Endothelin-1 and Endothelin-3 Promote Invasive Behavior via Hypoxia-Inducible Factor-1α in Human Melanoma Cells

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Abstract

Endothelin (ET) B receptor (ET_BR), which is overexpressed in human cutaneous melanomas, promotes tumorigenesis upon activation by ET-1 or ET-3, thus representing a potential novel therapeutic target. Hypoxia-inducible factor-1α (HIF-1α) is the transcriptional factor that conveys signaling elicited by hypoxia and growth factor receptors. Here, we investigated the interplay between ET axis and hypoxia in primary and metastatic melanoma cell lines. We report that under normoxic conditions, ET_BR activation by ET-1/ET-3 enhances vascular endothelial growth factor (VEGF) up-regulation, cyclooxygenase (COX)-1/COX-2 protein expression and COX-2 promoter activity, prostaglandin E_2 (PGE_2) production, and do so to a greater extent under hypoxia. Moreover, COX-1/COX-2 inhibitors block ET-induced PGE_2 and VEGF secretion, matrix metalloproteinase (MMP) activation, and cell invasion, indicating that both enzymes function as downstream mediators of ET-induced invasive properties. The ET_BR selective antagonist BQ788 or transfection with ET_BR small interfering RNA (siRNA) block the ET-mediated effects. ETs also increase HIF-1α expression under both normoxic and hypoxic conditions and its silencing by siRNA desensitizes COX-2 transcriptional activity, PGE_2 and VEGF production, and MMP activation in response to ET-3, implicating, for the first time, HIF-1α/COX as downstream targets of ET_BR signaling leading to invasiveness. In melanoma xenografts, specific ET_BR antagonist suppresses tumor growth, neovascularization, and invasiveness-related factors. Collectively, these results identify a new mechanism whereby ET-1/ET-3/ET_BR axis can promote and interact with the HIF-1α–dependent machinery to amplify the COX-mediated invasive behavior of melanoma. New therapeutic strategies using specific ET_BR antagonist could provide an improved approach to the treatment of melanoma by inhibiting tumor growth and progression. [Cancer Res 2007;67(4):1725–34]

Introduction

Recent studies have shown that endothelins (ETs) and endothelin (ET) B receptor (ET_BR) pathways play a relevant role in melanocyte transformation and melanoma progression (1–5). The ET family is composed of three isopeptides, ET-1, ET-2, and ET-3, which bind to two distinct subtypes of G protein–coupled receptors [i.e., ET A receptor (ET_AR) and ET_BR]. The ET_AR is highly specific for ET-1 and ET-2, whereas it binds ET-3 with low affinity. On the contrary, ET_BR is a nonselective receptor, which binds ET-1, ET-2, and ET-3 with similar affinity (6), and is the major subtype expressed by normal and transformed melanocytes (4). Gene expression profiling of human melanoma biopsies and cell lines indicated ET_BR as one of the genes overexpressed and associated with aggressive phenotype (7), and analysis of ET_BR expression in a representative panel of melanocyte lesions has identified this receptor as a tumor progression marker (8). ET-1, which is secreted by keratinocytes in response to UV, stimulates proliferation, chemotaxis, and pigment production in melanocytes through ET_BR (9–11). Moreover, ET-1 promotes melanocyte survival and inhibits the UV-induced apoptosis by activating the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (12). Down-regulation of E-cadherin expression by UV-induced ET-1 (13) results into an enhancement of melanoma invasive capability (14). Associated with loss of E-cadherin, activation of ET_BR increases expression of N-cadherin, matrix metalloproteinase (MMP)-2, MMP-9, and $\alpha_v\beta_3$ and $\alpha_2\beta_1$ integrins and inhibits intercellular communication by inducing phosphorylation of gap junctional protein connexin 43, allowing tumor cells to escape growth control and to invade (2). Downstream to ET_BR pathway, activation of focal adhesion kinase and extracellular signal-regulated kinase 1/2 signaling pathways occurs leading to enhanced cell proliferation, adhesion, migration, and MMP-dependent invasion. Hence, ET_BR has emerged recently as a potential therapeutic target for melanoma (2, 15).

Melanoma is an aggressive tumor that can metastasize early in the course of the disease and, most importantly, is resistant to most current therapeutic regimens. Thus, the identification of the genetic and environmental factors driving the natural history of this malignancy is essential for the development of new therapies (16). Among microenvironmental components, hypoxia represents a key tumor-promoting factor (17, 18), which has been associated with tumor progression (19–22). In melanoma hypoxic setting, the up-regulation of hypoxia-inducible factor-1 (HIF-1)α, the main transcriptional factor that allows cellular adaptation to hypoxia, is associated with neovascularization, vascular endothelial growth factor (VEGF) expression, poor prognosis, and resistance to therapy (23, 24). However, the role of hypoxia and HIF-1α in earlier stages of tumor development has not been systematically examined. Bedogni et al. (25) have elucidated how mildly hypoxia is essential for melanocyte transformation, showing that only in hypoxic condition the PI3K-Akt pathway can transform melanocytes through the stabilization of HIF-1α. These data argue for a more relevant role for local oxygen supplies in tumorigenesis, providing an example of how hypoxia can determine the level of aggression and invasion in response to oncogenic signaling pathways activation.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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HIF-1 is a heterodimeric transcription factor, composed by HIF-1α and the constitutively expressed HIF-1β. In normoxia, HIF-1α is hydroxylated at key proline residues facilitating von Hippel-Lindau protein binding, which in turn allows ubiquitination and subsequent proteosome-targeted degradation. Under hypoxic conditions, proline hydroxylation is inhibited, thereby stabilizing HIF-1α, which can then translocate into the nucleus and bind to costitutively expressed HIF-1β, forming the active HIF-1 complex (19). The HIF-1 complex recruits the transactivator p300/CBP, resulting in enhanced transcriptional activity. HIF-1 binds a conserved DNA consensus on promoters of its target genes known as the hypoxia-responsive element (HRE). HIF-1α activates the transcription of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism (18), and tumor invasion (21). HIF-1α controls the expression of several genes, including *VEGF, erythropoietin*, and *ET-1*, in response to hypoxia in different tumor cells (18, 26). Although hypoxia is the major inducer of HIF-1α, other stimuli, such as growth factors, including insulin, insulin-like growth factor (IGF)-I, transforming growth factor-α (TGF-α), platelet-derived growth factor, and epidermal growth factor, and cytokines, such as interleukin-1; oncogenic activation; or loss of tumor suppressor function, hormones, nitric oxide, and reactive oxygen species, are able to regulate HIF-1α expression (18, 19). Several growth factors, such as *IGF-II* and *TGF-α*, are also HIF-1 target genes. Binding of these factors to their cognate receptors stimulates the expression of HIF-1α, which in turn activates the transcription of gene that encodes IGF-II and TGF-α through an autocrine mechanism (18).

Cyclooxygenase (COX)-2 is overexpressed in various types of cancers, including melanoma (27), and compelling evidence supports a role for COX-2 and COX-2–derived prostaglandin E_2 (PGE_2) in angiogenesis (28, 29) and melanoma progression (30–32). However, the mechanisms that regulate transcriptional activation of COX-2, angiogenesis, and invasiveness in low-oxygen conditions have not been determined.

We hypothesized that COX-2 may be up-regulated by hypoxia and ETs as well as through HIF-1α. Because of the ET_BR relevance in melanoma progression, we also explored the role of ET_BR in ET-induced aggressive phenotype. Here, we report that ET-1 and ET-3 through ET_BR induce COX-1/COX-2, PGE_2, and VEGF in melanoma cells grown under normal oxygen conditions and that this mechanism may be responsible for invasive behavior of primary and more so of metastatic melanoma. These effects are amplified under hypoxia. At molecular levels, through ET_BR activation, ETs mimic cellular hypoxia inducing HIF-1α, which is involved in mediating ET-induced COX-2 promoter activity, COX-1/COX-2 and PGE_2 expression, and MMP activity. These findings indicate that targeting HIF-1α and related signaling through ET_BR blockade could effectively impair cutaneous melanoma progression.

Materials and Methods

Cells and cell culture conditions. The human cutaneous melanoma cell line 1007 was derived from primary melanoma (33). The melanoma cell lines SK-Mel 28 (American Type Culture Collection, Rockville, MD), M10, and Mel120 were derived from metastatic lesions (34). Cells were grown in RPMI 1640 containing 10% FCS. All culture reagents were from Invitrogen (Paisley, Scotland, United Kingdom). To expose cells to hypoxia, a modular incubator was used with an atmosphere setting of 5% CO_2, 95% N_2, and 1% O_2. In all experiments, cells were grown to 70% to 80% confluence on 100-mm glass dishes. Melanoma cells were starved for 24 h in serum-free medium and then incubated for indicated times with ET-1 or ET-3 (Peninsula Laboratories, Belmont, CA). The antagonist BQ788 (Peninsula Laboratories) was added 15 min before agonists, whereas pretreatment with NS-398, SC-560, or indomethacin (Cayman Chemical, Ann Arbor, MI) was done for 30 min before the addition of ETs.

Reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) was done using a SuperScript One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. Briefly, 1 μg RNA was reverse transcribed. The primers sets were as follows: 5′-GGCTCTAGATCGGG-CCTCCGAAACCAT-3′ and 5′-GGCTCTAGAGCGCAGAGTCTCCTCTTC-3′ (VEGF), 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ and 5′-GTGCATCAACA-CAGGCGCCTCTTC-3′ (COX-1), 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-TAGATCATCTCTGCCTGAGTATCTT-3′ (COX-2), 5′-TCAACACGG-TGGTGTCCTGC-3′ and 5′-ACTGAATAGCCACCAATCTT-3′ (ET_BR), and 5′-TGAAGGTCGGTGTCAACGGA-3′ and 5′-GATGGCATGGACTGTGGTCAT-3′ [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)]. Thirty-five cycles of amplification were done under the following conditions: melting at 95°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 30 s. The PCR products were analyzed by electrophoresis on a 2% agarose gel, and the relative intensity of signals was quantified using NIH image (Scion Corp., Frederick, MD).

RNA interference. Serum-starved melanoma cells were transfected with 100 nmol/L small interfering RNA (siRNA) duplexes against ET_BR, COX-2, or HIF-1α mRNA (SMARTpool) or with scrambled mock siRNA obtained commercially (Dharmacon, Lafayette, CO). siRNA transfection using LipofectAMINE reagent (Invitrogen) was done according to the manufacturer's protocol. Cell media were replaced with fresh serum-free media 48 h later and exposed to ET-3 or vehicle for 24 h. RNA and protein were then extracted for ET_BR COX-2 or HIF-1α analysis. Transfection with COX-2 promoter construct was done 24 h after HIF-1α siRNA transfection. Cells were then treated with ET-3, and after 24 h, COX-2 promoter activity was analyzed.

Western blot analysis. Whole-cell lysates or homogenized M10 tumor specimens were subjected to SDS-PAGE and analyzed by Western blotting using antibodies to HIF-1α (Transduction Laboratories, Lexington, KY); COX-1 and COX-2 (Cayman Chemical); anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA); and ET_BR (Alexis, San Diego, CA). Blots were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The membranes were reprobed with anti–β-actin to assure the equal amount of protein (Oncogene, CN Biosciences, Inc., Darmastadt, Germany).

ELISA. The VEGF protein levels in the conditioned media were determined in duplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D Systems, Minneapolis, MN). The sensitivity of the assay is <5.0 pg/mL. Intra-assay and interassay variations were 5.4% and 7.3%, respectively. Levels of PGE_2 released into the cell conditioned media were measured by ELISA using the PGE_2 High-Sensitivity Immunoassay according to the manufacturer's instructions (R&D Systems). The sensitivity of the assay is <8.25 pg/mL. Intra-assay and interassay variations were 9.5% and 10.9%.

Transfection of reporter construct and luciferase assay. Reporter construct phPES2 (−1432/+59) containing the 5′-flanking region of the human *COX-2* gene (35) was kindly provided by Dr. DuBois (Vanderbilt University Medical Center, Nashville, TN). For transient transfection, 1×10^5 cells were plated in six-well plates 48 h before transfection. The cells were cotransfected with 0.5 μg COX-2 firefly luciferase plasmid construct and with 0.05 μg of the pCMV-β-galactosidase plasmid (Promega, Madison, WI) using LipofectAMINE reagent following 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. For each experiment, the mean of three independent experiments done in triplicate was reported.

Gelatin zymography. The melanoma cell supernatants were electrophoresed for analysis in 9% SDS-PAGE gels containing 1 mg/mL gelatin as described previously (36). Briefly, the gels were washed for 30 min at 22°C in 2.5% Triton X-100 and then incubated in 50 mmol/L Tris-HCl (pH 7.6), 1 mmol/L $ZnCl_2$, and 5 mmol/L $CaCl_2$ for 18 h at 37°C. After incubation, the gels were stained with 0.2% Coomassie blue.

Chemoinvasion assay. Chemoinvasion was assessed using a 48-well modified Boyden's chamber (NeuroProbe, Pleasanton, CA) and 8-μm pore polyvinyl pyrrolidone–free polycarbonate Nucleopore filters (Costar, New York, NY) as described previously (36). The filters were coated with an even layer of 0.5 mg/mL Matrigel (Becton Dickinson, Bedford, MA). The lower compartment of chamber was filled with chemoattractant (100 nmol/L ET-3) and/or inhibitors (27 μL/well). Serum-starved 1007 cells (0.5×10^6/mL) were harvested and placed in the upper compartment (55 μL/well). Where specified, cells were preincubated for 30 min at 37°C with the indicated concentrations of COX-1 or COX-2 inhibitors. After 6 h of incubation at 37°C, the filters were removed and stained with DiffQuick (Merz-Dade, Dudingen, Switzerland), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

M10 melanoma xenografts. Female athymic (nu^+/nu^+) mice, 4 to 6 weeks of age (Charles River Laboratories, Milan, Italy), were handled according to the institutional guidelines under the control of the Italian Ministry of Health. Mice were injected s.c. on one flank with 1.5×10^6 viable M10 cells expressing ET_BR. The mice were randomized in groups (n = 10) to receive treatment i.p. for 21 days with A-192621 (10 mg/kg/d; Abbott Laboratories, Abbott Park, IL; ref. 2), and controls were injected with 200 μL drug vehicle (0.25 N $NaHCO_3$). The treatments were started 7 days after the xenografts, when the tumor was palpable. Each experiment was repeated thrice. Tumor size was measured with calipers and calculated using the formula $\pi / 6 \times$ larger diameter $\times$ (smaller diameter)^2.

Immunohistochemical analysis. Indirect immunoperoxidase staining was carried out on acetone-fixed 4-μm frozen tissue sections. The avidin biotin assays were done using the Vectastain Elite kit (for nonmurine

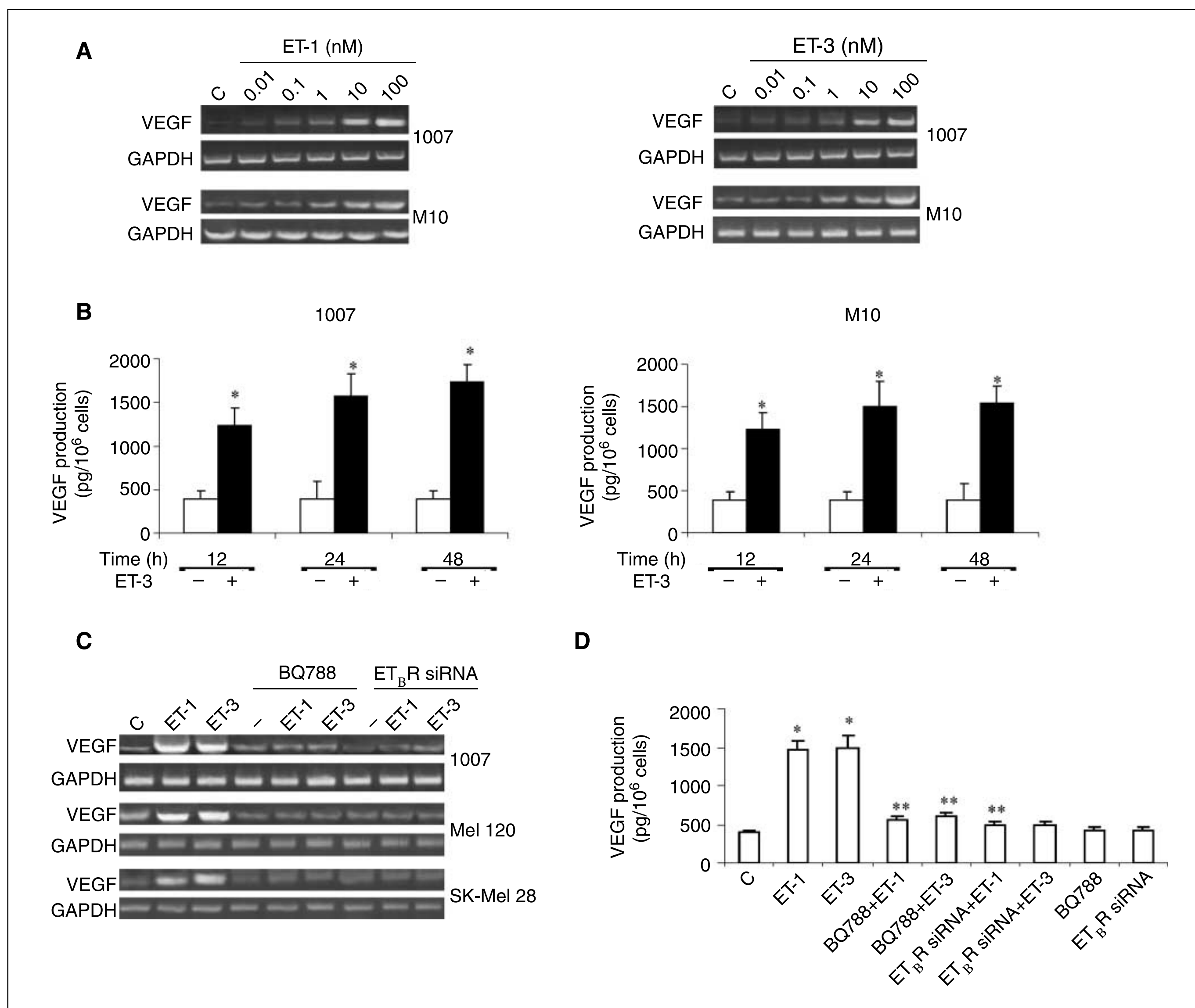


Figure 1. ET-1 and ET-3 promote VEGF expression through ET_BR in melanoma cell lines. *A,* serum-starved 1007 and M10 cells were stimulated with increasing concentrations of ET-1 or ET-3 for 6 h, and total RNA was extracted and analyzed by RT-PCR for VEGF mRNA expression. Primers for GAPDH mRNA were used as loading control. *B,* serum-starved 1007 and M10 cells were cultured for the indicated times in the presence of 100 nmol/L ET-3, and conditioned media were analyzed by ELISA for VEGF production. *Columns,* VEGF production; *bars,* SD. *, $P < 0.005$ compared with the control. *C,* total RNA from serum-starved 1007, SK-Mel 28, and Mel 120 cells cultured for 6 h in the presence of 100 nmol/L ET-1 or ET-3 alone or in combination with 1 μmol/L BQ788 or from cells transfected with ET_BR siRNA was extracted and analyzed by RT-PCR for VEGF transcripts. Primers for GAPDH mRNA were used as loading control. *D,* conditioned media from serum-starved 1007 cells cultured for 24 h in the presence of 100 nmol/L ET-1 or ET-3 alone or in combination with 1 μmol/L BQ788 or from cells transfected with ET_BR siRNA were collected and analyzed by ELISA for VEGF secretion. *Columns,* VEGF production; *bars,* SD. *, $P < 0.001$ compared with the control; **, $P < 0.004$ compared with ET-1 or ET-3.

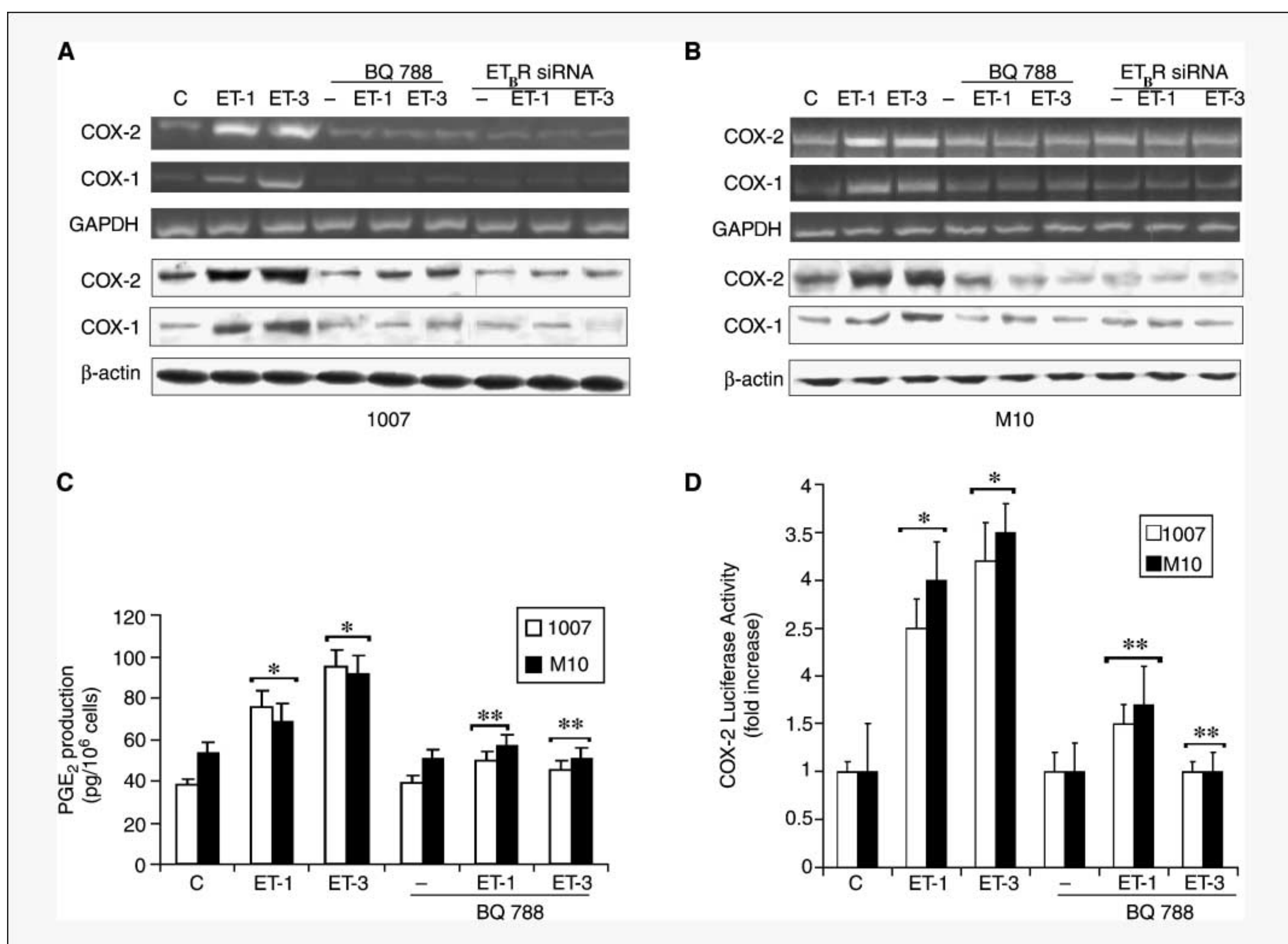


Figure 2. ET-1 and ET-3 induce COX-1 and COX-2 expression, COX-2 promoter activity, and PGE_2 production through ET_BR. Total RNA (*top*) or whole-cell lysates (*bottom*) from serum-starved 1007 (*A*) and M10 (*B*) cells cultured in the presence of 100 nmol/L ET-1 or ET-3 alone or in combination with 1 μmol/L BQ788 or from cells transfected with ET_BR siRNA was extracted after 6 or 24 h, respectively. COX-1 and COX-2 mRNA or protein expression was analyzed by RT-PCR or Western blotting. GAPDH primers and anti–β-actin were used as loading controls. *C,* conditioned media from serum-starved 1007 cells cultured for 24 h in the presence of 100 nmol/L ET-1 or ET-3 and/or 1 μmol/L BQ788 were collected and analyzed for PGE_2 production by ELISA. *Columns,* PGE_2 production; *bars,* SD. *, $P < 0.001$ compared with the control; **, $P < 0.005$ compared with ET-1 or ET-3. *D,* COX-2 promoter activity was measured in 1007 and M10 cells transiently transfected with a COX-2 promoter (phPES2, −1432/+59) construct and treated as in (*C*). Luciferase activity was expressed as fold increase after normalization with galactosidase activity. *Columns,* COX-2 luciferase activity; *bars,* SD. *, $P < 0.001$ compared with the control; **, $P < 0.005$ compared with ET-1 or ET-3.

primary antibodies) and the Vector MOM immunodetection kit (for murine primary antibodies) obtained from Vector Laboratories (Burlingame, CA). Mayer's hematoxylin was used as nuclear counterstain. Sections incubated with isotype-matched immunoglobulins or normal immunoglobulins served as negative control. The primary antibodies used were as follows: mouse anti–COX-2 (1:200; Cayman Chemical), anti-VEGF (1:200), monoclonal rat antimouse CD31 (platelet/endothelial cell adhesion molecule 1; 1:20; generously donated by Dr. A. Mantovani, Mario Negri Institute, Milan, Italy), anti-Ki67 MoAb (clone MIB1; 1:20; Ylem, Rome, Italy), and a monoclonal anti–MMP-2 (1:20; Oncogene Research Products, Cambridge, MA). The evaluation of microvessel density (MVD) was done by two independent observers on a ×200 magnification counted at least in five fields as reported previously (37). Ki67 score was expressed as tumor cells with nuclear staining counted at least in five separate ×40 microscopic fields.

Statistical analysis. Statistical analysis was done using the Student's *t* test. Repeated measures ANOVA followed by Scheffe post hoc testing for multiple comparisons was used to evaluate the statistical significance of observed differences (SSPS, Chicago, IL). All statistical tests were two sided. $P < 0.05$ was considered statistically significant.

Results

ET-1 and ET-3 increase VEGF expression through ET_BR in human melanoma cells. The effect of ET-1 and ET-3 on VEGF expression was investigated in primary (1007) and metastatic (M10) melanoma cell lines. RT-PCR analysis for VEGF revealed that in both cell lineages, ET-1 and ET-3 increased the VEGF transcript levels in a concentration-dependent manner (Fig. 1*A*). As shown by ELISA, VEGF release was induced by ET-3 in a time-dependent fashion, reaching the maximum stimulation (4-fold above control levels) after 48 h in both cell lines (Fig. 1*B*). To investigate the functional relevance of ET_BR blockade on VEGF production, we used pharmacologic and molecular approaches inactivating ET_BR in primary 1007 and metastatic Mel 120 and SK-Mel 28 melanoma cells. BQ788, as well as silenced ET_BR by specific siRNA (Supplementary Fig. S1), blocked ET-1/ET-3–induced VEGF mRNA expression and secretion (Fig. 1*C* and *D*), clearly showing that in melanoma cells, ET-1 and ET-3 induce VEGF through the binding with ET_BR and that blockade of ET_BR significantly inhibits VEGF production.

ET-1 and ET-3 increase COX-1 and COX-2 expression, COX-2 promoter activity, and PGE_2 production through ET_BR. We next investigated whether ET-1 and ET-3 could affect COX-1 and COX-2 expression and PGE_2 production in the 1007 and M10 melanoma cells. As shown by RT-PCR and Western blot analysis, ET-1 and ET-3 significantly induced COX-1 and COX-2 mRNA and protein up-regulation (Fig. 2*A* and *B*). ET-1 and ET-3 stimulation resulted also in a significant increase of PGE_2 production (>2-fold above the control; Fig. 2*C*). In the presence of BQ788, or in silenced ET_BR-cells, these effects were significantly inhibited (Fig. 2*A–C*). To determine whether ETs may regulate COX-2 promoter activity, cells were transiently transfected with the human COX-2 promoter (phPES2, −1432/+59) reporter and pCMV-β-galactosidase plasmids. Treatment with ET-1 or ET-3 for 24 h induced 3-fold increase in luciferase activity compared with untreated cells, which was fully prevented by BQ788 (Fig. 2*D*), showing that ET_BR-mediated pathways regulate COX-1 and COX-2 expression, COX-2 promoter, and PGE_2 release in melanoma cells.

COX-1 and COX-2 mediate ET-induced PGE_2, VEGF production, MMP activation, and melanoma cell invasion. To assess whether COX-mediated pathway may regulate ET-induced PGE_2 and VEGF production, as well as invasiveness, we used selective or nonselective COX inhibitors in 1007 melanoma cells. The COX-2 inhibitor, NS-398, or silenced COX-2 by specific siRNA (Supplementary Fig. S2), as well as the COX-1 inhibitor, SC-560, and the non-COX isotype selective inhibitor, indomethacin, significantly blocked both VEGF and PGE_2 production after 24 h of ET-3 treatment (Fig. 3*A* and *B*). These data indicate that both enzymes, although by a different extent, participate to ET-mediated PGE_2 and VEGF production in these COX-1/COX-2–positive melanoma cells. Impairment of COX pathways by selective inhibitors has been shown to reduce melanoma cell invasiveness (27). Because we showed previously that ETs promote MMP activity and invasion in melanoma cells (2), we analyzed the contribution of ET-3–induced COX enzymes to invasive activity. By using gelatin zymography and chemoinvasion assays, we showed that treatment with COX inhibitors reduced ET-3–induced MMP-9 and MMP-2 activation as well as cell invasion (Fig. 3*C* and *D*). Similar results were obtained in 1007 melanoma cells stimulated with ET-1 (data not shown). These results show that ET-1/ET-3 signaling elicits an ET_BR-dependent activation of PGE_2 pathway promoting cell invasiveness, MMP activity, and VEGF production through the activation of both COX enzymes.

ET-1– and ET-3–induced VEGF expression is mediated by HIF-1α. To analyze more in-depth the mechanisms by which ET-1 and ET-3 may influence the cellular hypoxic response, we

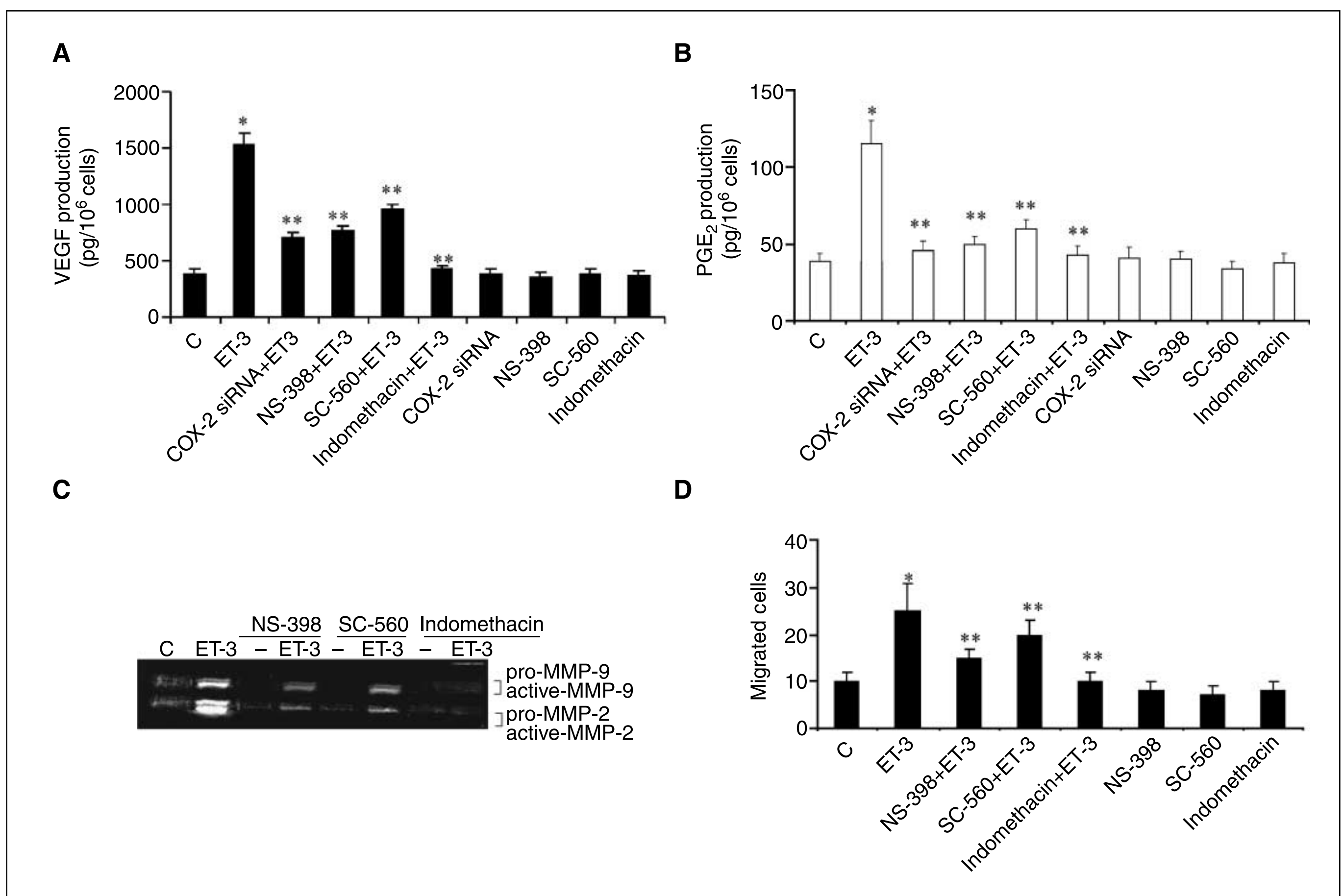


Figure 3. ET-induced VEGF and PGE_2 production, MMP activity, and invasion occur via both COX enzymes. Conditioned media were collected from serum-starved 1007 cells stimulated with 100 nmol/L ET-3 and/or COX-2 inhibitor, NS-398 (1 μmol/L), COX-1 inhibitor, SC-560 (9 nmol/L), or non-COX isotype selective inhibitor, indomethacin (26 μmol/L), or from cells transfected with COX-2 siRNA for 24 h and analyzed for VEGF (*A*) and PGE_2 (*B*) production by ELISA. *Columns,* PGE_2 production; *bars,* SD. *, $P < 0.001$ compared with the control; **, $P < 0.005$ compared with ET-3. *C,* gelatin zymography was used to determine MMP-2 and MMP-9 activities in conditioned media from 1007 cells treated as in (*A*). *D,* serum-starved 1007 cells were treated as in (*A*) and cell invasion was measured using a Boyden's chamber invasion assay. *Columns,* migrated cells; *bars,* SD. *, $P < 0.005$ compared with the control; **, $P < 0.001$ compared with ET-3.

investigated the effects of its major mediator HIF-1α. Although in normoxic conditions, HIF-1 α protein levels were barely detectable in 1007 and M10 melanoma cells, following exposure to ET-1 or ET-3 rapidly increased its expression (Fig. 4*A*). Under hypoxic conditions, the level of HIF-1α induction by ET-1 and ET-3 was increased to an even greater extent than that induced by hypoxia alone (Fig. 4*B*). Both in normoxic and hypoxic melanoma cells, ET-3–induced accumulation of HIF-1α protein paralleled the ET-3–induced VEGF production (Fig. 4*C*). At the end of 24 h of stimulation with ET-3, the VEGF production in 1007 and M10 melanoma cells reached a level (∼3.5-fold increase) higher than that observed under hypoxic treatment alone (∼3-fold increase; Fig. 4*C*). To address the functional role of ET_BR blockade on HIF-1α protein accumulation, we examined the effect of ET_BR antagonist, BQ788, or ET_BR siRNA under normal oxygen and low-oxygen environment. ET_BR blockade inhibited the ET-1– and ET-3–induced HIF-1α protein expression in both conditions (Fig. 4*A* and *B*), indicating that ET-1 and ET-3 mimic and cooperate with hypoxia to induce HIF-1α and to enhance VEGF expression through ET_BR. To evaluate whether HIF-1α was required in hypoxia and ET-mediated VEGF up-regulation, HIF-1α protein levels were silenced by the use of a specific siRNA (Supplementary Fig. S3). This treatment decreased the capacity to up-regulate HIF-1α under hypoxia and abolished hypoxia and ET-3–mediated VEGF induction under both normoxic and hypoxic stimuli (Fig. 4*D*), showing that ET- and hypoxia-inducible expression of VEGF is mediated by HIF-1α.

HIF-1α mediates ET-driven COX and PGE_2 pathway and MMP activity. The mechanism leading to COX-2 overexpression and induction by hypoxia in melanoma is unknown. To assess whether COX-1 and COX-2 expression could be modulated by hypoxia, we measured the expression of both enzymes in different oxygen conditions. As shown in Fig. 5*A*, exposure to hypoxia increased the intracellular levels of COX-1/COX-2 and this effect was amplified in the presence of ET-3. To address the role of HIF-1α in hypoxia- and ET-3–mediated COX-1/COX-2 expression, we transfected 1007 cells with HIF-1α siRNA. The reduced HIF-1α protein levels resulted into inhibition of ET-3– and hypoxia-induced COX-1 and COX-2 expression (Fig. 5*A*; Supplementary Fig. S3), showing for the first time that both hypoxia and ET-3 increase COX-2 expression, through HIF-1α under normoxic and hypoxic condition. To achieve a greater understanding of the regulatory mechanism underlying the HIF-1α–mediated COX-2 up-regulation, we transfected 1007 cells with COX-2 promoter alone or in combination with HIF-1α siRNA. Exposure to hypoxia for 24 h resulted in an ∼3-fold increase in COX-2 promoter activity compared with normoxia (Fig. 5*B*) comparable with that induced by ET-3 in normoxic conditions. Under hypoxic environment, 1007 cells treated with ET-3 displayed a further 4-fold increase of COX-2 promoter activity compared with the hypoxic cultured cells, suggesting that COX-2 can be transcriptional enhanced by exposure to hypoxia and that ET-3 and hypoxia may share a common transcriptional mechanism to potentiate COX-2 up-regulation. Cotransfection with HIF-1α siRNA and COX-2 promoter

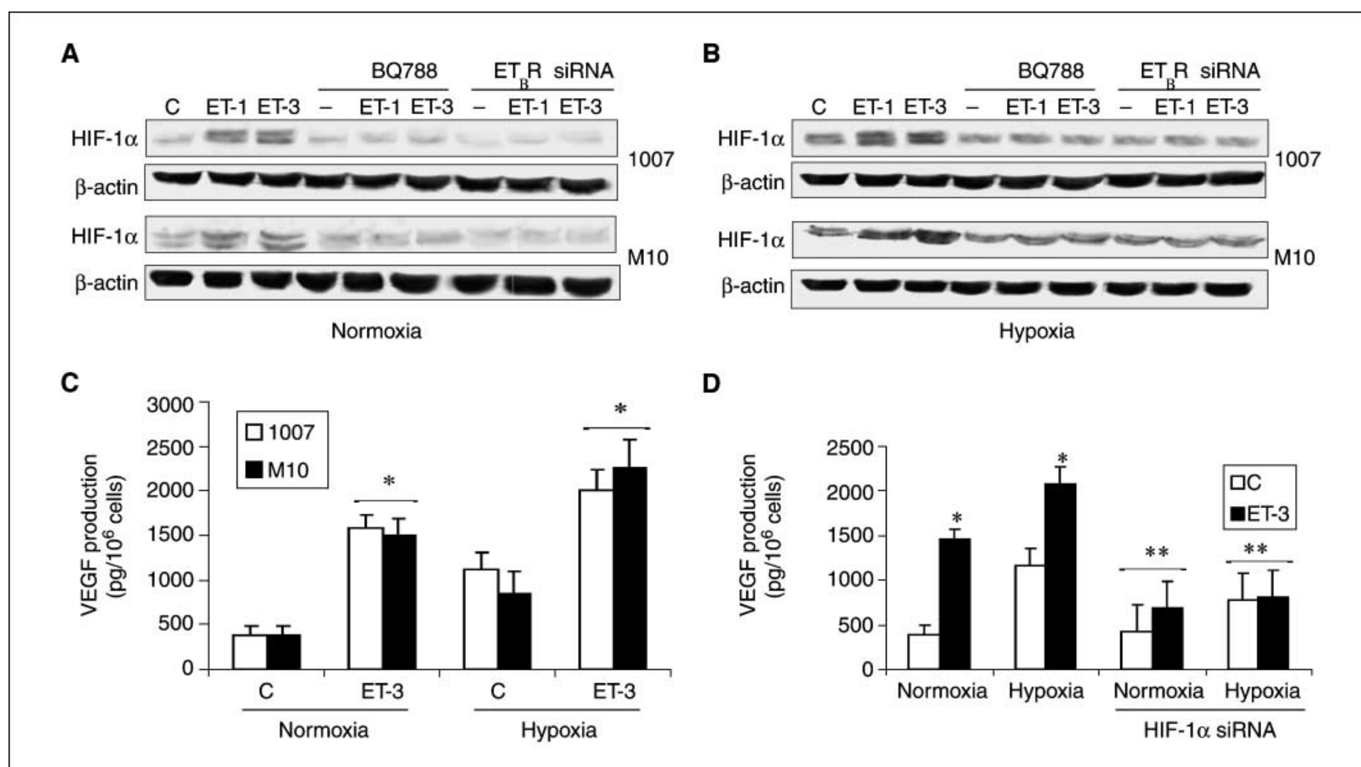


Figure 4. ET-1 and ET-3 increase HIF-1α–mediated VEGF secretion through ET_BR in normoxic and hypoxic conditions. Whole-cell lysates from serum-starved 1007 and M10 cells cultured under normoxic (*A*) or hypoxic (*B*) conditions in the presence of 100 nmol/L ET-1 or ET-3 and/or 1 μmol/L BQ788 or from cells transfected with ET_BR siRNA were extracted and analyzed for HIF-1α protein expression by Western blotting analysis. The filters were reprobed with the specific anti–β-actin as internal control. *C,* conditioned media from serum-starved 1007 and M10 cells treated with 100 nmol/L ET-3 under normoxic and hypoxic conditions for 24 h were analyzed by ELISA for VEGF secretion. *Columns,* VEGF production; *bars,* SD. *, $P < 0.005$ compared with control. *D,* 1007 cells were transfected with HIF-1α siRNA for 48 h and cultured for additional 24 h under normoxic or hypoxic conditions alone or in the presence of ET-3. Cell conditioned media were analyzed by ELISA for VEGF secretion. *Columns,* VEGF production; *bars,* SD. *, $P < 0.005$ compared with control; ** $P < 0.001$ compared with untransfected cells stimulated with ET-3 or hypoxia.

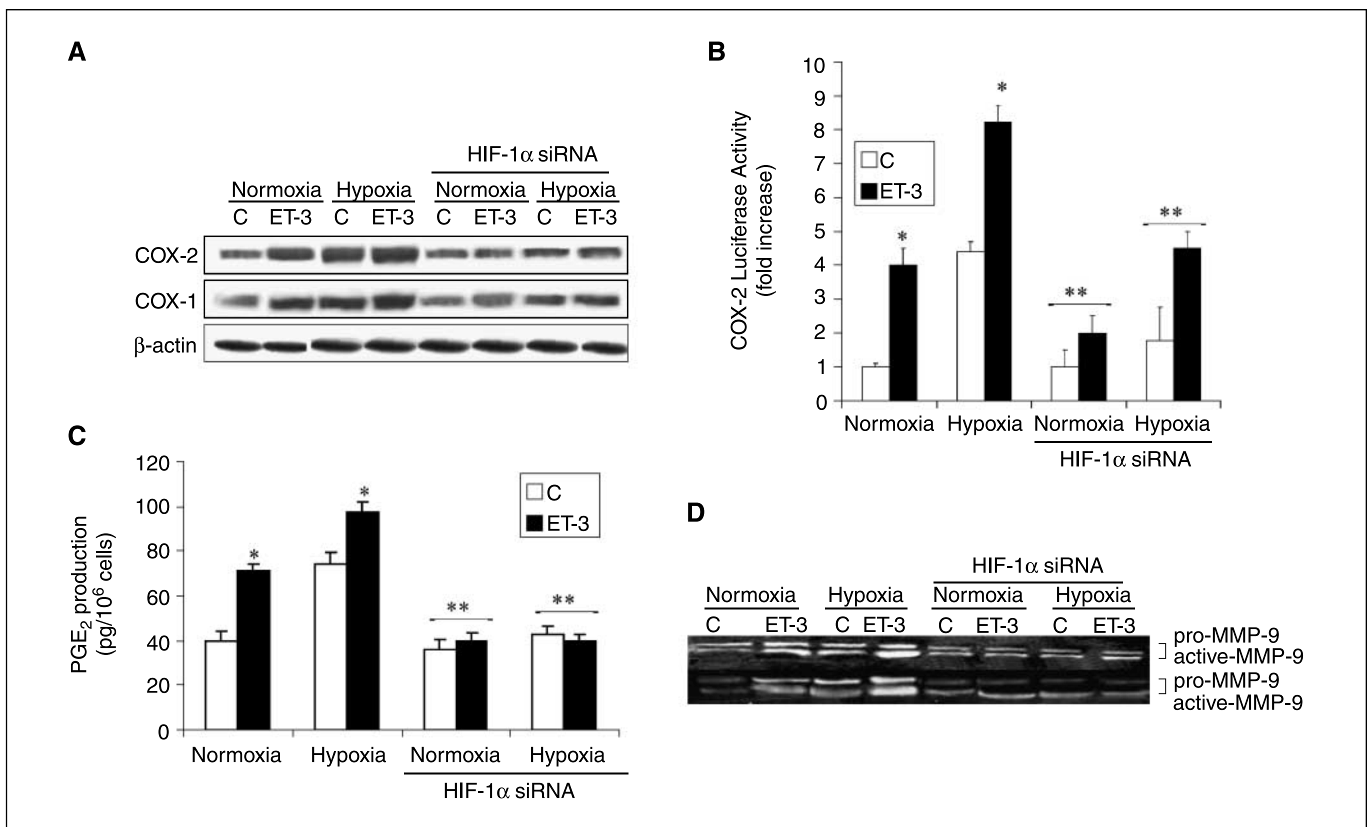


Figure 5. ETs induce COX-1/COX-2 expression, COX-2 transcriptional activation, PGE_2 production, and MMP activity via HIF-1α. *A,* 1007 cells were transfected with HIF-1α siRNA for a 48 h, cultured for additional 24 h under normoxic or hypoxic conditions either alone or in the presence of ET-3, and analyzed for COX-1 and COX-2 protein expression by Western blot. Anti–β-actin was used as loading control. *B,* HIF-1α silenced or nonsilenced 1007 cells were transfected with COX-2 promoter (phPES2, −1432/+59) construct and treated for 24 h under normoxic or hypoxic conditions with or without ET-3. Luciferase activity was measured and reported as fold increase after normalization with galactosidase activity. *Columns,* COX-2 luciferase activity; *bars,* SD. *, $P < 0.005$ compared with the control; **, $P < 0.001$ compared with HIF-1α siRNA untransfected cells stimulated with ET-3 or hypoxia. *C,* 1007 cells were transfected with HIF-1α siRNA for a 48 h and cultured for additional 24 h under normoxic or hypoxic conditions alone or in the presence of ET-3. Cell conditioned media were analyzed by ELISA for PGE_2 secretion. *Columns,* PGE_2 production; *bars,* SD. *, $P < 0.005$ compared with control; **, $P < 0.001$ compared with HIF-1α siRNA untransfected cells stimulated with ET-3 or hypoxia. *D,* conditioned media from 1007 cells treated as in (*C*) were analyzed by gelatin zymography to determine MMP activity.

significantly inhibited ET-3– and hypoxia-induced COX-2 transcriptional activity (Fig. 5*B*), indicating that HIF-1α is a major mediator of ET-3–driven COX expression and reporter gene activation in both oxygen-deprived and normoxic conditions. In hypoxia, ET-3 treatment potentiated the enhanced effect of hypoxia on PGE_2 production. In the presence of HIF-1α siRNA, hypoxia- and ET-3–induced PGE_2 levels were inhibited (Fig. 5*C*), indicating that in response to both stimuli, a HIF-1α–dependent increase in PGE_2 production occurs. We finally investigated the pathways whereby ET-3 and hypoxia could affect the invasive behavior of melanoma cells. ET-3 and hypoxia induced MMP activity, and in hypoxic conditions, the presence of ET-3 promoted MMP activity to a greater extent than hypoxia alone that was inhibited by HIF-1α siRNA (Fig. 5*D*). In conclusion, these data indicate that in melanoma cells, ET-3 signaling implies ET_BR-dependent COX-2 activity leading to VEGF and PGE_2 production and tumor protease activation through HIF-1α pathway in both normoxic and hypoxic environment.

ET_BR antagonist-induced inhibition tumor growth *in vivo* is associated with reduction in neovascularization and invasion-related factor expression. We next determined whether ET_BR blockade resulted in tumor growth inhibition and in the reduction of angiogenesis and invasive effectors *in vivo* by treating mice bearing established M10 tumors with A-192621, a selective nonpeptide ET_BR antagonist (2). Treatment with A-192621 (10 mg/kg/d) produced a 60% inhibition of tumor growth on day 40 after tumor injection (Fig. 6*A*) and it was generally well tolerated, with no detectable signs of acute or delayed toxicity. As shown in Fig. 6*B*, a marked reduction of COX-2, HIF-1α, and VEGF expression was observed in A-192621–treated mice compared with the control. Immunohistochemical evaluation revealed a significant and homogenous decrease of cytoplasmatic COX-2, MMP-2, and VEGF staining in tumors from treated mice, which paralleled the ability of A-192621 to reduce tumor vascularization, quantified as MVD, and proliferation index, evaluated as Ki-67–positive cells (Fig. 6*C*). Thus, MVD in untreated xenografts was significantly higher (41 ± 4; $P < 0,0004$) than in treated xenografts (10 ± 3), and Ki-67 score in untreated tumors was significantly higher (81 ± 5; $P < 0.0006$) than in the corresponded A-192621 tumors (44 ± 4; Fig. 6*D*).

These data indicate the physiologic relevance of ET_BR blockade in the regulation of tumor growth, neovascularization, and ET_BR-related signaling cascade, resulting in down-regulation of COX-2, VEGF, MMP-2, and HIF-1α expression.

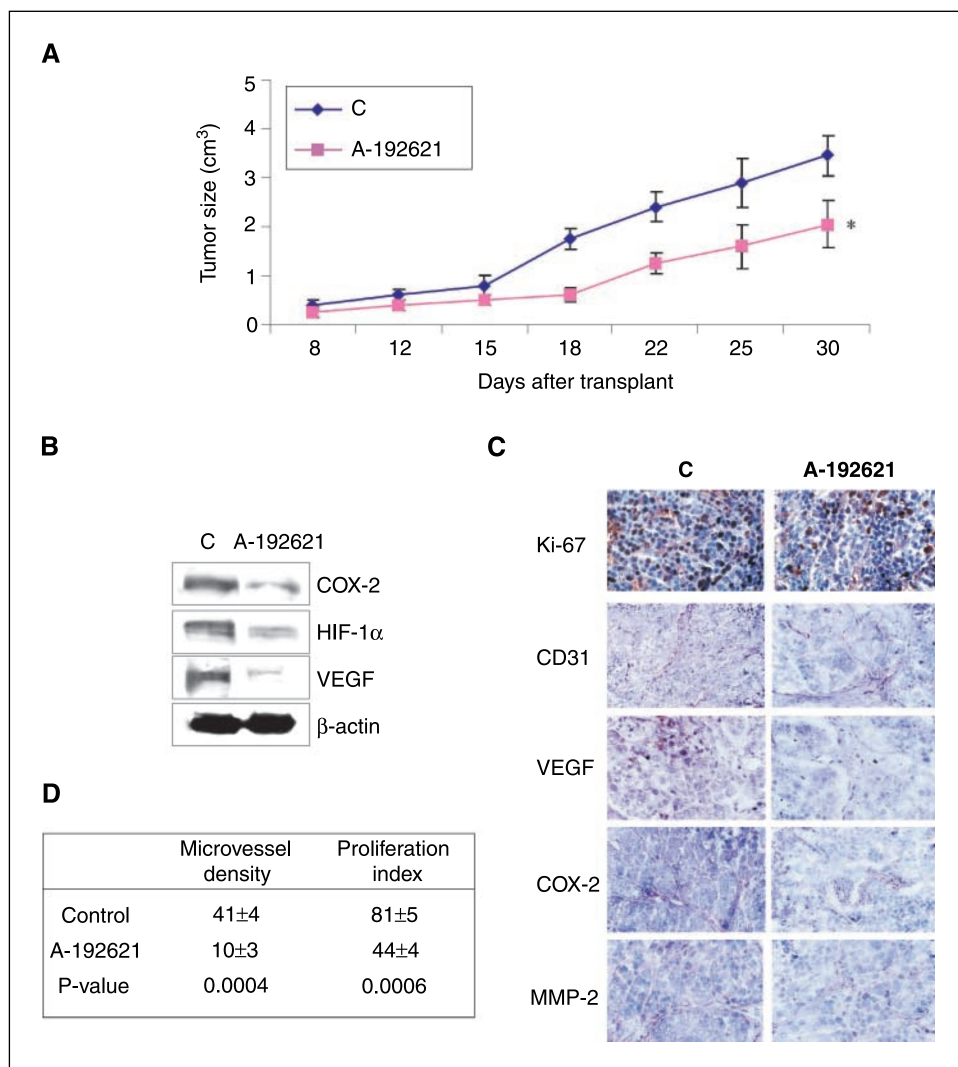
Discussion

The steady increase of melanoma incidence in the last decades, the early metastasization of the tumor, and the resistance of advanced melanoma to current treatment regimens underscore the

importance of acquiring a better understanding of the pathogenesis of this disease (16). Research in this area has identified ET axis as one of the key regulators of melanoma progression (2–4), suggesting that the inhibition of ET_BR signaling pathway may improve treatment of this malignancy. In this study, we investigated as to whether ETs activities may be influenced by the key microenvironmental factor, hypoxia, in modulating the invasive behavior of melanoma cells. Here, we show that ET_BR activation triggers a HIF-1α– mediated up-regulation of VEGF levels in primary and metastatic human melanoma cells. Moreover, HIF-1α seems to act in concert to ETs also in inducing COX-1/COX-2 expression, COX-2 transcriptional activity, PGE_2 production, MMP activity, and cell invasion, indicating a central role for ETs to potentiate hypoxia-induced melanoma progression through HIF-1α. Finally, blockade of ET_BR inhibits tumor growth, neovascularization, and invasive molecular determinants.

Neoangiogenesis and invasion in melanoma is strictly dependent on the interplay of a variety of stimuli, including local hypoxia (38). In addition to the classic hypoxia-mediated induction of HIF-1α, different growth factors, including ET-1, which is capable of inducing an angiogenic phenotype on endothelial cells and tumor neovascularization (37, 39), have been shown to enhance HIF-1α stabilization with resulting accumulation and activation (18–22, 40). Recent studies have provided evidence that the epidermal microenvironment of melanocytes is hypoxic and that a low oxygen level is required for melanocyte transformation initiated by Akt through a HIF-1α—dependent mechanism (25). In the present study, we show that ET-1 and ET-3 are inducers of HIF-1α expression equipotent to hypoxia and behave so even to a greater extent under hypoxic conditions, indicating that ETs and hypoxia exert additive effects on HIF-1α–dependent machinery to promote melanoma angiogenic determinants and cell invasion, which occur at early stages of melanomagenesis.

The COX-1 and COX-2 enzymes are involved in tumor progression by inducing proliferation, survival, angiogenesis, invasion, and metastasis in several solid tumors (27–32, 41–44). To elucidate the regulatory mechanisms that underlie COX-1/COX-2 regulation, we identify COX enzymes as downstream signals of ETs/ET_BR pathway, providing evidence that ET-1 and ET-3 induced COX-2 promoter activity and COX-1/COX-2 expression with resulting PGE_2 production. Furthermore, the decrease of ET-3–induced VEGF production, MMP activation, and cell invasion by COX inhibitors shows that COX-mediated pathway by ET_BR stimulates angiogenesis-related factor expression and migratory activities, thus identifying a novel mechanism responsible for ETs/ET_BR tumor-promoting properties. Differently from results of



	Microvessel density	Proliferation index
Control	41±4	81±5
A-192621	10±3	44±4
P-value	0.0004	0.0006

Figure 6. Blockade of ET_BR by A-192621 inhibits tumor growth, neovascularization, and invasion-related marker expression *in vivo. A,* antitumor activity of ET_BR antagonist treatment on established M10 human melanoma xenografts. Mice received injection s.c. with 1.5×10^6 cells. Seven days when tumor became palpable, mice were treated i.p. for 21 d with vehicle or with A-192621 (10 mg/kg/d). *Points,* averages of three different experiments; *bars,* SD. The comparison of time course of tumor growth curves by two-way ANOVA with group and time as variables showed that the group-by-time interaction for tumor growth was statistically significant. *, $P < 0.001$. *B,* immunoblotting for COX-2, HIF-1α, and VEGF expression in M10 tumor xenografts. Anti–β-actin was used as loading control. *C,* comparative immunohistochemical analysis of Ki-67, CD31, VEGF, COX-2, and MMP-2 expression in M10 tumor xenografts. Original magnification, ×250 and ×160 for CD31. *D,* quantitative assessment of immuonohistochemical analysis for MVD and proliferation index.

Denkert et al. (27), indicating that in melanoma, COX-2 is the major source of PGE_2, we showed by using selective COX-1/COX-2 inhibitors that both enzymes are involved in PGE_2-mediated invasiveness of these tumor cells. Our findings show that ETs and hypoxia contribute to COX-1/COX-2 up-regulation in melanoma cells. We further provide evidence that HIF-1α plays a major role in melanoma response to hypoxia as well as to ETs, demonstrating that down-regulation of HIF-1α in ET-stimulated cells inhibits the capability to induce COX-2 expression and transcriptional activity, VEGF and PGE_2 production, and tumor protease activity. Furthermore, ET-3 and hypoxia have additive effects on the induction of these events through HIF-1α. These data agree with recent results showing a close relationship between hypoxia and *COX-2* gene induction. In ovarian carcinoma, the effect of PGE_2 on VEGF is potentiated by hypoxia and is associated with HIF-1α expression (45), suggesting that COX-dependent prostanoids may play an important role in the regulation of hypoxia-induced VEGF expression. In hypoxic lung cancer, COX-2 is up-regulated in a HIF-1α–dependent manner, thus providing the first evidence that *COX-2* is a target gene of HIF-1α (46). In addition, while this report was in preparation, Kaidi et al. (47) reported that HIF-1α directly binds a specific HRE located at −506 on the COX-2 promoter, highlighting the biological significance of COX-2 up-regulation during hypoxia in colorectal cancer cells. Recent data show that PGE_2 can directly induce expression of HIF-1α protein (48, 49). These findings suggest the possibility of an autocrine stimulation, in which high PGE_2 levels due to increased COX-2 overexpression stimulate expression of HIF-1 responsible of continuous COX-2 expression. Our findings implicate, for the first time, HIF-1α/COX-2 as downstream checkpoints of finely tuned interconnected signals induced by ET axis and hypoxia capable of modulating tumor growth since the early stages of melanoma progression. Because the regulation of all these molecular effects is critical in melanoma progression, one can envision that melanoma hypoxia can activate HIF-1α enhancing the transcriptional activity of target genes, such as *ET-1*, which through the binding of its cognate receptor activates HIF-1α transcription resulting into promotion of invasiveness. As shown for several growth factors (18), HIF-1α therefore contributes to autocrine signaling pathways that are crucial for cancer progression. Collectively, the present results evidence that signaling pathways associated with angiogenesis and invasiveness can be activated by HIF-1α in melanoma cells exposed to ET-1/ET-3, disclosing a yet unidentified regulatory mechanism, which relays on the convergence of microenvironmental hypoxia and ETs, influencing the behavior of melanoma cells through HIF-1α-COX signaling cascade (Supplementary Fig. S4).

Gaining a better understanding of the complexities of tumor context can improve the development of more effective antitumor treatments. In this regard, ET_BR seems clinically relevant because by connecting with hypoxia, it can modulate melanoma progression. This is also supported by a gene array profiling of melanoma that identified ET_BR as one of the genes associated with multiple aggressive phenotypes, including the plasticity of melanoma cells to engage in vasculogenic mimicry (7). The invasive melanoma cells that are capable of generating tubular networks *in vitro* expressed in fact both MMPs and ET_BR (50). Immunohistochemical and immunoblot analysis of melanoma xenografts provides *in vivo* evidence for this concept because that treatment with ET_BR antagonist induces a significant tumor growth inhibition associated with a reduction of MVD, VEGF, COX-2, HIF-1α, and MMP-2 expression. Therefore, the antitumor effect of ET_BR antagonist on melanoma cell growth *in vivo* and *in vitro* (2, 15) is likely to result also from its interference with the formation of microvascular channels lined by tumor cells overexpressing ET_BR and MMPs. In conclusion, the present study delineates the link between hypoxia and ET_BR-triggered molecular events producing the activation of other signaling molecules, such as COX-2 and its downstream targets, to expanding the cellular communication network responsible for the invasive phenotype. In view of these findings, ET_BR antagonists, which have shown to induce concomitant antitumor activity and suppression of neovascularization *in vivo*, may represent a promising HIF-1α–targeted therapeutic approach in the treatment of melanoma.

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