

# Endothelin-1 Stimulates PAI-1 Protein Expression via Dual Transactivation Pathway Dependent ROCK and Phosphorylation of Smad2L

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## Abstract

**Objective:** In addition to the carboxy region, Smad2 transcription factor can be phosphorylated in the linker region as well. Phosphorylation of Smad2 linker region (Smad2L) promotes the expression of plasminogen activator inhibitor type 1 (PAI-1) which leads to cardiovascular disorders such as atherosclerosis. The purpose of this study was to evaluate the role of dual transactivation of EGF and TGF- $\beta$  receptors in phosphorylation of Smad2L and protein expression of PAI-1 induced by endothelin-1 (ET-1) in bovine aortic endothelial cells (BAECs). In addition, as an intermediary of G protein-coupled receptor (GPCR) signaling, the functions of ROCK and PLC were investigated in dual transactivation pathways.

**Materials and Methods:** The experimental study is an *in vitro* study performed on BAECs. Proteins were investigated by western blotting using protein-specific antibodies against phospho-Smad2 linker region residues (Ser245/250/255), phospho-Smad2 carboxy residues (465/467), ERK1/(Thr202/Thr204), and PAI-1.

**Results:** TGF (2 ng/ml), EGF (100 ng/ml) and ET-1 (100 nM) induced the phosphorylation of Smad2L. This response was blocked in the presence of AG1478 (EGFR antagonists), SB431542 (TGFR inhibitor), and Y27632 (Rho-associated protein kinase (ROCK) antagonist). Moreover, ET-1-increased protein expression of PAI-1 was decreased in the presence of bosentan (ET receptor inhibitor), AG1478, SB431542, and Y27632.

**Conclusion:** The results indicated that ET-1 increases the phosphorylation of Smad2L and protein expression of PAI-1 via induced the transactivation pathways of EGFR and TGFR. This study is the first attempt to scrutinize the significant role of ROCK in the protein expression of PAI-1.

**Keywords:** Atherosclerosis, ROCK, Smad2, Transactivation

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## Introduction

Endothelin-1 (ET-1) is a strong vasoconstrictor peptide that is synthesized by endothelial cells, probably causing the promotion of endothelial dysfunction (1-4). The effect of ET-1 is exerted through G-protein-coupled receptors (GPCRs): ET<sub>A</sub> and ET<sub>B</sub> (5). GPCR family is the biggest group of cell surface receptors participating in a number of physiological or pathological circumstances (6). Therefore, understanding the different dimensions of GPCR signaling is essential for therapeutic purposes. GPCRs-driven signaling pathways include the classic pathway via direct binding of ligand to GPCR on the cell membrane leading to activation of heterotrimeric G proteins and multiple signaling pathways. In recent years, transactivation pathways of protein tyrosine kinase receptors (PTK) such as epidermal growth factor receptor (EGFR), as well as protein serine/threonine kinase receptors (PS/TK) like transforming growth factor receptor (TGFR) have been identified as part of the GPCR signaling (7-9). Recent studies have demonstrated that different GPCR agonists such as thrombin, ET-1, and

AngII can contribute to transactivation of EGFR and TGFR (10-12). According to our previous study, it has been determined that ET-1 results in TGFR transactivation endothelial cells (13).

TGF $\beta$  receptors are a group of serine/threonine kinase receptors whose biological roles are performed by type I and type II receptor complexes (ALK5). TGF- $\beta$  signaling is launched by interaction of a ligand to the T $\beta$ RII/type I heterogenic complex leading to phosphorylation of the carboxy region of Smad proteins (14). Smad proteins are transcriptional factors that play a serious role in the TGF $\beta$ -superfamily signals (15, 16). The Smads have three distinct regions: two conserved regions including N-terminal (MH1) and C-terminal (MH2) regions, and one non-conserved region -linker region- that links MH1 and MH2 regions. Besides the carboxy region, the linker region can be phosphorylated as well (16-18). In the Smad-dependent TGF- $\beta$  signaling pathway, phosphorylation of C-terminal region occurs immediately by binding of TGF- $\beta$  to the cell surface receptor. However,

in non-Smad signaling, phosphorylation of Smad2 linker region (Smad2L) occurs indirectly by an activated serine/threonine kinase such as ERK1/2, p38, or JNK. Recent studies have shown that in addition to TGF- $\beta$ , GPCR agonists result in phosphorylation of Smad2L which can play a significant part in regulation of Smad's function (14). Phosphorylation of Smad2L increases the expression of proteoglycan synthesizing genes. It has been demonstrated that TGF- $\beta$ /Smad pathway increases plasminogen activator inhibitor type 1 (PAI-1) expression in different cell types (19, 20). PAI-1 is a member of the superfamily of serine-protease inhibitors (serpin) that may cause vascular disorders such as endothelial dysfunction (21, 22). Studies have shown that growth factors such as TNF- $\alpha$ , TGF, GPCR agonists such as thrombin, and angiotensin II can lead to increased mRNA expression of PAI-1 (16, 21, 23). In 1996, it was shown for the first time that angiotensin II (Ang II) can induce transactivation pathways. Subsequently, some comprehensive researches have focused on understanding the underlying mechanism of transactivation pathway in different cell types. However, the details of this pathway and the signaling molecules that participate in transactivation pathways induced by ET-1 are not very clear in bovine aortic endothelial cells (BAECs). Therefore, in the current study and for the first time, not only the role of dual transactivation pathways induced by ET-1 were evaluated in phosphorylation of Smad2L and PAI-1 expressions in BAECs, but also the role of ROCK assessed in the ET-1 induced PAI-1 expression.

## Materials and Methods

This experimental study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IRAJUMS.REC.1396.1.4). fetal bovine serum (FBS), penicillin-streptomycin solution, and low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Invitrogen, Carlsbad, CA, USA). EGF, ET-1, Y27632, AG1478, SB431542, and neomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant transforming growth factor- $\beta$ , HRP anti-rabbit IgG-peroxidase antibody produced in goat, anti-phospho-Smad2L (ser245/250/255) rabbit polyclonal antibody, anti-phospho-Smad2C (ser465/467) rabbit polyclonal antibody, anti-phospho-ERK1/2(The202/204), PAI-1 antibody, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA).

## Cell culture

Bovine aortic endothelial cells (BAEC) were gifted by Professor Peter J Little (School of Pharmacy, The University of Queensland, Australia). BAECs were cultured according to a previously-described procedure (13). In brief, the cells were cultivated in DMEM with 1 g/l glucose containing 10% FBS and 1% antibiotic; and when cells reached about 80 % confluence, they were pretreated with specific inhibitors in certain intervals. In the next step, ET-1 was added to the culture medium. BAECs were incubated with TGF $\beta$  (2 ng/ml) for 1 hour

and with EGF (100 ng/ml) for 5 minutes, once alone and once in combination with each other (13, 24). To investigate the effects of ET-1 on phosphorylation of Smad2L, the BAECs were treated with ET-1 (100 nM), and then harvested at 5 and 15 minutes, 2, 4, and 8 hours intervals. In order to evaluate Smad2C phosphorylation, BAECs were treated with ET-1 (100 nM) and harvested at 1, 2, and 4 hours intervals (13). In order to evaluate ERK phosphorylation, BAECs were treated with ET-1 (100 nM) and were subsequently harvested at 5, 15 and 30 minutes, 1, 2, 4, and 8 hours intervals (25). The effects of SB431542 (10  $\mu$ M for 30 minutes) and AG1474 (10  $\mu$ M for 30 minutes) (24) inhibitors on pSmad2L were tested by pretreating the cells with them. Thereafter, ET-1 (100 nM) was added to the culture medium. The neomycin (100  $\mu$ M for 1 hour) (26) and Y27632 (10  $\mu$ M for 30 minutes) (13) inhibitors were tested on pSmad2L via pre-incubation of the cells prior to addition of ET-1 (100 nM) to the culture medium. To investigate the effects of ET-1 on protein expression of PAI-1, the BAECs were treated with ET-1 (100 nM) and then harvested at 30 minutes, 1, 2, 4, and 8 hours intervals (13). The effects of SB431542 (10  $\mu$ M for 30 minutes) (18) and AG1474 (10  $\mu$ M for 30 minutes) (24) inhibitors on protein expression of PAI-1 were tested by pretreating the cells with them prior to adding ET-1 (100 nM) to the culture medium. The neomycin (100  $\mu$ M for 1 hours) (26) and Y27632 (10  $\mu$ M for 30 minutes) (13) inhibitors were tested on protein expression of PAI-1 via preincubation of the cells prior to addition of ET-1 (100 nM) to the culture medium. The cells were harvested after 4 hours.

## Western blot

Proteins were determined using the method of Seif et al. (4). Briefly, harvested cells were lysed in RIPA buffer. Then, the proteins were separated on 10% SDS-PAGE and transferred to a membrane (PVDF). After blocking steps, the membranes were incubated with primary antibodies. The membranes were washed and then exposed with a secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase. The labeled antibodies were detected with chemiluminescence exposure.

## Statistical analysis

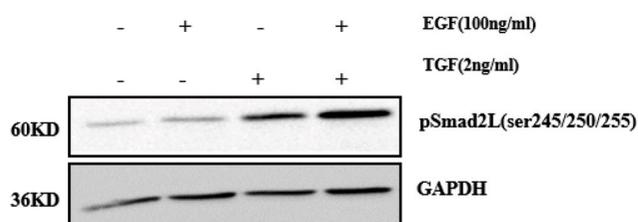
The results are presented as mean  $\pm$  SEM of three individual experiments. Statistical significance was estimated by one-way ANOVA, followed by the least significant difference post-hoc analysis (LSD).  $P < 0.05$  or  $P < 0.01$  considered as statistically significant. Fold change was calculated by dividing all the measured values from the intensity of each area by their controls (for both target and internal control). The areas were obtained using Image J software program. Then, the values of target groups were divided by the values of their control. Graph Pad Prism software program was used for drawing the graphs.

## Results

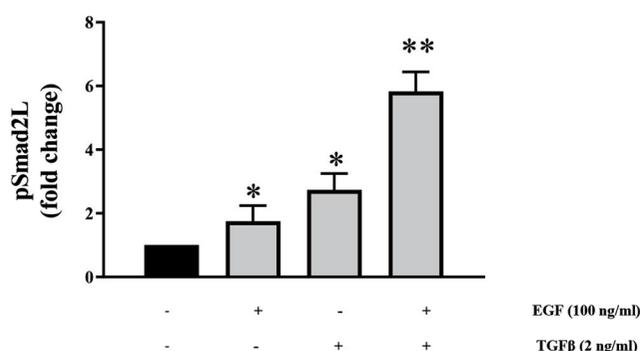
### TGF and EGF induced Smad2L phosphorylation in BAEC

To investigate the role of TGF $\beta$  and EGF in phosphorylation of Smad2L (ser245/250/255), BAECs were incubated with TGF $\beta$  (2 ng/ml) and EGF (100 ng/ml) for 1 hour and 5 minutes, respectively, once alone and once in combination with each other. TGF $\beta$  ( $P < 0.05$ ) and EGF ( $P < 0.05$ ) stimulated Smad2L phosphorylation, and the effects of combination of TGF and EGF could be additive to Smad2L phosphorylation ( $P < 0.01$ , Fig.1). This data demonstrates that both EGF and TGF $\beta$  are individually involved in the phosphorylation of Smad2L through two distinct pathways.

A



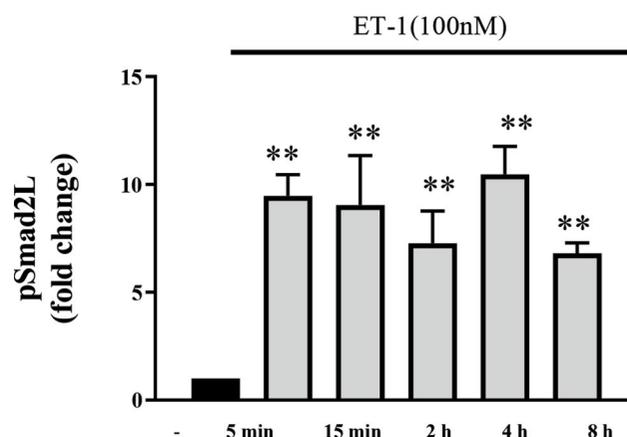
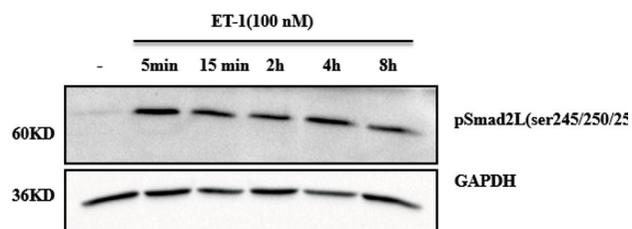
B



**Fig.1:** TGF $\beta$  and EGF lead to phosphorylation of Smad2L. BAECs were incubated with TGF $\beta$  (2 ng/ml) and EGF (100 ng/ml) for 1 hour and 5 minutes, respectively. Values are presented as mean  $\pm$  SEM of three individual experiments. TGF $\beta$ ; Transforming growth factor beta, EGF; Epidermal growth factor, \*,  $P < 0.05$ , and \*\*,  $P < 0.01$  vs. untreated.

### ET-1 stimulated Smad2L phosphorylation in BAECs

The effects of ET-1 on phosphorylation of Smad2L (ser245/250/255) were investigated in different times. BAECs were exposed to ET-1 (100 nM) and phosphorylation of Smad2L measured in a period of 5 minutes to 8 hours. ET-1 induced the phosphorylation of Smad2L (ser245/250/255) ( $P < 0.01$ , Fig.2). This result showed that ET-1 strongly stimulates Smad2L phosphorylation in BAEC.



**Fig.2:** ET-1 stimulates the phosphorylation of Smad2L. BAECs were incubated with ET-1 (100 nM) for periods of 5 minutes up to 8 hours. Values are presented as mean  $\pm$  SEM of three individual experiments. ET-1; Endothelin-1, \*\*,  $P < 0.01$  vs. untreated, min; Minutes, and h; Hours.

### ET-1 mediated dual transactivation of EGFR and TGF $\beta$ in BAECs

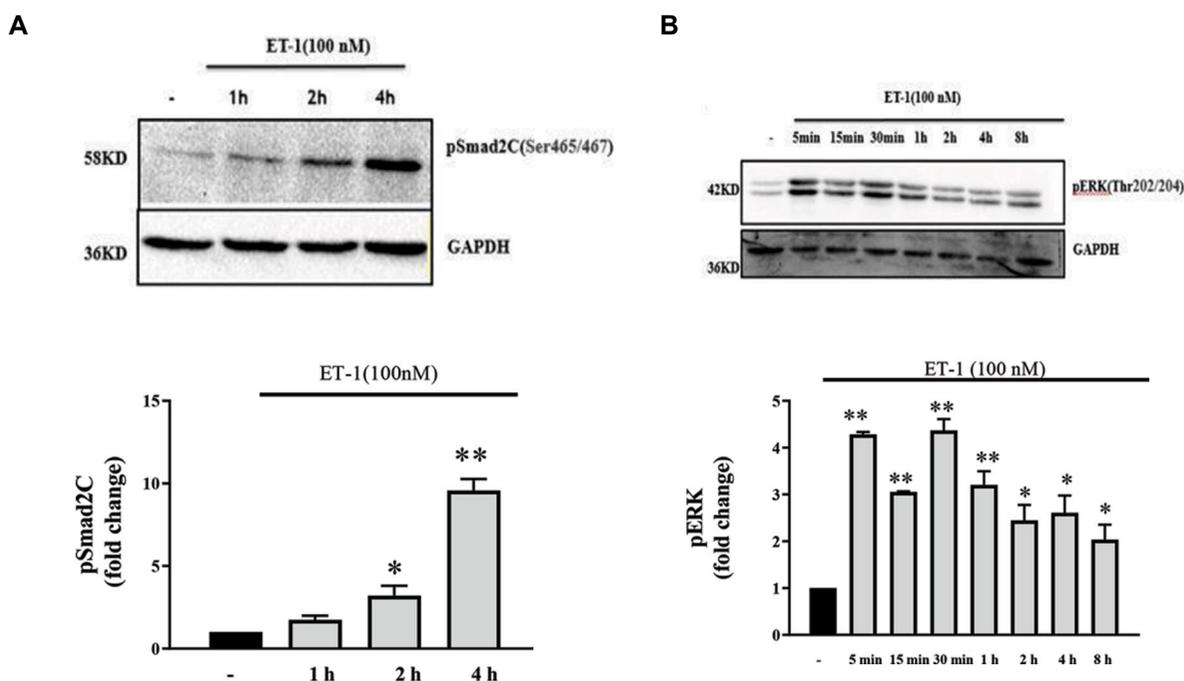
The question here is that whether there is evidence that the transactivation signaling pathways induced by GPCR are directly involved in phosphorylation of Smad2L? In order to evaluate the role of ET-1 in TGF $\beta$  transactivation, the phosphorylation of Smad2C (Ser465/467) was investigated as the instant downstream mediator of TGF $\beta$  activation. BAECs were treated with ET-1 (100 nM) in a period of 1-4 hours. ET-1 led to time-dependent increase in Smad2C phosphorylation at hours two ( $P < 0.05$ ) and four ( $P < 0.01$ , Fig.3A). These results demonstrated that by induction of TGF $\beta$  transactivation, ET-1 can stimulate Smad2C phosphorylation in BAEC in a time-dependent manner. Moreover, to examine the role of ET-1 in EGFR transactivation, the phosphorylation of ERK1/2 was investigated as the instant downstream mediator of EGFR. BAECs were incubated with ET-1 (100 nM) in certain intervals in a period from 5 minutes to 8 hours. ET-1 stimulated ERK1/2 phosphorylation in different points in time between 5 minutes and 1 hours ( $P < 0.01$ ) and between 2 and 8 hours ( $P < 0.05$ , Fig.3B). These results suggest that by transactivation of EGFR, ET-1 can lead to phosphorylation of ERK1/2 in BAEC.

### ET-1 stimulates Smad2L phosphorylation through dual transactivation of TGF $\beta$ and EGFR, as well as ROCK activity in BAECs

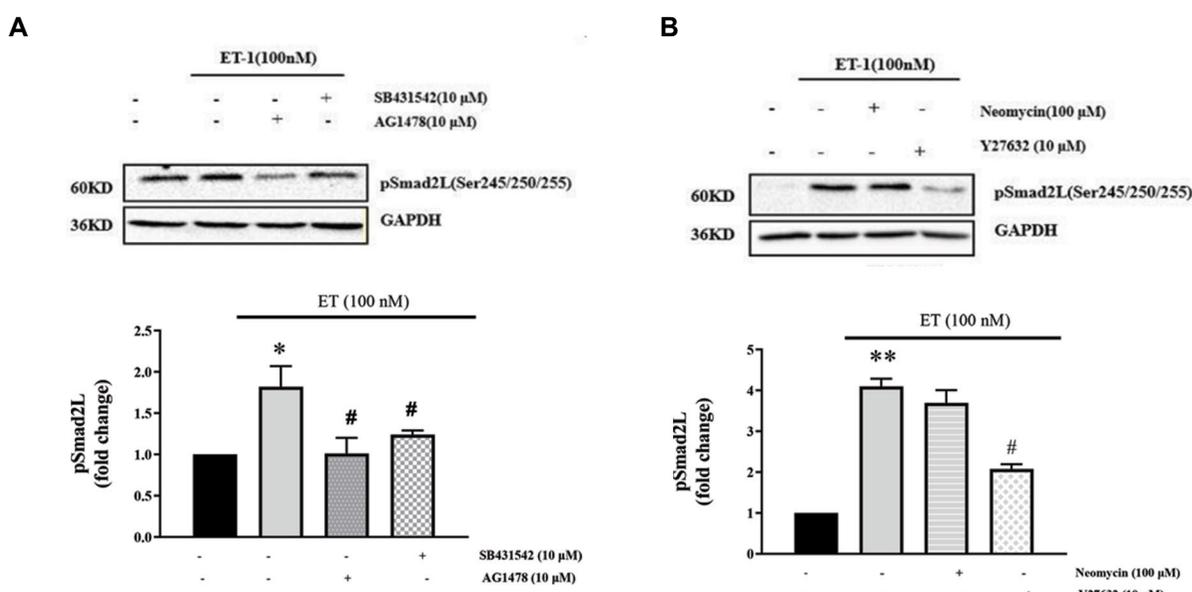
Subsequently, to explore the role of ET-1 induced transactivation pathways in phosphorylation of Smad2L, cells were evaluated in the presence of EGFR antagonist, AG1478 (10  $\mu$ M) and TGF $\beta$  antagonist, SB431542 (10  $\mu$ M)

for 30 minutes prior to treatment with ET-1 (100 nM) for 4 hours. Phosphorylation of Smad2L (ser245/250/255) was markedly alleviated in the presence of AG1478 and SB431542 ( $P < 0.05$ , Fig.4A). The results of this experiment indicate that ET-1 can induce the phosphorylation of Smad2L via transactivation of EGFR and TGFR. Furthermore, the roles of ROCK and PLC were examined in ET-1-induced phosphorylation of Smad2L (ser245/250/255). Neomycin (100  $\mu$ M), the specific inhibitor of PLC $\beta$ , was used as the

downstream mediator of G $\alpha_q$  for 1 hours prior to treatment with ET-1 (100 nM) for 4 hours, and Y27632 (10  $\mu$ M), a potent inhibitor of ROCK was used as the downstream mediator of G12/13 for 30 minutes prior to treatment with ET-1 (100 nM) for 4 hours. The results of this work showed that Y27632 can reduce Smad2L phosphorylation ( $P < 0.05$ ), but neomycin cannot do the same (Fig.4B). This shows that stimulation of Smad2L phosphorylation by ET-1 is dependent on ROCK activity.



**Fig.3:** ET-1 leads to phosphorylation of Smad2C and ERK1/2. BAECs were incubated with ET-1 (100 nM). ET-1; Endothelin-1, \*;  $P < 0.05$ , \*\*;  $P < 0.01$  vs. untreated, min; Minutes, and h; Hours. Values are presented as mean  $\pm$  SEM of three individual experiments.



**Fig.4:** ET-1 leads to phosphorylation of Smad2L via both dual transactivation and the ROCK activity. **A.** BAECs were preincubated with SB431542 (10  $\mu$ M) for 30 minutes and AG1478 (10  $\mu$ M) for 5 minutes before stimulation with ET-1 (100 nM) for 4 hours. **B.** BAECs were preincubated with neomycin for 1 hours (100  $\mu$ M) and with Y27632 (10  $\mu$ M) for 30 minutes before stimulation with ET-1 (100 nM) for 4 hours. ET-1; Endothelin-1, \*;  $P < 0.05$  vs. untreated, #;  $P < 0.05$  vs. ET-1 treated. Values are presented as mean  $\pm$  SEM of three individual experiments.

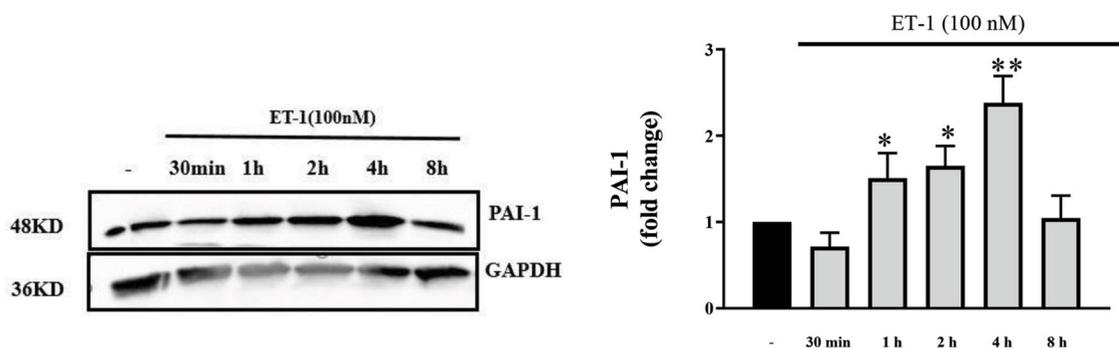
### ET-1 stimulated the protein expression of PAI-1 in BAEC

The effects of ET-1 were examined on the protein expression of PAI-1. BAECs were exposed to ET-1 (100 nM) at certain points in time from 30 minutes to 8 hours. ET-1 induced the protein expression of PAI-1 at hours one and two ( $P<0.05$ ) and four ( $P<0.01$ , Fig.5). Overall, the results showed that ET-1 increases the protein expression of PAI-1 in BAEC. we chose four hours' incubation time with ET-1 for the next experiments.

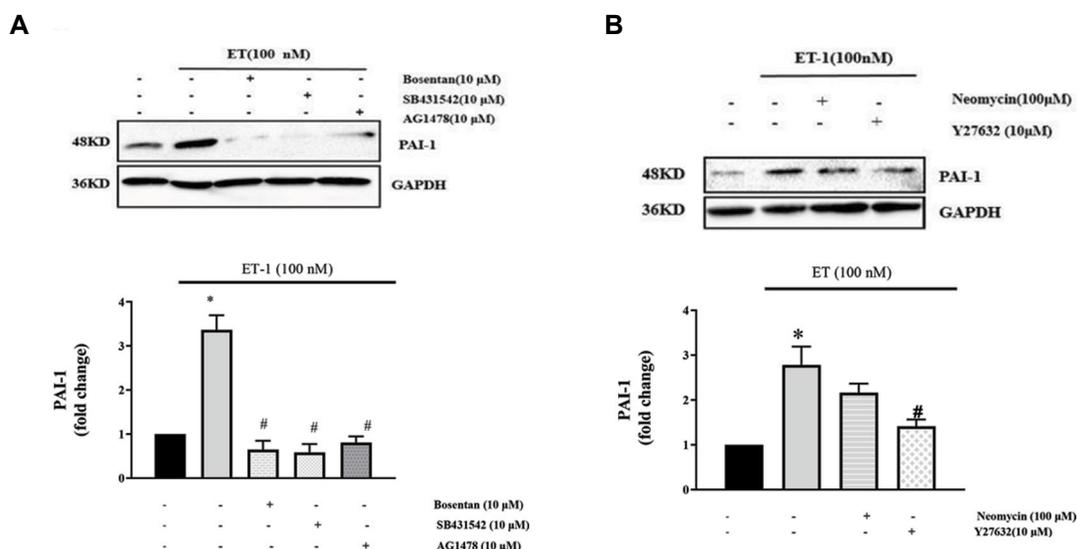
### ET-1 stimulates the protein expression of PAI-1 in BAECs through dual transactivation of TGFR and EGFR, as well as the ROCK activity

To assess whether ET-1 leads to an increase in protein expression of PAI-1 via its receptor with induction of dual transactivation, AG1478 (10  $\mu$ M) and SB431542 (10  $\mu$ M) as EGFR and TGFR inhibitors, respectively, and Bosentan (10  $\mu$ M) as ET receptor inhibitor were

utilized for 30 minutes prior to treatment with ET-1(100 nM) for 4 hours. The results showed that ET-1-increased protein expression of PAI-1 was reduced in the presence of AG1478 ( $P<0.05$ ), SB431542 ( $P<0.05$ ), and Bosentan ( $P<0.05$ , Fig.6A). The present work's data showed that via its receptor, ET-1 can transactivate EGFR and TGFR in order to stimulate the protein expression of PAI-1. Moreover, in order to study the roles of ROCK and PLC as mediators of the transactivation pathway in protein expression of PAI-1, neomycin (100  $\mu$ M) for 1 hours and Y27632 (10  $\mu$ M) for 30 minutes as inhibitors of PLC $\beta$  and ROCK were used before being stimulated with ET-1 (100 nM) for 4 hours. The results showed the significant reduction of the protein expression of PAI-1 in the presence of Y27632 ( $P<0.05$ ), while neomycin could not inhibit the protein expression of PAI-1 (Fig.6B). From this data, it was concluded that ET-1 increased the protein expression of PAI-1, which was dependent on ROCK activity.



**Fig.5:** ET-1 leads to an increase in the protein level of PAI-1. BAECs were incubated with ET-1 (100 nM) for 30 minutes to 8 hours. Values are presented as mean  $\pm$  SEM of three individual experiments. ET-1; Endothelin-1, \*;  $P<0.05$ , \*\*;  $P<0.01$  vs. untreated, min; Minutes, and h; Hours.



**Fig.6:** ET-1 leads to an increase in the protein level of PAI-1 via induction of dual transactivation pathways, as well as the ROCK activity. **A.** BAECs were preincubated with SB431542 (10  $\mu$ M), AG1478 (10  $\mu$ M), and Bosentan (10  $\mu$ M) 30 minutes before being stimulated with ET-1 (100 nM) for 4 hours. **B.** BAECs were preincubated with neomycin (100  $\mu$ M) for 1 hours and Y27632 (10  $\mu$ M) for 30 minutes before stimulation with ET-1 (100 nM) for 4hours. ET-1; Endothelin-1, \*;  $P<0.05$  vs. untreated, #;  $P<0.05$  vs. ET-1 treated. Values are presented as mean  $\pm$  SEM of three individual experiments.

## Discussion

In this study, the role of ET-1-induced dual transactivation pathways of EGFR and TGF $\beta$ R were investigated in phosphorylation of Smad2L, as well as the protein expression of PAI-1. Here, it was demonstrated that ET-1 stimulates Smad2L phosphorylation and increases the level of PAI-1 protein through transactivation of EGFR and TGF $\beta$ R, and that ROCK has a central role in this pathway. TGF $\beta$ -1, alone and in combination with EGF, induced the phosphorylation of Smad2L, which is consistent with the data put out by Kamato et al. (10). Phosphorylation of Smad2L was increased by EGF and TGF $\beta$ , indicating the presence of the active pathways of these growth factors in induction of Smad2L phosphorylation. Recent studies have shown that in addition to EGF and TGF $\beta$ , GPCR agonists result in activation of kinases such as NOX, P38, and ERK through induction of TGF $\beta$ R and EGFR transactivation (27). Our results showed that ET-1 increased the phosphorylation of Smad2L in BAECs. Kamato et al. (10) presented the evidence that thrombin leads to phosphorylation of Smad2L in VSMCs via transactivation-dependent signaling pathways. In this study, the focus was on the signaling pathways causing the phosphorylation of Smad2L. It was demonstrated that ET-1 stimulated the phosphorylation of Smad2C via TGF $\beta$ R transactivation, as well as ERK1/2 via EGFR transactivation, in BAEC. In a study recently published by the authors, it has been shown that ET-1 stimulates the phosphorylation of Smad2C via TGF $\beta$ R transactivation in BAEC (13). Burch et al. (28) showed that via PAR-1, thrombin can not only lead to EGFR transactivation, but also TGF $\beta$ R transactivation in VSMCs.

It was found that AG1478 (EGFR antagonist) and SB431542 (TGF $\beta$ R antagonist) reduced the effect of ET-1 on phosphorylation of Smad2L, suggesting that ET-1 mediated the phosphorylation of Smad2L through dual transactivation of EGFR and TGF $\beta$ R. Kamato et al. (24) demonstrated that thrombin stimulated the phosphorylation of Smad2L through transactivation of both EGFR and TGF $\beta$ R in VSMC. GPCRs are the biggest cell-surface receptors without any enzymatic activity. These receptors associate with G proteins including G $\alpha$ , G $\beta$ , and G $\gamma$ . Activated G proteins interact with diverse mediators and can regulate signaling responses (8). Signaling pathways that are activated by G proteins are comprised of the following: phospholipase C $\beta$ , adenylate cyclase (AC), and cyclic adenosine monophosphate (cAMP) pathways, as well as Rho kinase (ROCK) (29).

There have been several studies on the roles of these mediators in transactivation pathways. EGFR transactivation is stimulated by Ang II via increasing intracellular Ca<sup>2+</sup> and activation of PLC/IP3 pathway (30). On the other hand, another study concluded that EGFR transactivation was induced by Ang II, independent of intracellular calcium concentration and PLC/IP3 pathway (31). ROCK signaling leads to transactivation of RSTK in the epithelial cells of mouse lung (32). Therefore, in this study the roles of ROCK and PLC

were assessed in Smad2L phosphorylation. It was found that Y27632 (ROCK antagonist) decreased the phosphorylation of Smad2L that was induced by ET-1, but neomycin (PLC antagonist) had no effect on Smad2L phosphorylation. According to the previous studies, ROCK leads to phosphorylation of Smad2C through TGF $\beta$ R transactivation (13, 28, 33). Therefore, based on the results of this study, it is suggested that ROCK has an important role in ET-1 transactivation pathways and subsequently Smad2L phosphorylation. This result is consistent with earlier studies, showing that thrombin stimulated the phosphorylation of Smad2L which is dependent on MMP and ROCK activities in VSMCs (23). Wang et al. (34) showed that PAI-1 is a remarkable prognosticator of cardiovascular disease -dependent death. PAI-1 is a significant factor in the pathophysiology of vascular sclerosis. PAI-1 is mostly expressed by endothelial cells as well as tissues with elevated TGF $\beta$ -1 (35). Multiple studies have confirmed that TGF $\beta$ -1-induced PAI-1 expression occurs via stimulation of EGFR transactivation in vascular, epithelial, and endothelial cells (35, 36). The current study demonstrated that ET-1 increased the level of PAI-1 expression in BAECs in four hours after treatment with ET-1; however, this response was decreased eight hours after the treatment. Therefore, it is possible that deactivation of ET-1 occurred in eight hours. The same pattern can be seen in Smad2L phosphorylation, thus verifying that these pathways are related together. Cell lines alter in morphology, response to stimuli, growth rates, gene and protein expression in different passage numbers (37). The changes observed in the protein expression particularly in the control group in various experiments, may be influenced by different passage numbers, which can be considered as a limitation of this study. In addition, Cockell et al. (38) showed that thrombin induces antigen, natural activity, and mRNA expression of PAI-1 in baboon aortic smooth muscle cells (BASMC). Kerins et al. (39) indicated that Ang IV can stimulate the endothelial expression of PAI-1 via induction of an endothelial receptor.

The protein expression of PAI-1 that is stimulated by ET-1 is decreased in the presence of SB431542 and AG1478. It is suggested that induction of PAI-1 by ET-1 is intervened by transactivation of EGFR and TGF $\beta$ R. Chaplin et al. (33) reported that thrombin transactivated EGFR and TGF $\beta$ R, which can phosphorylate Smad2L and ERK1/2, increase the gene expression of CHSY1 enzymes in VSMCs. The ET-1-stimulated protein expression of PAI-1 was blocked in the presence of ET receptor antagonist (bosentan), strongly suggesting that this response is mediated via the ET-1 receptor. This study has been the first attempt to scrutinize the significant role of ROCK in protein expression of PAI-1 in BAECs. To examine the importance of ROCK and PLC as mediators of activated G proteins, the level of PAI-1 protein was investigated in the presence of Y27632 (ROCK antagonist) and neomycin (PLC antagonist). In a previous study by the authors, it was shown that ET-1 receptor can transactivate the TGF $\beta$ R and then phosphorylate Smad2C.

It was demonstrated that Rho/ROCK kinase plays an important role in mediating the transactivation of TGFR and phosphorylation of Smad2C (Ser465/467) induced by ET-1 (13). Moreover, in another study by the authors that has not yet been published, the role of Rho/ROCK kinase is investigated in ET-1-induced EGFR transactivation. In the current study, a decrease in PAI-1 protein expression was observed in the presence of Y27632 (ROCK antagonist) that could inhibit Smad2L phosphorylation. Based on the results obtained from this study, Rho/ROCK kinase (as a mediator of the transactivation pathway) has a role in Smad2L phosphorylation and PAI-1 protein expression.

In this study, it was shown that ROCK is involved in PAI-1 protein expression for the first time. Observations of the current work strongly suggest that ROCK induced Smad2L phosphorylation via transactivation and affected the enhancement of PAI-1 protein expression. TGF- $\beta$ 1 increases the PAI-1 expression in aortic endothelial cells via P38 and CDK activations, resulting in phosphorylation of Smad2L (16). Similarly, Talati et al. (40) described that thrombin induced the phosphorylation of the linker region of Smad2 and the increase of the mRNA expression of PAI-1 via transactivation of EGFR in keratinocytes. The results showed that EGFR and TGFR transactivation pathways that are induced by ET-1 are in fact independent pathways. Whereas, Smad2L phosphorylation is the common pathway between dual transactivation pathways. Therefore, this study suggests that Smad2L can be considered as a therapeutic target. However, further studies are required in order to identify GPCRs signaling more comprehensively.

## Conclusion

The current study demonstrated that ET-1 stimulated the phosphorylation of Smad2L, and this reaction was blocked by AG1478 and SB431542, suggesting that ET-1 leads to Smad2L phosphorylation via induction of dual transactivation of EGFR and TGFR. According to the results of previous studies, ROCK has a key role in inducing transactivation pathways. Hence, induction of Smad2L phosphorylation through dual transactivation of EGFR and TGFR is dependent on ROCK signaling. Furthermore, it was demonstrated that ET-1 increased the level of PAI-1 protein via transactivation of EGFR and TGFR, which is associated with promoting the intravascular thrombosis and atherosclerosis. Moreover, this cellular response is also dependent on ROCK signaling. Therefore, it can be concluded that Smad2L phosphorylation and promotion of PAI-1 protein level may be related together. However, further studies are needed to identify this signalling.

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## Authors' Contributions

F.S.; Performed all *in vitro* experiments, analyzed the data, and wrote the manuscript. H.B.-R., A.Kh.; Contributed to concept and design, financial support, and final approval of the manuscript. All the authors read and approved the final manuscript.

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