Published in final edited form as:

Cell Signal. 2010 February ; 22(2): 325–329. doi:10.1016/j.cellsig.2009.09.039.

Endothelin-1 induces p66Shc activation through EGF receptor transactivation: Role of β_1 Pix/ $G\alpha_{i3}$ interaction

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Abstract

Endothelin-1 (ET-1) is a vasoconstrictor peptide known to be a potent mitogen for glomerular mesangial cells. We have shown that ET-1 stimulates the adaptor protein p66Shc through Rac/Cdc42 guanine nucleotide exchange factor β_1 Pix. In this study, we demonstrate that ET-1-induced serine phosphorylation of p66Shc is mediated through $G\alpha_{i3}$. Pertussis toxin treatment of cells induced a significant decrease in the interaction between β_1 Pix and ET_A -R, and an increase in the binding of $G\alpha_{i3}$ and $G\beta_1$ to β_1 Pix. Activation of heterotrimeric G proteins by AlF_4^- resulted in an increase of $G\alpha_{i3}$ binding to β_1 Pix, which was significantly disrupted in cells expressing β_1 Pix dimerization deficient mutant, β_1 Pix Δ (602–611). In cells expressing β_1 Pix Δ (602–611), ET-1-induced p66Shc activation was also significantly decreased. Specific inhibition of EGF receptor by AG1478 blocked ET-1-induced p66Shc activation and the binding of p66Shc and $G\alpha_{i3}$ to β_1 Pix. Inhibition of Erk1/2 blocked p66Shc activation induced by ET-1. Altogether, our results indicate that ET-1 activates p66Shc through EGF receptor transactivation, leading to the activation of $G\alpha_{i3}$, β_1 Pix and Erk1/2.

Keywords

Endothelin receptor; β_1 Pix; $G\alpha_{i3}$; p66Shc; Erk1/2; EGF receptor; Transactivation

1. Introduction

Endothelin-1 (ET-1) is the best characterized member of the endothelin family of vasoactive peptides. ET-1 binds to specific G protein-coupled receptors and evokes a wide variety of cellular responses via activation of tyrosine and serine-threonine protein kinases. ET-1-derived intracellular signals are also transduced by channel and transport proteins, as well as by various phospholipases, all of which cooperate in amplifying the biological properties of this peptide [1,2].

β_1 Pix (Pak-interacting exchange factor) is a guanine nucleotide exchange factor (GEF) for Rac 1 and Cdc42 [3,4]. GEF proteins contain a pleckstrin homology (PH) domain and the catalytic Dbp homology (DH) domain. In addition to these domains, β_1 Pix contains a Src homology 3 (SH3) domain that binds with high affinity to a polyproline stretch in Pak1 [3]. β_1 Pix also binds GIT1 through a GIT1-binding domain [5]. The leucine zipper (LZ) domain at the C-terminus

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mediates β_1 Pix homodimerization [6]. There is mounting evidence showing that Pix functions as an integrator of signaling pathways controlling adhesion and cytoskeletal organization. Recently we have shown that endothelin-1 (ET-1) induces β_1 Pix translocation to focal adhesions through a PKA- dependent pathway [7]. Pix was found to form a complex with paxillin, Pak, and p95PKL, which regulates cytoskeletal remodeling [8]. Moreover, the Pak-Pix-GIT complex has been shown to target and regulate focal adhesions [5]. We have shown recently that β_1 Pix binds 14-3-3 β resulting in the inhibition of β_1 Pix GEF activity toward Rac1 [9]. Moreover, ET-1 induces β_1 Pix and p66Shc up-regulation through Erk1/2 activation resulting in cell proliferation independently of Akt [10]. β_1 Pix was also found to enhance mast cell secretion through G_i -dependent mechanism [11].

The adaptor protein Shc exists in three isoforms with relative molecular masses (K_d) of 46, 52, and 66 [12]. They consist of a phosphotyrosine binding domain (PTB), a collagen homology domain (CH1) and a C-terminal Src homology 2 domain (SH2). In addition, p66Shc has a collagen homology domain 2 (CH2) at the N-terminus in which Ser36 is located. The serine 36 is phosphorylated in response to oxidative stress and appears to be critical for coupling Shc to stress response leading to apoptosis [13]. p66Shc knockout mice had a 30% increase in average lifespan when compared with control animals [14]. This increased longevity has been linked to enhanced resistance to oxidative stress. We have previously demonstrated the crucial role of p52Shc tyrosine phosphorylation in ET-1-mediated Ras activation in mesangial cells [15]. This phosphorylation enables and stabilizes the formation of a Shc-Grb2 complex and its subsequent association with Ras exchange factor Sos (Son of sevenless) facilitates the biphasic activation of Ras [15].

2. Materials and Methods

2.1. Materials

Cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA). Endothelin-1 was purchased from Calbiochem (La Jolla, CA). Anti-ET_A-R was from Millipore (Billerica, MD). Anti-Myc, anti-G α_{i3} , and anti-G β_1 antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho-Erk1/2 was purchased from Cell Signaling (Danvers, MA). Anti-p66Shc was from Invitrogen and anti-phosphoSer36p66Shc was purchased from Calbiochem. Anti-SHC was from BD transduction Laboratories (San Jose, CA).

2.2. Cell culture and transfection

Human mesangial cells were cultured in RPMI 1624 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a 37°C humidified incubator with 5% CO₂. Transient transfection of cells with mammalian expression vectors was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.3. Plasmids

The Myc-tagged β_1 Pix and β_1 Pix Δ (602–611) plasmids have been described [7,9,10].

2.4. Immunoprecipitation and Western blot analysis

Cells were transfected with the appropriate construct for 24 h. Cells were washed twice in phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, and 1 μ g/ml leupeptin. Equal amounts of proteins were separated by using 7.5% sodium dodecyl sulfate-

polyacrylamide gel electrophoresis, electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore), immunoblotted with the appropriate antibody, and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences, Inc.). For immunoprecipitation, antibodies against $G\alpha_{i3}$ or p66Shc were added to the cell lysate (500 μ g) for 2 h of incubation, followed by addition of protein A- or protein G-agarose beads for an additional hour. The beads were washed three times in PBS. The immunoprecipitated proteins were released from the beads by boiling in 1 $\times$ sample buffer for 5 min and subsequently analyzed by Western blotting. Total cell lysate (TCL) was run to assess the over-expression of the constructs. Expression of recombinant Myc- β_1 Pix and its mutant was verified by immunoblotting with anti-Myc antibody.

3. Results

3.1. Endothelin-1 induces serine phosphorylation of p66Shc through $G\alpha_{i3}$

We have previously shown that exposure of rat mesangial cells to ET-1 stimulation induces ERK1/2 and p66Shc activation [16]. In this study, we have used lysates of human mesangial cells (HMCs) expressing Myc-tagged β_1 Pix for immunoprecipitation with anti-p66Shc antibody. Results showed that β_1 Pix alone induced sizeable p66Shc activation as revealed by anti-phospho-p66ShcSer36 antibody and this activation was further increased by ET-1 stimulation (Fig. 1). Treatment of the cells with pertussis toxin almost completely blocked p66Shc activation induced by β_1 Pix over-expression and by ET-1 stimulation (Fig. 1), indicating that ET-1 stimulates p66Shc through $G\alpha_{i3}$ signaling pathway.

3.2. Pertussis toxin decreased ET_A -R binding to β_1 Pix and increases the binding of $G\alpha_{i3}$ and $G\beta_1$ to β_1 Pix

Pertussis toxin ADP-ribosylates the $G\alpha_{i3}$ C-terminal and prevent the GPCR from interacting and activating $G\alpha_{i3}$ [17,18]. We, therefore, investigated the effect of pertussis toxin on the interaction between ET_A -R and β_1 Pix. Our result showed that pertussis toxin treatment induced a significant decrease in the interaction between ET_A -R and β_1 Pix (Fig. 2a), suggesting that β_1 Pix may interact with ET_A -R through $G\alpha_{i3}$ proteins. Indeed, we found that pertussis toxin induces a strong increase in the binding of both $G\alpha_{i3}$ and $G\beta_1$ to β_1 Pix (Fig. 2b, c). This result is in agreement with the fact that by preventing the interaction between all the receptors coupled to $G_{i/o}$ including ET_A -R and $G\alpha_{i3}$, pertussis toxin will inevitably increase the interaction between $G\alpha_{i3}$ and β_1 Pix because of the increased local concentration of receptor free $G\alpha_{i3}\beta\gamma$.

3.3. Binding of $G\alpha_{i3}$ is dependent on its activation state and requires β_1 Pix dimerization

We have previously shown that dimerization is required for β_1 Pix interaction [9,10]. To evaluate the potential interaction between $G\alpha_{i3}$ and β_1 Pix, cells were transfected with empty vector or Myc- β_1 Pix. Using anti- $G\alpha_{i3}$ antibody to immunoprecipitate endogenous $G\alpha_{i3}$, we found a detectable interaction between $G\alpha_{i3}$ and β_1 Pix (Fig. 3a). To determine if the interaction between $G\alpha_{i3}$ and β_1 Pix is dependent on the $G\alpha_{i3}$ activation state, we immunoprecipitated endogenous $G\alpha_{i3}$ in the absence or presence of AMF, which mimics the transition state of GTP hydrolysis. In the presence of AMF, the basal interaction between $G\alpha_{i3}$ and β_1 Pix was markedly increased (Fig. 3a), indicating that the binding of $G\alpha_{i3}$ to β_1 Pix is dependent on $G\alpha_{i3}$ activation state observed after $G\alpha_{i3}$ activation through the receptor. In Contrast, in cells expressing dimerization deficient mutant β_1 Pix Δ (602–611) the binding of $G\alpha_{i3}$ to β_1 Pix was minimally increased in the presence of AMF (Fig. 3a), confirming the crucial role of dimerization in β_1 Pix interaction with other proteins. Because β_1 Pix dimerization is important to its interaction with $G\alpha_{i3}$ and p66Shc [9,10], we asked whether β_1 Pix dimerization deficient mutant will affect p66Shc activation by ET-1. After transfection of cells with β_1 Pix (wt) or β_1 Pix Δ (602–611), lysates were immunoprecipitated using anti-p66Shc antibody. We found that ET-1 stimulation induced a strong activation of p66Shc as measured by its Ser36 phosphorylation (Fig. 3b). In

cells expressing $\beta_1\text{Pix}\Delta$ (602–611), p66Shc activation by ET-1 was partially but strongly inhibited indicating that $\beta_1\text{Pix}$ dimerization plays an important role in p66Shc activation.

3.4. The binding of $G\alpha_{i3}$ and p66Shc to $\beta_1\text{Pix}$ is mediated through EGFR transactivation

To examine possible receptor cross-talk between ET_A and EGF receptors, we used a specific inhibitor of epidermal growth factor receptor kinase AG1478. Pretreatment of cells expressing $\beta_1\text{Pix}$ with AG1478 strongly inhibited ET-1-induced p66Shc Ser36 phosphorylation, whereas PDGF antagonist AG1296 had no effect (Fig. 4a, top panel). Similarly, inhibition of EGF receptor by AG1478 also blocked the binding of p66Shc to $\beta_1\text{Pix}$ whereas AG1296 had no effect (Fig. 4a, second panel from the top); suggesting that p66Shc activation and its binding to $\beta_1\text{Pix}$ are regulated through EGF receptor transactivation. Furthermore, we found that ET-1 stimulation did not increase the interaction between $G\alpha_{i3}$ and $\beta_1\text{Pix}$. However, pretreatment of cells with AG1478 significantly blocked the binding of $G\alpha_{i3}$ to $\beta_1\text{Pix}$ (Fig. 4b, top panel), whereas AG1296 had a less inhibitory effect. Inhibition of MEK by PD98059 did not affect the interaction between p66Shc and $\beta_1\text{Pix}$ but it did completely block p66Shc activation by ET-1 (Fig. 4a, top panel), indicating that p66Shc is downstream of Erk1/2. This result confirms our previous finding that Erk1/2 inhibition suppresses ET-1-induced p66Shc activation [15].

4. Discussion

These studies have demonstrated that serine 36 phosphorylation of p66Shc induced by ET-1 is mediated through PTx-sensitive $G\alpha_{i3}$ protein of $G_{i/o}$ family. We provide evidence that both $\beta_1\text{Pix}$ and ET-1 induced p66Shc activation through $G\alpha_{i3}$ -dependent mechanism. We show that PTx treatment decreased the binding of $G\alpha_{i3}$ to $\text{ET}_A\text{-R}$ and at the same time increased the binding of $G\alpha_{i3}$ and $G_{\beta 1}$ to $\beta_1\text{Pix}$, strongly suggesting that $\beta_1\text{Pix}$ interacts with $\text{ET}_A\text{-R}$ through $G\alpha_{i3}$ since PTx is known to block the interaction between the receptor and the C-terminal of $G\alpha_{i3}$ [17,18]. Therefore the local concentration of free $G_{\alpha\beta\gamma}$ is increased and bind to $\beta_1\text{Pix}$ to produce non-productive complex. Using co-immunoprecipitation, we found that endogenous $G\alpha_{i3}$ binds to $\beta_1\text{Pix}$ and this binding is significantly increased upon activation of $G\alpha_{i3}$ by AMF (which mimics the activation induced by the receptor), suggesting that $\beta_1\text{Pix}$ can recognize the conformational changes associated with $G\alpha_{i3}$ activation. $\beta_1\text{Pix}$ is not related to p115RhoGEF family that has been shown to activate RhoA through $G_{12/13}$ [19,20]. Active $G\alpha_{i3}$ has been shown to directly interact and activate p115RhoGEF [20], while another member of p115RhoGEF, LARG, interacts with $G\alpha_q$ and $G\alpha_{12/13}$ to activate RhoA [21]. These findings provide a mechanistic explanation of how signals are transmitted from the GPCR to p115RhoGEF and therefore to RhoA.

The expression of $\beta_1\text{Pix}\Delta$ (602–611) which disrupts $\beta_1\text{Pix}$ dimerization severely impaired AMF-induced binding of $G\alpha_{i3}$ to $\beta_1\text{Pix}$. This result confirms our previous studies showing that $\beta_1\text{Pix}$ dimerization is required for its interaction with p66Shc [10] and 14-3-3 β [9]. In addition to $\beta_1\text{Pix}$ -GEF activity that plays crucial role in regulating the activation of small GTPases Cdc42 and Rac [3], $\beta_1\text{Pix}$ also possesses a scaffolding activity that enables it to interact and regulates other proteins, therefore providing different possibilities of cross-talks that take place downstream of GPCR activation. Indeed, we found that $\beta_1\text{Pix}$ dimerization deficient mutant, $\beta_1\text{Pix}\Delta$ (602–611), strongly blocked p66Shc activation by ET-1. Furthermore, our studies demonstrate that ET-1-induced p66Shc activation is dependent on EGF-R transactivation as indicated by the effect of EGF-R kinase inhibition by tyrphostin AG1478. This result adds to the growing list of GPCR signaling that induce EGF-R transactivation [22–24] and highlights the role of EGF-R transactivation in physiological and pathophysiological condition such as vascular tone, cancer and hypertrophic growth [25,26]. The adaptor protein Shc (66, 52 and 46 isoforms) has been demonstrated to link tyrosine kinase receptors to the activation of MAPK cascades [27]. We and others have provided evidence that the recruitment of Shc is required

for GPCR-dependent Ras-Raf-MEK-ERK pathway activation [15,28]. In addition to tyrosine phosphorylation, we have previously shown that p66Shc can be activated through Ser36 phosphorylation when stimulated by ET-1 and that this stimulation is blocked when Erk1/2 is inhibited [16,10] indicating that Erk1/2 acts upstream of p66Shc. However, Erk1/2 activation is not required for p66Shc binding to β_1 Pix since Erk1/2 inhibition did not affect the interaction between β_1 Pix and p66Shc. Transactivation of EGF-R is also involved in the regulation of the binding of both p66Shc and $G\alpha_{i3}$ to β_1 Pix which corroborates our hypothesis suggesting that β_1 Pix scaffolding activity enables it to act as a platform to interact with active $G\alpha_{i3}$ (through ET_A-R stimulation) resulting in the sequential activation of Erk1/2 and p66Shc.

Transactivation of EGF-R is a mechanism thought to mediate Erk1/2 activation by GPCR [29]. Our present data extend this mechanism to include p66Shc activation by ET-1 and the regulation of β_1 Pix interaction with $G\alpha_{i3}$. This interaction may be crucial to EGF-R transactivation induced by ET-1 since in COS-7 cells; EGF-R transactivation requires the over-expression of $G\alpha_i$ and $\beta\gamma$ subunits [30]. ET-1 is known to induce mitogenic effects on human ovarian carcinoma cells [23] through EGF-R transactivation which may explain the up-regulation of growth factors usually found in these tumor cells.

Acknowledgments

This work was supported in part by the National Institutes of Health Grant HL22563.

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Figure 1.

ET-1 activates p66Shc through pertussis toxin-dependent G_i protein. HMCs were transfected with empty vector or Myc- β_1 Pix for 24 h. Cells were left untreated or treated with pertussis toxin (0.2 μ g/ml) overnight. After stimulation of cells with ET-1 (100 nM) for 5 min, lysates were subjected to immunoprecipitation using anti-p66Shc antibody and immunoprecipitated proteins were probed using anti-phosphop66ShcSer36, anti-Myc or anti-p66Shc antibodies. TCL: total cell lysate. Lower panel shows densitometric analysis of the bands corresponding to Phospho-p66ShcSer36. Each column represents the means $\pm$ S.E.M. of three independent determinations. * Significant difference from cells stimulated with ET-1 in the absence of PTx ($P < 0.05$).

Figure 2.

Effect of pertussis toxin on β_1 Pix interaction with ET_A-R, $G\alpha_{i3}$ and $G\beta_1$. Cells expressing β_1 Pix were left untreated or treated with pertussis toxin (0.2 μ g/ml) overnight. Cell lysates were immunoprecipitated with anti-ET_A-R (A) anti- $G\alpha_{i3}$ (B) or anti- $G\beta_1$ and subjected to Western blot analysis. The bottom panels indicate the expression of Myc- β_1 Pix. Lower panels show densitometric analysis of the bands corresponding to Myc β_1 Pix binding to ET_A-R (A), Myc β_1 Pix binding to $G\alpha_{i3}$ (B) or Myc β_1 Pix binding to $G\beta_1$ (C). Each column represents the means $\pm$ S.E.M. of three independent experiments. * Significant difference compared to cells with no PTx treatment ($P < 0.05$).

B

Figure 3.

Activation state-dependent binding of $G\alpha_{i3}$ to β_1 Pix. (A) HMCs cells were transfected with empty vector, Myc- β_1 Pix or Myc- β_1 Pix Δ (602–611) for 24 h. Cells were lysed in the absence or presence of AMF (AlCl₃, 50 μ M; MgCl₂, 10 mM; NaF, 5 mM) and immunoprecipitated using anti- $G\alpha_{i3}$ antibodies followed by Western blotting using specific antibodies against each component as indicated. Lower panel shows densitometric analysis of the bands corresponding to Myc β_1 Pix or Myc- β_1 Pix Δ (602–611) binding to $G\alpha_{i3}$ (A). Each column represents the means $\pm$ S.E.M. of three independent experiments. * Significant difference compared to cells expressing Myc- β_1 Pix(wt) in the presence of AMF ($P < 0.05$). (B) Cells were transfected as described above and lysates were immunoprecipitated with anti-p66Shc antibodies followed by Western blotting analysis as indicated. Lower panel shows densitometric analysis of the bands corresponding to Phospho-p66ShcSer36. Each column represents the means $\pm$ S.E.M. of three independent experiments. * Significant difference compared to cells expressing Myc- β_1 Pix(wt) in the presence of ET-1 ($P < 0.05$).

Figure 4.

EGF-R transactivation mediates ET-1-induced p66Shc activation. Cells were transfected with empty vector or Myc- β_1 Pix for 24 h and left untreated or treated with AG1296 (10 μ M), AG1478 (2 μ M) or PD98059 (20 μ M) for 30 min before stimulation with ET-1 (100 nM) for 5 min. Cell lysates were immunoprecipitated with anti-p66Shc (A) or anti- $G\alpha_{i3}$ (B) and Western blots were analyzed with the indicated antibodies. These data are representative of similar results obtained in three independent experiments. Quantitative analysis of the bands corresponding to Phospho-p66ShcSer36 (A) or Myc- β_1 Pix/ $G\alpha_{i3}$ interaction (B) was obtained by densitometry. Results are expressed as means $\pm$ S.E.M. of three independent experiments. Statistical significance between the groups was analyzed using the Student's *t* test. *, $P < 0.05$

compared with Phospho-p66ShcSer36 levels measured in cells stimulated with ET-1 alone (A) or with Myc- β_1 Pix/ $G\alpha_{i3}$ interaction in the presence of ET-1 alone (B).