Bombesin and Glucocorticoids Stimulate Human Breast Cancer Cells to Produce Endothelin, a Paracrine Mitogen for Breast Stromal Cells

Michael P. Schrey, Kirti V. Patel, and Nikolaos Tezapsidis

ABSTRACT

Human breast cancer cells have been recently reported to produce endothelin (ET) 1. To investigate the potential regulation of ET production in breast cancer cells, we have measured the release of ET-like immunoreactivity from the T47D cell line in response to various paracrine/endocrine factors. Bombesin (0.1 μM) and cortisol (1 μM) stimulated maximal respective increases in IR-ET release to 580 and 369% of basal values after 6 h. The responses to cortisol and bombesin were additive. The response to bombesin was dose dependent with a median effective dose around 1 nM and was inhibited by the receptor antagonist [Leu⁸-Cys²,CH₂NH-Leu⁴] bombesin. Pretreatment of T47D cells with pertussis toxin had no effect on bombesin-induced inositol lipid hydrolysis but inhibited ET-like immunoreactivity release in response to bombesin in the presence of glucocorticoid, by 56%. ET-1 (10 nM) and insulin-like growth factor (10 ng/ml) stimulated modest separate increases in DNA synthesis in human breast fibroblasts of 35 and 71%, respectively, but together exhibited a strong synergistic response to 905% of control values. This in vitro study demonstrates the potential for bombesin and glucocorticoid to regulate ET production in human breast cancer cells, which may in turn have a paracrine influence on neighboring stromal cell function.

INTRODUCTION

It is now apparent that the vasoactive peptide ET³ 1 originally isolated from porcine endothelial cells (1) is produced by a wide variety of nonendothelial cultured cell types, including vascular smooth muscle cells (2), renal epithelial cells (3), and normal human breast epithelial cells (4). A recent study has also reported the presence of ET-1 in the conditioned medium from several human cancer cell lines, including four mammary carcinoma cell lines (5). Although ET-receptors were not detectable in breast epithelial cells, breast stromal cells do possess ET-receptors which are coupled to an inositol lipid-specific phospholipase C (4), suggesting a possible paracrine role for this peptide in the breast.

The expression of ET-1 mRNA and the secretion of immunoreactive ET-1 has been shown to be regulated by a wide variety of hormones and regulatory polypeptides, including interleukin 1β, transforming growth factor β, and phorbol ester in renal epithelial cells (3); vasopressin and angiotensin II in vascular smooth muscle cells (2); prolactin and transforming growth factor β in normal breast epithelial cells (4).

We have previously reported activation of inositol lipid hydrolysis and intracellular Ca²⁺ mobilization by bombesin in human breast cancer cells (6). However, this stimulation of cellular signalling by bombesin was not accompanied by a mitogenic response (6). Since bombesin-like peptides induce mitogenic responses in a variety of cell types (7, 8), we have, in the present study, used the T47D breast cancer cell line to investigate the potential regulation of ET production in these cells by bombesin and various other endocrine/paracrine factors. We have also investigated the effect of ET-1 on human breast stromal fibroblast DNA synthesis to assess a potential mitogenic paracrine role for ET-1 in the breast.

MATERIALS AND METHODS

Materials. Bombesin and bombesin antagonist, IGF-1 and ET-1 were purchased from Bachem Ltd. (United Kingdom). Human prolactin was a gift from Dr. S. Lynch, Birmingham and Midland Hospital for Women, Birmingham, United Kingdom. Human epidermal growth factor was a gift from Dr. P. Scholes, ICI Pharmaceuticals, Macclesfield, United Kingdom. [*H]Jinositol (15.8 Ci/mmol) and [*H]thymidine (40-60 Ci/mmol) were purchased from Amersham International (United Kingdom). All other biochemicals and reagents were obtained from Sigma Co. (United Kingdom). Cell culture materials were purchased from Flow Laboratories (United Kingdom).

Cell Culture. T47D cells were obtained from the National Cell Bank at Porton Down, United Kingdom. MCF-7 cells were obtained from Dr. M. Lippman (National Cancer Institute, Bethesda, MD). Both cell lines were grown in EMEM containing 5% fetal calf serum and were routinely passaged as previously described (6). After reaching approximately 70% confluence, cell monolayers in 25-cm² flasks were washed in serum-free medium and were serum starved for 24 h prior to experimentation in EMEM containing 0.5% BSA. This procedure minimizes any influential effect of serum on ET production. The cells were then incubated in 2 ml of EMEM containing 0.5% BSA in the presence or absence of bombesin and/or glucocorticoids (see figure legends for details). Incubations were terminated by removal of the medium and cell numbers were determined by counting released cell nuclei as previously described (6). The release of IR-ET into the medium overlay was measured by radioimmunoassay (as described below).

Endothelin Radioimmunoassay. Endothelin was measured in 50-μl aliquots of conditioned medium overlay (see "Results" for experimental details), using an ET-1,2 radioimmunoassay kit according to the procedure detailed by the manufacturer (Amersham International). Assays were performed on unextracted aliquots of conditioned medium and standard curves were generated in the presence of equivalent volumes of the appropriate culture media. The sensitivity of the assay under these conditions, as defined by the amount of ET-1 needed to reduce zero dose binding by 2 SD, was 1.9 fmol/tube. The cross-reactivity of the antibody was determined as: ET-1, 100%; ET-2, 204%; ET-3, 0.0024%; big ET-1, 37.9%. No agents used in this study interfered with the assay at the concentrations used. The mean intraassay precision was 5.6%. All samples within the same experiment were measured in the same assay.

Identification of IR-ET by HPLC. Conditioned medium (10 ml) from dexamethasone (100 nm) treated T47D cells, grown in 75-cm² flasks, was collected after a 24-h incubation period. This medium was immediately applied to a Sep-Pak C₁₈ cartridge (Waters Associates, Milford, MA) and a 5-ml wash of water plus 0.1% TFA applied. ET was then extracted with 2 ml of 80% acetonitrile in 0.1% TFA. The sample was dried on a Speedvac vacuum centrifuge and then resuspended in 150 μl of buffer A (20% acetonitrile in 0.1% TFA). The sample was injected on a SuperPac Pep-S 5-μm column (Pharmacia; 4 x 250 mm) and eluted, at a flow rate of 1.25 ml/min, using a discontinuous gradient, starting with 100% buffer A and varying buffer B (80%...
acetonitrile in 0.1% TFA) as follows: 0–10% buffer B (0–5 min); 10–20% buffer B (5–85 min); and 20–46% buffer B (85–94 min). Fractions (1.25 ml) were collected, dried on a Speedvac vacuum centrifuge, and subsequently resuspended in 150 μl of 0.02 M borate buffer, pH 7.4, containing 15 mM sodium azide and assayed for ET. The recovery of total IR-ET from the column was 93%.

Inositol Lipid Hydrolysis. 

Preparation of Human Breast Fibroblasts. Human breast fibroblasts were prepared by a modification of methods previously described (10) from surgically removed malignant breast tumors. Tissue obtained at surgery was immediately transported to the laboratory in ice-cold phosphate-buffered saline, twice with 0.5 ml 10% trichloroacetic acid, and once with 0.5 ml methanol. The resulting precipitated material was solubilized over a 20-min period at 24°C with 0.5 M NaOH. After neutralization with 0.5 M HCl, aliquots were removed and counted for radioactivity.

Expression of Data. All values are presented as means ± SD from individual representative experiments. Statistical evaluation of the data when comparing control groups with treatment groups was by Student's t test. Comparisons between the individual means of different treatment groups were analyzed by analysis of variance followed by a t test.

RESULTS

Bombesin and Glucocorticoids Stimulate ET Production in Human Breast Cancer Cells. A serial dilution curve of extracted conditioned medium obtained from T47D cells exhibited parallelism with that of standard ET-1 in the radio-immunoassay (Fig. 1A), indicating that these cells do indeed release immunoreactive ET-1 activity. The immunoreactive profile following reverse phase HPLC of this T47D cell-conditioned medium is depicted in Fig. 1B and shows a major peak in the position of ET-1. A smaller peak in the region of ET-2 was also apparent. Basal release of IR-ET from T47D cells exhibited a temporal increase over a 24-h period with no detectable production after 2 h, the levels at 6 h and 24 h being 7.2 ± 0.4 and 46.4 ± 4.2 fmol/10⁵ cells, respectively (Fig. 2A). Both bombesin (0.1 μM) and cortisol (1 μM) stimulated IR-ET release after 6 h to 580% (P < 0.01) and 369% (P < 0.02) of basal values, respectively (Fig. 2A). The level of bombesin-stimulated release declined after 24 h, whereas that due to cortisol was maintained (Fig. 2A). Bombesin (0.1 μM) and cortisol (1 μM) together exhibited an additive effect on IR-ET release at these concentrations (Fig. 2A). Bombesin stimulated a dose-dependent increase in IR-ET release with a maximal effect between 10 and 100 nM bombesin and a median effective dose around 1 nM. Dexamethasone (0.1 μM) also stimulated IR-ET production (Fig. 2B) and demon-

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Effect of Pertussis Toxin on ET Production and Inositol Lipid Hydrolysis. Pretreatment of T47D cells with pertussis toxin increased basal IR-ET release by 34% \((P < 0.02)\), while decreasing both the individual and combined response to bombesin and dexamethasone by 51, 32, and 66%, respectively (Fig. 4A). Although pertussis toxin decreased basal inositol phosphate accumulation \((P < 0.02)\), the dose-related increase in inositol lipid hydrolysis in response to bombesin was unaffected by the toxin (Fig. 4B).

Effect of ET-1 on DNA Synthesis in Human Breast Fibroblasts. In serum-free conditions, ET-1 alone was weakly mitogenic for human breast fibroblasts causing a 35% increase in[^H]thymidine incorporation into DNA at 10 nM (Table 1). DNA synthesis was also stimulated in the presence of fetal calf serum (10%, v/v), EGF (10 ng/ml), IGF-1 (10 ng/ml), and bombesin (100 nM) to 2737, 185, 171, and 131% of control values, respectively (Table 1). Basic fibroblast growth factor was without effect (data not shown). The mitogenic response to ET-1 was greatly amplified in the presence of IGF-1 (Table 1). Thus, the observed increase in[^H]thymidine incorporation into DNA in response to the combined presence of both ET-1 and IGF-1 (8910 ± 997) was significantly potentiated 7.6-fold compared with the calculated additive effects of ET-1 plus IGF-

<table>
<thead>
<tr>
<th>HT-thymidine incorporation (cpm/well)</th>
<th>IGF-1 (10 ng/ml)</th>
<th>EGF (10 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1107 ± 181</td>
<td>1897 ± 361*</td>
</tr>
<tr>
<td>Fetal calf serum, 10%</td>
<td>30294 ± 3859A</td>
<td>2051 ± 153A</td>
</tr>
<tr>
<td>ET-1 (10 nM)</td>
<td>1496 ± 137E</td>
<td>10017 ± 997*</td>
</tr>
<tr>
<td>Bombesin (100 nM)</td>
<td>1452 ± 333F</td>
<td>2695 ± 362</td>
</tr>
<tr>
<td>ET-1 plus bombesin</td>
<td>1860 ± 371*</td>
<td>2695 ± 362</td>
</tr>
</tbody>
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[^A] \(P < 0.01\);[^B] \(P < 0.001\);[^C] \(P < 0.02\);[^D] \(P < 0.05\), compared with control values.

**Figure 2.** (A) Release of IR-ET from T47D cells as a function of time. Cells were incubated for various times in the absence (□) or presence of bombesin (0.1 μM, ■); cortisol (1.0 μM, ◊); or bombesin plus cortisol (○). Points, mean of triplicate incubations; bars, SD. (B) Dose-dependent release of IR-ET in response to bombesin in the absence (□) and presence (○) of dexamethasone (0.1 μM). Incubations were for 24 h. Points, mean of triplicate determinations, except at 1 and 10 nM bombesin in the presence of dexamethasone, where \(n = 2\); bars, SD.

**Figure 3.** Effect of various treatments on IR-ET production in human breast cancer cells. T47D cells (A–C) or MCF-7 cells (D) were incubated for 24 h in the absence (C, □) or presence of various agents, including bombesin (B, ■), dexamethasone (□), and prolactin (PRL; 200 ng/ml). (D) Effect of bombesin (0.1 μM) and dexamethasone (0.1 μM) on IR-ET production in MCF-7 cells. Columns represent mean of triplicate incubations; bars, SD. ▲, \(P < 0.05\); ▲▲, \(P < 0.01\) compared to control values by Student's t-test.

**Figure 4.** Effect of pertussis toxin on (A) IR-ET release and (B) total inositol phosphate (IP) production in T47D cells. Cells were pretreated with or without pertussis toxin (100 ng/ml) for 16 h prior to the measurement of IR-ET release during a 6-h incubation or IP production during a 1-h incubation (for details see “Materials and Methods”). (A) IR-ET release was measured in the absence (C) or presence of bombesin (BN; 0.1 μM) or dexamethasone (DX; 0.1 μM) or both. Hatched bars represent IR-ET release above respective control values. ▲, \(P < 0.02\) for stimulation by pertussis toxin (PT); ▲▲, \(P < 0.02\); ▲▲▲, \(P < 0.001\) for inhibition of the BN and DX responses in the presence of PT. (B) Dose-dependent increase in IP production in response to bombesin in cells pretreated without (C) or with pertussis toxin (□). All values represent the means ± SD, \(n = 3\).
ET-1 (Table 1). The majority of this immunoreactivity coeluted with authentic ET-1 following HPLC. Whether the peak approaching to the position of ET-2 standard is indeed authentic ET-2 is unknown.

The present demonstration of the ability of bombesin to stimulate a dose-dependent increase in IR-ET production in human breast cancer cells. This increase in IR-ET release exhibited a temporal lag between 2 and 6 h and required de novo protein synthesis, a finding consistent with regulation of ET production at the level of peptide synthesis and/or ET-mRNA expression as has been demonstrated in various other cell types (2-4). In contrast, human prolactin, which has been previously shown to cause ET release and ET-mRNA induction in normal human breast epithelial cells (4), as well as induce the expression of specific proteins in T47D cells in the presence of glucocorticosteroids (11), had no effect on IR-ET release from T47D cells in the present study. The reason for the differences in efficacy of bombesin to stimulate ET-1 production in T47D versus MCF-7 cells is unknown. In this respect both cell lines exhibit the same sensitivity and maximal responses to bombesin with regard to activation of cellular signaling (6).

The production of IR-ET by both T47D cells and MCF-7 cells was also stimulated by glucocorticosteroids, implying that induction of ET in breast cancer cells can be mediated by diverse intracellular pathways. This suggestion is further supported by the observed additivity of the separate responses to bombesin and glucocorticoid and the temporal differences in these two responses. However, a degree of synergism between dexamethasone and bombesin was evident in some experiments (Fig. 2B, Fig. 3A), suggesting some interaction between these pathways. Glucocorticoids have also been recently shown to stimulate ET release from vascular smooth muscle cells (12).

We have previously reported the activation of inositol lipid hydrolysis by bombesin in human breast cancer cells (6), whereas glucocorticoids have no effect on this pathway. Whether this signal transduction pathway is instrumental in the stimulation of ET production by bombesin in these cells is unknown. However, protein kinase C activation has been implicated in the induction of ET production in renal epithelial cells (3). Our observations on the effect of pertussis toxin on bombesin action in T47D cells are consistent with a role for a pertussis toxin-sensitive guanine nucleotide regulatory protein in the control of ET production distal to and possibly independent of inositol lipid signaling. Similarly, bombesin-stimulated inositol lipid hydrolysis in Swiss 3T3 cells is also unaffected by pertussis toxin (13), whereas later bombesin-activated events such as enhanced c-myec expression and DNA synthesis in response to bombesin both operate via a pertussis toxin-sensitive mechanism(s) in these cells (14).

The levels of stimulated IR-ET production in T47D cells in the present study (see Fig. 2A) are sufficient to maintain a concentration of ET (approximately 85 and 225 pm after 6 and 24 h, respectively), which is well within the biologically effective range for this peptide, and which is similar to that reported in human vascular smooth muscle cells in response to vasopressin and angiotensin II (2) in normal breast epithelial cells (4), and in other human breast cancer cell lines (5).

The pathophysiological significance of our findings in relation to a possible autocrine/paracrine role for ET and bombesin-related peptides in breast cancer is open to speculation. We have recently reported the presence of both these peptides in breast cyst fluid (15). Furthermore, we have found significantly elevated breast cyst fluid levels of bombesin-related peptides in women with apocrine breast cysts (15), a group which has an increased risk of developing breast cancer. Although the function of ET produced by breast epithelial cells is unclear, a potential paracrine action on neighboring stromal and smooth muscle cells has been previously proposed (4).

Endothelin is mitogenic for certain cultured cell types, including Swiss 3T3 cells (16), Rat-1 fibroblasts (17), and vascular smooth muscle cells (18). In this respect ET could potentially act as an angiogenic factor in breast tumors. In the present study, ET-1 (10 nm) alone caused a modest increase in DNA synthesis in human breast fibroblasts. However, ET-1 elicited a greatly enhanced mitogenic response in the presence of IGF-1. This synergism with ET-1 was specific for IGF-1 and was not observed in the presence of fibroblast growth factor, EGF, or bombesin. Synergistic effects on DNA synthesis between regulatory peptides and polypeptide growth factors are now well established (19). Indeed, ET-1 also potentiates DNA synthesis in Swiss 3T3 cells in the presence of EGF, fibroblast growth factor, bombesin (16), and IGF-1 (20). Although the specific mechanisms underlying this synergism are unknown, they presumably comprise the interaction of diverse mitogenic signaling pathways used by different competence and progression growth factors.

Human fibroblasts produce IGF-1 (21) and indeed, human breast stromal cells express IGF-1 mRNA (22). IGF-1 may thus function as an autocrine mitogen as well as having a paracrine effect on adjacent breast epithelial cells. In the present study we have shown that breast cancer cells can be stimulated to produce levels of ET-1 which are potentially mitogenic for breast stromal cells in the presence of IGF-1. Extrapolation of these in vitro findings to the tumor in vivo situation must obviously be exercised with caution. Nonetheless, this mitogenic action of ET-1 on breast fibroblasts may partly contribute to the marked desmoplastic response characteristic of human breast tumors.

Considerable evidence indicates that the mammary stroma exercises a paracrine influence on the neighboring epithelial cell population (for review see Ref. 23). Thus the presence of fibroblasts augments the estrogenic response of normal murine mammary epithelial cell growth in vitro (24, 25) as well as MCF-7 cell growth in vivo (26). The mechanisms mediating such effects are unknown. However, in addition to secreting paracrine mitogens such as IGF-1, breast stromal cells also secrete an extracellular matrix which may also have an environmental influence on breast epithelial cell function (23). Hence, although human breast epithelial cells appear not to possess receptors for ET-1 (4), ET-1 could indirectly affect breast cancer cell growth consequent to its action on breast stromal cell growth and/or function.

M. P. Schrey, K. V. Patel, and T. Tezapsidis, unpublished observations.
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