Activation of Mitogenic Signaling by Endothelin 1 in Ovarian Carcinoma Cells

Anna Bagnato, Raffaele Tecca, Valeriana Di Castro, and Kevin J. Catt
Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute, Via delle Messi d'Oro 156—158, 00158, Rome, Italy [A. B., R. T., V. D. C.], and Endocrinology and Reproduction Research Branch, NIH, Bethesda, Maryland 20892 [K. J. C.]

ABSTRACT

Endothelin 1 (ET-1) is produced in ovarian cancer cell lines and has been shown to act through ETA receptors as an autocrine growth factor to promote tumor cell proliferation in vitro. In OVCA 433 cells, the efficacy of ET-1 as a stimulus of [3H]thymidine incorporation was equivalent to that of epidermal growth factor. ET-1 also stimulated the rapid expression of c-fos, an action mediated by ETA receptors. The mitogenic action of ET-1 was not mediated by a pertussis toxin-sensitive G protein. Analysis of the effects of inhibition and depletion of protein kinase C (PKC) on mitogenic responses demonstrated that PKC was necessary but not sufficient for maximal stimulation by ET-1. In quiescent OVCA 433 cells, ET-1-induced stimulation of [3H]thymidine incorporation was prevented by two structurally distinct inhibitors of tyrosine kinase, herbimycin A and genistein. These results indicate that both PKC and protein tyrosine kinase participate in ET-1-stimulated mitogenic signaling. ET-1 rapidly stimulated tyrosine phosphorylation of several cellular proteins, among which p125FAK and p42 mitogen-activated protein kinase were identified. The additivity between the potent mitogenic actions of ET-1 and epidermal growth factor is consistent with the independence of their signal transduction pathways in ovarian cancer cells. These findings also indicate that intracellular signaling between the ETA receptor and a yet unidentified tyrosine kinase is involved in the mitogenic response to ET-1.

INTRODUCTION

Recent studies on ovarian cancer, which is a major cause of death from gynecological malignancy, have focused on factors that regulate the growth of neoplastic ovarian cells (1). The identification of these factors might reveal novel and potentially more effective approaches to the treatment of ovarian tumors. ET-1, a potent vasoconstrictor peptide initially isolated from endothelial cells (2), is also produced by a variety of human cancer cells (3—6). ET-1 has been suggested to be involved in the pathogenesis of certain types of cancer through an autocrine or paracrine mechanism (7, 8). The physical effects of ET-1 are mediated by two distinct, GPCRs: a selective ETA receptor that binds ET-1 and ET-2 with high affinity and ET-3 with low affinity, and a nonselective ETB receptor that binds all three ET isopeptides with equal affinity. These receptors trigger a common set of early signaling events, including activation of phospholipase C and inositol trisphosphate-dependent increases in intracellular Ca2+ levels, activation of PKC, and rapid expression of c-jun and c-fos (9, 10).

In the porcine ovary, ET-1 acts through ETB receptors as an endogenous regulator of granulosa cell function (11). However, we have recently demonstrated that ET-1 is produced by human ovarian cancer cells and acts through ETA receptors as an autocrine growth factor to stimulate calcium signaling and proliferative responses (12).

These findings support the view that ET-1 could participate in the development and/or progression of human ovarian tumors. As a growth regulatory peptide, ET-1 influences cell proliferation directly and can also act synergistically with growth factors that have been implicated in cancer progression (13, 14). The intracellular signaling pathways involved in the regulation of cell growth and division in response to mitogenic activation of GPCRs have not been clearly defined. Recent studies have focused on the roles of intermediates such as cyclic AMP and βγ subunits (15), and associated intracellular signaling proteins (16), in the tyrosine kinase cascade activated by GPCRs. Both PKC and protein tyrosine kinase activity have been implicated in mitogenic signaling pathways activated by ET-1 in specific cell types, but their individual contributions remain unclear. Although such pathways are independently controlled, the existence of cross-talk between them has been observed in cells stimulated by ET-1 (17, 18).

To investigate the proliferative actions of ET in ovarian carcinoma cells, we compared the activation of mitogenic signal pathways induced by ET-1, which binds to a GPCR, and EGF, which activates a receptor tyrosine kinase. In OVCA 433 cells, ET-1 was found to be as effective as EGF as a mitogenic stimulus and rapidly induced c-fos gene expression. Both PKC and tyrosine phosphorylation are involved in the ET-1-induced mitogenic signaling response. The binding of ET-1 to ETA receptors also rapidly stimulated tyrosine phosphorylation of cellular proteins, including focal adhesion kinase (p125FAK) and MAP kinase. It is possible that mitogenic signaling by ET-1 involves cross-talk between GPCRs and growth factors such as EGF, leading to increased proliferation of ovarian cancer cells.

MATERIALS AND METHODS

Cell Culture. The human ovarian carcinoma cell line OVCA 433 (19) was a generous gift from Dr. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy). Cells were cultured in DMEM (Whittaker Bioproducts, Inc., Walkersville, MD) containing 1% penicillin-streptomycin and 10% FCS in 75-cm² plastic flasks at 37°C under 5% CO2—95% air. When the cells reached 70—80% confluence, the cultures were serum deprived by incubation for 24 h in DMEM.

Thymidine Incorporation Assay. Cells were seeded in 96-well plates at approximately 80% confluence (2 × 10⁵ cells/well) and incubated in serum-free medium for 24 h to induce quiescence. Mitogenic stimuli were then added, and after 18 h, when the cells were confluent, 1 μCi of [methyl-3H]thymidine (6.7 Ci/mmol; DuPont, New England Nuclear Research Products, Wilmington, DE) was added to each well. Six h later the culture media were removed and the cells were washed three times with PBS, treated with 10% trichloroacetic acid for 15 min, washed twice with 100% ethanol, and solubilized in 0.4 N sodium hydroxide. The cell-associated radioactivity was then determined by liquid scintillation counting. Responses to all agents were assayed in sextuplicate and results were expressed as the means of three separate experiments.

RNA Extraction and Northern RNA Blot Analysis. Total cellular RNA was isolated from quiescent or ET-1-stimulated cells using the acid guanidinium isothiocyanate method (20). Ten to 20 μg of RNA were electrophoresed on 1% agarose-formaldehyde gels, transferred to Nytran membranes by capillary blotting, and immobilized by UV cross-linking. The filters were treated for 4 h at 42°C in 50% formamide prehybridization buffer containing 200 μg/ml denatured salmon sperm DNA and torula yeast RNA. The filters were hybridized with 32P-labeled cDNA probes containing inserts from plasmid clones of fos (Clontech, Palo Alto, CA). Subsequently, rehybridization

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: ET, endothelin; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; PTX, pertussis toxin; PKC, protein kinase C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; FAK, focal adhesion kinase; TPA, 12-O-tetradecanoyl-phorbol 13-acetate; OAG, oleoyl-2-acyl-sn-glycerol; MBP, myelin basic protein.

10 ICANCER RESEARCH57. 1306-1311, April 1, 1997
was performed with a cDNA probe for human GAPDH (Clontech) to control for variations in gel loading and transfer efficiency. The cDNA probes were labeled at specific activities of 0.5-1 × 10^6 dpm/pg with [32P]dCTP (6000 Ci/mmol) using a random hexanucleotide priming kit. After hybridization in a 50% Northern blot hybridization buffer at 42°C for 12 to 16 h, the blots were washed at a final stringency of 0.1× SSC-0.1% SDS at 55°C and exposed to Kodak films at -70°C with intensifying screens for 12 to 24 h. Levels of gene expression were quantitated with a Phosphorlmager and Imagequant software (Molecular Dynamics, Sunnyvale, CA) and were normalized to those of GAPDH, whose mRNA remained essentially constant.

Immunoprecipitation. OVCA 433 cells were grown to 80-90% confluence in 100-mm plastic Petri dishes and then serum starved for 24 h. After addition of agonists to the dishes for selected periods, the cells were rapidly washed with ice-cold PBS and scrapped into 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 1 mM o-vanadate, 0.06 units of aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). The lysates were preclarified for 30 min at 4°C with protein A-Sepharose, and immunoprecipitation was performed with anti-p125FAK polyclonal antibody (a gift from Dr. G. Tarone, University of Turin, Italy) for 1 h at 4°C. The precipitates were washed six times with lysis buffer and analyzed by electrohoresis on 7.5% polyacrylamide gels followed by immunoblotting.

Immunoblotting. For immunoblot analysis of the mobility shift of MAP kinase, cells were stimulated, treated with lysis buffer as above, and separated on 12.5% polyacrylamide gels (acrylamide/bis, 30:0.2). Blotting of cell extracts or p25 immunoprecipitates to nitrocellulose was performed at 0.5 A/mg at room temperature. The blots were then incubated for 1 h with anti-phosphotyrosine monoclonal antibody (0.5 μg/ml, clone 4G10; Upstate Biotechnology, Inc.) bound to protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). Immunocomplexes were recovered by centrifugation and washed three times in lysis buffer and twice in kinase buffer and then incubated for 30 min at 30°C with 5 μg of MBP, 10 μg of ATP, and 10 μCi of [γ-32P]ATP. The reactions were terminated by the addition of 20 μl of Laemmli’s buffer and boiling for 3 min, and samples were analyzed by SDS-PAGE in 12.5% acrylamide gels followed by autoradiography to visualize the phosphorylation of MBP.

RESULTS

Effects of ET-1 on DNA Synthesis in Ovarian Cancer Cells. Assays for ET-1-induced mitogenic responses were performed on OVCA 433 cells, which express abundant high-affinity receptors for ET-1 and for EGF (K_d 0.10 nm and 45,500 receptors/cell for ET-1 versus K_d 0.25 nm and 52,700 receptors/cell for EGF as determined by Scatchard analysis). ET-1 has been reported to act as a comitogen that requires low concentrations of serum, insulin, or polypeptide growth factors for maximum mitogenic activity (13). However, we recently observed that ET-1 alone can stimulate dose-dependent increases in [3H]thymidine incorporation and proliferation in OVCA 433 cells, and these actions were blocked by BQ 123 (12), an ET_A-selective receptor antagonist (21). A comparison of the mitogenic effects of ET-1 and EGF on OVCA 433 cells is shown in Fig. 1. In this study, ET-1 was equipotent with EGF in stimulating mitogenesis. When quiescent OVCA 433 cells were treated with 10 nm ET-1 in the presence of 10 ng/ml EGF, additive thymidine responses were observed (Table 1). However, at a 10-fold higher ET-1 concentration, slightly greater than additive effects (+16%) were observed.

Effect of ET-1 on c-fos Gene Expression. Mitogens that stimulate increases in intracellular Ca^{2+} levels and activate PKC often induce expression of immediate response genes (22). When added to OVCA 433 cells, ET-1 increased the level of c-fos mRNA with the time course shown in Fig. 1B. The low levels of c-fos found in quiescent cells showed a substantial increase after stimulation with 100 nm ET-1 to reach a maximum at 30 min and returned to the basal level by 4 h (Fig. 2B). The induction of c-fos mRNA was almost completely abolished in the presence of a 10-fold excess of BQ 123, demonstrating that this response to ET-1 is mediated by ET_A receptors (Fig. 3).

Effect of PTX on ET-1-Induced DNA Synthesis. To determine whether mitogenic signaling by ET-1 utilizes a PTX-sensitive (23) or insensitive (18) G protein, ovarian cancer cells were preincubated with PTX prior to hormone stimulation. Treatment of quiescent cells with 100 ng/ml PTX for 6 or 16 h had no effect on ET-1-induced
Role of Protein Kinase C in ET-1-induced DNA Synthesis. To analyze the contribution of PKC to mitogenic signaling by ET-1, quiescent OVCA 433 cells were treated with 800 nM GF 109203X, a PKC-selective bisindolylmaleimide inhibitor. This compound, when applied at a concentration known to inhibit DNA synthesis induced by PKC-activating mitogens (24), markedly attenuated the subsequent stimulation of $[^3H]$thymidine incorporation by ET-1 but not that induced by EGF (Fig. 5A). The latter finding indicates that inhibition of the mitogenic response to ET-1 was specific and not due to cytotoxicity. Similarly, addition of 0.1 nM staurosporine, a potent but less selective inhibitor of PKC, markedly inhibited ET-1 induced $[^3H]$thymidine incorporation (data not shown). Furthermore, depletion of PKC by pretreatment of cells with TPA reduced the subsequent stimulation of $[^3H]$thymidine incorporation by ET-1 but had no effect on the response to the EGF (Fig. 5B). Although these results indicate that mitogenic signaling by ET-1, but not by EGF, requires PKC, they suggest that activation of PKC is not alone sufficient to mediate this response. The extent to which stimulation of PKC can mimic the
The effects of ET-1 was examined in OVCA 433 cells treated with the PKC activators OAG and TPA. These agents only slightly increased \[^{3}H\]thymidine incorporation and did not reconstitute the growth factor-like action of ET-1 on this response (Fig. 5B).

**Effects of Tyrosine Kinase Inhibitors on ET-1-Stimulated DNA Synthesis.** ET-1 has been shown to increase tyrosine phosphorylation of cellular proteins, but it is not clear whether this response is linked to mitogenesis (25, 26). To evaluate the functional significance of ET-1-stimulated tyrosine phosphorylation, the \[^{3}H\]thymidine incorporation response of cells stimulated by ET-1 was measured after pretreatment with herbimycin A, a benzoquinoid inhibitor of cellular tyrosine kinases (27). As shown in Fig. 6A, herbimycin A prevented \[^{3}H\]thymidine incorporation in response to all concentrations of ET-1 tested. Furthermore, genistein, a chemically and functionally dissimilar inhibitor of tyrosine kinase activity (28), also completely prevented \[^{3}H\]thymidine incorporation in cells treated with mitogenic concentrations of ET-1 (Fig. 6B). These data are consistent with the hypothesis that stimulation of tyrosine phosphorylation is necessary for mitogenic signaling by ET-1.

**ET-1 Stimulates Tyrosine Phosphorylation of FAK (p125FAK).** The observation that tyrosine-phosphorylated substrates in the 115–130-kDa region were present in protein extracts from ET-1-stimulated cells (data not shown) led us to test whether p125FAK was phosphorylated during agonist action in OVCA 433 cells. p125FAK is a newly identified tyrosine kinase that becomes rapidly phosphorylated on tyrosine residues after integrin-mediated cell spreading and adhesion (29) and also when cells are stimulated by mitogenic peptides or transfected by v-src (30). Immunoprecipitation of p125FAK demonstrated that the enzyme was tyrosine phosphorylated in unstimulated OVCA 433 cells (Fig. 7) and that ET-1 induced significant increases in its phosphoryrosine content, with an initial peak at 5 min that fell toward basal values at 30 min. In contrast, EGF had no significant effect on p125FAK phosphorylation in these cells (data not shown).

**ET-1 Induces Phosphorylation and Activation of MAP Kinase.** MAP kinases (or extracellular signal-regulated kinases) are activated by numerous growth factors and calcium-mobilizing agonists, and several lines of evidence have suggested their essential role in cell cycle progression from G0-G1 to S (31, 32). ET-1-induced activation of MAP kinase is readily detectable on immunoblots, where the phosphorylated form of p42 MAP kinase displays lower electrophoretic mobility. As shown in Fig. 8A, both EGF (10 ng/ml) and ET-1 (100 nm) induced a rapid shift in the mobility of p42 MAP kinase, and the activation induced by ET-1 was inhibited by BQ 123.

To confirm that the phosphorylation of MAP kinase was accompanied by increased enzyme activity, we performed immunocomplex kinase assays using MBP as a substrate. Stimulation of OVCA 433 cells with either ET-1 or EGF caused enhanced phosphorylation of MBP in MAP kinase immunoprecipitates (Fig. 8B), demonstrating that MAP kinase phosphorylation parallels increased enzymatic activity. Pretreatment of cells with the ETA receptor antagonist, BQ 123, prevented the stimulation of MBP phosphorylation induced by ET-1, confirming that the ETA receptor subtype mediates the activation and phosphorylation of MAP kinase.

**DISCUSSION**

The downstream signals that mediate growth responses secondary to activation of GPCRs have been extensively analyzed in normal and immortalized cells, but have been little explored in human cancer cells. We have previously shown that ET-1 activates calcium signaling and proliferative responses in ovarian cancer cells. The present findings demonstrate the induction of immediate early gene expression in such cells, one of the earliest genomic responses to ET-1 (10). This transcriptional response, which is an important nuclear signal...
ET-1-induced phosphorylation and activation of MAP kinase. A, OVCA 433 cells were incubated for 5 min with vehicle (C) EGF (10 ng/ml), ET-1 (100 nM) or ET-1 (100 nM) treated with BQ 123 (1 μM) and analyzed for phosphokinase activity using MBP as substrate, as described in "Materials and Methods." B, quiescent OVCA 433 cells were incubated with anti-ERK2 antibody. The phosphorylated and activated form of p42 MAP kinase exhibits reduced electrophoretic mobility.

Several ligands that bind to GPCRs have been found to utilize PKC and tyrosine kinases as major downstream effectors. ET-1 also stimulates the tyrosine phosphorylation of p125FAK, which is predominantly localized in focal adhesions and is rapidly phosphorylated by integrins, neuropeptides, and oncogenes (41). Recent studies have shown that p60src forms a stable association with the tyrosine-phosphorylated form of p125FAK through its SH2 domain, suggesting a role for p125FAK in signal transduction (42). Furthermore, increased levels of p125FAK were found to accompany changes in epithelial and mesenchymal tumors during their progression to an invasive phenotype (43). The convergence of these various properties of p125FAK suggests that this protein participates in a variety of cellular processes, including a diverse set of normal and abnormal functions such as cell adhesion, cell motility, and, ultimately, cell proliferation.

ET-1 causes phosphorylation and activation of p42 MAP kinase, which is regulated by an upstream MAP kinase (MEK) that is thought to serve as a point of convergence of diverse signaling pathways, including the phospholipase C/PKC cascade and the receptor tyrosine kinase cascade (32). Activation of PKC and tyrosine kinases has been implicated in signaling from ETA receptors to MAP kinase in several cell types including fibroblasts (38, 44), mesangial cells (18, 45), vascular smooth muscle cells (46), and astrocytes (16, 36, 47). The present findings are in agreement with these reports and, along with our previous observations, indicate that ET-1 acts as a tumor growth factor by activating G protein-mediated signal transduction pathways in ovarian cancer cells. Binding of ET-1 to the ETA receptor subtype results in activation of a PTX-insensitive G protein that stimulates phospholipase C activity and promotes Ca2+/PKC signaling. Furthermore, ET-1 enhances mitogenesis through at least two pathways that utilize PKC and tyrosine kinases as major downstream effectors. ET-1 also stimulates the tyrosine phosphorylation of p125FAK, which is thought to transduce signals involved in tumor cell invasion. However, the role of this tyrosine kinase in mitogenic signal transduction is not yet known. The activation of MAP kinases by ET-1 is followed by increases in immediate-early gene expression and mitogenic responses that typically accompany growth factor activity.

A comparison of mitogenic signaling by ET-1 and EGF suggests not only the possibility of common intermediates in the signaling pathways of receptor tyrosine kinases and GPCRs, but also the activation of yet unidentified tyrosine kinases through cross-talk between these intracellular signaling cascades. Identification of such putative tyrosine kinase(s) is an obvious challenge for future studies. Recently, Daub et al. (48) have demonstrated a role for receptor tyrosine kinases as downstream mediators in GPCR mitogenic signaling via a ligand-independent mechanism of EGF receptor transactivation. The present findings clearly demonstrate that ET-1 is a potent mitogen in ovarian cancer cells and has additive actions with EGF that do not appear to regulate the Gq/Gi transition, is mediated by the ETA receptor. In the present study, ET-1 was found to stimulate DNA synthesis in ovarian cancer cells with the same efficacy as EGF, a typical growth factor, and at maximally effective concentrations its effect was additive to that of EGF. In contrast with the synergistic effects of ET-1 and polypeptide growth factors in certain cell types (33), only slight potentiating between the two hormones was observed on the growth responses of ovarian cancer cells. ET-1 has been found to potentiate DNA synthesis in Swiss 3T3 and NRK cells in the presence of EGF (34), and additive effects were observed on the proliferation of prostate cancer cells (4) and breast stromal cells (8). The mechanisms underlying the synergism between ET-1 and EGF are not well defined, but presumably depend on interactions between specific components of their mitogenic signaling pathways. In the present case, the predominately additive actions of ET-1 and EGF on thymidine incorporation at saturating agonist concentrations reflect the independence between their individual signaling mechanisms.

Several ligands that bind to GPCRs have been found to utilize PTX-sensitive G proteins to initiate growth responses in their target cells (35). However, ET-1 is known to stimulate phospholipase C activity and calcium signaling via a PTX-insensitive G protein in several cell types (18, 36, 37). Our data in ovarian cancer cells are consistent with the role of Gs or a related PTX-insensitive G protein in the ET-1-stimulated signal transduction cascade that regulates cell growth. Previous evidence has implicated PKC in mitogenic signaling by ET-1 (17, 38–40), but it is unclear whether PKC activation is sufficient for the proliferative response and whether the requirement for PKC is unique to GPCR agonists. In OVCA 433 cells, the effects of inhibition and depletion of PKC provide evidence that PKC is necessary for ET-1-induced growth responses. However, this was not the case for EGF, indicating the different requirements of the two agonists for activation of PKC to induce mitogenesis. Furthermore, TPA and the cell permeant diacylglycerol analogue, OAG, did not reproduce the effects of ET-1 on [3H]thymidine incorporation. Thus, other signals must be required in concert with PKC to mediate ET-1-induced mitogenesis.

Tyrosine kinases and their phosphorylated substrates are essential components in the mitogenic actions of numerous growth factors (15, 16). Tyrosine phosphorylation has also been found to occur during signaling responses associated with cell activation by phospholipase C-linked mitogenic peptides. The present data reveal a characteristic pattern of protein tyrosine phosphorylation following cell stimulation with ET-1. The importance of tyrosine phosphorylation in mitogenic signaling by ET-1 was indicated by the manner in which two chemically and functionally dissimilar tyrosine kinases inhibitors, herbimycin A and genistein, prevented the stimulation of [3H]thymidine incorporation by ET-1. It is clear that, in addition to PKC, mitogenic signaling by ET-1 requires an as yet unidentified tyrosine kinase(s). In the present study, ET-1 rapidly stimulated tyrosine phosphorylation of multiple cellular proteins. Among these were identified p125FAK kinase, which is predominantly localized in focal adhesions and is rapidly phosphorylated by integrins, neuropeptides, and oncogenes (41). Recent studies have shown that p60src forms a stable association with the tyrosine-phosphorylated form of p125FAK through its SH2 domain, suggesting a role for p125FAK in signal transduction (42). Furthermore, increased levels of p125FAK were found to accompany changes in epithelial and mesenchymal tumors during their progression to an invasive phenotype (43). The convergence of these various properties of p125FAK suggests that this protein participates in a variety of cellular processes, including a diverse set of normal and abnormal functions such as cell adhesion, cell motility, and, ultimately, cell proliferation.
involve receptor transactivation. It is likely that interaction or convergence of diverse mitogenic signaling pathways is necessary for expression of the mitogenic activity of ET-1. Further investigation of this issue should clarify the functional role of ET-1 and its receptor in the regulation of cell growth and in the pathophysiology of ovarian cancer.

ACKNOWLEDGMENTS


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