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Endothelin-1 Induces Vascular Endothelial Growth Factor by Increasing Hypoxia-inducible Factor-1α in Ovarian Carcinoma Cells*

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Angiogenesis is an essential prerequisite for tumor growth, invasion, and metastasis. In ovarian carcinoma cells, endothelin-1 (ET-1) stimulates the secretion of vascular endothelial growth factor (VEGF), a major mediator of tumor angiogenesis. In OVCA 433 and HEY ovarian carcinoma cell lines, ET-1 treatment increases VEGF mRNA expression and induces VEGF protein levels in a time- and dose-dependent fashion, and do so to a greater extent under hypoxic conditions. ET-1 also increases hypoxia-inducible factor-1α (HIF-1α) accumulation and activates the HIF-1 transcription complex under both normoxic and hypoxic conditions, suggesting a role for HIF-1 in the induction of VEGF expression. These effects are inhibited by the selective ET_A receptor (ET_AR) antagonist, BQ123. The ET-1-induced increase in HIF-1α protein levels is due to the enhanced HIF-1α stabilization. These results implicate HIF-1α in the induction of VEGF expression in ET-1-stimulated ovarian carcinoma cells, and provide a mechanism whereby ET-1 acting selectively through ET_AR can interact with the HIF-1α-dependent machinery of angiogenesis. Our results suggest that new therapeutic strategies using specific ET_AR antagonists could provide an additional approach to the treatment of ovarian carcinoma by inhibiting neovascularization as well as tumor cell growth.

Tissue hypoxia represents a physiological stimulus for angiogenesis by enhancing the production of several autocrine angiogenic mediators (1–3). Endothelin-1 (ET-1),[1] one such mediator, is produced by endothelial cells, by vascular smooth muscle cells, and by tumor cells (4). ET-1 receptors, ET_A and ET_B, belong to the family of G-protein-coupled receptors (GPCR) and bind ET-1 with equal affinity (5). Aberrant ET-1-induced cell proliferation and/or survival is implicated in the pathophysiology of a number of malignancies, including ovarian carcinoma (6–7). In this tumor, engagement of ET_AR by ET-1 triggers activation of signaling pathways linked to tumor cell proliferation (8), apoptosis protection (9), invasiveness (10), and neoangiogenesis (11–12). In this regard, we demonstrated previously (13) that elevated expression of ET-1 and its receptors was significantly associated with microvessel density and vascular endothelial growth factor (VEGF) expression, known to be correlated with poor survival in ovarian cancer patients. High levels of ET-1 were detected in the majority of ascitic fluids of these patients and significantly correlated with VEGF ascitic concentrations (12). Whereas growth factors like VEGF and their cognate tyrosine kinase receptors are the best characterized mediators of angiogenesis, a number of GPCR have also been shown to play a role in angiogenesis (14). Thus, ET-1 modulates different stages of neovascularization including proliferation, migration, invasion, protease production, and morphogenesis of human umbilical vein endothelial cells and stimulates neovascularization *in vivo* (11, 15). Furthermore, it has been shown that GPCR agonists can also indirectly modulate angiogenesis through the induction of VEGF (16). In vascular smooth muscle cells, angiotensin II, thrombin, and ET-1 have been shown to induce VEGF expression (17, 18). In the ovarian carcinoma cell line, OVCA 433, we demonstrated that ET-1 stimulated VEGF production through ET_AR by an extent comparable with hypoxia, indicating that ET-1 could modulate tumor angiogenesis through direct angiogenic effects on endothelial cells and through the VEGF stimulation (12). Transcriptional up-regulation of the hypoxia-inducible factor-1 (HIF-1) has been shown to play a major role in VEGF gene induction (19). HIF-1 is composed of HIF-1α and HIF-1β that bind as dimers to the hypoxia-responsive element (HRE) (20). HIF-1 controls the expression of a number of pro-angiogenic genes such as VEGF, erythropoietin, and ET-1 in response to hypoxia in different tumor cell lines (1–3, 19). HIF-1 is up-regulated in a broad range of malignancies, where it correlates with tumor grade and vascularity (21). Although the HIF-1β protein is readily found in all cells, HIF-1α, which is virtually undetectable under normal oxygen conditions, is strongly and rapidly induced by hypoxia. Oxygen-dependent proline hydroxylation regulates the binding of von Hippel-Lindau suppressor protein, the recognition component of a ubiquitin-protein ligase that targets HIF-1α for ubiquitination and proteosomal degradation (22–24). Although hypoxia has been shown to be the major inducer of HIF-1α in all cells tested, other stimuli, such as growth factors, hormones, nitric oxide, transition metals, and iron chelators, are able to induce VEGF expression in an HIF-1-dependent manner in normoxic cells (25–29). We hypothesized that ET-1 promotes VEGF production through HIF-1α and that this mechanism may be responsible for the increasing

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[1] The abbreviations used are: ET-1, endothelin-1; ET_AR, endothelin A receptor; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; GPCR, G-protein coupled receptor; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CHX, cycloheximide; ELISA, enzyme-linked immunosorbent assay; HRE, hypoxia-responsive element.

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angiogenesis resulting in ovarian tumor growth. Therefore, we used HEY and OVCA 433 ovarian carcinoma cell lines as a model to investigate the stimulation of VEGF expression by ET-1 and the mechanisms involved in ET-1 induction of VEGF. Because ET-1 stimulates ovarian tumor cell proliferation and survival selectively through ET_AR, which is the receptor subtype predominantly expressed in these tumor cells, we also explored which receptor is involved in the ET-1-induced VEGF expression. Here we report that ET-1 induces VEGF expression in ovarian cancer cells grown under normal oxygen conditions. ET-1-increased VEGF expression was mediated by ET_AR and was associated with the induction of HIF-1α protein, suggesting a role for HIF-1 in this effect. Furthermore, we found that ET-1 enhanced HIF-1α stability leading to HIF-1α protein accumulation, and these effects were amplified under hypoxic conditions. Our results clearly demonstrated that ET-1 mimics cellular hypoxia inducing HIF-1α protein that can form the HIF-1 transcription complex inducing higher levels of HIF-1 DNA binding activity in ovarian carcinoma cells. These findings suggest that this ET_AR-activated pathway should play a major role in vascular VEGF production and neovascularization in ovarian carcinoma.

EXPERIMENTAL PROCEDURES

Cell Cultures—OVCA 433 and HEY human ovarian carcinoma cell lines were the generous gifts from Prof. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy) and were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cells were routinely cultured in 5% CO_2, 95% air (normoxic conditions) at 37 °C. To expose cells to hypoxia, cells were placed into an air-tight chamber (Modular Incubator Chamber, Billups-Rothenberg, CA) with inflow and outflow valves and were infused with a mixture of 5% CO_2, 95% N_2 for 20 min. In all experiments cells were grown to 70–80% confluence on 100-mm glass dishes. Cells were then serum-deprived by incubation for 24 h in serum-free medium and exposed to normoxia or hypoxia in the presence or in the absence of ET-1 or BQ123, a specific ET_AR antagonist (Peninsula Laboratories, Belmont, CA). All culture reagents were from Invitrogen. When the effects of ET_AR antagonist were studied, BQ123 was added 15 min before the agonist. Pretreatment with cycloheximide (100 μM, Sigma) was performed 2 h prior to the addition of ET-1 for *de novo* protein synthesis of VEGF. For the HIF-1α stability study, cycloheximide was added after 24 h of ET-1 or hypoxic treatment, and the cells were further incubated under normoxia for varying times.

Reverse Transcription-PCR—Total RNA from HEY and OVCA 433 cells was extracted using the TRIzol (Invitrogen) method according to the manufacturer's instructions. RT-PCR was performed on 1 μg of total RNA using a Superscript One-step RT-PCR System (Invitrogen) according to the manufacturer's instructions. The primer sets were as follows: VEGF, 5′-GGCTCTAGATCGGGCCTCCGAAACCAT-3′ and 5′-GGCTCTAGAGCGCAGAGTCTCCTCTTC-3′; GAPDH, 5′-TGAAGGTCGGTGTCAACGGA-3′and 5′-GATGGCATGGACTGTGGTCAT-3′. Thirty five cycles of amplification with a PerkinElmer Life Sciences 9700 thermocycler were performed under the following conditions: melting at 95 °C for 30 s; annealing at 60 °C for 45 s; extension at 72 °C for 30s for VEGF amplification, and for GAPDH amplification a 55 °C annealing temperature was used. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide and visualized and photographed under UV light. In all experiments, two control reactions, one containing no mRNA and another containing mRNA but no reverse transcriptase, were included.

ELISA—OVCA 433 and HEY cells were seeded at 1×10^6 cells/dish in complete medium and serum-starved for 24 h. Cells were cultured under normoxic and hypoxic conditions. After varying times conditioned media were collected, centrifuged, and frozen for subsequent use. The VEGF protein levels in the media were determined in duplicate by ELISA using the reagents and the protocol supplied with the Quantikine Human VEGF immunoassay kit (R & D Systems, Minneapolis, MN).

Western Blotting—Cells were washed with ice-cold phosphate-buffered saline and lysed by scraping with a rubber policeman in lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 0.15% Triton X-100, 1 mM orthovanadate, 0.06 units of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). Protein concentrations were determined by the BCA Protein assay (Pierce). Protein cell extracts were subjected to SDS-polyacrylamide gel (7.5%) and transferred to nitrocellulose. The filters were blocked with 5% bovine serum albumin in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween) and incubated with VEGF polyclonal (Sigma) or HIF-1α monoclonal antibody (Transduction Laboratories, Lexington, KY) for 3 h. After washing with TBST, blots were incubated with peroxidase-conjugated anti-goat IgG or anti-mouse IgG for VEGF or HIF-1α, respectively, washed again, and subjected to the ECL (Amersham Biosciences) procedure. The presence of VEGF was also assayed in HEY cell-conditioned medium and analyzed by Western blotting.

Northern Blotting—Total RNA from HEY cells was extracted using the TRIzol (Invitrogen) method according to the manufacturer's instructions, separated by electrophoresis on 2% denaturing formaldehyde-agarose gel, and transferred to a nylon membrane. The membranes were UV cross-linked and hybridized in the QuickHyb hybridization solution (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The cDNA probe used for analysis of the VEGF and GAPDH mRNA was prepared using RT-PCR products. Probes were labeled with [α-^{32}P]dCTP using a random primer Oligolabeling kit (Amersham Biosciences) according to the manufacturer's instructions. Densitometric scanning was performed with a Mustek MFS-6000 CX apparatus, and the data were analyzed with Phoretix 1D software and normalized to those of GAPDH.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay—HEY cells were grown as before and lysed in buffer A (50 mM HCl, 0.5% Nonidet P-40, 25 mM Hepes, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM dithiothreitol). The lysates were pelleted and resuspended in buffer B (500 mM HCl, 25 mM Hepes, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μM dithiothreitol, 10% glycerol). Nuclear debris was removed by centrifugation, and aliquots of the supernatant were stored at −80 °C.

Oligonucleotide (130 ng) corresponding to the HRE sequence of the VEGF gene (5′-TCGACCACAGTGCATACGTGGGCTCCAACAGGTCCTCTTC-3′) was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) (30). Nuclear extracts (5 μg of protein) were preincubated (15 min at room temperature) together with poly(dI-dC) (Sigma) in 20 μl of buffer consisting of Tris-HCl (pH 7.5, 10 mM), 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol. The labeled probe (3×10^4 cpm) was then added and incubation continued for an additional 15 min. For competition assay, the labeled probe was mixed with a 100-fold molar excess of unlabeled oligonucleotide and then added to the nuclear extract. For supershift assays HIF-1α antibody (Transduction Laboratories) was added to the nuclear extract at a final dilution of 1:3 immediately after addition of the probe, and the mixture was kept on ice for 20 min. Reaction mixtures were then loaded onto 5% non-denaturing polyacrylamide gels. Electrophoresis was performed in 0.5× TBE (40 mM Tris-HCl, 45 mM boric acid, and 2.5 mM EDTA). Gels were vacuum-dried and autoradiographed overnight at −80 °C.

Statistical Analysis—All statistical analyses were assessed using a two-tailed Student's *t* test and performed by the Inplot software system (GraphPad Software Inc., San Diego, CA).

RESULTS

ET-1 Increases VEGF mRNA and Protein Level in Ovarian Carcinoma Cells—We first performed RT-PCR analysis to determine whether ET-1 affects the levels of VEGF mRNA in HEY and OVCA 433 cells, which produce elevated amount of ET-1 and express high affinity ET_A receptors (31, 32). Ovarian carcinoma cells were incubated with different concentrations of ET-1 ranging from 0.01 to 100 nM. ET-1 increased the three VEGF transcript levels corresponding to $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ in a concentration-dependent manner (Fig. 1). VEGF transcript levels were markedly enhanced by 1 nM ET-1, with a further increase induced by 10 nM reaching maximal stimulation by 100 nM.

We next assessed whether induction of VEGF mRNA levels by ET-1 resulted in a parallel increase of VEGF protein in a time- and concentration-dependent manner. Conditioned medium from OVCA 433 and HEY cells incubated in the presence of various concentrations of ET-1 and for different times was assayed for secreted VEGF by ELISA analysis. After ET-1 stimulation, secreted VEGF levels increased progressively with

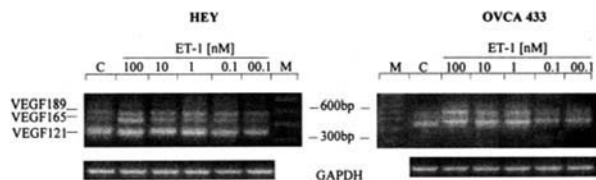

FIG. 1. **ET-1 induces VEGF mRNA expression in human ovarian carcinoma cells in a concentration-dependent manner.** Expression of 360-, 500-, and 590-bp mRNA transcripts for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, respectively, was detected by RT-PCR analysis. Primers for the amplification of GAPDH gene were used as controls. HEY and OVCA 433 cells were incubated with increasing concentrations of ET-1 (0.01, 0.1, 1, 10, and 100 nM) for 24 h before total RNA extraction. Data shown are PCR products for VEGF and GAPDH as visualized by ethidium bromide. *M*, size marker; *C*, control.

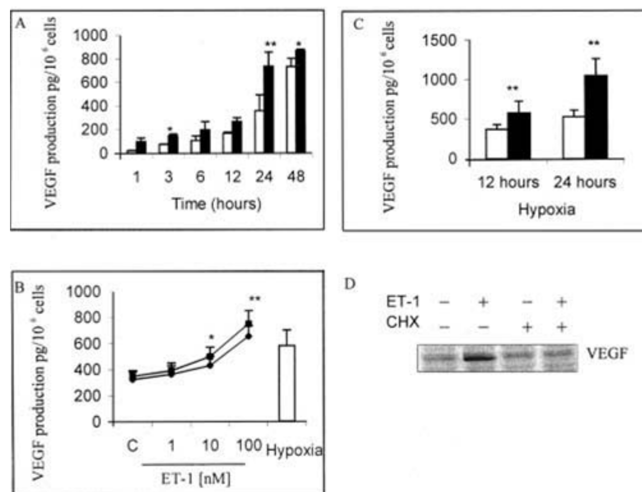

FIG. 2. **ET-1 increases VEGF protein secretion in a time- and concentration-dependent fashion.** *A*, ELISA of secreted VEGF protein in the conditioned media of HEY cells. Cells were incubated for 1, 3, 6, 12, 24, and 48 h in the absence (*open bar*) or in the presence of 100 nM ET-1 (*closed bar*) before collection of conditioned media. Data expressed are means of results from three experiments each performed in triplicate. *Bars*, ± S.D. *, $p \leq 0.02$ compared with control. **, $p \leq 0.005$ compared with control. *B*, OVCA 433 (♦) and HEY (■) cells were incubated with increasing concentrations of ET-1 (1, 10, and 100 nM) or in hypoxia for 24 h (*open bar*) before collection of conditioned media. VEGF production is reported as means of results from three ELISA determinations each performed in duplicate. *Bar* ± S.D. *, $p \leq 0.02$ compared with control (*C*). **, $p \leq 0.005$ compared with control. *C*, HEY cells were cultured under hypoxic conditions for 12 or 24 h in the absence (*open bars*) or in the presence (*closed bars*) of 100 nM ET-1 before collection of conditioned media. VEGF production is reported as means of results from three ELISA determinations each performed in duplicate. *Bars* ± S.D. **, $p \leq 0.005$ compared with control. *D*, OVCA 433 cells were exposed for 24 h to ET-1 (100 nM) in the absence or presence of cycloheximide (CHX, 100 μM) or to cycloheximide alone. VEGF protein levels were evaluated in whole cell lysates by Western blotting.

detectable production as early as 1 h and maximum stimulation (2.5-fold above control levels) at 24 h ($p \leq 0.005$, Fig. 2*A*). VEGF production increased progressively when HEY and OVCA 433 cells were treated with different concentrations of ET-1, reaching maximal stimulation at a concentration of 100 nM ($p \leq 0.005$, Fig. 2*B*). After 24 h of incubation with 100 nM ET-1, VEGF production was stimulated to an extent comparable with that induced by hypoxia (Fig. 2*B*), a recognized potent stimulus of VEGF.

We next investigated whether ET-1 cooperated with hypoxia, increasing the induction of VEGF production. After 12 and 24 h of hypoxic stimulus in HEY cells, ET-1-induced VEGF production increased progressively up to 1.5- and 2.0-fold above control levels, respectively (Fig. 2*C*). These results indicated that ET-1 induced VEGF production in a manner comparable with hypoxia and that under hypoxic conditions ET-1 cooperated to hypoxia amplifying the VEGF production.

To analyze whether ET-1-induced expression of VEGF was dependent on *de novo* protein synthesis, we determined the effect of the protein synthesis inhibitor cycloheximide (CHX) alone or in combination with ET-1. As shown in Fig. 2*D*, induction of VEGF protein upon ET-1 treatment in OVCA 433 cells was strongly inhibited by CHX, suggesting that induction of VEGF expression by ET-1 was dependent on ongoing protein synthesis.

ET-1 Induces VEGF Expression through ET_A Receptor—To characterize the ET-1 receptor subtype involved in the up-regulation of VEGF mRNA, we performed Northern blotting. Densitometric analysis of Northern blot autoradiographic bands and comparison with the intensity of the bands of the GAPDH expression indicated that VEGF expression after 6 h of stimulation with ET-1 (100 nM), under normoxic conditions, was increased up to 2.6-fold above control levels to an extent comparable with that induced by hypoxia. In addition, under hypoxic conditions ET-1 increased VEGF mRNA expression 1.7-fold above the control levels (Fig. 3*A*). Pretreatment of cells with the ET_AR antagonist BQ123 (1 μM) completely blocked the ET-1-stimulated VEGF mRNA expression under normoxic and hypoxic conditions (Fig. 3*A*).

To define the receptor-mediated action of ET-1 on VEGF protein production, we also analyzed conditioned media collected from HEY cells cultured for 24 h with ET-1 (100 nM) by Western blotting and by ELISA (Fig. 3, *B* and *C*). As evaluated by ELISA, ET-1-induced VEGF secretion increased up to 1.7- and 1.9-fold above control levels under normoxic and hypoxic conditions, respectively. After 24 h of incubation, the VEGF stimulation by ET-1 was completely blocked by BQ123 under both conditions (Fig. 3, *B* and *C*). These results indicated that ET-1-induced VEGF expression and secretion were mediated by the ET_AR.

ET-1-induced VEGF Expression Is Associated with the HIF-1α Protein Accumulation—To investigate whether ET-1-induced VEGF expression may be mediated by HIF-1α, we analyzed the HIF-1α protein levels upon ET-1 treatment in HEY cells cultured under normoxic or hypoxic conditions by Western blotting. The HIF-1α protein expression under normal oxygen conditions was undetectable, while exposure of HEY cells to hypoxia rapidly increased HIF-1α protein levels. At the end of 24 h stimulation with ET-1 (100 nM) in normoxic HEY cells, the HIF-1α protein expression was strongly induced to a level that was more elevated than the hypoxic treatment (Fig. 4*A*). When HEY cells were stimulated with 100 nM ET-1 under hypoxic conditions, the level of HIF-1α induction was increased to an even greater extent than that induced by hypoxia. We then evaluated whether HIF-1α accumulation induced by ET-1 was associated with the VEGF protein expression. Both in normoxic or hypoxic HEY cells, ET-1-induced accumulation of HIF-1α protein paralleled the ET-1-induced VEGF production (Fig. 4*B*).

ET-1 Induces the Stabilization of HIF-1α Protein—The undetectable expression of HIF-1α in non-stimulated HEY cells suggested that the HIF-1α protein was unstable under normoxic conditions in this cell line. To assess whether ET-1 regulated the expression of HIF-1α protein by inhibiting its degradation, we monitored the levels of ET-1-induced HIF-1α protein after blocking protein synthesis by cycloheximide. To this end, we investigated the decay of hypoxia-stabilized HIF-1α after transfer of cells from hypoxia to normoxia. A rapid decay of HIF-1α protein was observed within 15 min and was completely undetectable by the end of 60 min under normoxia (Fig. 5*A*). In contrast, ET-1-induced HIF-1α protein levels under normoxia remained constant for 60 min after cycloheximide

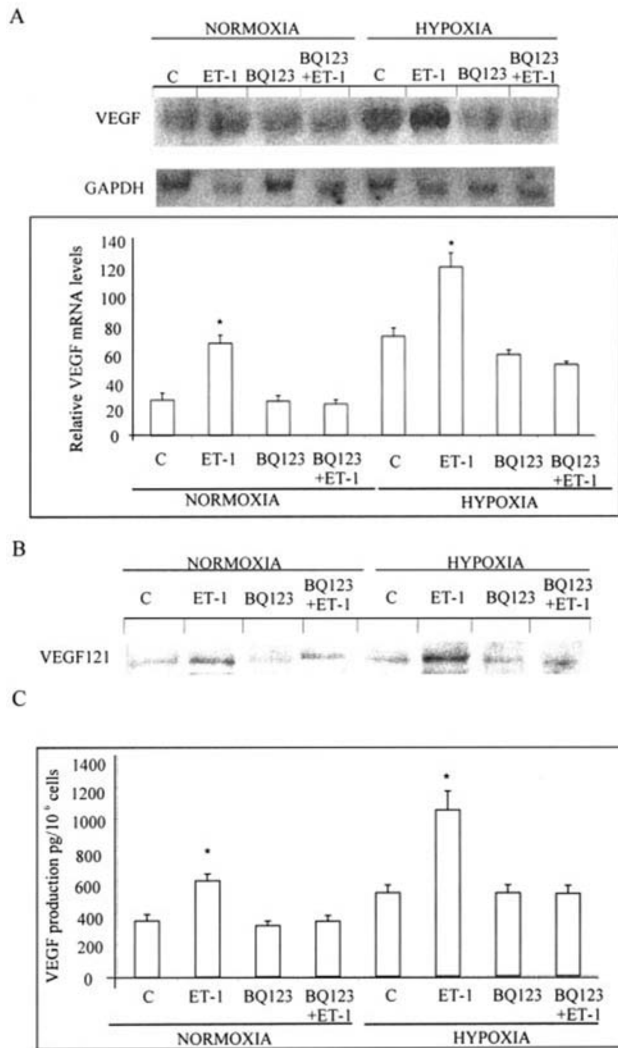

FIG. 3. **ET-1-induced VEGF expression is mediated through ET_AR.** HEY cells were cultured either under normoxic or hypoxic conditions in the presence of a selective ET_AR antagonist, BQ123 (1 μM) alone or in combination with ET-1 (100 nM), and total RNA and conditioned media were collected to analyze VEGF mRNA expression and VEGF production, respectively. *A*, total RNA (20 μg) extracted from cells treated for 6 h with ET-1 was analyzed by Northern blotting using VEGF cDNA probes. The filters were also hybridized with a GAPDH probe, as a control for RNA loading. The *lower panel* shows the relative amounts of VEGF mRNA expression calculated by densitometric analysis of the bands. The relative amounts of VEGF mRNA were quantified and normalized to the corresponding GAPDH amounts. Data expressed are means of results from three experiments. *Bars* ± S.D. *, $p \leq 0.005$ compared with control (*C*). *B*, conditioned media from HEY cells treated with 100 nM ET-1 for 24 h were tested for VEGF by Western blotting. *C*, VEGF secretion was measured in conditioned media from HEY cells treated with 100 nM ET-1 for 24 h by ELISA. Data expressed are means of results from three experiments each performed in duplicate. *Bars* ± S.D. *, $p \leq 0.005$ compared with control (*C*).

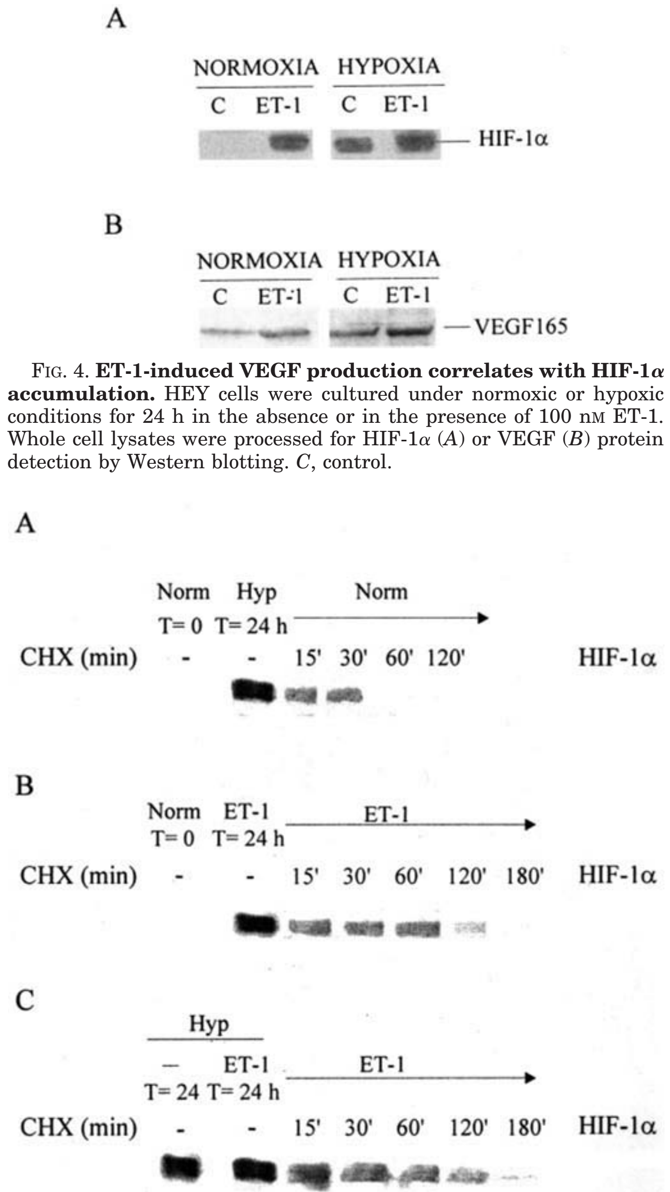

FIG. 4. **ET-1-induced VEGF production correlates with HIF-1α accumulation.** HEY cells were cultured under normoxic or hypoxic conditions for 24 h in the absence or in the presence of 100 nM ET-1. Whole cell lysates were processed for HIF-1α (*A*) or VEGF (*B*) protein detection by Western blotting. *C*, control.

FIG. 5. **ET-1 induces the stabilization of HIF-1α protein.** HIF-1α expression was induced by exposure HEY cells to hypoxia (*Hyp*; *A*) or to 100 nM ET-1 (*ET-1*; *B*) or to exposure of 100 nM ET-1-treated cells to hypoxia for 24 h (*C*). After that cycloheximide (*CHX*) was added in the media to a final concentration of 100 μM, and cells were further incubated under normoxia. Cell lysates were extracted at the indicated time points. HIF-1α protein levels were assessed by Western blotting with an HIF-1α-directed antibody.

addition and decreased relatively slowly to ~40% within 120 min thereafter (Fig. 5*B*). Moreover, the decay of hypoxia-stabilized HIF-1α after transfer of cells from hypoxia to normoxia in the presence of ET-1 was found to occur at a greater extent than hypoxia alone and with kinetics in which the levels of HIF-1α protein were still detectable and clearly above basal levels after 3 h of cycloheximide exposure. These results clearly indicated that ET-1 induced HIF-1α protein in normoxic and hypoxic HEY cells by slowing down its degradation. The finding that increased levels of VEGF expression were associated with the stabilization of HIF-1α protein in ET-1-treated HEY cells may be indicative of a role for HIF-1α in the induction of VEGF expression by ET-1.

ET-1 Activates the HIF-1 Transcription Complex—To be active, HIF-1α must form the HIF-1 transcription complex with HIF-1β and bind to the specific HRE DNA sequence (20). Therefore, we evaluated HIF-1 DNA binding activity with electrophoretic mobility shift assay experiments. When a 40-bp ^{32}P-labeled oligonucleotide probe containing the HIF-1-binding site was incubated with nuclear extracts from ET-1-treated HEY cells, under normoxic and hypoxic conditions, a number of protein-DNA complexes were increased (Fig. 6). The identity of HIF-1 in these complexes was confirmed by competition experiments and the use of a specific antibody. A 100-fold excess of unlabeled oligonucleotide competed with the probe for the binding of HIF-1α. A specific monoclonal antibody against HIF-1α was able to supershift the hypoxia-inducible complexes without

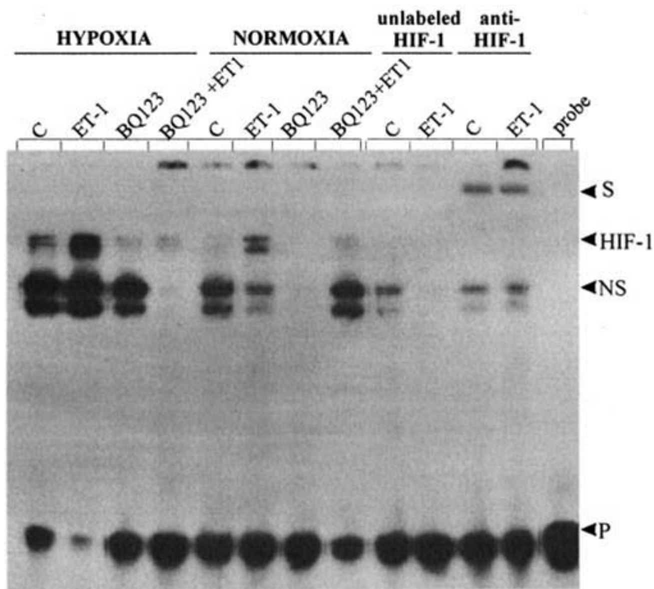

FIG. 6. **ET-1 induces HIF-1-DNA complex through ET_AR.** Nuclear extracts were prepared from HEY cells preincubated without (*C*) or with ET-1 (100 nM) or BQ123 (1 μM) or BQ123 plus ET-1 under normoxia or hypoxia for 24 h. Nuclear extracts were incubated with a ^{32}P-radiolabeled oligonucleotide probe, and DNA-protein complexes were then resolved by electrophoresis. For specificity control nuclear extracts from untreated (*C*) or treated HEY cells were incubated with a 100-fold molar excess of unlabeled HIF-1α oligonucleotide. For supershift, nuclear extracts were preincubated in the presence of the monoclonal antibody against HIF-1α (anti-HIF-1α). Supershifted complexes are indicated as *S*. The probe in the absence of any added protein was loaded in the *last lane* as control. The specific HIF-1 complex is indicated as HIF-1. Nonspecific protein DNA complexes are indicated as *NS*. Free probe is indicated as *P*.

affecting constitutive DNA-protein complexes. This induction was mediated through ET_AR binding, as demonstrated by the inhibitory effect on the induction of HIF-1 DNA complex by BQ123 under both hypoxic and normoxic conditions. These results indicated that ET-1 mimics hypoxia to induce HIF-1α protein that can form the HIF-1 complex with HIF-1β and bind the HRE sequence and that this mechanism is mediated through ET_AR.

DISCUSSION

In addition to the classical hypoxia-mediated induction of VEGF, a number of angiogenic stimuli including several tyrosine kinase receptors and GPCR agonists have also been shown to induce VEGF expression through HIF-1α (25, 27, 33–34). Among the latter we have reported (11, 12) that ET-1 induces an angiogenic phenotype on endothelial cells and neovascularization in ovarian carcinoma. In this study we demonstrated that ET-1 enhanced VEGF mRNA and protein levels in ovarian carcinoma cell lines via ET_AR. This modulation is associated in HEY cells with the accumulation of HIF-1α protein indicating a role for HIF-1 in the induction of VEGF expression. ET-1 induced HIF-1α stability and potentiated the hypoxia-mediated induction of HIF-1α and VEGF expression. Furthermore, ET-1 shares with hypoxia the ability to induce a complex that binds HRE as demonstrated by electrophoretic mobility shift assay. Steady-state levels of HIF-1α protein were regulated at the level of synthesis and stability. Many activators of HIF-1α, including hypoxia, have been shown to induce HIF-1α protein expression by inhibiting ubiquitination and degradation (24). To investigate whether ET-1 induced HIF-1α protein accumulation through a similar mechanism in HEY cells, we examined the kinetics of the decay of ET-1-induced HIF-1α protein in the absence of protein synthesis. As a reference for the stability of HIF-1α under normoxia in non-treated HEY cells, we investigated the decay of hypoxia-stabilized HIF-1α protein upon reoxygenation in the same manner. Analysis of HIF-1α protein stability in cycloheximide-treated cells showed that the degradation of HIF-1α was decreased in ET-1-treated cells compared with control under both hypoxic and normoxic conditions, supporting the notion that the induction of HIF-1α protein level by ET-1 was due to enhanced HIF-1α stability. Our results confirmed findings reporting that HIF-1α protein was unstable in normoxic OVCAR-3 and HI34 ovarian carcinoma cell lines and demonstrated the induction of VEGF expression and HIF-1α protein by the oxidative stressor arsenite (35). In this study we have shown that after stimulation with ET-1 increased HIF-1α protein levels accumulated in the cells, and the HIF-1 transcription complex was formed and could bind to the HRE-binding site. Therefore, ET-1-induced HIF-1 accumulation in ovarian carcinoma cell lines activated all the signals necessary for a full HIF-1 response.

These findings indicate that under normoxic conditions ET-1 was able to activate the hypoxia response pathway, which is the strongest physiological regulator of VEGF expression implying a direct involvement of ET-1 in the elicitation of the angiogenic phenotype. The HIF-1α-mediated transcription of VEGF by ET-1 under normoxic conditions pointed to a general mechanism through which oncogenes and growth factors may up-regulate VEGF and could synergize with hypoxia during tumor growth. In human cancers, HIF-1α was overexpressed as a result of intratumoral hypoxia and genetic alterations affecting key oncogenes and tumor suppressor genes. HIF-1α overexpression in biopsies of brain, breast, cervical, esophageal, oropharyngeal, and ovarian cancer was correlated with treatment failure and mortality (36). Overexpression of HIF-1α protein, as demonstrated by immunohistochemistry, was observed in 68.6% of epithelial ovarian cancer specimens (37). Necrotic areas, most likely induced by hypoxia, were more frequently observed in poorly differentiated ovarian tumor as compared with well differentiated carcinomas. Stronger expression of HIF-1α in well differentiated ovarian tumors than poorly differentiated tumors suggested that overexpression of HIF-1α in these tumors cannot be attributed to hypoxia alone. Birner *et al.* (37) further demonstrated a correlation between HIF-1α overexpression and increased vascularization in human ovarian cancer. In this regard, we previously reported (12) that ET-1 was expressed in 84% of the ovarian carcinomas and demonstrated for the first time a strong correlation between ET-1 and neovascularization and VEGF expression. These findings suggest that ET_AR activation by ET-1 participates in ovarian carcinoma pathogenesis, driving tumor cells growth and angiogenesis in a paracrine fashion by inducing the expression and secretion of VEGF acting on HIF-1α. Because increased HIF-1 activity may also influence tumor progression independently from its regulation of VEGF expression (38), inhibition of HIF-1α could represent a novel approach to cancer therapy especially for those cancer types, such as ovarian tumors, in which a high level of HIF-1 overexpression is correlated with mortality. Our findings, besides giving further insights into the role of the ET-1 receptor in cancer, may be clinically relevant. Addition of a specific ET_AR antagonist, BQ 123, blocked the ET-1-induced up-regulation of VEGF expression and secretion as well as the ET-1-induced activation of HIF-1 transcription complex, indicating a direct involvement of ET_AR in the control of tumor angiogenesis. Because the regulation of VEGF production is a critical event in tumor angiogenesis, one can envision that under pathological conditions such as cancer, ET-1 may be up-regulated by hypoxia (3) and that ET-1, in turn, might exert an angiogenic effect increasing

VEGF production through the HIF-1α-dependent mechanism. Thus, under hypoxic conditions ET-1 cooperates with hypoxia-amplifying HIF-1α stability and VEGF production. The present studies indicating ET-1 as a novel regulator of HIF-1α stability added further information on the overall importance of ET-1/ET_AR in regulating tumor angiogenesis. Thus ET_AR blockade in ovarian tumor cells prevents the growth-promoting activity of ET-1 by inhibiting cell proliferation (8) and survival (9) and cell migration and invasiveness (10) but also the VEGF-mediated angiogenesis. In conclusion, these results defined a novel mechanism that could act with a hypoxic independent pathway and/or could enhance the hypoxic dependent molecular machinery resulting in tumor angiogenesis and identified ET_AR as novel target for the development of anti-angiogenic therapies.

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