Published in final edited form as:

J Steroid Biochem Mol Biol. 2006 December ; 102(1-5): 79–88. doi:10.1016/j.jsbmb.2006.09.026.

Mechanisms of endothelin-1-induced MAP kinase activation in adrenal glomerulosa cells

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Abstract

G protein-coupled receptors (GPCRs) such as angiotensin II, bradykinin and endothelin-1 (ET-1) are critically involved in the regulation of adrenal function, including aldosterone production from zona glomerulosa cells. Whereas substantial data are available on the signaling mechanisms of ET-1 in cardiovascular tissues, such information in adrenal glomerulosa cells is lacking. Bovine adrenal glomerulosa (BAG) cells express receptors for endothelin-1 (ET-1) and their stimulation caused phosphorylation of Src (at Tyr416), proline-rich tyrosine kinase (Pyk2 at Tyr402), extracellularly regulated signal kinases (ERK1/2), and their dependent proteins, p90 ribosomal S6 kinase (RSK-1) and CREB. ET-1 elicited these responses predominantly through activation of a G_i -linked cascade with a minor contribution from the G_q /PKC pathway. Whereas selective inhibition of EGF-R kinase with AG1478 caused complete inhibition of EGF-induced ERK/RSK-1/CREB activation, it caused only partial reduction (30–40%) of such ET-1-induced responses. Consistent with this, inhibition of matrix metalloproteinases (MMPs) with GM6001 reduced ERK1/2 activation by ET-1, consistent with partial involvement of the MMP-dependent EGF-R activation in this cascade. Activation of ERK/RSK-1/CREB by both ET-1 and EGF was abolished by inhibition of Src, indicating its central role in ET-1 signaling in BAG cells. Moreover, the signaling characteristics of ET-1 in cultured BAG cells closely resembled those observed in clonal adrenocortical H295R cells. The ET-1-induced proliferation of BAG and H295R cells was much smaller than that induced by Ang II or FGF. These data demonstrate that ET-1 causes ERK/RSK-1/CREB phosphorylation predominantly through activation of G_i and Src, with a minor contribution from MMP-dependent EGF-R transactivation.

Keywords

Endothelin-1; adrenal glomerulosa cells; EGF; MAP kinase; Src kinase; CREB; RSK-1

Introduction

Aldosterone, the mineralocorticoid synthesized and secreted by adrenal zona glomerulosa cells, is an essential regulator of sodium/potassium and fluid balance and acid-base homeostasis. It regulates epithelial fluid and electrolyte excretion, and thus blood volume and pressure (28). In addition to such physiological actions, aldosterone has deleterious

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effects on cardiovascular function (28). Aldosterone secretion by glomerulosa cells is stimulated by extracellular K^+ , angiotensin II (Ang II), corticotrophin and other G protein-coupled receptors (GPCRs), such as endothelin-1 (ET-1), as well as receptor tyrosine kinase (RTK) agonists. The role of GPCRs in the modulation of aldosterone synthesis has been extensively studied, and RTK activation by epidermal growth factor (EGF) has been shown to enhance the stimulatory actions of GPCRs (19, 23). However, little information is available on the signaling pathways involved in the activation of mitogen-activated protein kinases (MAPKs) by GPCRs and RTKs in adrenal glomerulosa cells. Moreover, the extent to which cross-communication between GPCRs and RTKs occurs in these cells has not been established (9).

MAPKs and their target proteins (e.g., RSK-1) and transcription factors such as the cAMP response element binding protein (CREB) are involved in cell survival, growth, secretion, chemotaxis, motility and memory. In glomerulosa cells, MAPKs participate in the regulation of growth and proliferation of glomerulosa cells by activation of GPCRs and RTKs (43). Cells utilize a wide variety of pathways in transducing signals from plasma membrane receptors to MAPKs, especially the extracellularly regulated signal kinases 1 and 2 (ERK1/2). Recent studies have revealed that activation of MAPKs by external stimuli, such as GPCR agonists, cytokines, growth hormones, steroids and environmental stresses, often occurs through transactivation of RTKs, in particular the EGF receptor (35, 49). While the mechanism of ERK1/2 activation by RTKs is well defined, the steps leading from GPCR stimulation to RTK tyrosine phosphorylation are only partially understood (49).

ET-1 receptors (ET_A and ET_B) are expressed in many tissues including the heart, blood vessels, adrenal cortex, and brain. ET-1 exerts multiple biologic effects that include vasoconstriction, hypertension, cardiac hypertrophy and fibrosis and inflammation. Its proinflammatory responses in the heart are produced by recruiting leukocytes, stimulating production of adhesion molecules, and inducing cytokine expression (46). Moreover, the levels of ET-1 are increased in patients with pulmonary arterial hypertension (51). ET-1 promotes prostaglandin E₂-dependent expression of vascular endothelial growth factor, leading to proangiogenic and invasive responses of ovarian carcinoma cells (44). It also stimulates the secretion of aldosterone from adrenal glomerulosa cells and potentiates the effects of other secretagogues such as adrenocorticotrophin releasing hormone (ACTH) and angiotensin II (2, 4, 14, 22).

There is substantial evidence that GPCR-mediated MAPK activation is often dependent on transactivation of the EGF receptor (6, 15, 26). However, ET-1-induced phosphorylation of ERK1/2 can be both dependent (6, 15, 26) and independent (27) of EGF-R activation in a cell-type specific manner. In VSMCs, EGF-R activation has a major role in the cell proliferation induced by ET-1 (16). ET-1 receptors are expressed in bovine adrenal glomerulosa (BAG) cells, but their signaling mechanisms therein have not been investigated. We observed that ET-1-induced phosphorylation of ERK1/2, RSK-1 and CREB in BAG cells is predominantly mediated by activation of Src and occurs through activation of both G_q/PKC- and G_i-linked pathways, with the partial involvement of MMP-dependent transactivation of the EGF-R.

Materials and Methods

Chemicals

ET-1, PKC inhibitors, PP2, wortmannin, LY294202, CRM197, AG1478, GM6001, pertussis toxin, anti-phospho-EGF receptor (Y845 and Y1173), and anti-phospho-Pyk2 (Y402) antibodies were purchased from Calbiochem (San Diego, CA); human recombinant EGF was from Invitrogen Life Technologies (Carlsbad, CA) or Biosource Int. (Camarillo, CA).

DMEM, Medium 199 (M199); donor horse serum (DHS), fetal bovine serum (FBS), and antibiotic solutions were from Invitrogen Life Technologies. Recombinant HB-EGF and anti-HB-EGF antibody were from R & D Systems, Inc (Minneapolis, MN). A dominant negative EGF-R plasmid was provided by Dr. Yosef Yarden, PKC isoform mutants by Dr. J-W. Soh, Columbia University, NY (41) and Csk and dominant negative Pyk2 by Dr. Zvi Naor, Tel Aviv University. Dominant negative H-Ras (S17N) and anti-phospho-Src (Y416) antibody were from Upstate Biotechnology (Lake Placid, NY). Antibodies to phospho-RSK1, RSK-1, actin, EGF receptor and Src were from Santa Cruz Biotechnology (Santa Cruz, CA), or Cell Signaling Technology (Beverly, MA). Pyk2 antibodies were from BD Biosciences (San Jose, CA) or Cell Signaling Technology. Anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-CREB (Ser133) and anti-ERK1/2 antibodies were from Cell Signaling Technology. Secondary antibodies conjugated to horse-radish peroxidase were from Kirkegaard and Perry Laboratories (Gaithersburg, MD), and ECL reagents were from Pierce (Rockford, IL). All other reagents were purchased from Sigma.

Cell culture

Primary cultures of adrenal glomerulosa cells were prepared from bovine adrenal glands as previously described (45). Cells were cultured in 6-well plates in DMEM containing 10% (v/v) donor horse serum, 2% fetal bovine serum (FBS), 100 µg/ml streptomycin, 100 IU/ml penicillin, 5 µg/ml fungizone, and 25 µg/ml gentamycin. Cells cultured in a humidified atmosphere of 5% CO₂ in air at 37 °C for 2-3 days formed confluent monolayers, and were rendered quiescent by withdrawal of serum for 24 h before use. After stimulation for the time periods indicated in the individual experiments, cells were washed with ice-cold PBS, lysed with Laemmli buffer, and frozen at -70 °C before analysis.

Cell Transfections

Cells were transfected with plasmid DNA using Nucleofector (Amaxa Biosystems) using specific Nucleofector Solutions and applying specific electrical parameters optimized for epithelial cells. Cells were grown in 75 cm flasks, washed with sterile HBS and collected after trypsinization in DMEM. After centrifuging at 1000 rpm for 10 min, the supernatant was discarded and the cell pellet was resuspended in Basic Nucleofector Solution (Amaxa Biosystems) to a final concentration of 10⁶ cells/100 µl and mixed with 1 µg of DNA. The nucleofection sample was transferred to cuvette and treated in the nucleofector using an appropriate program. After transfection, cells were transferred to 6-well plates for 2 days and incubated in serum-free medium for 16 h before experimental treatments.

Immunoprecipitation

After treatment with inhibitors and drugs, cells were placed on ice and washed twice with ice-cold PBS, then lysed in RIPA lysis buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM Na-pyrophosphate, 5 mM EDTA, 1% NP-40, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml pepstatin and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and probe-sonicated (Sonifier Cell Disruptor). Solubilized lysates were clarified by centrifugation at 8000 g for 10 minutes, precleared with agarose, and then incubated with specific antibodies and protein A or G agarose. The immunoprecipitates were collected, washed four times with lysis buffer, and dissolved in Laemmli buffer. After heating at 95 °C for 5 minutes, the samples were centrifuged briefly and the supernatants were analyzed by SDS-PAGE on 8-16% gradient gels.

Immunoblot analysis

Cells were grown in 6-well plates and at 60-70% confluence were serum-starved for 24 h before treatment at 37 °C with selected agents. The media were then aspirated and the cells were washed twice with ice-cold PBS and lysed in 100 µl Laemmli sample buffer. The samples were briefly sonicated, heated at 95 °C for 5 minutes, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8-16%) gradient gels and transferred to PDVF membranes. Blots were incubated overnight at 4 °C with primary antibodies and washed 3 times with TBST before probing with horseradish peroxidase-conjugated secondary antibodies for one hour at room temperature. Blots were then visualized with ECL (enhanced chemiluminescence reagent; Amersham Pharmacia or Pierce) and quantitated with a scanning laser densitometer. In some cases, blots were stripped and reprobed with other antibodies.

Ras activation assay

The active form of Ras was determined using the EZ-Detect™ Ras Activation Kit (Pierce). The active GTP-bound Ras was extracted from cell lysates with the GST/Raf1/Ras-binding domain (RBD), and the pulled-down active Ras was detected by Western blot analysis using anti-Ras antibody.

Cell Proliferation assay

Cell proliferation was measured by addition of CellTiter 96^R AQueous One Solution Reagent (Promega, Corp.), which contains a tetrazolium compound [MTS^(a)-tetrazolium] and an electron coupling reagent [phenazine ethosulphate; PES]. Briefly, cells were grown in 96-well microtiter plates and changed to serum-free media at 50-60% confluence. Inhibitors were added 30 min before the addition of agonists and left for 24 h. The CellTiter 96^R AQueous reagent (20 µl) was added and plates were kept in a humidified incubator at 37 °C for 2-3 h, then absorbance was measured at 490 nm.

Results

Stimulation of BAG cells with ET-1 caused rapid phosphorylation of ERK1/2 with a maximum effect at 5 min and a decline after 15 min (Fig. 1A). Agonist activation of the endogenous EGF receptors in BAG cells also caused rapid phosphorylation of ERK1/2 (Fig. 1B). In many cell types, the growth-promoting effects of GPCRs are primarily mediated by transactivation of the EGF-R, with subsequent activation of signaling pathways such as Ras/Raf/MEK/ERK cascade and PI3K/Akt (35). However, no information is available on the role of EGF-R in ET-1 signaling in adrenal glomerulosa cells. The selective EGF-R kinase antagonist, AG1478, completely abolished EGF-induced activation of ERK1/2 and its dependent protein p90 ribosomal S6 kinase (RSK-1), but only partially (40%) inhibited ET-1-induced responses (Fig. 2 A, B). These findings indicate that the stimulatory effects of ET-1 on ERK1/2 and RSK-1 phosphorylation in BAG cells are largely independent of EGF-R transactivation. GPCR-mediated transactivation of the EGF-R is generally mediated by metalloproteinase (MMP)-dependent release of heparin-binding EGF (HB-EGF) or other soluble ligands which bind to and activate the EGF-R, culminating in activation of the ERK1/2 cascade (26, 35). Blockade of MMP action by the broad-spectrum inhibitor, GM6001, had a partial (35-40%) inhibitory effect on ET-1-induced ERK1/2 activation, but had no effects on EGF-induced responses (Fig. 3A, B). Consistent with this, the selective HB-EGF antagonist, CRM, and a neutralizing antibody against HB-EGF, also had partial inhibitory effects on ERK1/2 phosphorylation by ET-1, but not on EGF-induced responses. These data indicate that ERK1/2 phosphorylation induced by ET-1 is partly mediated by activation of MMP-dependent generation of HB-EGF in glomerulosa cells (Fig. 3C).

We next determined the upstream signaling molecules involved in ET-1-induced ERK1/2 activation. Blockade of G_i with pertussis toxin (PTX; 100 ng/ml for 16 h) significantly diminished ET-1-induced activation of ERK1/2 by 70%. However, PKC inhibition by Go6983 (1 μ M) caused a partial decrease (40%) in ET-1 responses. Simultaneous blockade of G_i and PKC completely abolished ET-1 responses in BAG cells (Fig. 4A). In contrast, stimulation of BAG cells with PMA (200 nM) caused marked phosphorylation of ERK1/2 and RSK-1 that was abolished by PKC inhibition, but not by inhibition of G_i with PTX (Fig. 4B). These data suggest that although G_i activation is critical in transducing ET-1 receptor signaling, a significant component of PKC-mediated signaling also contributes to this process.

The effects of ET-1 are mediated primarily through its interaction with two major GPCR types, ET_A and ET_B , which interact with G_q /PKC, G_s and G_i , and phospholipase A_2 cascades in a tissue and species-specific manner (7, 42). To examine the relative contribution of these receptor types in ET-1 signaling, we pretreated cells with the selective ET_A receptor antagonist, BQ610, and the ET_B antagonist, BQ788 prior to stimulation with ET-1. The results shown in Fig. 4C demonstrate that blockade of ET_A and ET_B impaired ET-1 responses in BAG cells. These data demonstrate that ET-1 causes ERK1/2 phosphorylation through two distinct and parallel signaling cascades comprising of G_q /PKC and G_i in BAG cells.

Previous studies have shown an important role of non-receptor tyrosine kinases, such as Src and proline-rich tyrosine kinase (Pyk2), during GPCR-mediated ERK 1/2 signaling in various cell types (34). An examination of the roles of these signaling proteins in BAG cells revealed that ET-1 caused phosphorylation of Src at Tyr416 and Pyk2 at Tyr 402. The selective Src inhibitor, PP2, blocked the stimulatory effects of ET-1 on phosphorylation of Src and Pyk2 as well as ERK1/2 (Fig. 5A, B), confirming the critical role of Src during ET-1 signaling in these cell.

Activation of G_i is known to mediate downstream signals through activation of phosphoinositide 3-kinase (PI3K) in specific cell types (10, 13, 52). The extent to which ET-1 utilizes the PI3K/Akt cascade in BAG cells is not known. ET-1 caused phosphorylation of Akt at Ser473 as early as 2 min which was significantly attenuated by inhibition of Src by PP2 (Fig. 6A). Moreover, treatment of BAG cells with varying concentrations of wortmannin, PI3K inhibitor, had no significant effects on ERK1/2 activation by ET-1, but completely abolished the phosphorylation of Akt at Ser473 (Fig. 6B). These data indicate a critical role of Src in the activation of ERK1/2 as well as PI3K/Akt, however, the latter pathway does not contribute to agonist-induced ERK1/2 activation. Substantial data indicate that ET-1 enhances DNA synthesis and cell proliferation in cardiovascular tissues (16, 51), adrenal cortex of rat (22) and humans (29). An analysis of the data revealed that ET-1-induced proliferation of BAG cells was less effective (15%) than that elicited by EGF (30%).

In general, GPCRs cause ERK1/2 activation through recruitment of adaptor molecules such as Shc, Grb and Sos, which cause activation of Ras/Raf/MEK/ERK cascade. However, ERK1/2 phosphorylation can also be independent of Ras activation. To determine if ET-1-induced responses are mediated through activation of Ras, we measured ERK1/2 activation in BAG cells overexpressing dominant negative Ras (dnRas; S17N). As shown in Fig. 7A, dnRas markedly reduced ET-1 and EGF-stimulated phosphorylation of ERK1/2, indicating that both ET-1 and EGF exert their effects in BAG cells largely through a Ras-dependent pathway. An analysis of the mechanism of Ras activation indicates that ET-1-induced Ras activation is primarily dependent on activation of G_i and Src, but is largely independent of

MMP/EGF-R activation (Fig. 7B). These data suggest that ET-1 elicits its effects predominantly through sequential activation of G_i , Src and Ras in BAG cells.

Our results show that ET-1 causes activation of ERK1/2 and RSK-1. Both of these signaling molecules are known to translocate to the nucleus to mediate transcriptional changes in target proteins such as CREB (17, 37). We next determined whether ET-1 signaling results in activation of CREB in BAG cells. As shown in Fig. 7C, ET-1 stimulation caused phosphorylation of CREB at Ser133, and this was significantly attenuated by inhibition of G_i , Src and MEK by PTX, PP2 and U0126, respectively. However, inhibition of EGF-R by AG1478 had minor effects.

Discussion

Bovine adrenal cells, which express several GPCRs (including those for LPA, Ang II, bradykinin, and ET-1), as well as RTKs for EGF and FGF (9, 43), provide a physiological model for investigation of the mechanism(s) of cross-communication between these receptors during ERK1/2 activation. One of the major mechanisms mediating cross-communication between GPCRs and RTKs is the activation of MMPs that cause release of several ligands such as heparin binding-EGF (HB-EGF), amphiregulins, transforming growth factor- α (TGF- α), fibroblast growth factor (FGF) and tumor necrosis factor- α (TNF- α), which bind to and activate the EGF-R (26, 32, 35, 38). The ADAM family of MPs has been implicated in the transactivation of EGF-R and cell proliferation by GPCR agonists such as Ang II, ET-1, phenylephrine, bombesin and LPA in cardiovascular and other tissues (3, 12, 31, 36, 38, 50). Much of this work has been performed in cancer cells and cell lines, and the sparsity of information in primary native cells raises a question about the physiological relevance of these observations. It has been shown that signaling pathways may be altered in cell lines and transformed cells, and do not necessarily reflect the intrinsic biochemical mechanisms of native cells.

Our data in cultured BAG cells show that ERK1/2 phosphorylation by both ET-1 and EGF is primarily mediated by activation of Src kinase, which has been implicated in agonist-induced induction of MMPs and subsequent EGF-R transactivation in several cell types (11, 25, 36, 38). However, the MMP/EGF-R pathway makes only a minor contribution in transducing downstream signals of ET-1. Interestingly, the signaling characteristics of ET-1 in native BAG cells closely resemble to those observed in clonal H295R cells. These data indicate that the signals emanating from ET-1 and EGF stimulation converge on Src kinase, which causes activation of the Ras/Raf/MEK cascade. In contrast to the critical role of MMP-dependent EGF-R transactivation in numerous cell lines and tumor cells, and some cardiovascular tissues (16, 18), ET-1 signaling is largely independent of this cascade in native as well as clonal adrenal cells. The potent EGF-R antagonist, AG1478, which completely abolishes the effects of EGF, causes at most 40% inhibition of ET-1-induced ERK1/2 activation in BAG cells (Fig. 2). These and other data (38) indicate that the involvement of a multiplicity of signaling pathways during agonist-induced ERK1/2 activation is not uncommon. For example, ERK1/2 activation by bradykinin in COS-7 cells occurs via a dual signaling pathway involving the independent activation of PKC and transactivation of the EGF-R (1). In contrast, more recent studies showed that ERK1/2 activation in HEK293 cells by angiotensin II, a G_q /PKC-coupled GPCR, is independent of EGF-R transactivation (38) but is dependent on both PKC and β -arrestin2 (47).

The present studies in BAG cells demonstrate that a G_i -linked cascade is the predominant pathway mediating the effects of ET-1 on phosphorylation of Src/Pyk2 and ERK1/2, whereas activation of PKC makes only a minor contribution to ET-1 signaling. GPCRs coupled to G_i -linked cascades are known to cause activation of Src and PI3K through

interaction with released $\beta\gamma$ subunits (10, 13, 52). Our data in BAG cells showed that ET-1 causes ERK1/2 phosphorylation through G_i and Src, with the additional involvement of the EGF-R. Interestingly, EGF also exerts its effects on ERK1/2 activation through Src in BAG cells, but this is independent of PKC (33). Thus, signals emanating from ET-1 and EGF stimulation appear to converge on Src, leading to activation of the Ras/Raf/MEK/ERK cascade in BAG cells.

Src and Pyk2, the non-receptor protein tyrosine kinases, are important intermediates of signal transduction cascades, controlling pathways as diverse as cell growth, differentiation, migration, secretion and genome maintenance (21). These kinases are also involved in regulation of the cytoskeleton due to their ability to associate with various cytoskeletal proteins, including focal adhesion kinase (FAK). Pyk2 belongs to the FAK family, and is involved in cell proliferation and migration (39, 53). Src and Pyk2 are activated by GPCRs, integrins, cytokines and growth factors, and appears to be indispensable for EGF-R activation (34). An important role of Src has been observed in the modulation of aldosterone secretion. The Src inhibitor, PP2, has been shown to inhibit basal as well as agonist-mediated aldosterone production in response to stimulation with angiotensin II, K^+ and dibutyryl-cAMP in adrenocortical H295R cells, possibly through inhibition of early steps in steroidogenesis (40, 43). Our data in BAG cells indicate that ET-1 causes rapid Src-dependent phosphorylation of Pyk2 at Tyr402, and inhibition of Src attenuates ERK1/2 and CREB phosphorylation (Fig. 7). However, overexpression of dominant negative Pyk2 had no effects on ET-1 responses (data not shown), excluding its involvement in the activation of ERK1/2 and CREB. Thus, Src provides a bifurcation point during ET-1-induced activation of Pyk2 and ERK1/2.

Our results also show that ET-1 causes rapid activation of Akt in a PI3K-dependent manner (Fig. 6). Activation of EGF-R and PI3K by GPCR agonists has been implicated in cell proliferation, migration and invasion (8, 30). To evaluate the impact of ET-1 signaling on the growth of glomerulosa cells, we examined the role of PI3K/Akt in these cells. This revealed that ET-1-induced BAG cell proliferation is of lesser magnitude than that stimulated by EGF (15 vs 30%). In general, ET-1 causes substantial stimulation of proliferation of VSMCs and other cell types (16). Interestingly, earlier studies showed that ET-1 inhibits the proliferation of calf adrenal glomerulosa cells, primarily due to agonist-induced downregulation of the ET-1 receptors (5). The reason for this differential effect is not known. It is possible that the experimental conditions and the timing of the proliferation studies contribute to such differences. Our previous studies have shown that Ang II is the most potent proliferative and mitogenic GPCR agonist in BAG cells, and that Ang II-induced increase in [3 H]-thymidine incorporation is evident only between 8-11 days in cultured BAG cells (45).

The 43 kDa transcription factor, CREB, was originally thought to be solely phosphorylated by activated cAMP-dependent PKA, but, subsequent studies revealed that several stimuli, including PKC, calcium, and RTKs can also cause phosphorylation of CREB (17). Our results show that ET-1 caused phosphorylation of CREB via G_i , Src but largely independent of activation of EGF-R and PKA (Fig. 7). Phosphorylation of CREB at Ser133 is known to regulate diverse processes including neuronal signaling, memory formation, cell proliferation and apoptosis. Our results show that inhibition of G_i and Src caused marked reduction in ET-1-induced CREB phosphorylation (Fig. 7). However, inhibition of PKA by CMI had negligible effects on ET-1 responses (data not shown), thus excluding the involvement of G_s /PKA in ET-1 signaling in BAG cells.

Previous studies have shown that expression and activation of signaling molecules is altered in transformed and cancer cells. For example, MMP levels are low in normal liver, but

many-fold higher in hepatocellular carcinomas (20). Hence, MAPK activation by a GPCR agonist, angiotensin II, is dependent on MMP-mediated transactivation of the EGF-R in clonal C9 hepatic cells (38), but is independent of these proteins in native rat hepatocytes (24, 48). Similarly, PI3K/Akt activity is high in H295R cells derived from human adrenal cortical carcinoma tissue, but low in primary cultures of BAG cells (54). Since many studies on cross-communication between GPCRs and RTKs have been conducted on cell lines and transformed cells, we examined the signaling characteristics of ET-1 in clonal H295R cells. Our results show that the time-courses of the activation of ERK1/2 and RSK-1 following ET-1 and EGF stimulation were similar to those observed in BAG cells. Moreover, ET-1-induced responses were largely independent of MMP and EGF-R activation in H295R cells (B.H. Shah, unpublished results). Thus, ET-1-stimulated MAPK signaling in H295R cells closely resembles to that in native BAG cells. In summary, the present findings indicate that Src activation is essential for ET-1-induced phosphorylation of ERK1/2, RSK1 and CREB, whereas MMP-dependent EGF-R activation has a minor role in this cascade in cultured BAG cells.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute of Child Health and Human Development, NIH, Bethesda, MD.

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Abbreviations

EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
ERK1/2	extracellularly signal regulated kinases 1 and 2
GPCR	G protein-coupled receptor
PI3K	phosphoinositide 3-kinase
PMA	phorbol 12-myristate 13-acetate
Pyk2	proline-rich tyrosine kinase
RTK	receptor tyrosine kinase

Fig. 1. ET-1 and EGF cause transient activation of ERK1/2

A, Time-course of the effects of ET-1 and EGF on phosphorylation of ERK1/2. Serum-starved BAG cells were treated with ET-1 (100 nM) and EGF (20 ng/ml) for the time periods indicated, lysed in Laemmli sample buffer and analyzed by SDS-PAGE using phospho-specific antibodies against ERK1/2 (Thr202/Tyr204). The blots were stripped and reprobed with ERK1/2. ERK1/2 phosphorylation in unstimulated cells at time zero min (control) was taken as 1 and agonist-induced increases in phosphorylation were compared to control as arbitrary units (a.u.). The data shown are representative of 3 experiments.

Fig. 2. ET-1-induced ERK1/2 phosphorylation in BAG cells is partly dependent on transactivation of the EGF-R

A,B, Concentration-dependent inhibitory effects of EGF-R kinase inhibition on ERK1/2 phosphorylation by ET-1 and EGF. Cells were treated with increasing concentrations of AG1478 for 20 min before stimulation with ET-1 (100 nM) and EGF (20 ng/ml) for 5 min.

Fig. 3. Effects of metalloproteinase inhibitor, GM6001, on agonist-induced ERK1/2 activation in BAG cells

A,B, Cells were treated with increasing concentrations of GM6001 for 20 min and stimulated with ET-1 (100 nM) and EGF (20 ng/ml) for 5 min. C, Effects of HB-EGF antagonist, CRM (10 μ g/ml), on agonist-induced ERK1/2 activation.

Fig. 4. Roles of G_i and PKC in agonist-induced phosphorylation of ERK1/2

A,B, Cells were treated with G_i inhibitor, pertussis toxin (PTX ; 100 ng/ml) for 16 h, and PKC inhibitor, Go6983 (1 μ M), for 20 min followed by stimulation with ET-1 (100 nM) and PMA (100 nM) for 5 min. **C,** Effects of ET_A and ET_B antagonists on ET-1-induced ERK1/2 activation. BAG cells were treated with ET_A antagonist, BQ610 (---) and ET_B antagonist, BQ788 (---) for 20 min followed by stimulation with ET-1 for 5 minutes. Quantitation of data is shown (n=3-4).

Fig. 5. A central role of Src in agonist-induced ERK1/2 activation in BAG cells

A, Concentration-dependent inhibitory effects of Src inhibitor, PP2, on ET-1-induced phosphorylation of ERK1/2 and Src (Y416) and Pyk2 (Y402). BAG cells were treated with increasing concentrations of PP2 for 20 min and stimulated with ET-1 (100 nM for 5 min). Cells were lysed in Laemmli sample buffer and analyzed by SDS-PAGE. **C**, The quantitation of data is shown (n=3).

Fig. 6. Role of PI3K in agonist-induced phosphorylation of Akt and ERK1/2

A, Concentration-dependent effects of Src inhibition on phosphorylation of Akt at Ser473.

B, Effects of PI3K inhibition by wortmannin on phosphorylation of Akt at Ser473 and ERK1/2 by ET-1. Cells were treated with varying concentrations of wortmannin and stimulated with ET-1 (100 nM) for 5 min, then lysed in Laemmli sample buffer and analyzed by SDS-PAGE. **C**, The quantitation of data is shown (n=3).

Fig. 7. ET-1 signaling occurs through Ras activation in BAG cells

A, Overexpression of dominant negative Ras (dnRas; S17N) plasmid cDNA (1 μ g) impairs agonist-induced ERK1/2 activation. Cells were transfected with cDNA as described in Methods. B, Mechanisms involved in Ras activation by ET-1. BAG cells were treated with PTX (100 ng/ml) for 16 h, and GM6001 (20 μ M), PP2 (10 μ M), and AG1478 (100 nM) for 20 min followed by stimulation with ET-1 (100 nM) for 5 min. Agonist-stimulated Ras activation was measured as described in Methods. C, Mechanisms involved in ET-1-induced phosphorylation of CREB at Ser133 in BAG cells. BAG cells were treated with PTX (100 ng/ml) for 16 h, and PP2 (10 μ M), AG1478 (100 nM) and UO126 (1 μ M) for 20 min followed by stimulation with ET-1 (100 nM) for 5 min.