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Endothelin Stimulates *sis*-Inducing Factor-like DNA Binding Activity in CHO-K1 Cells Expressing ET_A Receptors

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ET-1, a member of the family of peptides known as endothelins, binds to a G-protein-coupled receptor, ET_A, and stimulates a variety of cellular responses, including contraction, growth, and mitogenesis. ET-1 stimulation of a chinese hamster ovary cell line stably transfected with the ET_A receptor (CHO/ET_A) induced formation of SIF (*sis*-inducing factor), a key component of the STAT (*S*ignal *T*ransducers and *A*ctivators of *T*ranscription) pathway, in a concentration-dependent manner. SIF induction was blocked by a specific inhibitor of ET_A, BQ610, and by genistein, a tyrosine kinase inhibitor. This report demonstrates that ET-1 stimulates the STAT pathway of signal transduction through a G-protein-coupled receptor, ET_A, in this stably transfected cell line.

Endothelins are a family of peptides that were first identified by their powerful vasoconstrictor activity [1]. They have since been shown to act as growth factors in a number of different tissues [2–4]. ET-1, one of three related endothelin peptides (ET-1, ET-2, and ET-3), is produced by the cleavage of a precursor protein, big ET-1, by an endothelin converting enzyme. The active 21 amino acid ET-1 peptide binds to a G-protein-coupled receptor, which has been cloned and found to be present in at least 3 subtypes (ET_A, ET_B, and ET_C) [5–7]. In several cell types, ET-1 binding to ET_A rapidly stimulates phospholipase C, through a G-protein intermediate, to hydrolyze phosphatidylinositol (4,5)-bisphosphate, generating inositol phosphates and diacylglycerol [3,8,9]. Inositol phosphates and diacylglycerol both ultimately lead to increased concentrations of intracellular Ca^{2+}, which are responsible for many of the short-term cellular responses to endothelin, such as vascular smooth muscle contraction. ET-1 binding to the ET_A receptor also results in stimulation of the mitogen-activated protein kinase (MAPK) signaling cascade in cardiac myocytes [10], which may play a role in long term responses to endothelin, such as cell hypertrophy.

A number of cytokines and growth factors have recently been shown to transduce signals from the receptor directly to the nucleus by way of the STAT pathway (*S*ignal *T*ransducers and *A*ctivators of *T*ranscription) [11–19]. In this pathway, receptor binding leads to the tyrosine phosphorylation of proteins in the cytoplasm, which translocate to the nucleus and act as transcription factors. A number of STAT proteins that are tyrosine phosphorylated in response to stimulation have been identified, including p91 (Stat1α/Stat91), p84 (Stat 1β/Stat84), p92(Stat3/Stat92) [17] and others [20,21]. The phosphorylated proteins associate to form *sis*-inducing factor (SIF) in the cytoplasm, which then translocates to the nucleus and interacts with *sis*-inducing elements (SIE) in the promoter regions of a number of different genes [13,14,17]. The STAT signal transduction pathway provides a direct link between cell surface receptors and target genes in the nucleus. It has recently been shown that receptor binding of angiotensin II, a peptide hormone that acts through a G-protein-coupled receptor (like endothelin), stimulates the STAT pathway in cardiac fibroblasts [22] and in rat aortic smooth muscle cells [23].

To test whether ET-1 binding also stimulated the STAT pathway, we used a chinese hamster

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ovary cell line (CHO-K1) stably transfected with the ET_A receptor [24]. Endothelin stimulated that STAT pathway in this stably transfected cell line, suggesting that this signal transduction pathway may be involved in endothelin-induced growth responses.

MATERIALS AND METHODS

Cell culture. Chinese hamster ovary cells (CHO-K1) stably transfected with the ET_A receptor (CHO/ET_A) were kindly provided by Dr. Patricia Rose [24]. The CHO/ET_A cells were maintained in media containing α-Minimum Essential Media (α-MEM), 10% fetal bovine serum, 1% antibiotic/antimycotic, and 200 μg/ml G418 (geneticin) (all components from Gibco/BRL). The cells were serum-starved for 12 to 16 hours before treatment by changing the media to F-12 Nutrient Mixture (Gibco/BRL). Cells were treated with endothelin-1 (human, porcine sequence; synthetic; Sigma), and in some experiments, cells were pretreated with the endothelin receptor antagonist BQ 610 (Peninsula Laboratories), the tyrosine kinase inhibitor genistein (Biomol Research Lab, Inc), or cycloheximide (CHX; Sigma).

Nuclear extracts. Following treatment, cells were harvested and nuclear extracts prepared as previously reported [22]. Cultures of cells were rinsed in cold phosphate-buffered saline, and then harvested in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol), containing protease and phosphatase inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM NaF). The cell suspensions were incubated for 10 min on ice, sedimented at 1,000 × g for 10 min, the pellets resuspended in the same buffer, Dounce homogenized, and sedimented in a microfuge (13,500 × g) for 3 min. The pelleted nuclei were incubated for 30 min at 4°C in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (see above), and sedimented in a microfuge for 5 min. The supernatant was designated as the nuclear extract. The extracts were dialyzed against low salt buffer (20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA) containing protease and phosphatase inhibitors (see above) for 4 h, protein concentration determined by Bradford assay [25], and stored at −80°C.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays were performed as described earlier [26] with some modifications [22]. Five μg of nuclear extract protein was incubated with 1 μg of poly (dI-dC) poly (dI-dC) in 20 μl of 10 mM HEPES, pH 7.9, 50 mM NaCl, 1mM EDTA, 10% glycerol, for 20 min, at 25°C. The samples were incubated with 1 or 2 fmol of radiolabeled probes (~5000 cpm) for 10 min, at 25°C. The sequence of the probe used in this study has been reported previously [27](SIE-DNA, 5′-CAGTTCCCGTCAATC-3′). Binding reactions were resolved by polyacrylamide gel electrophoresis (4% native gel) containing 0.5 × Tris borate EDTA (TBE) buffer (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA). Gels were run at 150 V in a cold room (4–8°C) for 2–3 h in 0.5 × TBE buffer, dried, and exposed to x-ray film for 12–24 h.

RESULTS

To determine whether ET-1 binding stimulated the formation of SIF complexes, we treated CHO/ET_A cells with various concentrations of ET-1, and using nuclear extracts from treated and control cells, determined whether complexes formed between proteins in the nuclear extracts and ^{32}P-labeled SIE probe molecules. The formation of protein-DNA complexes was determined using an electrophoretic mobility shift assay (EMSA). Fig. 1 shows the concentration dependence of the SIF response to ET-1. The induction of SIF binding factor was half maximal at 10 nM ET-1, which is consistent with the Kd of the ET_A receptor [28].

The time course for the ET-1-induced SIF response was also determined. Fig. 2 shows that maximal induction of SIF occurred after 2 h of stimulation with ET-1, and that the level of stimulation remained high after 6 h of exposure to ET-1. In order to determine if the SIF induction in response to ET-1 was specific, we pretreated the cells with an ET_A receptor antagonist, BQ 610. As the results in Fig. 3 show, pretreatment with BQ 610 inhibited the stimulation of SIF activity by ET-1, demonstrating the specificity of the SIF response to ET-1. The tyrosine kinase inhibitor genistein also inhibited the SIF induction by ET-1 (Fig. 3). Cycloheximide pretreatment had no effect on the ET-1 induction of SIF, indicating that protein synthesis was not necessary for the response to occur (Fig. 3).

DISCUSSION

The mechanisms by which ligand binding to a receptor on the cell surface triggers specific cellular responses has been an area of intense research. A recent addition to the signal transduction pathways recognized in cells has been the STAT signaling system [11–19]. This pathway is unique

among known signal transduction pathways in that it provides a direct link between a cell surface receptor and target genes in the nucleus. The STAT pathway was first described in conjunction with cytokine and growth factor receptors. However, recent reports have shown that angiotensin II, acting through a G-protein-coupled receptor, also stimulates the STAT pathway [22,23].

Endothelin is a vasoactive peptide that has been shown to have mitogenic activity [2–4]. Since endothelin also binds to a G-protein-coupled receptor, we determined whether it stimulated the STAT signal transduction pathway. Our results show that in the stably transfected cell line utilized in this study (CHO/ET_A), ET-1 activated the STAT signal transduction pathway. ET-1 stimulated the formation of a SIF complex, the level of which was dependent on the concentration of ET-1, with maximal stimulation at 10^{-8} M (Fig. 1). The concentration of ET-1 required for half-maximal stimulation of SIF was similar to the Kd previously reported for the ET_A receptor [28]. IL-6 also stimulated an increase in the formation of SIF in these cells, which comigrated with the SIF induced by ET-1 (data not shown). Since the SIF induced by low concentrations of IL-6 has been shown to be SIF complex A [14], this suggests that ET-1 also induces predominantly SIF complex A.

A timecourse of the SIF response to ET-1 showed that maximal stimulation occurred 1 to 2 hours after addition of ET-1 (Fig. 2), as opposed to the rapid response to PDGF and other growth factors, where maximal stimulation of the STAT pathway occurs within minutes after addition of the factor. To test whether the delay in SIF response to ET-1 is caused by a need for protein synthesis, cells were pretreated with cycloheximide. This pretreatment did not inhibit the ET-1-induced SIF response (Fig. 3), indicating that protein synthesis is not required for ET-1 to stimulate SIF activity. Angiotensin II also stimulates the SIF response in this delayed manner, suggesting that this timecourse of induction of SIF may be characteristic of G-protein-coupled receptors [22].

The SIF response by ET-1 was blocked by the ET_A receptor antagonist BQ610 (Fig. 3), demonstrating that the SIF response was specific for the ET_A receptor. SIF formation by ET-1 required the action of a tyrosine kinase stimulated by the ET_A receptor, since genistein blocked the SIF complex formation (Fig. 3). In this aspect, SIF activated by ET-1 is similar to the previously described STAT pathways stimulated by cytokines and growth factors [18,19].

Recent work has shown that AII stimulation of the STAT pathway is mediated through a member of the Janus kinase family of soluble tyrosine kinases, JAK2 [23]. In addition to increased kinase activity, JAK2 was also rapidly phosphorylated on tyrosine residues following AII stimulation. JAK2 also coprecipitated with the AII receptor (AT_1) following AII binding, suggesting that the enzyme physically associates with the G-protein-coupled receptor upon ligand binding. It was

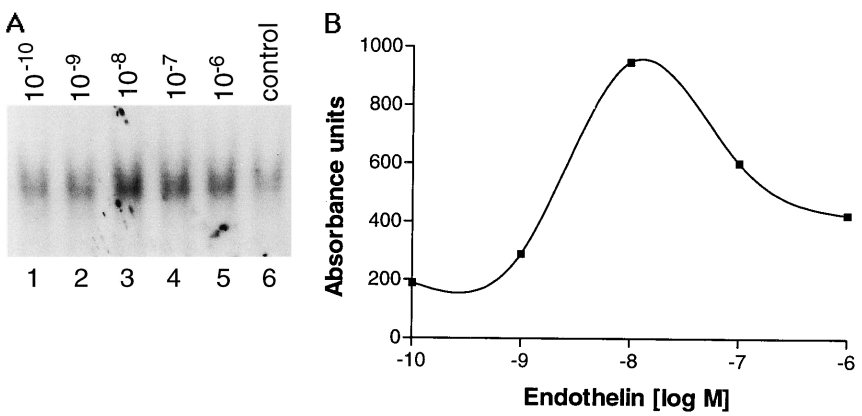

FIG. 1. Concentration dependence of ET-1-induced stimulation of SIF in CHO/ET_A cell nuclear extracts. **A.** Cells were treated with various concentrations of ET-1 for 2 h (lanes 1–5), or vehicle control (lane 6). **B.** Graphic representation of data in A. The intensities of the complexes in A were densitometrically scanned and the absorbance units plotted against the respective ET-1 concentration. Similar results were obtained in a replicate experiment.

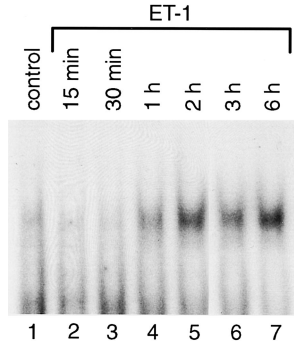

FIG. 2. Timecourse for ET-1-induced stimulation of SIF in CHO/ET_A cell nuclear extracts. Cells were treated with 10^{-7} M ET-1 for the times indicated. Similar results were obtained in a replicate experiment.

suggested that association between the ligand-bound receptor and JAK2 resulted in JAK2 phosphorylation and activation, with JAK2 subsequently phosphorylating STAT proteins, which then translocate to the nucleus [23]. Like AT_1, ET_A may couple to the STAT pathway through a similar mechanism, since it is also a G-protein-coupled receptor.

In conclusion, we have shown that endothelin can stimulate SIF complex formation in this stably transfected cell line. The STAT pathway may be responsible for the induction of genes involved in the mitogenic responses to ET-1, since this pathway has been shown to induce mitogenic genes in response to cytokines and growth factors. In addition, the similarities between the timecourse of SIF responses to angiotensin II and endothelin suggest that the delayed induction of SIF may be a general characteristic of G-protein-coupled receptors.

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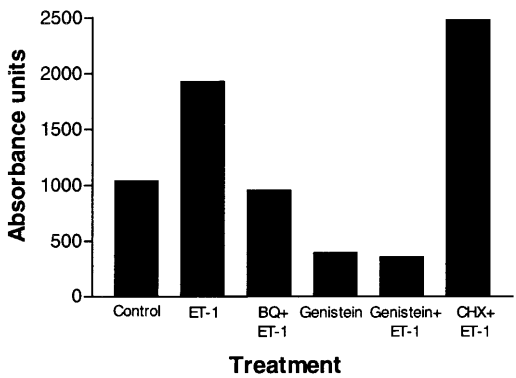

FIG. 3. Effects of pretreatments on ET-1-induced stimulation of SIF in CHO/ET_A cell nuclear extracts. The intensities of SIF complexes were densitometrically scanned and the absorbance units plotted. ET-1 treatment was at a concentration of 10^{-7} M for 2 h. BQ (BQ610) pretreatment was at a final concentration of 10^{-5} M for 15 min, followed by treatment with 10^{-7} M ET-1 for 2 h. Genistein pretreatment was at a final concentration of 100 μg/ml for 15 min, followed by treatment with 10^{-7} M ET-1 for 2 h. CHX (cycloheximide) pretreatment was at a final concentration of 2 μg/ml for 5 min, followed by treatment with 10^{-7} M ET-1 for 2 h. Similar results were obtained in a replicate experiment.

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