $PGE_2$ released into the cells medium were measured by the $PGE_2$-Correlate-EIA (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's instructions. The sensitivity of the assay is 13.4 pg/ml for $PGE_2$. Intraassay variation is 5.8% for $PGE_2$ and interassay variation is 5.1% for $PGE_2$.

**Transfection of Reporter Construct and Luciferase Assay.** Reporter construct phPES2 (−1432/+59) containing the 5′-flanking region of the human *COX*-2 gene (11) were kindly provided by Dr. DuBois (Vanderbilt University Medical Center, Nashville, TN). For transient transfection, $1 \times 10^5$ cells were plated in 6-well plates 48 hrs before transfection. The cells were cotransfected with 0.5 μg of COX-2 luciferase plasmid construct and with 0.05 μg of the pCMV–β-galactosidase plasmid (Promega), using LipofectAMINE reagent (Invitrogen), as described in the manufacturer's protocol. The cells were lysed and their luciferase activities were measured (Luciferase assay system; Promega). The results were normalized to β-galactosidase activity.

**Statistical Analysis.** Results are representative of at least three independent experiments, each performed in triplicate. All statistical analysis was assessed using a two-tailed Student's *t* test.

## Results

**Green Tea and EGCG Inhibit $ET_AR$ and ET-1 Expression.** Sustained $ET_AR$ signaling caused by an autocrine ET-1/$ET_AR$ loop has been implicated in ovarian tumor growth and progression (2, 6). To evaluate the effect of green tea polyphenols on the ET-1/$ET_AR$ axis, we examined $ET_AR$ and ET-1 expression in ovarian carcinoma cells, which secrete high levels of ET-1, in the range required for activation of $ET_AR$ in an autocrine fashion. HEY and OVCA 433 cells were treated with different concentrations of green tea and of the polyphenol EGCG, the principal mediator of the green tea antitumor effect. Total RNA or conditioned media were collected after 6 hrs and 24 hrs, respectively. RT-PCR and ELISA demonstrated that green tea and EGCG induced a dose-dependent inhibition of $ET_AR$ and ET-1 expression at the mRNA (Fig. 1A) and protein (Fig. 1B) levels. These results demonstrate that green tea and the polyphenol EGCG are able to reduce the expression of the ET-1/$ET_AR$ axis in ovarian carcinoma cells.

**EGCG Inhibits ET-1–Induced COX-1 and COX-2 Expression and $PGE_2$ Production.** Because in HEY and OVCA 433 cells, ET-1 induces expression of both COX-1 and COX-2, which contribute to $PGE_2$ and VEGF

production and cell invasion (10, 11), we examined the ability of EGCG to modulate the basal and the ET-1–induced COX-1 and COX-2 expressions. We, therefore, treated HEY cells for 6 hrs with different concentrations of EGCG alone or in combination with ET-1 and analyzed *COX*-1 and *COX*-2 mRNA expression by RT-PCR. Treatment with EGCG resulted in a dose-dependent reduction of the basal and ET-1–induced COX-1/2 levels (Fig. 2A). Moreover, to evaluate whether the reduction of COX-1 and COX-2 expression results in an inhibition of their enzymatic activity, we measured the basal and the ET-1–induced $PGE_2$ production after treatment with EGCG. HEY cells were treated with different concentrations of EGCG and with the selective $ET_AR$ antagonist, BQ 123, alone or in combination with ET-1. As shown in Figure 2B, EGCG treatment resulted in a dose-dependent reduction of the basal and the ET-1–induced $PGE_2$ production, comparable to that induced by BQ 123. These results demonstrate that EGCG is able to reduce the $ET_AR$-mediated COX-2–derived $PGE_2$ production in ovarian carcinoma cells.

**EGCG Inhibits ET-1–Induced COX-2 Promoter Activity.** ET-1 controls COX-2 expression at transcriptional and posttranscriptional levels (11). To determine whether EGCG regulates the basal and the ET-1–induced COX-2 promoter activity, HEY cells transfected with the human COX-2 promoter (phPES2–1432/+59), were pretreated with different concentrations of EGCG alone or in combination with ET-1. After 24 hrs, luciferase activity was measured and normalized to β-galactosidase activity. As shown in Figure 2C, ET-1 treatment induced a 3.1-fold increase in luciferase activity that was significantly reduced by EGCG in a dose-dependent manner. Moreover, EGCG

was able to reduce the basal level of COX-2 promoter activity. These results indicate that EGCG inhibits the ET-1–mediated regulation of the COX-2 promoter in ovarian cancer cells.

## Discussion

Epidemiologic and clinical studies have clearly shown that the COX-1 and COX-2 enzymes play a key role in the progression of ovarian carcinoma (12, 14). In these malignancies, COX-1– and COX-2–derived $PGE_2$ have been implicated in tumor angiogenesis (11, 14), and in the invasive and migratory capacity (10). We have previously demonstrated that *in vivo* blockade of $ET_AR$ autocrine pathway using a highly specific $ET_AR$ antagonist results in tumor growth reduction associated with a significant inhibition of microvessel density, expression of VEGF, MMP-2, and COX-2, and increased tumor apoptosis (11, 12), suggesting that the $ET_AR$ signaling pathway may represent a potential preventive and therapeutic tumor target (15). The limited treatment options of ovarian cancer have prompted the need for developing alternative strategies for the management of this disease. Because tumor chemoprevention and therapy with green tea or green tea polyphenols seem to offer new approaches to block tumor growth and progression, the study of green tea and EGCG mechanisms of action is becoming essential from the biologic as well as from the therapeutic point of view. Epidemiologic studies have shown that green tea consumption can enhance the survival of patients with epithelial ovarian cancer (16), and EGCG has been reported to have a growth inhibitory effect on ovarian tumor cell lines (17). In this study, we addressed whether ET-1/$ET_AR$–induced
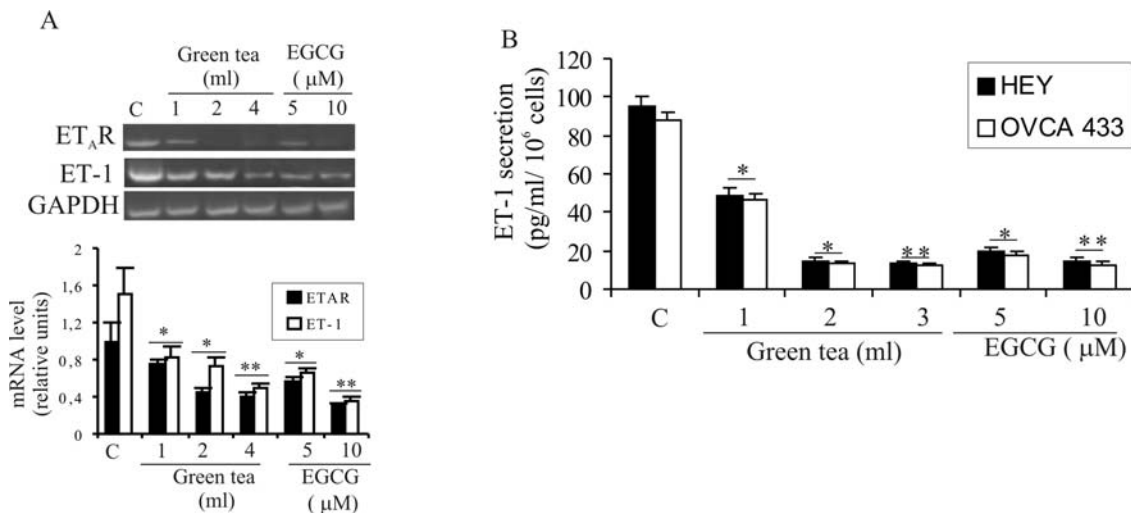


**Figure 1.** Green tea and EGCG inhibit $ET_AR$ and ET-1 expression. (A) Serum-starved HEY cells were treated with the indicated concentrations of green tea or EGCG for 6 hrs and total RNA was extracted. $ET_AR$ and ET-1 expression was evaluated by RT-PCR. Primers for the amplification of the *GAPDH* gene were used as control for the RT-PCR experiments. Densitometric analysis of RT-PCR products was evaluated by Scion image. Data were statistically analyzed and represented as the average value of three independent experiments. Bars, ± SD. *$P <$ 0.006; **$P <$ 0.005, compared with the control. (B) HEY and OVCA 433 cells were treated with the indicated concentrations of green tea or EGCG for 24 hrs and conditioned media were collected. ET-1 secretion was measured by ELISA. Results were representative of at least three independent experiments, each performed in triplicate. Bars, ± SD. *$P <$ 0.005; **$P <$ 0.001, compared with the control.
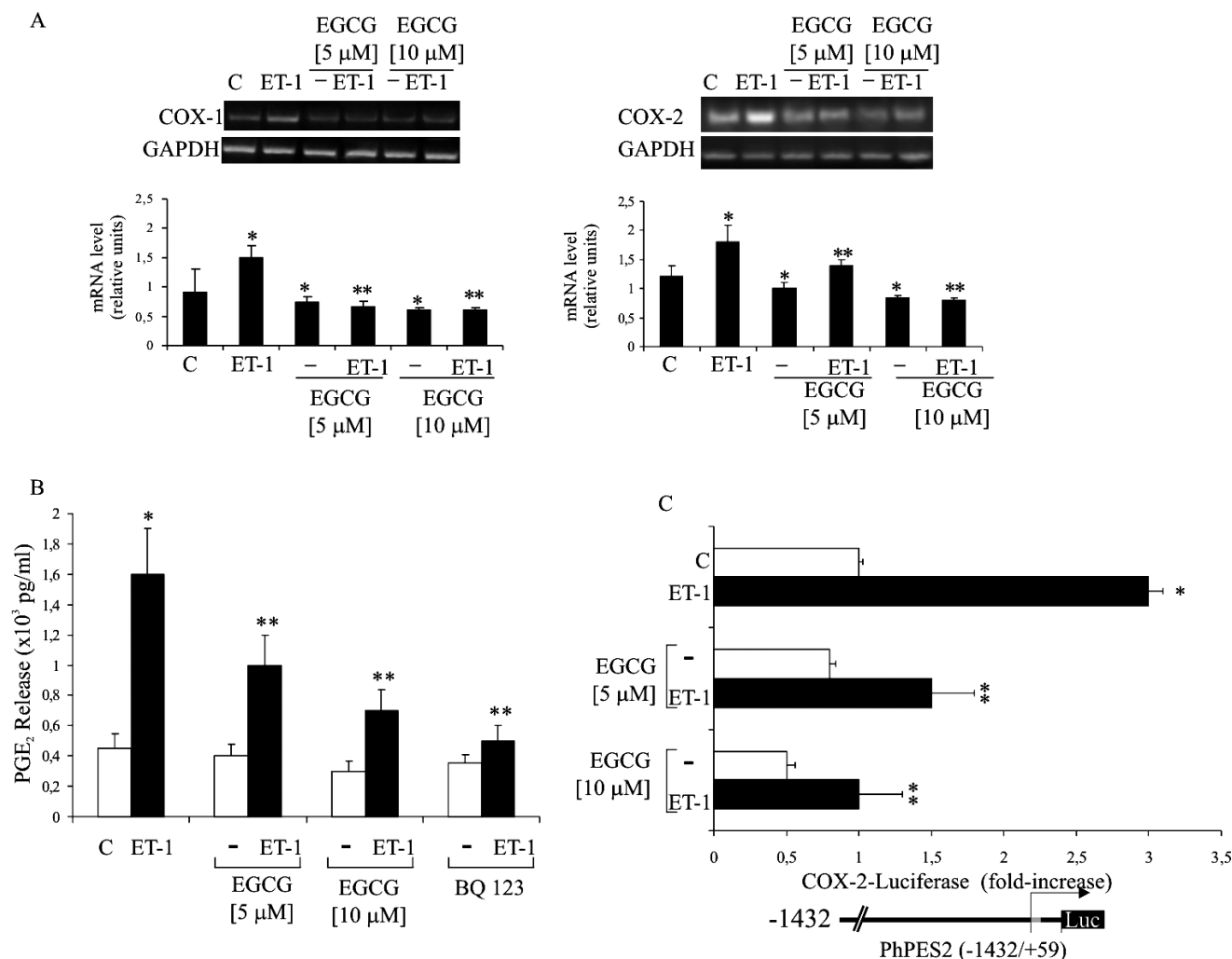
**Figure 2.** EGCG inhibits ET-1–induced COX-1 and COX-2 expression. Serum-starved HEY cells were treated with different concentrations of EGCG alone or in combination with 100 n$M$ ET-1 for 6 hrs or 24 hrs and total RNA (A) or conditioned media (B) were collected. COX-1 and COX-2 mRNA were analyzed by RT-PCR by using specific primers for COX-1 and COX-2. Primers for the amplification of the GAPDH gene were used as controls. (A) Densitometric analysis of RT-PCR products was evaluated by Scion image. Data were statistically analyzed and represented as the average value of three independent experiments. Bars, $\pm$ SD. *$P < 0.006$ compared with the control; **$P < 0.005$ compared with ET-1. (B) PGE$_2$ production was measured in conditioned media by ELISA. Bars, $\pm$ SD; *$P < 0.001$, compared with control; **$P < 0.005$, compared with ET-1. (C) HEY cells were transfected with the phPES2 ($-1432/+59$) construct of the human COX-2 promoter coupled with a luciferase reporter gene, as indicated in the bottom of the figure, and incubated for 12 hrs with different concentrations of EGCG alone or in combination with 100 n$M$ ET-1. COX-2 promoter activity was examined using the Luciferase Assay System in four independent experiments and was expressed as fold-increase relative to control. *$P < 0.006$ compared with control; **$P < 0.004$ compared with ET-1.

autocrine signaling pathways are modulated by green tea polyphenols, and whether this treatment inhibits the expression of molecules involved in angiogenesis and invasiveness. There is a growing body of compelling evidence that targeted inhibition of abnormal or improper overexpression or activity of COX-2 and COX-1 is valuable for preventing ovarian cancer. In this study, we analyzed the capacity of green tea and EGCG to regulate the ET-1 axis and the ET-1–mediated COX-2/COX-1 pathways. We demonstrated that in ovarian carcinoma cells, treatment with green tea infusion or with the polyphenol EGCG significantly inhibited the expression of ET-1 and ET$_A$R mRNA expression. These results are in agreement with several studies showing that the inhibition of growth factor

receptor expression and pathways is one of the possible mechanisms of actions of green tea and EGCG (18). Concordant with these reports, we found that green tea and EGCG inhibited the ET-1–induced COX-1 and COX-2 expression that resulted in a reduction of PGE$_2$ secretion. These results are, therefore, of biologic and clinical relevance for understanding the molecular mechanism underlying the antitumor activity of green tea polyphenol, which may be useful in developing preventive as well as therapeutic protocols.

Because high levels of ET-1 are associated with a number of malignancies (15), the impairment of ET$_A$R signaling by green tea could be of broad therapeutic relevance.

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