Original Article

G α_{13} Induces PreproET-1 Gene Expression *via* JNK

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The endothelin B receptor (ET_BR) has been shown to mediate autoinduction of endothelin-1 (ET-1). We previously reported that the ET_BR interacts with G₁₃, a member of the heterotrimeric GTP-binding protein family. In the present study, we examined whether G₁₃ induces preproET-1 (ppET-1) gene transcription, which could result in ET-1 autoinduction in a renal epithelial cell line. We generated a reporter gene construct under control of the ppET-1 promoter region. The construct was transiently expressed in COS-7 cells. Transient expression of ET_BR increased the promoter activity of ppET-1 following treatment with 100 nmol/l of ET-1. Expression of G_{13Q226L} or G_{13Q209L}, constitutively active forms of G₁₃ and G_q, also activated the ppET-1 promoter. ET_BR-stimulated ppET-1 promoter activity was partially diminished by the expression of dominant negative forms of c-Jun N-terminal kinase (JNK1APF) or MAPK/ERK kinase (MEK_{K97M}). Expression of JNK1APF also inhibited G_{13Q226L}-induced ppET-1 promoter activation. These findings indicate that G₁₃ can induce ppET-1 gene expression through a JNK-mediated pathway. Our results also suggest that this G₁₃-coupled signaling pathway may play an important role in a sustained ET-1 autoinduction loop in various pathophysiological conditions. (*Hypertens Res* 2002; 25: 427–432)

Key Words: G-protein, endothelin, endothelin receptor, transcription

Introduction

G α_{13} , a member of the G₁₂ family of guanine nucleotide-binding proteins (G-proteins), is ubiquitously expressed and regulates multiple complex cellular effects. G α_{13} is activated by thrombin, thromboxane, thyroid-stimulating hormone, and angiotensin II AT_{1A} receptors (1–5). In addition, we previously demonstrated that endothelin B receptor (ET_BR) activates G α_{13} (6). G α_{13} regulates Na/H exchanger activity (7, 8), extracellular signal regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK) (9–11), nuclear factor κ B (NF- κ B) (12) and the activity of the low molecular weight GTPase, Rho (13–15), and is also capable of neoplastic transformation, induction of apoptosis, and stimulation of actin re-

arrangement (9, 13, 14, 16–20). However, the details of these G α_{13} -induced cell responses have yet to be elucidated.

Endothelins (ETs) are versatile bioactive proteins that interact with two homologous G-protein coupled receptors, endothelin A receptor (ET_AR) and ET_BR. Both ET receptors are expressed in a wide variety of tissues and cultured cells. The fact that the wide distribution of both ET receptors is paralleled by a similar overlapping localization of ET production implies the potential importance of autocrine and/or paracrine functions for the ET-dependent signaling systems (21, 22). The effects of ET include inhibition of adenylyl cyclase, presumably through the activation of G α_i ; activation of phospholipase C, presumably through the activation of G_{q/11}; stimulation of tyrosine phosphorylation by an undetermined mechanism; and activation of the mitogen-activated

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protein kinases (ERK, JNK, and p38) (23, 24). Because of its vasoconstrictor and growth-promoting action, numerous studies indicate that ET-1 is involved in the pathogenesis of a broad spectrum of renal and cardiovascular diseases (21, 22, 25–27). Previous studies have demonstrated that ET-1 induced ET-1 production through ET_AR and/or ET_BR-mediated pathways, suggesting that a sustained ET-1 autocrine loop contributes to the progression of cardiovascular and renal diseases (28–32).

ET and G α_{13} share a number of effects on cells, including activation of JNK, the Na/H exchanger and Rho, and stimulation of cellular proliferation and differentiation (1, 7–11, 13–15, 21, 23, 24, 33–38). We previously demonstrated that ET_BR activates G α_{13} (6). In the present study, we have examined whether G α_{13} could activate the preproET-1 (ppET-1) gene promoter to take part in the ET-1 autoinduction loop. We show that the activated G α_{13} is able to induce ppET-1 gene expression *via* JNK dependent pathway.

Methods

Materials

COS-7 cells were obtained from the American Type Culture Collection (Rockville, USA). Tissue culture medium and fetal bovine serum (FBS) were obtained from GIBCO-BRL (Rockville, USA); ET-1 from Calbiochem (San Diego, USA); restriction and DNA modifying enzymes from GIBCO-BRL or TaKaRa (Kusatsu, Shiga, Japan); and tissue culture plastic wares from Falcon (Becton Dickinson Labware, Bedford, USA).

Cell Culture

COS-7 cells were grown in Dulbecco's modified Eagle's medium with 10% FBS in a 10% CO₂–90% air incubator. Culture medium was replaced every 3 days. Cells were passed using 0.01% ethylenediamine tetraacetic acid (EDTA)-trypsin.

Plasmids

A 2.6 kb fragment of human ET-1 promoter region (from -2456 to +168, a gift from G.A. Visner, University of Florida, Gainesville, USA) (39) was subcloned into a luciferase reporter plasmid, pGL3 basic (Promega, Madison, USA). The cDNA encoding the human ET_BR was a gift from M Yanagisawa (University of Texas Southwestern Medical Center, Dallas, USA) (40). The cDNA constructs of α_{5Q227L} , α_{2Q205L} , $\alpha_{13Q226L}$, and α_{4Q209L} were described previously (8, 41, 42). An expression construct of a dominant negative form of *c-Jun* N-terminal kinase (JNK1APF) was provided by GL Johnson (University of Colorado, Denver, USA) (43) and a cDNA coding for the dominant negative form of MAPK/ERK kinase (MEK_{K97M}) was a gift from MH Cobb

Fig. 1. Activation of ppET-1 promoter through ET_BR in COS-7 cells. COS-7 cells were transfected with a ppET-1 promoter/luciferase construct in the presence or absence of ET_BR as described in the Methods section. Twenty-four hours after transfection, ET-1 was added to the culture media to a final concentration of 100 nmol/l. After an additional 24 h incubation, cells were harvested to measure ppET-1 promoter activity by luciferase assay. Values are the fold-increase over the control and expressed as the mean $\pm$ SEM (n = 4). * p < 0.05.

(University of Texas Southwestern Medical Center, Dallas, USA). cDNAs were subcloned into pcDNA3 (Invitrogen, Carlsbad, USA). The sequence of the resulting construct was confirmed using restriction and sequencing analysis.

Transfection and Luciferase Assay

COS-7 cells (4.0×10^5 cells/well) were seeded the day before transfection in 6-well plates. The cells were transfected using the Ca-PO₄ co-precipitation method. Each transfection mixture contained 0.25 μ g of a reporter construct and 0.5 μ g of other cDNA construct(s) as indicated. The total amount of transfected DNA was adjusted to 2 μ g by adding an empty vector, pcDNA3.

After incubating the cells with DNA/Ca-PO₄ precipitates for 4–6 h at 37°C, the cells were washed three times with phosphate buffered saline, and incubated with Dulbecco's modified eagle medium (DMEM) containing 0.2% FBS. The transfected cells were harvested 48 h after transfection. Where indicated, ET-1 (100 nmol/l) were added 24 h before the harvest. Luciferase activity was measured using a Luciferase Assay System (Promega) and a TD-20/20 Luminometer (Turner Designs, Sunnyvale, USA) according to the manufacturer's instruction.

Statistical Analysis

Statistical significance was evaluated using the two-tailed, unpaired Student's *t*-test for comparisons between 2 means, or analysis of variance (ANOVA) analysis followed by the Newman-Keuls method for more than 2 means. A value of

Fig. 2. *G*-protein-mediated activation of ppET-1 promoter. COS-7 cells were transfected with a ppET-1 promoter/luciferase construct and pcDNA3 encoding $G\alpha_{13}Q226L$, $G\alpha_qQ209L$, $G\alpha_sQ227L$ or $G\alpha_{12}Q205L$. Forty-eight hours after transfection, luciferase activity was assayed. Values are the fold-increase over the control and expressed as the mean $\pm$ SEM (n = 5). *p < 0.05.

p < 0.05 was regarded as statistically significant.

Results

The ET_BR Activates ppET-1 Promoter Following ET-1 Treatment in COS-7 Cells

Previous studies have demonstrated ET_BR-mediated autoinduction of ET-1 in endothelial and mesangial cells (29–31). However, in a study of human keratinocytes, ET-1 treatment decreased ET-1 production presumably through ET_BR (44), suggesting that ET_BR-mediated induction of ET-1 may be cell type-dependent. In the current study, we examined whether activation of ET_BR induces ppET-1 gene expression in COS-7 cells. The ET_BR was transiently expressed, and ET-1 (100 nmol/l) was added to the culture medium 24 h after transfection. Transfected cells were harvested 48 h after transfection and luciferase activity was measured. As shown in Fig. 1, treatment with 100 nmol/l ET-1 increased ppET-1 transcription by approximately 2.5-fold in COS-7 cells expressing ET_BR, while expression of ET_BR itself had no significant effect on ppET-1 promoter activity. These data indicate that ET-1 is able to induce ppET-1 transcription through the activation of ET_BR in COS-7 cells.

The Effects of Constitutively Active G-Protein Subunits on ppET-1 Promoter Activity

The ET_BR is known to activate G-proteins that are members of the $G\alpha$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12}$ families (6). To investigate which G-protein α subunit family is responsible for the activation of ppET-1 gene transcription through the ET_BR, we coexpressed constitutively active forms of four α subunits, $G\alpha_{13}$ ($\alpha_{13}Q226L$), $G\alpha_q$ (α_qQ209L), $G\alpha_s$ (α_sQ227L) or $G\alpha_{12}$ ($\alpha_{12}Q205L$),

Fig. 3. Involvement of JNK- and MEK-dependent pathways in ET_BR-mediated ppET-1 promoter activation. COS-7 cells were transfected with a ppET-1 promoter/luciferase construct and ET_BR in the presence or absence of either JNK1APF or MEK_{K97M}. Twenty-four hours after transfection, cells were treated with 100 nmol/l ET-1 for 24 h. Then cells were harvested and assayed for luciferase activity. Values are the fold-increase over the control and expressed as the mean $\pm$ SEM (n = 4). *p < 0.05.

Fig. 4. Involvement of JNK in $G\alpha_{13}$ -mediated ppET-1 promoter activation. COS-7 cells were transfected with a ppET-1 promoter/luciferase construct and $G\alpha_{13}Q226L$ with or without JNK1APF. Cells were harvested 48 h after transfection and luciferase activity was measured. Values are the fold-increase over the control and expressed as the mean $\pm$ SEM (n = 6). *p < 0.05.

and the ppET-1 promoter construct in COS-7 cells. Luciferase activity was measured 48 h after transfection. Expression of $\alpha_{13}Q226L$ and α_qQ209L increased ppET-1 promoter activity with similar potency, while α_sQ227L and $\alpha_{12}Q205L$ had no effect on the ppET-1 transcription (Fig. 2). These results indicate the possibility that $G\alpha_{13}$ - and/or $G\alpha_q$ -coupled signaling systems might be involved in the ET_BR-mediated autoinduction of ET-1.

Involvement of a JNK-Dependent Signaling Pathway in the Activation of ppET-1 Promoter through ET_BR and G₁₃

Since the ET_BR activates multiple signaling pathways including JNK and MEK, we investigated the involvement of JNK- and MEK-dependent pathways in ET_BR-induced ppET-1 promoter activation. COS-7 cells were transiently transfected with ET_BR in the presence or absence of either the JNK1APF or MEK (MEK_{K97M}) expression vectors. ET-1 (100 nmol/l) was added to the culture media 24 h before harvest and reporter gene activity was measured 48 h after transfection. Figure 3 shows that expression of JNK1APF or MEK_{K97M} partially inhibited ppET-1 promoter activity following the ET_BR stimulation. These findings strongly demonstrate that both JNK- and MEK-dependent signaling pathways are involved in the autoinduction of ET-1 through ET_BR. Next, we examined the effect of dominant negative JNK on G₁₃-induced activation of the ppET-1 promoter, since G₁₃ activates JNK-dependent pathways in a variety of cells. As shown in Fig. 4, expression of JNK1APF partially blocked the G₁₃Q226L-induced ppET-1 promoter activity in COS-7 cells. Taken together, our results suggest that autoinduction of ET-1 through ET_B receptor activation involves a G₁₃-dependent signaling system that is partially mediated by JNK in COS-7 cells.

Discussion

In the present study, we demonstrated that activated ET_BR and constitutively active forms of G₁₃ as well as G_q activate ppET-1 gene transcription in COS-7 cells. Activation of ppET-1 promoter by G₁₃ was partially mediated by a JNK-dependent pathway. ET_BR activated G₁₃ when they were co-expressed in HEK293 cells (6). Taken together, our previous and current results suggest a role of G₁₃ in ET_BR-mediated ET-1 autoinduction and other ET-1-mediated pathophysiological reactions. It is well known that endothelin production is up-regulated by various stimuli such as TGF- β , tumor necrosis factor (TNF)- α , interleukins, insulin, nor-epinephrin, angiotensin II (via AT₁ receptors), thrombin, and other physicochemical factors (22, 23, 27). Since G₁₃ interacts with AT₁ receptors and thrombin receptors (3, 5), G₁₃ could play an important role in induction of endothelin by these peptides. Recently, Offermanns and associates showed that a dominant negative form of G₁₃ prevented ET-1-mediated vascular smooth muscle cell (VSMC) contraction *in vitro* and that intracellular injection of activated G₁₃ evoked VSMC contraction (45), which suggests the involvement of G₁₃ not only in the regulation of ET-1 production, but also in a broad range of ET-1 bioactivities, including vascular tone regulation.

In our system, G₁₃ induces ET-1 transcription presumably through activating protein (AP-1). Because activation of a small GTPase, Rho, is one of the downstream conse-

quences of G₁₃ activation (13–15), and because a recent study demonstrated that Rho activates the ppET-1 promoter in bovine aortic endothelial cells (46), Rho might be involved in the autoinduction of ET-1 that is mediated by G₁₃. Moreover, since G_q has been shown to activate JNK and Rho (47, 48), it is likely that G_q induces ET-1 production not only through protein kinase C (PKC)-mediated pathways but also through JNK and/or Rho. Further studies are needed to elucidate the contribution of the Rho-dependent signaling system in the JNK-mediated autoinduction of ET-1 in our system.

We previously demonstrated that ET_AR does not activate G₁₃ (6). A previous study reported that a blockade of ET_AR that was able to activate G_q did not inhibit ET-1 production stimulated by ET-1 in rat mesangial cells (29). On the other hand, in a study using a rat model of congestive heart failure, the ET_AR antagonist BQ-123 normalized the increased expression level of ppET-1 mRNA (32). In addition, the constitutively active form of G₁₂ was not able to increase ppET-1 promoter activity in our system, although G₁₂ has been demonstrated to activate JNK and Rho in HEK293 cells (49). These results suggest that the nature of the signaling pathways involved in the autoinduction of ET-1 may vary in different tissues.

In conclusion, we demonstrated that G₁₃ induces ppET-1 promoter activation partially through a JNK dependent pathway in COS-7 cells, which suggests a possible role of G₁₃ in ET-1 autoinduction. Further investigation is required to complete the picture of the ET-1 autoinduction pathways and G-protein-mediated signaling cascades that are downstream of ET receptors.

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