

## Inhibition of Hypoxia Inducible Factor-1 $\alpha$ by Dihydroxyphenylethanol, a Product from Olive Oil, Blocks Microsomal Prostaglandin-E Synthase-1/Vascular Endothelial Growth Factor Expression and Reduces Tumor Angiogenesis

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### Abstract

**Purpose:** 2-(3,4-dihydroxyphenyl)-ethanol (DPE), a polyphenol present in olive oil, has been found to attenuate the growth of colon cancer cells, an effect presumably related to its anti-inflammatory activity.

**Experimental Design:** To further explore the effects of DPE on angiogenesis and tumor growth we investigated the *in vivo* efficacy of DPE in a HT-29 xenograft model and *in vitro* activities in colon cancer cells exposed to interleukin-1 $\beta$  (IL-1 $\beta$ ) and prostaglandin E-2 (PGE-2).

**Results:** DPE (10 mg/kg/day for 14 days) inhibited tumor growth, reducing vessel lumina and blood perfusion to tumor, and diminished expression of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), vascular endothelial growth factor (VEGF), and microsomal prostaglandin-E synthase-1 (mPGEs-1). *In vitro*, DPE (100  $\mu$ mol/L) neither affected cell proliferation nor induced apoptosis in HT-29 and WiDr cells. DPE prevented the IL-1 $\beta$ -mediated increase of mPGEs-1 expression and PGE-2 generation, as it did the silencing of HIF-1 $\alpha$ . Moreover, DPE blocked mPGEs-1-dependent expression of VEGF and inhibited endothelial sprouting induced by tumor cells in a coculture system. PGE-2 triggers a feed-forward loop involving HIF-1 $\alpha$ , which impinges on mPGEs-1 and VEGF expression, events prevented by DPE via extracellular signal-related kinase 1/2. The reduction of PGE-2 and VEGF levels, caused by DPE, was invariably associated with a marked decrease in HIF-1 $\alpha$  expression and activity, independent of proteasome activity, indicating that the DPE effects on tumor growth and angiogenesis are dependent on the inhibition of HIF-1 $\alpha$  translation.

**Conclusions:** We show that the *in vivo* DPE antitumor effect is associated with anti-inflammatory and antiangiogenic activities resulting from the downregulation of the HIF-1 $\alpha$ /mPGEs-1/VEGF axis. *Clin Cancer Res*; 16(16): 4207–16. ©2010 AACR.

The tumor microenvironment influences both tumor growth and progression, limits the access of therapeutics to the tumor, and contributes to the development of drug resistance. Hypoxia, inflammation, and angiogenesis, which are major processes of the tumor microenvironment, promote malignancy through an array of intertwined signals. Among these, hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is a key player as it activates the transcription of

multiple genes involved in tumor progression, which include vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2), hallmarks of angiogenesis and inflammation, respectively (1–4).

The existence of a close link among hypoxia, angiogenesis, and inflammation in tumors is exemplified by colon carcinoma (5). In colon carcinoma, both the intrinsic inflammatory output and that originating from the milieu influence many aspects of cancer progression, including angiogenesis and the ability to metastasize. COX-2 and prostaglandin E-2 (PGE-2) have been implicated in the control of colon cancer progression (6). The PGE-2 protumorigenic action has been attributed to its ability to promote both tumor growth and the angiogenic process (7, 8). We recently showed a synergistic interaction between PGE-2 angiogenic action and the activation of the fibroblast growth factor-2 proangiogenic signaling pathway in endothelial cells (9).

PGE-2 is synthesized by three different PGE-synthases (PGEs): one cytosolic PGEs (cPGEs) and two microsomal PGEs (mPGEs-1 and mPGEs-2), which differ in

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance of Results

Natural products derived from foodstuff have assumed a large role in the preventive approach in the treatment of colon cancer. A polyphenol of olive oil, 2-(3, 4-dihydroxyphenyl) ethanol (DPE), has been reported to possess scavenging properties, and anti-inflammatory and antithrombotic activities. Here, we delineate the molecular mechanism by which DPE may inhibit colon cancer progression, interfering with inflammation and angiogenesis. We provide the framework in which compounds possessing antioxidant, anti-inflammatory, and antiangiogenic properties might be combined with chemotherapeutic agents. DPE, through the inhibition of inflammatory (microsomal prostaglandin-E synthase-1) and angiogenic markers (hypoxia inducible factor-1 $\alpha$ , vascular endothelial growth factor) known to contribute to the aggressive behavior of colon cancer cells, seems to be a promising and attractive chemopreventive molecule.

intracellular localization, expression, and activity (10). Expression of mPGEs-1, highly inducible by growth factors and inflammatory stimuli, has been reported to correlate with high PGE-2 levels and colon cancer progression (10).

In this context, chemopreventive agents that influence hypoxia, inflammation, or angiogenesis could be potentially efficacious in controlling colon carcinoma. Among the preventive therapeutic approaches in colon cancer, those based on natural products derived from foodstuff have assumed a crucial role, probably because empirical observations have suggested a health-promoting effect for particular diet habits. A polyphenol of olive oil, 2-(3, 4-dihydroxyphenyl) ethanol (DPE), which possesses scavenging, anti-inflammatory, and antithrombotic activities, has been shown to reduce the tumor necrosis factor  $\alpha$ -induced activation of the inflammatory pathway in a model of colon cancer (11, 12).

In this study, we examined the *in vivo* efficacy of DPE in human HT-29 tumor xenografts and measured tumor biomarkers, including tumor microvessel density, tumor perfusion, cell proliferation, and apoptosis. DPE administration produced antiangiogenic effects and vessel remodeling, which may account for the antiproliferative and proapoptotic effects observed in HT-29 experimental tumors. In addition, DPE inhibited HIF-1 $\alpha$ , mPGEs-1, and VEGF expression in tumors, suggesting that these are potential targets of DPE activity. Accordingly, in cultured HT-29 and WiDr colon cancer cells we found that the proinflammatory cytokine interleukin-1 $\beta$  (IL-1 $\beta$ ) promoted HIF-1 $\alpha$  expression and activity, which in turn mediates PGE-2 production and VEGF secretion by inducing the expression of mPGEs-1. The prostanoid further increases HIF-1 $\alpha$  levels, generating a feed-forward loop, which amplifies gene transcription of mPGEs-1

and VEGF. DPE suppresses PGE-2 effects, preventing extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated HIF-1 $\alpha$  induction and, consequently, mPGEs-1/PGE-2 signaling activation and VEGF overexpression. Thus, the polyphenol might be regarded as a promising chemopreventive agent that acts by reducing the intrinsic proinflammatory and angiogenic potential of colon cancer cells.

### Materials and Methods

#### Reagents

Reagents were as follows: PGE-2, IL-1 $\beta$ , U0126, anti- $\beta$ -actin (Sigma); DPE, LY294002, anti-COX-2, anti-mPGEs-1, anti-mPGEs-2, and anti-cPGEs (Cayman Chemicals); neutralizing anti-VEGF (R&D Systems); anti-HIF-1 $\alpha$  (BD Transduction); anti-VEGF (RELIAtech), anti-cleaved caspase-3, anti phospho-p44/42 mitogen-activated protein kinase (MAPK), p44/42 MAPK, anti-phospho Akt, and anti-Akt (Cell Signalling); Hoechst 33342 (Invitrogen).

#### Cell lines

HT-29 and WiDr, human colorectal adenocarcinoma cells, were obtained from the American Type Culture Collection and were cultured as recommended. CVEC, postcapillary venular endothelial cells, were cultured as described (9).

#### Caspase-3 activity

Caspase-3 activity was measured using a commercially available kit (Molecular Probes, Invitrogen) following the manufacturer's instructions.

#### Western blotting

Cells ( $5 \times 10^5$ ) were plated in 60-mm dishes, serum deprived (0.1% FCS, 24 hours), then exposed to IL-1 $\beta$ , PGE-2, or hypoxia in the presence or absence of DPE. Experimental details are in Supplementary Materials.

#### Real-time PCR

Total RNA was obtained using the RNA mini kit (Qiagen). Details are in Supplementary Materials.

#### Luciferase activity

Cells were transiently transfected with a vector containing the luciferase reporter gene under control of the hypoxia responsive element (HRE) sequence (pGL2-HRE) as described (13), or of the mPGEs-1 promoter (mPGEs-1-LUC) containing a fragment from -895 to +30 of the 5' flanking region of the mouse mPGEs-1 promoter in the reporter gene vector pXP2. Details may be found in Supplementary Materials.

#### PGE-2 and VEGF immunoassays

PGE-2 was measured by an Enzyme Immuno Assay kit (Cayman Chemical). VEGF in the supernatant was determined using a Quantikine kit (R&D Systems). Details may be found in Supplementary Materials.

### Coculture assay

Cytodex microcarrier beads were arranged as described in Supplementary Materials.

### siRNA transfection and cell cotransfection

The small interfering RNA (siRNA) sequences (reported in Supplementary Materials) were from Qiagen. The day before transfection, cells were trypsinized, and  $3 \times 10^5$  cells were seeded in 6-well plates. Transient transfection of siRNA was carried out using HT-29 transfection reagent (AltoGen) according to the manufacturer's instructions. Cells were assayed 48 hours after transfection.

### In vivo tumor xenograft

Experiments were carried out in accordance with the European Community (EEC) guidelines and National Ethical Committee. Details are in Supplementary Materials.

### Statistical analysis

Results are expressed as means  $\pm$  SE. Statistical analysis was carried out using Student's *t* test, ANOVA, or Student-Newman-Keuls test for multiple comparison.  $P < 0.05$  was considered statistically significant.

## Results

### DPE modifies vessel morphology by reducing VEGF, HIF-1 $\alpha$ , and mPGEs-1 expression in HT-29 tumor xenograft

In light of studies showing that DPE induced growth arrest and apoptosis in HT-29 human colon carcinoma cells (14), we investigated the DPE action in an *in vivo* model of tumor growth.

To study the effects of DPE on colon cancer growth, HT-29 cells were inoculated in nude mice, and treatment with vehicle control (10% ethanol) or DPE (10 mg/kg/day  $\times$  14 days) was started 4 days after implantation, a time at which tumors were measurable ( $\sim$ 3 mm diameter). Tumor growth and body weight were measured up to 14 days after the start of treatment. Tumor size in the control group increased steadily, reaching an average volume of 1,000 mm<sup>3</sup>, 19-fold higher at day 14, relative to day 4. DPE administration reduced tumor growth by 50% starting from day 8 ( $P < 0.01$ ), relative to the vehicle-treated group (Fig. 1A). During the course of treatment neither body weight loss nor signs of toxicity were observed. To study whether DPE possessed antiangiogenic effects, we examined intratumoral microvessel density by immunohistochemical analysis. Tumors from DPE-treated mice displayed a density of vessels similar to control ones (Fig. 1B, left), although a significant decrease in vessel luminal size in DPE-treated mice was observed (Fig. 1B, right). We then investigated vessel morphology by double immunostaining for CD31 with either  $\alpha$  smooth muscle actin ( $\alpha$ SMA) or NG2, a pericyte marker. In vehicle-treated mice, blood vessels appeared leaky, tortuous, dilated, and saccular, and endothelial cells displayed an aberrant morphology characterized by loosely attached or absent

pericytes (Fig. 1C, top left).  $\alpha$ SMA-tagged cells were detected in both control and treated groups (data not shown). Notably, the pericyte marker NG2 was only partially associated with CD31 (endothelium) in specimens from untreated mice, whereas it was abundantly coexpressed with CD31 in DPE-treated mice (Fig. 1C, top right). Hoechst 3224 diffusion in tumors from DPE-treated mice indicated a reduced vascular perfusion (Fig. 1C, bottom right) compared with vehicle-treated mice (Fig. 1C, bottom left). Changes in pericyte coverage and tumor perfusion in treated mice are consistent with an antiangiogenic activity of DPE that may contribute to the observed tumor growth delay.

Solid tumors overexpress angiogenic factors, including VEGF, to recruit blood vessels and to maintain tumor vasculature. We therefore investigated whether DPE could influence VEGF expression. Indeed, we found lower VEGF levels in tumors from DPE-treated mice than in controls (Fig. 1D).

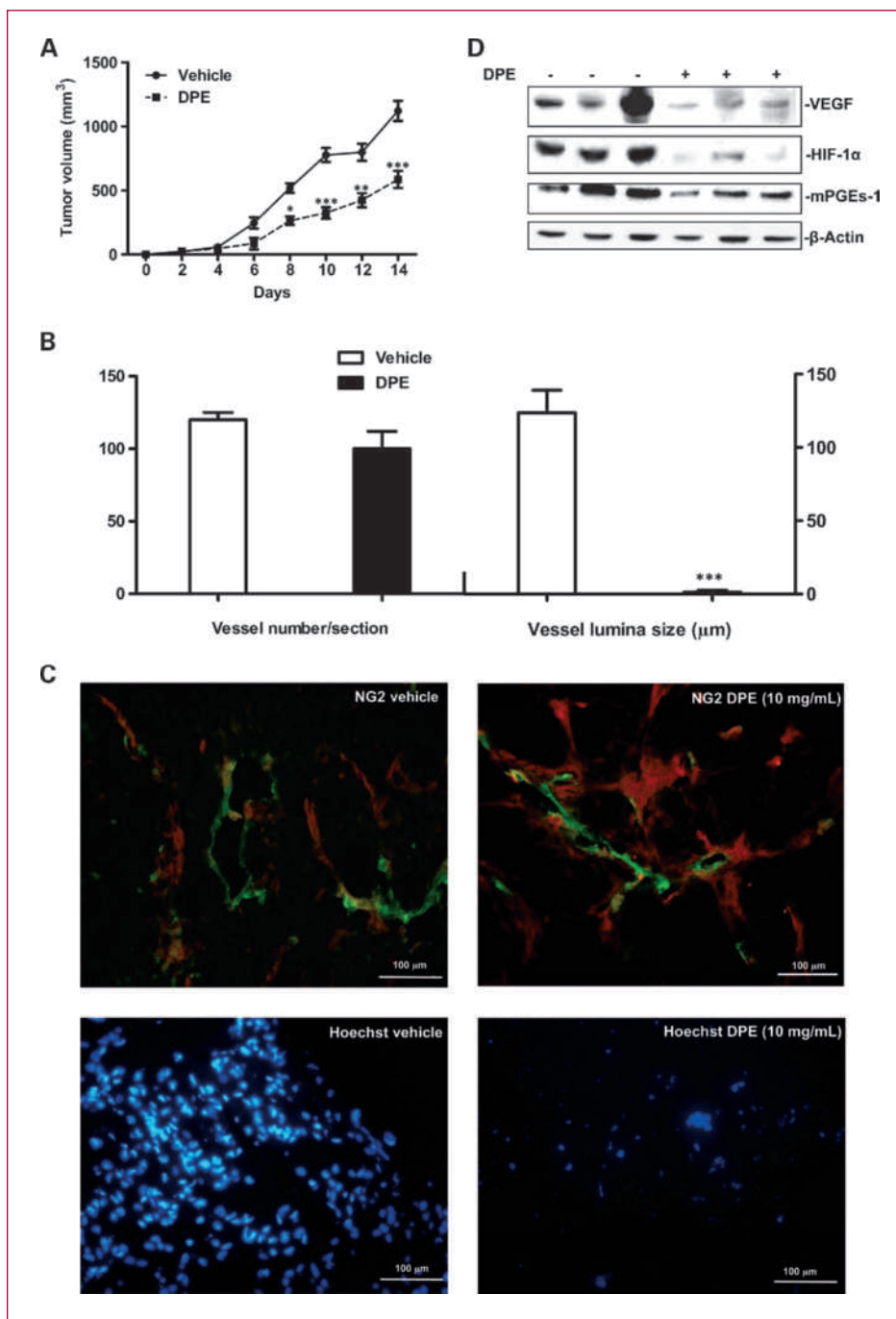
Because HIF-1 $\alpha$  mediates VEGF transcriptional activation in colon cancer (15), we measured its expression by immunohistochemical (Supplementary Fig. S1A) and immunoblot analysis (Fig. 1D). Vehicle-treated tumors displayed marked HIF-1 $\alpha$  expression, which was nearly abolished in tumors treated with DPE (Supplementary Fig. S1A and D). These findings indicate that DPE exerts its antiangiogenic action by interfering with HIF-1 $\alpha$  expression, resulting in diminished VEGF levels and impairment of perfusion.

Because mPGEs-1 is involved in colon tumor angiogenesis and progression (16), we investigated whether DPE influenced its expression. Indeed, mPGEs-1 expression was downregulated in tumor specimens from DPE-treated mice, relative to controls (Fig. 1D). Consistently, we found reduced lymphocytes infiltration (Supplementary Fig. S1B and C), a reduction of proliferative index (Ki67; 10% versus 40%), and increased levels of caspase-3 in tumors from DPE-treated mice, relative to controls (Supplementary Fig. S1D and E).

### DPE inhibits IL-1 $\beta$ -induced mPGEs-1 expression

In light of the *in vivo* results, we investigated the correlation/association between the inflammatory pathway mPGEs-1/PGE-2 and HIF-1 $\alpha$ /VEGF signaling, and the DPE effect on these systems in HT-29 and WiDr cells. We first investigated the direct toxicity and proapoptotic effects of DPE on these cancer cell lines. DPE exhibited no toxic effects at concentrations up to 100  $\mu$ mol/L (Supplementary Table S1), the highest concentration used for the subsequent experiments.

We used IL-1 $\beta$  to promote the upregulation of inflammatory signals in tumor cells (17). Exposure of HT-29 (Fig. 2A) and WiDr cells (Supplementary Fig. S2) to IL-1 $\beta$  (1 ng/mL, 9 hours) increased mPGEs-1 and COX-2 expression levels. The stimulation was specific for mPGEs-1, as other PGE synthase isoforms, mPGEs-2 and cPGEs, were not induced by IL-1 $\beta$  (Fig. 2A). DPE inhibited mPGEs-1 expression, but did not affect COX-2 expression (Fig. 2A). DPE inhibition of IL-1 $\beta$ -induced mPGEs-1 expression was concentration



**Fig. 1.** DPE affects vessel size and the growth of HT-29 tumor xenograft in athymic nude mice. A, DPE antitumor activity evaluated in nude mice inoculated with HT-29 cells and treated (14 days) after the onset of tumor growth (day 4 from inoculation). Data are reported as tumor volume ( $\text{mm}^3$ ); \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$  compared with vehicle (14 nude mice each different experimental group). B, vessel number and size in tumors. Quantification of human CD-31 and vessel lumina was done counting 10 random fields/section for slides, each slide having five sections. Number of vessels counted for section (left) or diameter ( $\mu\text{m}$ ) in tumors (right). \*\*\*,  $P < 0.001$  compared with vehicle. C, representative images of double-immunostaining for CD31 (green) and NG2 (red) in tumor sections from control (top, left) or DPE-treated (top, right) mice. Representative images of vessel perfusion, evaluated by Hoechst 32224 staining (blue), in control tumor (bottom, right) and DPE-treated tumor (bottom, left). Scale bars,  $100 \mu\text{m}$ . Images taken at  $\times 40$ . D, mPGEs-1, VEGF, and HIF-1 $\alpha$  protein expression in tumor sections from control or DPE-treated mice ( $n = 3$ ). In all images the gel shown is representative of three with similar results.

dependent, and maximal at  $100 \mu\text{mol/L}$  (Supplementary Fig. S3A). Inhibition of mPGEs-1 expression by DPE was also observed at the mRNA level (data not shown). Secretion of PGE-2, the product of mPGEs-1, was increased in HT-29 (3-fold; Fig. 2B) and WiDr cells (15-fold; Supplementary Fig. S2B) following IL-1 $\beta$  exposure. Coincubation with DPE abolished the IL-1 $\beta$  effect on both colon cancer cells (Fig. 2B and Supplementary Fig. S2B). However, DPE failed to reduce PGE-2 production when it was added to the

cells immediately before the prostanoid assay (1 hour; Supplementary Fig. S3B), indicating that the compound reduces the mPGEs-1 expression, rather than inhibiting its enzymatic activity.

#### DPE inhibits mPGEs-1-mediated VEGF production and angiogenesis

We then analyzed the relationship between the mPGEs-1/PGE-2 system and VEGF output. VEGF mRNA and

protein expression and production were found to be upregulated by IL-1 $\beta$ , and were reduced by DPE treatment (Fig. 2C and D and Supplementary Fig. S3C). PGE-2, either exogenously administered or endogenously produced through IL-1 $\beta$  upregulation of mPGEs-1, enhanced VEGF mRNA expression (2.9- and 3.9-fold, respectively; Fig. 2D). These increases were abolished by DPE, suggesting that IL-1 $\beta$  effects on VEGF expression are mPGEs-1 driven. Of note, DPE also inhibited the increase of VEGF mRNA expression elicited by hypoxia (Fig. 2D). Proof that mPGEs-1 regulates VEGF expression was obtained in HT-29 cells silenced for mPGEs-1, in which IL-1 $\beta$  challenge failed to upregulate VEGF expression (Fig. 2E).

In view of the large VEGF output induced by IL-1 $\beta$  in HT-29 cells, we investigated whether tumor-derived VEGF was responsible for endothelial cell-mediated pseudocapillary formation in an *in vitro* model of angiogenesis. In a transwell apparatus we cocultured HT-29 (upper compartment) and endothelial cells (CVEC; lower compartment), with the medium between compartments being diffusible. The aim was to monitor formation of pseudocapillaries induced by the HT-29-conditioned medium in CVEC seeded on coated beads and embedded in a fibrin gel. Under these conditions, sprouting of CVEC was increased by prior addition of PGE-2 to HT-29 cells (Fig. 3A, panel PGE-2 versus panel 0.1% CS). The addition of an anti-VEGF neutralizing antibody to the CVEC compartment (Fig. 3A, panel anti-VEGF) inhibited endothelial cell sprouting. Similarly,

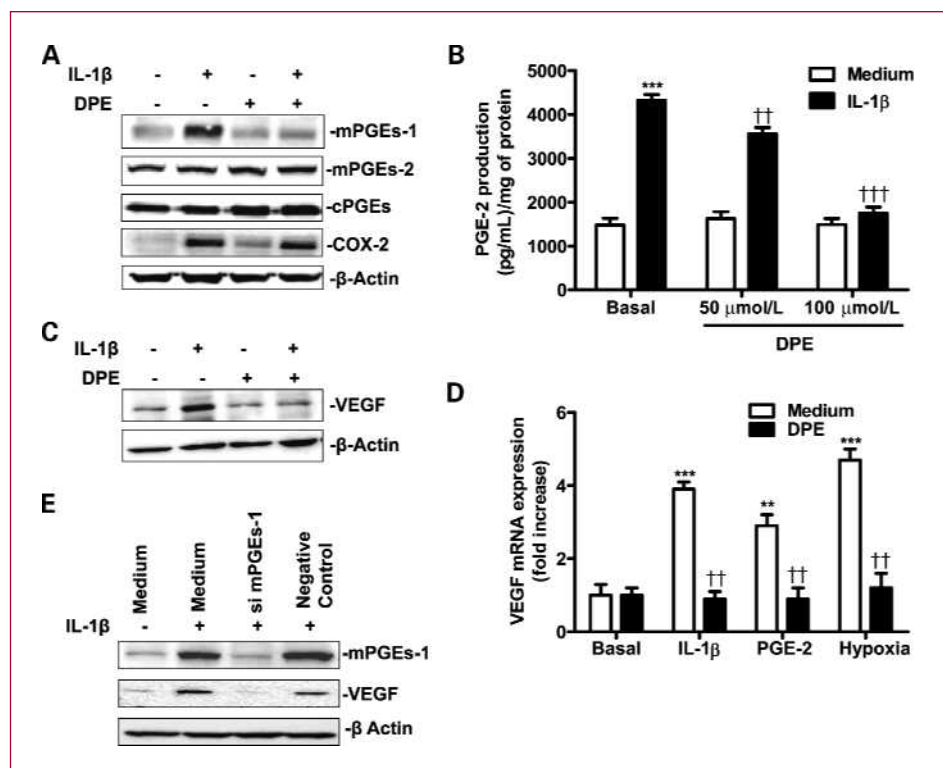
DPE added to HT-29 cells, either alone (data not shown) or in combination with PGE-2, significantly reduced sprouting ( $P < 0.001$ ; Fig. 3A, panel PGE-2+DPE and Fig. 3B), showing that DPE inhibits the PGE-2/VEGF-mediated pathway in colon cancer cells, ultimately leading to inhibition of tumor-associated angiogenesis.

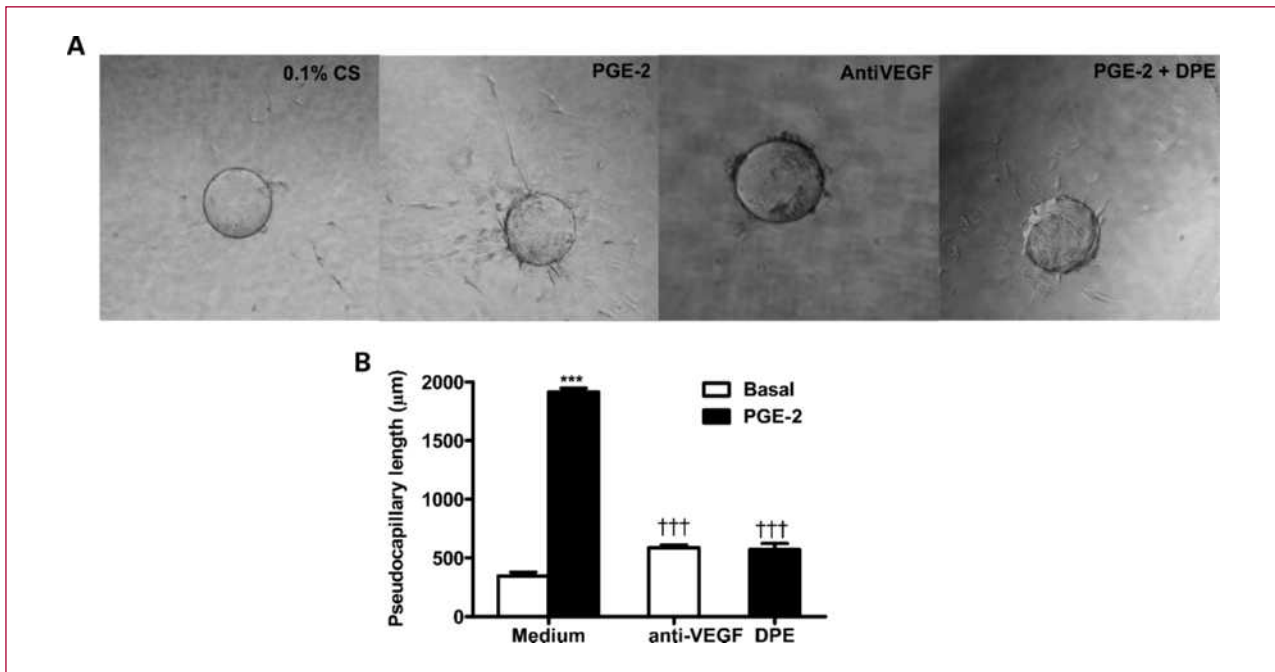
#### DPE inhibits PGE-2-induced HIF-1 $\alpha$ expression

Next we studied the role of HIF-1 $\alpha$  in the upregulation of VEGF mediated by the mPGEs-1/PGE-2 pathway, by silencing the transcription factor in HT-29 cells. Silencing HIF-1 $\alpha$  (Small interfering RNA) suppressed the PGE-2-induced VEGF expression and production (Supplementary Fig. S4A and B), indicating that HIF-1 $\alpha$  production is indeed required for the induction of VEGF by PGE-2, as previously shown (16).

We then investigated the relationship between the IL-1 $\beta$ -mediated mPGEs-1 and HIF-1 $\alpha$  expression by carrying out a time course experiment (Fig. 4A and B). IL-1 $\beta$  induced HIF-1 $\alpha$  protein expression, which peaked at 3 hours and was sustained up to 9 hours (Fig. 4A). In contrast, mPGEs-1 expression was delayed, being detectable at 6 hours and persisting up to 18 hours (Fig. 4B). That HIF-1 $\alpha$  is stringently required for the IL-1 $\beta$ -mediated activation of mPGEs-1 was shown by the reduced PGE-2 levels and the lowered expression of mPGEs-1 in HIF-1 $\alpha$ -silenced cells (Fig. 4C and D and Supplementary Fig. S4C). Further, HIF-1 $\alpha$  silencing suppressed the transcriptional activation of the mPGEs-1-LUC promoter,

**Fig. 2.** DPE inhibits IL-1 $\beta$ -mediated mPGEs-1 and VEGF expression in HT-29 cells. A, mPGEs-1, mPGEs-2, cPGEs, and COX-2 protein expression in cells exposed to IL-1 $\beta$  (1 ng/mL, 9 hours) with/without DPE (100  $\mu$ mol/L). B, Enzyme Immuno Assay (EIA) immunoassay for PGE-2 production in cells treated with IL-1 $\beta$  (18 hours) in the presence/absence of DPE (50 or 100  $\mu$ mol/L). C, VEGF expression in cells exposed to IL-1 $\beta$  (18 hours) with or without DPE (100  $\mu$ mol/L). D, VEGF mRNA expression in cells exposed to IL-1 $\beta$  (8 hours) or PGE-2 (1  $\mu$ mol/L, 8 hours) or hypoxia (1% O $_2$ ) with/without DPE (100  $\mu$ mol/L). Data are expressed as fold increase over untreated cells. E, mPGEs-1 and VEGF expression in cells silenced for mPGEs-1 and exposed to IL-1 $\beta$  (18 hours).



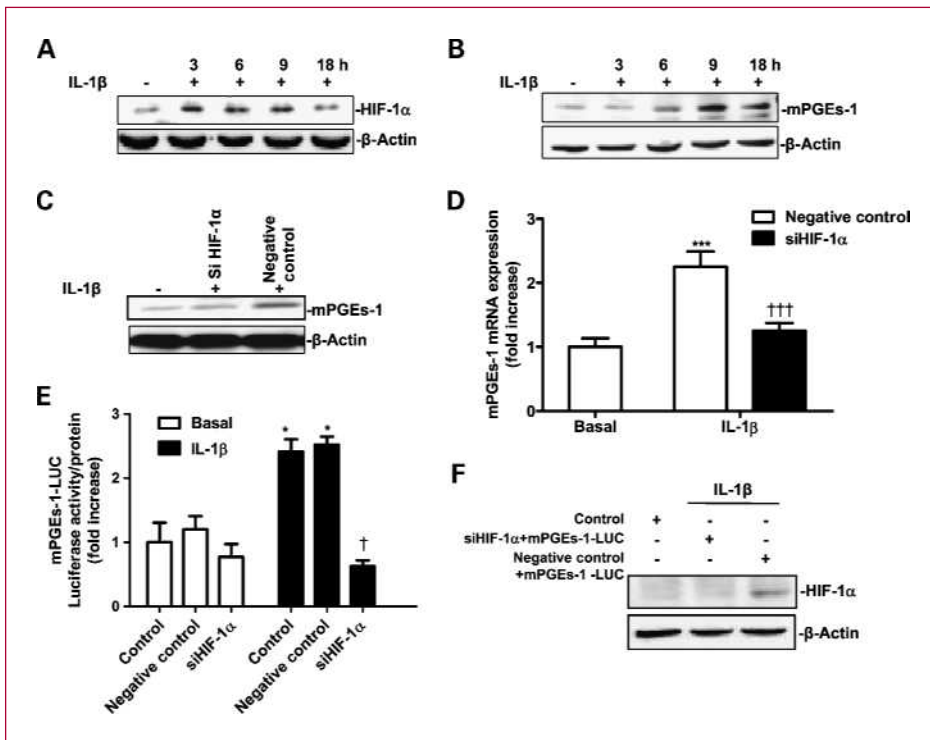


**Fig. 3.** PGE-2 induces angiogenesis in a coculture system. A, representative images of CVEC cocultured with HT-29 cells. B, data from five experiments run in triplicate are reported as quantification of total length of pseudocapillary like structures ( $\mu\text{m}$ ); \*\*\*,  $P < 0.001$ , compared with untreated cells. †††,  $P < 0.001$ ; ††,  $P < 0.01$  compared with treated cells.

substantiating the instrumental role of HIF-1 $\alpha$  in IL-1 $\beta$ -induced mPGEs-1 expression (Fig. 4E and F).

We explored whether PGE-2 would induce HIF-1 $\alpha$  expression/activity, generating a reinforcing feedback loop

for the amplification of VEGF expression and induction of angiogenesis. Indeed, exogenous PGE-2 induced HIF-1 $\alpha$  protein accumulation in HT-29 and WiDr cells, an effect detectable as early as 6 hours (Supplementary Fig. S5A



**Fig. 4.** IL-1 $\beta$  induces HIF-1 $\alpha$  expression which mediates mPGEs-1/PGE-2 pathway activation in HT-29 cells. A and B, time course expression of HIF-1 $\alpha$  and mPGEs-1, respectively, in cells exposed to IL-1 $\beta$  (1 ng/mL). C and D, mPGEs-1 expression (9 hours) and mRNA levels (6 hours), respectively, in cells silenced for HIF-1 $\alpha$  (siHIF-1 $\alpha$ ) and treated with IL-1 $\beta$  (1 ng/mL). E and F, transcriptional activity of mPGEs-1 in cells silenced for HIF-1 $\alpha$ , transiently transfected for the luciferase reporter plasmid of mPGEs-1 promoter (mPGEs-1-LUC), and treated with IL-1 $\beta$  (1 ng/mL, 6 hours). Data, from three experiments, are fold increase of luciferase activity (normalized for protein content and efficiency of transfection).

and 2C, respectively). Further, in HT-29 cells transiently transfected with the pGL2-TK-HRE vector, PGE-2 increased luciferase expression by 3.7-fold, whereas hypoxia increased it by 6-fold relative to cells cultured under normoxic conditions (Fig. 5B). HIF-1 $\alpha$  mRNA levels were unaffected by PGE-2 exposure (data not shown).

Having established that mPGEs-1/PGE-2 regulates HIF-1 $\alpha$ , we explored whether DPE could impair PGE-2-induced HIF-1 $\alpha$  expression and/or transcriptional activity. Coincubation of DPE with PGE-2 abrogated HIF-1 $\alpha$  expression (Fig. 5A), markedly reducing HIF-1 $\alpha$ -activity (Fig. 5B). Similar results were obtained in WiDr cells (Supplementary Fig. S2C).

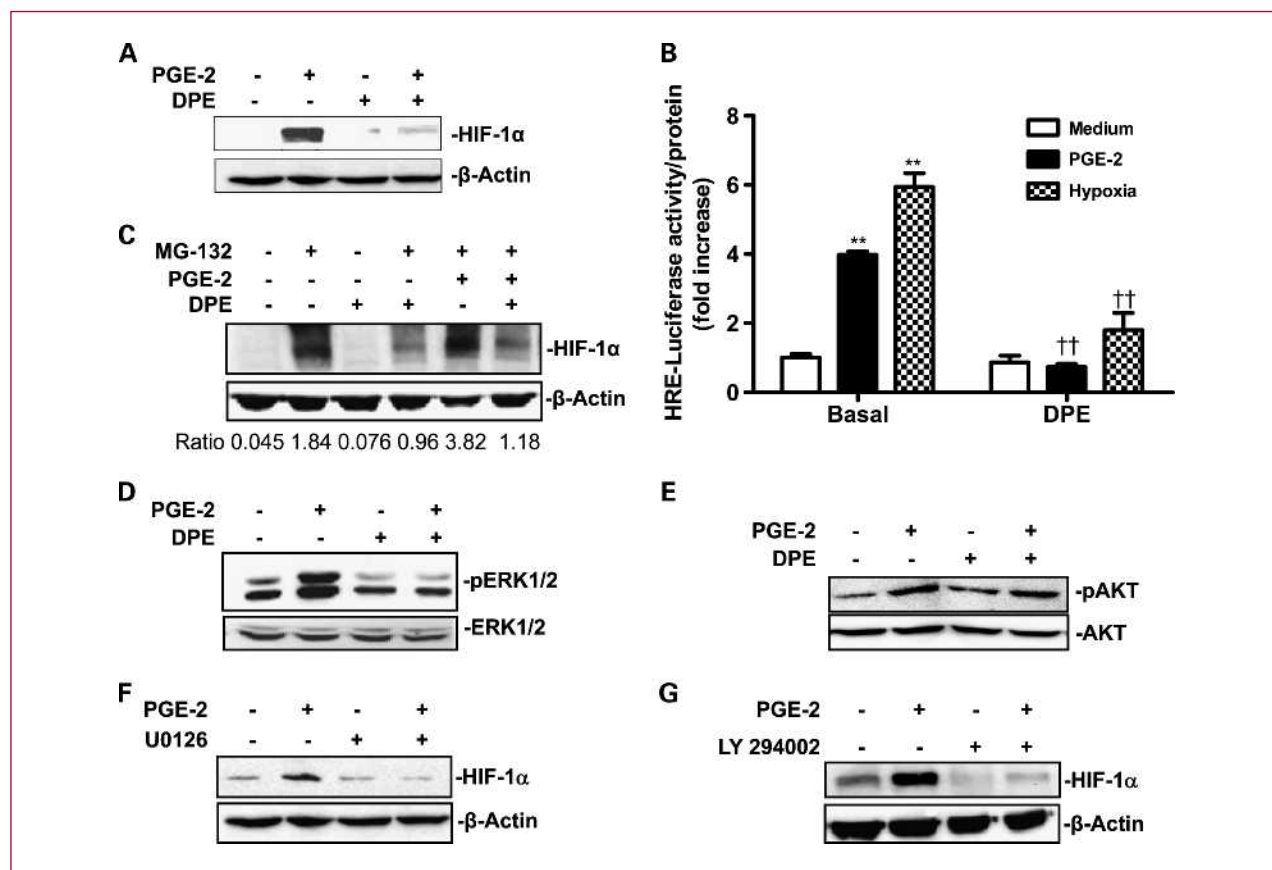
Of note, in agreement with the result showing that DPE downregulates the hypoxia-induced VEGF mRNA (Fig. 2D), we found that the polyphenol significantly reduced the hypoxic induction of the HRE-promoter transcriptional activity (Fig. 5B). DPE did not affect constitutive luciferase activity (data not shown), which shows that its inhibition of luciferase is HIF-1 $\alpha$  dependent.

### DPE inhibits HIF-1 $\alpha$ expression by targeting PGE-2-mediated ERK 1/2 phosphorylation

Given that DPE affects PGE-2-dependent HIF-1 $\alpha$  protein levels, we investigated whether the polyphenol impaired HIF-1 $\alpha$  protein synthesis or stability.

In HT-29 cells, PGE-2, unlike hypoxia, increased HIF-1 $\alpha$  protein synthesis, without affecting protein stability, as previously reported in HTC116 cancer cells (16). DPE prevented normoxic accumulation of HIF-1 $\alpha$  protein in the presence of the proteasome inhibitor MG-132, suggesting an effect on protein synthesis rather than protein degradation (Fig. 5C).

Next, we investigated signals downstream to PGE-2, namely ERK1/2 and Akt, potentially involved in HIF-1 $\alpha$  overexpression. Indeed, PGE-2 stimulated the phosphorylation of both ERK1/2 and Akt in HT-29 cells. Both signals seem to be involved in the HIF-1 $\alpha$  induction, as their specific inhibitors, U0126 (for MEK) and LY294002 (for PI3Kinase), abrogated HIF-1 $\alpha$  expression (Fig. 5F and G). DPE blocked only ERK1/2



**Fig. 5.** DPE blocks exogenous PGE-2-induced HIF-1 $\alpha$  expression in HT-29 cells. A, HIF-1 $\alpha$  expression in cells exposed to PGE-2 (1  $\mu$ mol/L, 9 hours) in the presence/absence of DPE (100  $\mu$ mol/L). B, HIF-1 $\alpha$  transcriptional activity in cells transfected with the plasmid pGL2TK-HRE and exposed to PGE-2 (1  $\mu$ mol/L, 3 hours) or hypoxia (1% O<sub>2</sub>). Data represent fold increase of luciferase activity (normalized for protein content and efficiency of transfection) compared with normoxic levels. C, HIF-1 $\alpha$  protein expression in cells exposed to the proteasome inhibitor MG-132 (20  $\mu$ mol/L, 6 hours) with or without PGE-2 (1  $\mu$ mol/L) and treated with DPE (100  $\mu$ mol/L); the ratio of HIF-1 $\alpha$  and  $\beta$ -actin expressed as arbitrary density unit is reported. D and E, ERK1/2 and Akt phosphorylation in cells exposed to PGE-2 (1  $\mu$ mol/L, 15 minutes), in the presence or absence of DPE (100  $\mu$ mol/L). F and G, HIF-1 $\alpha$  expression in cells exposed to PGE-2 (1  $\mu$ mol/L, 9 hours) in the presence/absence of MEK inhibitor (U0126 1  $\mu$ mol/L) or PI3kinase inhibitor (LY 294002 1  $\mu$ mol/L).

phosphorylation, leaving the Akt phosphorylation unaffected (Fig. 5D and E).

Taken together, these results show that DPE reduces the inflammatory input triggered by IL-1 $\beta$  by inhibiting the HIF-1 $\alpha$ /mPGEs-1 signaling pathway, which may account for the observed inhibition of tumor growth and antiangiogenic effects. Furthermore, DPE downregulates the PGE-2-dependent reinforcing feedback loop and affects HIF-1 $\alpha$  expression/activity leading to a functional impairment of the mPGEs-1/PGE-2/VEGF axis (Fig. 6).

## Discussion

The evidence presented in this study shows that DPE, an antioxidant from olive oil, shares with other flavonoids the ability to reduce experimental colon tumor growth. The mechanism underlying the DPE antitumor effect lies in its anti-inflammatory and antiangiogenic properties, as the compound selectively suppresses the expression of mPGEs-1 induced by IL-1 $\beta$ , the ensuing generation of PGE-2, and the enhanced tumor angiogenesis. We show that DPE downregulates the expression and activity of HIF-1 $\alpha$ , which in our model tightly regulates the inflammatory tumor microenvironment, and leads to a functional impairment of the mPGEs-1/PGE-2/VEGF axis.

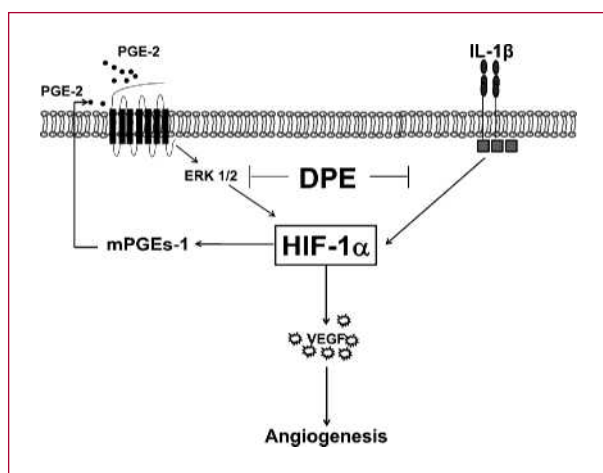
*In vivo*, DPE reduced HT-29 tumor growth by affecting vessel morphology and maturation, a feature reported to be related to VEGF inhibition (18). Molecular analysis showed that DPE, administered to tumor-bearing mice, downregulated markers of both inflammation (mPGEs-1) and angiogenesis (HIF-1 $\alpha$  and VEGF). Surprisingly, DPE did not affect microvessel density, but strongly impaired vascular function within tumors, as measured by Hoechst

3224 diffusion. Tumors expressing high VEGF levels have been reported to develop tortuous, leaky, and sacular blood vessels (18). Anti-VEGF therapy results in modifications of both vessel morphology, such as pericyte coverage of tumor capillaries, and vessel functionality (19, 20). DPE treatment, despite unchanged vessel number, reduced the tumor perfusion, diminished vessel size, and enhanced the perivascular coverage. Thus, the decreased proliferative index and the enhanced expression of apoptotic signaling, observed in DPE-treated tumor bearing mice, might be attributable to an impaired tumor blood supply, rather than to a direct antiproliferative effect of the compound.

In cultured colon cancer cells (HT-29 and WiDr) exposed to the proinflammatory cytokine IL-1 $\beta$ , DPE inhibited, in a concentration-dependent fashion, the induction of mPGEs-1 expression. Notably, DPE neither influenced IL-1 $\beta$ -induced expression of COX-2 nor mPGEs-2 and cPGES levels, thus showing a high degree of specificity. DPE treatment prevented the large PGE-2 output, yet it failed to inhibit prostanoid production when added at later stages following IL-1 $\beta$  treatment. This indicates that DPE influences mPGEs-1 expression, rather than its enzymatic activity. Similarly, DPE suppressed the IL-1 $\beta$ -induced upregulation of the VEGF system in all of its components (mRNA expression, protein synthesis and secretion). VEGF upregulation was also abrogated in tumor cells silenced for mPGEs-1, highlighting the stringent PGE-2 requirement for VEGF expression. It should also be noted that administration of PGE-2 to tumor cells increased VEGF output, promptly reduced by DPE.

PGE-2-induced VEGF overexpression has been previously reported to be mediated by HIF-1 $\alpha$  upregulation (15). Here, by silencing HIF-1 $\alpha$ , we show that its activity is required for the expression of both mPGEs-1 and VEGF in response to PGE-2, providing the first indication that DPE might exert antiangiogenic activity through inhibition of the mPGEs-1/PGE-2/VEGF signaling pathway resulting from downregulated HIF-1 $\alpha$ . In line with this hypothesis, we found that HIF-1 $\alpha$  expression was enhanced as early as 3 hours after IL-1 $\beta$  treatment, preceding by several hours the increase of mPGEs-1 expression. Thus, it seems that in colon cancer cells HIF-1 $\alpha$  acts as the transcriptional mediator for mPGEs-1 expression, a finding also shown in other cell lines (21, 22). However, HIF-1 $\alpha$  seems to be indirectly involved in the activation of mPGEs-1 promoter, as suggested by the lack of a canonical HRE sequence (RCGTG) in the mPGEs-1 promoter sequence (23). Recently, Lee et al. (22) suggested the presence of three potential HRE sites within the first intron of the mPGEs-1 gene, which may contribute to the HIF-1-dependent mPGEs-1 expression.

We found that PGE-2 strongly upregulates HIF-1 $\alpha$  expression and activity, triggering a positive feedback loop that amplifies and sustains both mPGEs-1 and VEGF transcription. DPE interrupts the flow of signals within the reinforcing loop by blocking MAPK activation and PGE-2 production, supporting the hypothesis that the polyphenol inhibits



**Fig. 6.** Schematic model of inflammatory-induced HIF-1 $\alpha$ /mPGEs-1 signaling. The scheme illustrates a reinforcing feedback loop existing between HIF-1 $\alpha$ /mPGEs-1, a pathway leading to angiogenesis through VEGF upregulation. DPE interrupts the flow of signals within the reinforcing loop by blocking MAPK activation and PGE-2 production.



mPGEs-1 signaling through downregulation of HIF-1 $\alpha$  transcriptional activity. Further support for the reinforcing feedback loop between HIF-1 $\alpha$  and the mPGEs-1/PGE-2 system in tumor angiogenesis was found in functional experiments in which endothelial cells were cocultured with HT-29 cells. The proangiogenic response was both HIF-1 $\alpha$  and VEGF dependent, as DPE inhibited endothelial cell sprouting, exerting an effect similar to that of an anti-VEGF antibody.

Regarding the mechanism by which DPE reduces HIF-1 $\alpha$  expression/activity, its known antioxidant and radical scavenger activities might contribute to the quenching of the transcription factor in IL-1 $\beta$ - or PGE-2-stimulated colon cancer cells (11, 24). Recently, the antioxidant-mediated decrease of Reactive Oxygen Species (ROS) level in ovary carcinoma cells has been shown to reduce HIF-1 $\alpha$  and VEGF expression (25). DPE is also capable of inhibiting ERK1/2 phosphorylation induced by PGE-2, a transduction pathway that controls HIF-1 $\alpha$  activity (26). Here, we show that PGE-2 induces HIF-1 $\alpha$  expression via ERK1/2 activation in HT-29 cells, and that DPE suppresses the PGE-2/ERK1/2/HIF-1 $\alpha$  signaling pathway by inhibiting ERK1/2 phosphorylation.

In conclusion, the delineation of the molecular mechanism by which DPE may interfere with the interplay among inflammation, angiogenesis, and tumor progression provides the framework in which a variety of compounds possessing antioxidant, anti-inflammatory,

and antiangiogenic properties (e.g., quercetin, resveratrol, and others) might be combined with chemotherapeutic agents. In this context, DPE, through the inhibition of HIF-1 $\alpha$  and its downstream target genes *VEGF* and *mPGEs-1*, which are known to contribute to the aggressive behavior of colon cancer cells, seems to be a promising and attractive chemopreventive molecule.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Clinical Cancer Research

## Inhibition of Hypoxia Inducible Factor-1 $\alpha$ by Dihydroxyphenylethanol, a Product from Olive Oil, Blocks Microsomal Prostaglandin-E Synthase-1/Vascular Endothelial Growth Factor Expression and Reduces Tumor Angiogenesis

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