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Endothelin-1 Induced Phosphorylation of Caveolin-1 and Smad2C in Human Vascular Smooth Muscle Cells: Role of NADPH Oxidases, c-Abl, and Caveolae Integrity in TGF-β Receptor Transactivation

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ABSTRACT

Caveolin-1(Cav-1) is one of the most important components of caveolae in the cell membrane, which plays an important role in cell signaling transduction, such as EGFR and TGF-β receptor transactivation. The purpose of this study was to evaluate the effect of c-Abl and NAD(P)H oxidases (NOX) on phosphorylation of Cav-1 and consequently their effect on phosphorylation of Smad2C induced by Endothelin-1 in human vascular smooth muscle cells (VSMCs). In this study, all experiments were performed using human VSMCs. The phosphorylation level of the Caveolin-1 and Smad2C proteins were assessed by western blotting using Phospho-Caveolin-1 (Tyr14) antibody and phospho-Smad2 (Ser465/467) antibody. The data were reported as mean ± SEM. The VSMCs treated with endothelin-1(ET-1) (100 nanomolar (nmol)) demonstrated a time-dependent increase in the pCav-1 level ($p<0.05$). The inhibitors of NOX (diphenyleneiodonium) ($p<0.05$), cholesterol depleting agent (beta-cyclodextrin) ($p<0.05$) and c-Abl inhibitor (PP1) ($p<0.0١$) were able to reduce the level of the phospho-Cav-1 and phospho-Smad2C induced by Et-1 ($p<0.05$). Our results proposed that caveolae structure, NOX, c-Abl played an important role in the phosphorylation of Cav-1 induced by ET-1 in the human VSMCs. Furthermore, our findings showed that phosphoCav-1 involved in TGFR transactivation. Thus, Et-1 via a transactivation-dependent mechanism can cause phosphorylation of Smad2C.

Keywords: Endothelin-1, Caveolin-1, c-Abl, NADPH oxidase, Smad2 protein

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Introduction

Atherosclerosis is a chronic inflammatory disorder characterized by the retention of oxidative-modified low-density lipoprotein (oxLDL) in the early stages of atherogenesis, a process known as the response to retention. The synthesis of extracellular matrix components, such as proteoglycans, appears to be involved in the process through the retention of oxLDL in the subendothelial layer of the coronary artery (1-3). Vascular smooth muscle cells (VSMCs) normally synthesize proteoglycans with natural length glycosaminoglycans (GAGs). However, growth factors such as Et-1, TGF-β, and EGF stimulate these cells to synthesize and secrete hyper-elongated GAGs (4).

Endothelin-1 (ET-1) is a peptide composed of 21 amino acids that acts as a vasoconstrictor and has a range of effects on vascular smooth muscle cells (VSMCs) by interacting with its receptors on the plasma membrane (1). ET-1 receptor is a member of the family of G-protein-coupled receptors (*GPCRs*) (2). ET-1, through two types receptors, exert its effects: type A and B (3). GPCRs families are the most abundant cell surface receptors that exert their effects through multiple signaling pathways. Numerous signaling pathways can be induced by GPCR agonists including the well-known classical, beta-arrestin pathways and transactivation pathway(4). Our previous study on endothelial cells showed that ET-1 via rearrangements of the Actin cytoskeleton can induce TGFR transactivation, thereby leading to phosphorylation of Smad2C. Carboxy-terminal phosphorylation of Smad2 drives the expression of proteoglycan synthesizing enzymes (5).

Many cytoskeletal ingredients and membrane proteins, as well as enzymes that control the cytoskeleton, centralize to caveolae, the rich cholesterol and sphingolipid plasma membrane surface (6, 7).caveolin-1(Cav-1) is concentrated to caveolae and considered as one of the most important structural ingredients of caveolae (8). Cav-1 reacts with various signaling molecules through its caveolin scaffolding domain (CSD). I*t* has been shown that caveolin-1 can regulate signaling molecules concentrated in the cell membrane. Changes in caveolin expression and its phosphorylation that may be contributed to the receptor tyrosine kinase signal transduction.(9, 10).

Caveolae membrane have abundant cholesterol and a cholesterol-depleting agent such as β-cyclodextrin, has been shown to affect the phosphorylation of caveolin-1 (Cav-1) and subsequently impact cellular signaling pathways. Specifically, treatment with β-cyclodextrin leads to disrupt the formation of caveolae and alter cellular signaling events that are dependent on these structures (6).

The objective of this study was to assess the involvement of signaling mediators, including NAD(P)H oxidases (NOX), c-Abl, and caveolae structure, in the phosphorylation of Cav-1. Additionally, the study aimed to investigate the impact of PCav-1 on the transactivation of TGF-β receptor and Psmads induced by endothelin-1.

Materials and methods

The following materials were purchased: Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12; Invitrogen), fetal bovine serum (FBS; Gibco, USA), antibiotic (Penicillin-Streptomycin;), Endothelin-1, beta-cyclodextrin, DPI, PP1, SB-431542, Sigma-Aldrich (St. Louis, MO, USA). Phospho-Caveolin-1(Tyr14)

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antibody, anti-PSmad2C-rabbit monoclonal antibody (ser465/467), HRP anti-rabbit IgG-peroxidase antibody produced in goat, were prepared from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human vascular smooth muscle cells (VSMCs) were obtained from the National Cell Bank at Pasteur Institute in Tehran, Iran. The VSMCs were cultured in DMEM/F12 growth medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere at 5% CO2. All cell culture media were supplemented with 100 units/mL penicillin and streptomycin to prevent bacterial contamination. VSMCs were plated 4×10^5 cells/ 3.5-mm culture plates that supplemented with 10% fetal bovine serum and 1% antibiotic at 37 °C in a 5% CO2 incubator. After 16 hours of starvation, the cells were treated with ET-1 and inhibitors.

VSMCs pretreated for 3 and 5 hours with β-cyclodextrin (4 mM), then treated with ET-1(1 and 2 hours). To investigate the role of c-Abl in phosphorylation of Cav-1 inducted by ET-1, VSMCs pretreated for 1 hour with PP1 (10 µM) as c-Abl Inhibitor, then treated with ET-1(1 and 2 hours).

Western Blot

Cellular protein was extracted with RIPA lysis buffer (50 mM Tris·HCl, 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4 and 1% cocktail protease inhibitor); protein assay was taken with BCA Protein Assay Kit (Pars Tous, Iran). After the addition of the sample loading buffer, 70 µg of total protein lysates were boiled, separated by SDS-PAGE at 100V for three hours till Bromophenol blue marker reached bottom of the gel. Then protein was transferred to PVDF membrane (PVDF membrane; Roche Diagnostics, Germany). Membranes were blocked with 3% skimmed milk powder in TBST for 2 h at room temperature and then incubated with phospho-Caveolin-1(Tyr14) antibody (1:500 dilutions with 5% BSA in TBST), anti-PSmad2C-rabbit monoclonal antibody (ser465/467) (1:1000 dilution with 3% BSA in TBST) overnight at 4 C. The secondary antibody horseradish peroxidase (HRP)-labelled (anti-rabbit IgG; 1:10000 dilutions with 3% skimmed milk in TBST) added to membranes for 1h at room temperature. Membranes were developed with the enhanced chemiluminescence (ECL) solution (Clarity Western ECL Substrate, Bio-Rad, Hercules, CA, USA), and the bands were appeared with using Bio-Rad ChemiDoc XRS+.

Statistical analysis

All results were reported as mean ± standard error of the mean (SEM), experiments are repeated independent 3 times. All groups were compared with one-way ANOVA. Results were considered significant at $P < 0.05$.

Results

ET-1 stimulates the phosphorylation of Cav-1 in VSMCs

First, we examined the effect of ET-1 on the *p*Cav-1 (Tyr14) at different time points. VSMCs were subjected to ET-1 (100 nM) and the level of pCav-1 was measured at time points 5 min-2 h. Our results showed that ET-1 increased the *p*Cav-1 ($P <0.05$) at 1 and 2 h (Figure 1) in a time-dependent manner. As a positive control, H_2O_2 (5 mM) stimulated the phosphorylation of Cav-1 at 1 h ($P <0.05$) (Figure 1).

The cholesterol depleting agent-β-cyclodextrin- decreased the ET-1 Mediated Phosphorylation of the Cav-1 in the Human VSMCs

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To investigate the role of caveolae structure in phosphorylation of Cav-1, we destroyed caveolae structure with the cholesterol-depleting agent, β-cyclodextrin. The VSMCs treated with ET-1 showed an

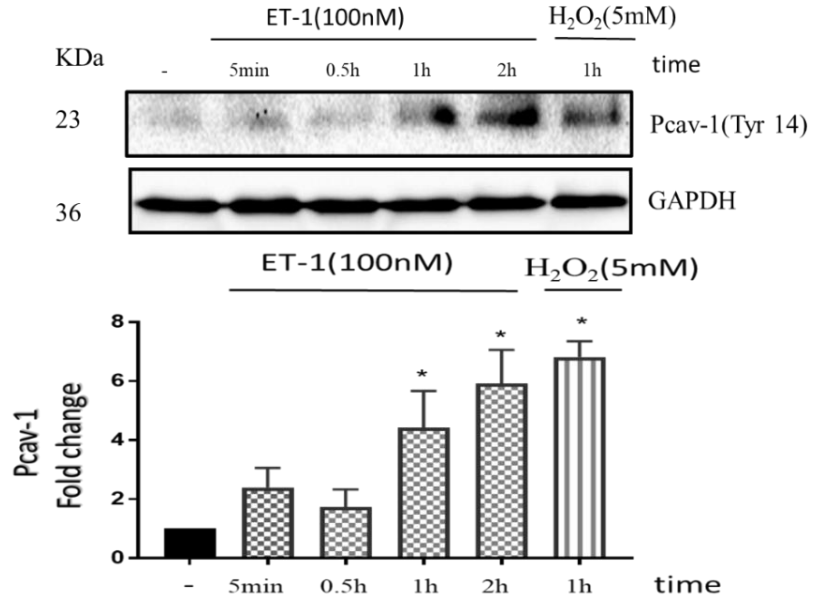

Fig.1. Endothelin-1 causes phosphorylation of Cav-1. VSMCs were exposed to Endothelin-1 (100 nM) for 5min-2 h. As a positive control, H_2O_2 (5 mM) stimulated the phosphorylation of Cav-1 at 1 h. *P < 0.05 compared with untreated. Values are the mean ± SEM.

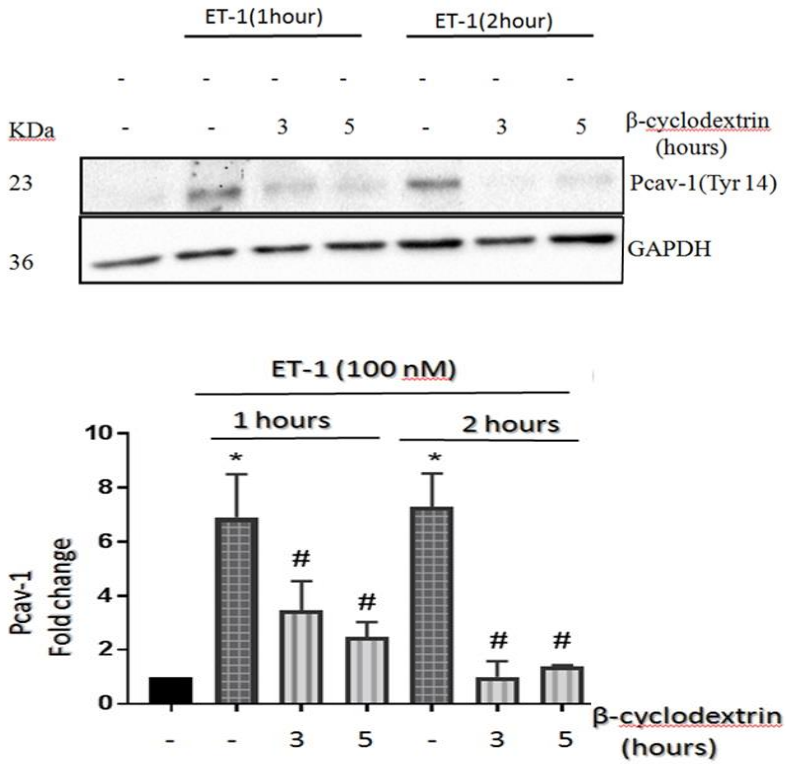

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Fig.2. Caveolae structure is important in phosphorylation of Cav-1. VSMCs were preincubated with β-cyclodextrin (4mM) at 3 and 5h before stimulation with ET-1 (100 nM) at 1 and 2h. *P < 0.05 compared with untreated, #p < 0.05 compared with ET-1 treated. Values are the mean ± SEM from triplicate experiments.

increase in the pCav-1 level at the both 1 and 2 hours (P < 0.05). At 1 and 2 hours treated with ET-1, β-cyclodextrin significantly reduced the level of the pCav-1 at 3 and 5 hours treat with β-cyclodextrin (P <0.05) (Figure 2). These data indicated the intact caveolae have critical role in signal transduction and phosphorylation of Cav-1 stimulated by ET-1.

The NOX Inhibitor (DPI) decreased the ET-1-Mediated Phosphorylation of Cav-1 in the Human VSMCs

To investigate the role of NOX in phosphorylation of Cav-1, VSMCs pretreated for 2 hours with DPI (10 µM), then treated with ET-1 (1 and 2 hours). Our result showed that DPI decreased pCAV-1 at 1 and 2 hours after stimulated with ET-1 (P < 0.05) (Figure 3). These data suggested that Et-1 increased phosphorylation of CAV-1 meditated by NOX, because of DPI, NOX inhibitor decreased pCAV-1 when induced by ET-1.

PP1 reduced ET-1-mediated phosphorylation of Cav-1 in human VSMCs

The VSMCs treated with ET-1 showed an increase in the pCav-1 level at 1 and 2 h (P < 0.01). In the presence of PP1, the level of phosphoCav-1 was decreased at 1 and 2 hours (P < 0.01) (Figure 4). These data demonstrated the importance role of c-Abl enzymes in the ET-1-mediated phosphorylation of Cav-1.

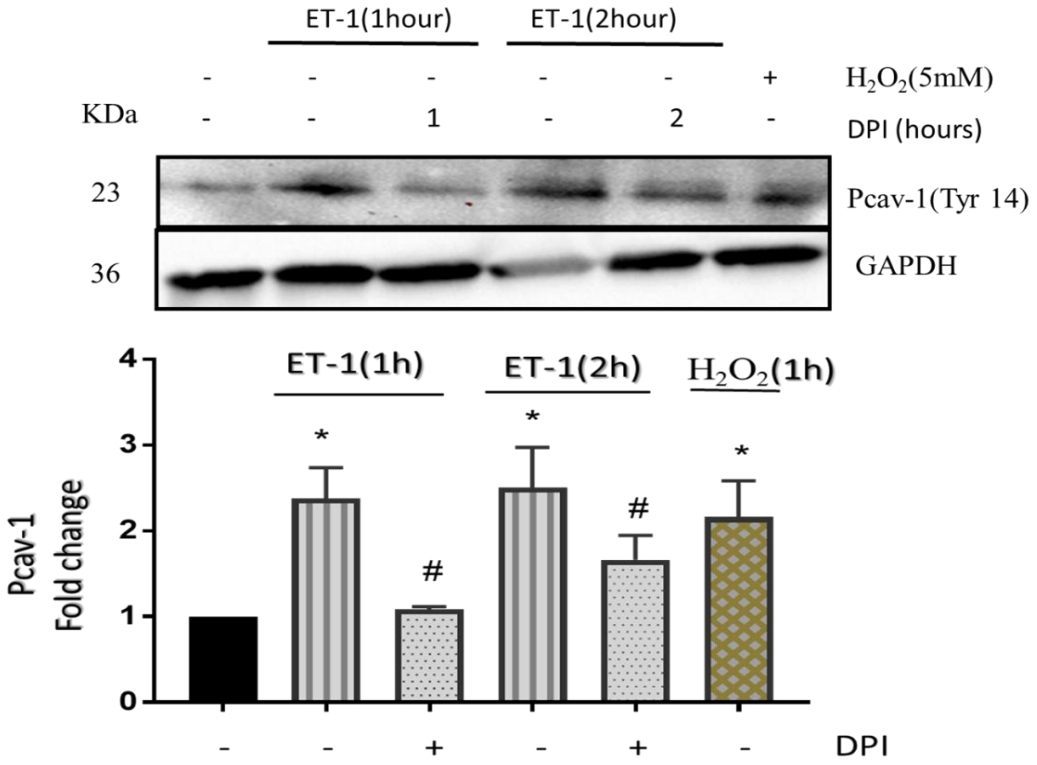

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Fig.3. ET-1 leads to phosphorylation of Cav-1 in an NOX dependent manner. VSMCs were preincubated with DPI (10 µM) at 2 h before stimulation with ET-1 (100 nM) at 1 and 2 h. *P < 0.05 compared with untreated, Values are the mean ± SEM from triplicate experiments.

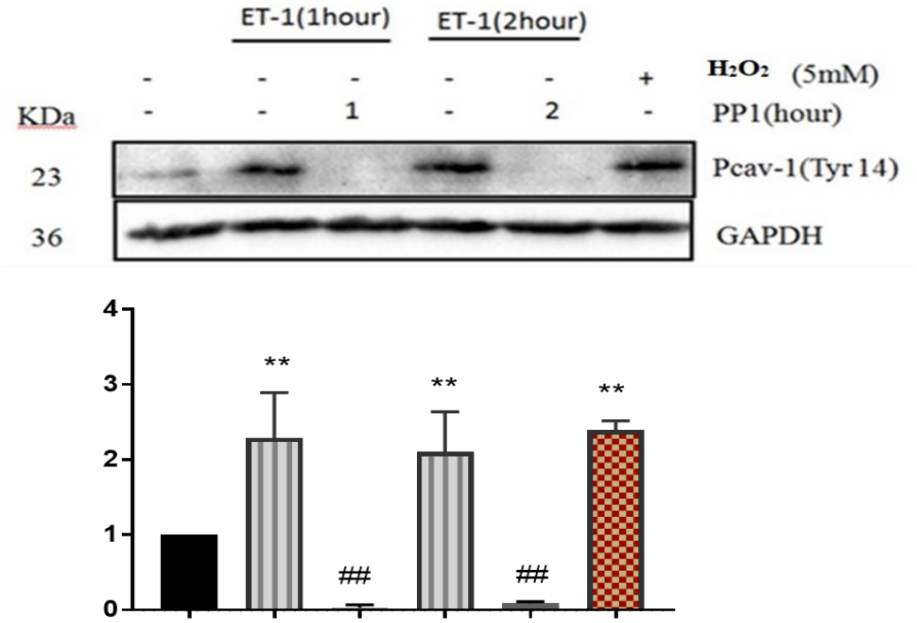

Fig.4. ET-1 leads to phosphorylation of Cav-1 in a c-Abl dependent manner. VSMCs were preincubated with PP1 (10 µM) at 1h before stimulation with ET-1 (100 nM) at 1 and 2 h. **P < 0.01 compared with untreated, ##p < 0.01 compared with ET-1 treated. Values are the mean ± SEM from triplicate experiments.

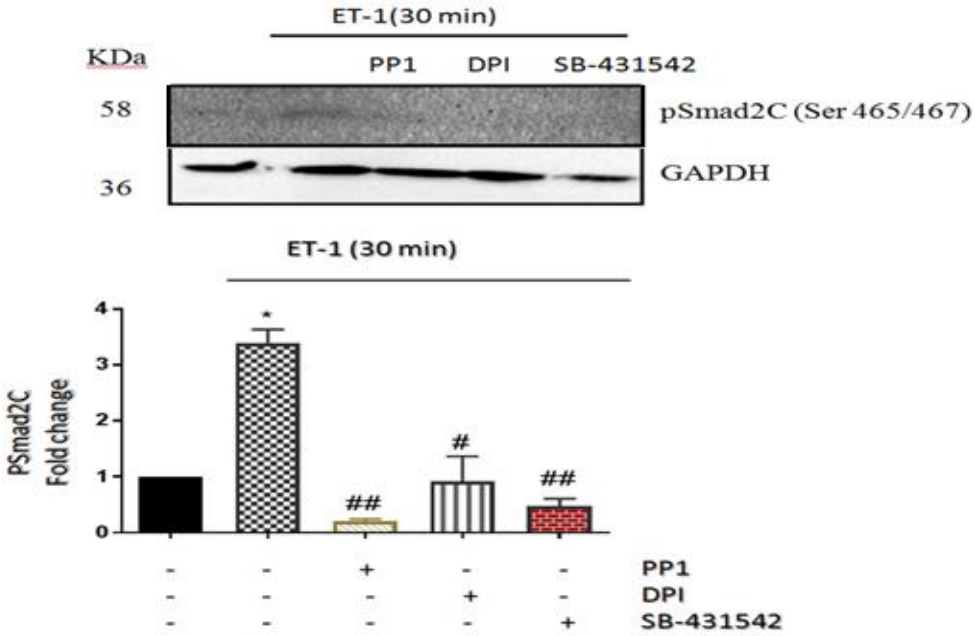

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Fig.5. ET-1 leads to phosphorylation of Smad2C in a c-Abl and NOX dependent manner. VSMCs were preincubated with DPI (10 µM) for 2 h, PP1 (10 µM) for 1 h, SB-431542 (10 µM) for 30 min before stimulation with endothelin-1 (100 nM) for 30 min. *P < 0.05 compared with untreated, #P < 0.05 and ##P < 0.01 compared with ET-1 treated. Values are the mean ±SEM from triplicate experiments.

pCav-1 mediates phosphorylation of Smad2C, through TGFR transactivation

To investigate the involvement of pCav-1 in the transactivation of TGF-β receptor by endothelin receptor, as well as the phosphorylation of Smad2C, vascular smooth muscle cells (VSMCs) were pretreated with PP1 (10 µM), DPI (10 µM), and SB-431542 (10 µM) for 1 hour, followed by incubation with ET-1 (100 nm) for 30 minutes. Our results showed that treatment with ET-1 led to a significant increase in the phosphorylation level of Smad2C ($P<0.05$). However, in the presence of PP1, DPI, and SB-431542, the phosphorylation level of Smad2C decreased significantly ($P<0.05$ and $P<0.01$) (Figure 5). These findings suggest that ET-1 activates the TGF-β receptor, leading to the phosphorylation of Smad2C, and that this activation is mediated by pCav-1. Furthermore, our results indicate that the activation of the TGF-β receptor is induced by the stimulation of c-Abl and NOX.

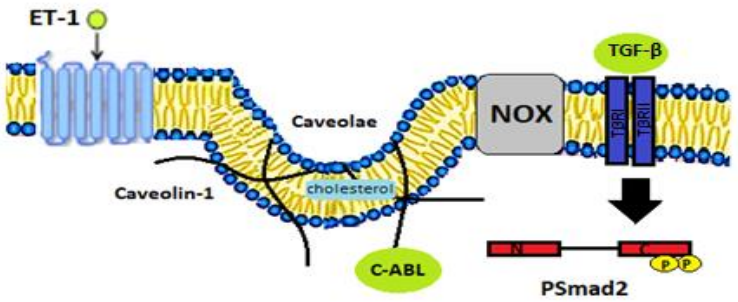

Fig.6. Schematic representation of the TβRI transactivation pathway induced by ET-1. ET-1 can induce phosphorylation of Cav-1 through c-ABL and NOX. Subsequently, PhosphoCav-1 causes transactivation of TGFR. As a result, these events lead to phosphorylation of Smad2c in vascular smooth muscle cell (VSMC).

Discussion

In this study, we have demonstrated that ET-1 can induce the phosphorylation of Cav1, a cytosolic scaffolding protein, through the use of protein-tyrosine kinase (c-Abl) and NOX. We have also discovered that this phosphorylation of Cav1 by ET-1 leads to transactivation of TGFR. Furthermore, we observed that this transactivation results in the phosphorylation of Smad2c in VSMCs, as shown in Figure 6.

In previous study, we have shown that ET-1 can induce TGFR transactivation pathway through the activation of cytoskeletal rearrangement, this resulted in increased the phosphorylation of Smad2C in BAECs (5).Also in 2013, Burch et al. showed that thrombin is capable of stimulation TGFR transactivation pathway through cytoskeletal rearrangement in VSMCs (11); but the detailed molecular mechanism of the TGFR transactivation remain unknown.

A main part of the cell membrane is caveolae that have an important role in the transduction of signaling pathways (12). Many caveolae-mediated functions carry-out via the caveolin protein (13). Some of the functions of caveolin in the membrane including the assistance of binding cell membrane to the cytoskeletal

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actin, and via its CDS domain can connect to different mediators cytosolic tyrosine kinase, Small GTPase, integrin (14, 15).For these functions, caveolin-1 may play an important role in transactivation pathway.

To investigate the role of Cav-1 in the transactivation of the TGFR receptor signaling, we first examined the effect of ET-1 on phosphorylation of Cav-1. Our results showed that in a time-dependent manner, ET-1 is able to induce the phosphorylation of Cav-1. In the following, we evaluated the role of NOX and C-Abl on phosphorylation of Cav-1 induced by ET-1 and subsequent TGFR transactivation.

Caveolae serve as signaling platforms by concentrating signaling molecules. The presence of intact caveolae in the plasma membrane is required for the localization of signaling molecules and the subsequent cellular response. Studies have shown that the integrity of cell membranes and caveolin-associated signaling mediators is compromised during cerebral ischemia (21).

We used a beta cyclodextrin (cholesterol eting agent) to evaluate the importance of integrity of caveolae for Cav-1 phosphorylation. Our results showed that degradation structure of caveolae cause decreased phosphorylation of Cav-1, which indicating the importance of the intact caveolae structure in ET-1 induced phosphorylation of Cav-1.

Different kinases such as c-Abl,c-SRC, NOX have been localized to the caveolae surface (16, 17). The c-Abl tyrosine kinase is implicated in various cellular activities including regulation of actin and microtubules cytoskeleton which leading to cytoskeleton rearrangement (18).

To explore the potential involvement of c-Abl in the phosphorylation of Cav-1 induced by ET-1, we utilized a specific c-Abl inhibitor (PP1). Our results demonstrated that treatment with PP1 led to a significant reduction in the level of Cav-1 phosphorylation induced by ET-1.

In previous study, we show that NOX enzymes plays an important role in induction of TGFR transactivation by ET-1(19). In this study, we used a specific NOX inhibitor, DPI, to evaluate the role of Nox enzymes on endothelin-induced phosphorylation of Cav-1.Our results indicated that the activity of Nox enzymes is effective in phosphorylation of Cav-1.

Next, to investigate the role of phosphorylation of Cav-1 in the TGFR transactivation pathway, we evaluated the phosphorylation of Smad2C in the presence of SB-431542 (specific TGFR inhibitor), PP1 and DPI. We reported that in VSMC, phosphorylation of Smad2C induced by ET-1 in presence SB-431542, PP1, DPI decreased. Our results showed that the c-ABL and NOX are needed to induci*ng* the TGFR transactivation, in addition we came to the conclusion that phosphorylation of Cav-1 involved in the pathways linking ET receptor to the phosphorylation of Smad2C. This finding is founded by the recent study who determined that for EGFR transactivation induced by Ang-II the caveolae and clathrin-coated pits also associated with phosphoCav-1 is required (20).

In summary, we provide evidence that integrity of caveolae, NAD(P)H oxidase and c-Abl have important role in phosphorylation of Cav-1, which is required for TGFR transactivation. We also found that phosphoCav-1 is involved in phosphorylation of Smad2C that leads to hyper- elongation of GAG chain and development of atherosclerotic plaque.

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