Endothelin-1 dependent expression of GAG genes involves NOX and p38 mediated Smad linker region phosphorylation

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Abstract
Endothelin-1 (ET-1) is implicated in the development of atherosclerosis and mediates glycosaminoglycan (GAG) chain hyperelongation on proteoglycans. Our aim was to identify the ET-1-mediated signalling pathway involving NADPH oxidase (NOX), p38 MAP kinsae and Smad2 linker region phosphorylation (phospho-Smad2L) regulate GAG synthesising enzymes mRNA expression (C4ST-1 and ChSy1) involved in GAG chains hyperelongation in human vascular smooth muscle cells (VSMCs). Signalling intermediates were detected and quantified by Western blotting and the mRNA levels of GAG synthesising enzymes were assessed by quantitative real-time polymerase chain reaction (qRT-PCR). ET-1 treatment of human VSMCs resulted in an increase in phospho-Smad2L level. The TGF-β receptor antagonist, SB431542 and the mixed ETA and ETB receptor antagonist bosentan, inhibited ET-1-mediated phospho-Smad2L level. In the presence of apocynin and diphenyleneiodonium chloride (DPI) (NOX inhibitors) and SB239063 (p38 inhibitor) ET-1-mediated phospho-Smad2L levels were inhibited. The gene expression levels of GAG synthesising enzymes post-ET-1 treatment were increased compared to untreated controls (p < 0.01). The ET-mediated the mRNA levels of these enzymes were blocked by the bosentan, SB431542, SB239063, DPI, apocynin and antioxidant N-acetyl-L-cysteine (NAC). ET-1-mediated signalling to GAG synthesising enzymes gene expression occurs via transactivation-dependent pathway involving NOX, p38 MAP kinsae and Smad2 linker region phosphorylation.

KEYWORDS
endothelin-1, GAG synthesising enzymes, NADPH oxidase, Smad2 linker region, transactivation

1 | INTRODUCTION

A critical initiating step of atherogenesis is sub-endothelial retention and deposition of atherogenic low-density lipoproteins (LDLs) in the arterial intima by the extracellular matrix molecules, particularly proteoglycans. LDLs bind to the chondroitin sulphate (CS) glycosaminoglycan (GAG) chains on proteoglycans in the intima via an ionic interaction between basic amino acid residues of apolipoproteins B100 and...
negatively charged sulphate and carboxyl groups on glycosaminoglycan side chains.\textsuperscript{1,2} The structural changes in CS GAG chains of proteoglycans is the initial pro-atherogenic step that leads to increase the retention of atherogenic lipids and progression of atherosclerosis.\textsuperscript{3} The increase in GAG chain length may be due to increases in the expression of the enzymes that synthesise the CS GAG chains. Recently, it has been shown that two Golgi enzymes, chondroitin 4-O-sulfotransferase-1 (C4ST-1) and chondroitin N-acetylgalactosaminyltransferase-2 (ChGn-2), regulate the CS chain length in the arterial wall during the progression of atherosclerosis.\textsuperscript{4} Therefore, the pathways controlling the expression and activity of these enzymes might be a novel therapeutic strategy for the prevention of atherosclerosis. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide associated with the development of atherosclerosis via its actions on all cells of the vasculature.\textsuperscript{5} The expression of ET-1 and its receptors is up-regulated in vascular cells cultured in vitro and in atherosclerotic human arteries.\textsuperscript{5–7} Vascular smooth muscle cells (VSMCs) express ET\textsubscript{A} and ET\textsubscript{B} receptors that these receptors are seven transmembrane G protein-coupled receptors (GPCRs).\textsuperscript{9} ET-1 acting through its GPCRs signals a plethora of diverse signalling pathways to activate pro-atherogenic cellular events such as proliferation, contraction, migration and extracellular matrix production.\textsuperscript{9,10} Cellular signalling by GPCR occurs via three major pathways: G protein mediated pathways, signalling mechanisms through β-arrestin molecules that form scaffold complexes and the transactivation-dependent pathway of GPCR-activated cell surface kinase receptors which result in regulated gene transcription and modifications of cellular function.\textsuperscript{11} Daub et al. in 1996, described that multiple GPCR agonists such as thrombin, ET-1 and angiotensin II (Ang II) can mediate the transactivation of protein tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR).\textsuperscript{12} More recently it has been discovered that GPCRs can also mediate the transactivation of protein serine/threonine kinase receptors such as that for transforming growth factor (TGF)-β receptor (T\textsubscript{R}1).\textsuperscript{13} The action of ET-1 on proteoglycan synthesis in human VSMCs is blocked by SB431542, suggesting that ET-1-stimulated proteoglycan synthesis is partially via transactivation of the T\textsubscript{R}1.\textsuperscript{14} ET-1 stimulates the synthesis and secretion of proteoglycans with longer GAG chains which enhanced LDL binding and the signalling for this response occurs via ET receptor-mediated transactivation of the T\textsubscript{R}1. The involvement of Smad2 transcription factor in this pathway has been described in human VSMCs.\textsuperscript{14,15} Smads are transcription factors that play a crucial role in the TGF-beta family signalling cascades, Smad2/3 then complexes with Smad4 for translocation into the nucleus where they regulate transcription of multiple genes including those associated with GAG chains elongation.\textsuperscript{32} Smad 2/3 and smad4 consist of three functional domains: The N-terminal DNA binding domain (MH1); a linker region; and a C-terminal domain (MH2). Recently, Kamato et al. showed that thrombin stimulation of human VSMCs leads to the transactivation of the T\textsubscript{R}1 to induce regulation of ChSy-1 and C4ST-1 mRNA expression involving Smad2 linker region phosphorylation.\textsuperscript{15,16} Smad linker region phosphorylation can be activated by multiple serine/threonine kinases, including mitogen-activated protein kinase (MAP kinase) cascades. The stimulation of Smad linker region phosphorylation by non-TGF-β and T\textsubscript{R}1 pathways is a clear example of pathway crosstalk.\textsuperscript{15–18} Our earlier study showed that TGF-beta regulated proteoglycan synthesis through Smad linker region that phosphorylated by p38 MAP kinase.\textsuperscript{19} p38 MAP kinase signalling pathway activated by Reactive oxygen species (ROS) as molecular signalling. It is known that NADPH Oxidase (NOX) is one of major source of intracellular ROS in the cells. Here, we have studied whether NOX has a role in GAG synthesising enzyme mRNA expression through p38 MAP kinase activation that resulted in increased phosphorylation of the Smad linker region. Very little is known about the effects of GPCR signalling on Smad linker region phosphorylation in VSMCs. In this study, we have investigated the effect of GPCR agonist and vasoactive compound ET-1 on the phosphorylation of the transcription factor Smad2 linker region. ET-1 treatment of human VSMCs leads to a time-dependent increase in Smad2 linker region phosphorylation levels. Additionally, we show that the mechanism of ET-1-stimulated phosphorylation of Smad2 linker region occurs via transactivation-dependent pathway involving NOX and p38 MAP kinase. We also demonstrate that ET-1-mediated signalling to GAG synthesising enzymes mRNA expression occurs via phosphorylation of Smads in the linker region.

2 | RESULTS

2.1 | ET-1 rapidly increases the phosphorylation of Smad2 linker region in human VSMCs

Previous study showed that the GPCR agonist ET-1 acting through its receptor, ET receptor, leads to the phosphorylation of the transcription factor Smad2 in its extreme carboxy termini, a response normally associated with T\textsubscript{R}1 activation.\textsuperscript{14} Smad linker region phosphorylation can occur via the TGF-β or alternatively many other agonists to regulate gene transcription.\textsuperscript{15,20} We performed a time course experiment (0–6 h) of human VSMCs to study the ET-1 (100 nM) stimulated Smad2 linker region phosphorylation. There was a rapid increase in phosphorylation of Smad2 linker region at 0.5 h (3.3-fold) (p < 0.01) following the addition of ET-1 (Figure 1). Phospho-Smad2L levels was increased up to 1 h (3-2 fold) (p < 0.01) after which it fell slightly to 2-fold at 4 h. These results demonstrate that phosphorylation of Smad2 linker region is mediated by the ET-1 signalling pathway in these cells. ET-1 phosphorylation of Smad2 linker region showed a temporal response with maximum phosphorylation at 0.5 h. This time point was chosen in subsequent experiments to investigate phosphorylation of Smad2 linker region (Figure 2).

2.2 | ET-1 mediated Smad2 linker region phosphorylation occurs via the transactivation of the EGFR and the T\textsubscript{R}1

To explore the pathway of ET-1 transactivation leading to Smad2 linker region phosphorylation, we asked the question whether or not the transactivation of ET-1 to EGFR or T\textsubscript{R}1 leads to phosphorylation of Smad2 linker region. We utilised the T\textsubscript{R}1 inhibitor, SB431542 (10 μM) and the EGFR inhibitor, AG1478 (5 μM). ET-1 (100 nM)
increased the phosphorylation of Smad2 linker region ($p < 0.05$). In the presence of SB431542, the ET-1 mediated Smad2 linker region phosphorylation was inhibited with a maximal inhibitory effect ($p < 0.01$) (Figure 3A). In the presence of AG1478 the phosphorylation of Smad2 linker region was inhibited ($p < 0.01$) (Figure 3B). To investigate if ET-1 can transactivate EGFR and TβR1 to cooperate in generating increased phosphorylation of Smad2 linker region, both of the EGFR and TβR1 inhibitors were used in VSMCs treated with ET-1 (100 nM).

2.3 ET-1 mediated Smad2 linker region phosphorylation involves NOX

In human VSMCs we found that ET-1 increases the phosphorylation of Smad2 linker region with a peak phosphorylation at 0.5 h. In order to elucidate the role of NOX in phosphorylation of Smad2 linker region, two inhibitors of NOX (DPI and apocynin) were used to block the TGF-β stimulated Smad2 linker region phosphorylation. VSMCs were stimulated with ET-1 (100 nM) for 0.5 h to ensure the maximal phosphorylation of Smad2 linker region. ET-1 had a 3.5-fold increase in the phosphorylation of Smad2 linker region (Figure 3A). Bosentan (20 μM) prevented the ability of ET-1 to stimulate linker region phosphorylation ($p < 0.05$). Inhibition of NOX with DPI (1 and 10 μM) totally blocked ET-1 stimulated phosphorylation of Smad2 linker region ($p < 0.01$). DPI is an inhibitor for flavoenzymes that widely used...
to block NOX but is not specific to NOX isoforms. We tested a more selective NOX inhibitor, apocynin, that by preventing translocation of p47phox (homologue of NOXO1 in NOX1) to plasma membrane interferes with NOX activation and was previously demonstrated to be an inhibitor of atherosclerotic lesion.\textsuperscript{21} ET-1 (100 nM) stimulated a 1.5-fold increase in phosphorylation of Smad2 linker region at 0.5 h ($p < 0.05$) and this response was completely abolished in the presence of apocynin (1 and 10 $\mu$M; Figure 3B).

### 2.4 | ET-1 mediated Smad2 linker region phosphorylation occurs via p38 MAP kinase

Smad linker region phosphorylation can be activated by multiple serine/threonine kinases, such as p38 MAP kinase.\textsuperscript{16,22} It is known that in human VSMCs TGF-\(\beta\) mediated Smad2 linker region phosphorylation requires p38 MAP kinase activity.\textsuperscript{16} To study the involvement of p38 MAP kinase in ET-1 induced Smad2 linker region phosphorylation, human VSMC were treated in the presence or absence of p38 MAP kinase inhibitor, SB239063 (10 and 20 $\mu$M). ET-1 (100 nM) treatment caused a 2.8-fold ($p < 0.01$) increase in Smad2 linker region phosphorylation after 0.5 h (Figure 4). Inhibition of p38 MAP kinase with SB239063 (10 and 20 $\mu$M) blocked ET-1 stimulated phosphorylation of Smad2 linker region ($p < 0.01$). These data suggest that Smad2 linker region phosphorylation specifically involves p38 MAP kinase signalling. TGF-\(\beta\) (2 ng/ml, 0.5 h) was used as a positive control. TGF-\(\beta\) treatment increased Smad2 linker region phosphorylation ($p < 0.01$) and this response was prevented in the presence of p38 inhibitor, SB239063 (10 $\mu$M).

### 2.5 | ET-1 mediated phosphorylation of p38 is downstream of NOX

To investigate the role of NOX in ET-1-induced p38 MAP kinase activation, we studied the effects of two NOX inhibitors on ET-1-induced p38 MAP kinase phosphorylation in human VSMCs. The cells were pretreated with DPI (10 $\mu$M) and apocynin (10 $\mu$M) for 2 h before the addition of ET-1 (100 nM) for 30 min. We found an increase in phosphorylation of p38 MAP kinase in human VSMCs treated with ET-1 compared to untreated cells ($p < 0.05$). ET-1-stimulated p38 MAP kinase phosphorylation was inhibited by these inhibitors. These data show that ET-1 mediated phosphorylation of p38 MAP kinase is downstream of NOX (Figure 5).

### 2.6 | ET-1 dependent C4ST-1 and ChSy-1 enzymes mRNA expression involves NOX and p38 mediated Smad linker region phosphorylation

In VSMCs, ET-1-stimulated elongation of CS GAG chains on proteoglycans is partially mediated via ET-1 receptor transactivation of
Tjp1. Here we asked the question whether or not the transactivation of ET-1 to Tjp1 leads to the mRNA expression of GAG synthesising enzymes, specifically C4ST-1 and ChSy-1. Initially we determined the response of human VSMCs to ET-1 treatment to stimulate the mRNA expression of C4ST-1, the enzyme that transfers a sulphate group from a donor molecule to N-Acetylgalactosamine (GalNAc) sugar of the repeating disaccharide units on the elongating GAG chain. Following ET-1 (100 nM) treatment of VSMCs a 2.2-fold increase (p < 0.01) in C4ST-1 mRNA expression was observed after 6 h (Figure 6A) and in the presence of bosentan (20 μM) was inhibited. To explore if ET-1 employs transactivation of the Tjp1 in mediating expression of C4ST-1 mRNA levels, we utilised the Tjp1 inhibitor, SB431542 (10 μM). In the presence of SB431542, the ET-1 mediated mRNA expression was inhibited with a maximal inhibitory effect (p < 0.01). These results indicate that in VSMCs ET-1 stimulated mRNA expression of C4ST-1 involves the ET receptor mediated transactivation of the Tjp1. To evaluate the role of the p38 MAP kinase and NOX in ET-1-mediated mRNA expression C4ST-1, we employed the p38 antagonist (SB239063), the NOX inhibitors (DPI and apocynin) and ROS scavenger (NAC). All inhibitors had an inhibitory effect on ET-1 mediated mRNA expression of C4ST-1 (Figure 6A). Chondroitin polymerisation of the CS GAG chain occurs via the action of chondroitin synthases, including ChSy-1. A similar result was seen for the mRNA expression of ChSy-1, where ET-1 (100 nM) elicited a 3-fold increase at 6 h and the response in the presence of bosentan (20 μM), was inhibited (Figure 6B). SB431542 (10 μM) had an inhibitory effect on ET-1 mediated mRNA expression of ChSy-1 (p < 0.01). These results indicate that in human VSMCs ET-1 stimulated mRNA expression of ChSy-1 involves the ET receptor mediated transactivation of the Tjp1. In the presence of SB239063 (20 μM) (p < 0.01), DPI (20 μM), apocynin (20 μM) and NAC (10 mM) (p < 0.01) the mRNA expression for ChSy-1 was completely inhibited (Figure 6B). These results suggest that ET-1 may up-regulate C4ST-1 and ChSy-1 mRNA expression via transactivation of Tjp1 involving NOX/ROS, p38 MAP kinase and subsequent phosphorylation of Smad2 in the linker region (Figure 7).

3 Discussion

The signalling pathways that are capable of regulating the length of GAG chains on proteoglycan and biglycan, may represent a potent target for the prevention of atherosclerosis. Here, we investigate a new signalling pathway from ET-1 to Smad2 linker region phosphorylation which leads to regulate the specific GAG synthesising enzymes in human VSMCs. ET-1 is a potent vasoconstrictor hormone which is expressed in atherosclerotic plaques and inhibition ET-1 production and its signalling is a target for prevention of atherosclerosis. The increase in GAG chain length of proteoglycans secreted by VSMCs...
such that they exhibit increased LDL binding is the critical initiating factor in the development of atherosclerotic plaque. To better understand of the complex signalling pathways controlling GAG chain hyperelongation, we explored the mRNA expression associated with the enzymes which mediate the elongation of GAG chains. We found that in human VSMCs treated with ET-1, the expression of ChSy-1 and C4ST-1 was increased. The action of ET-1 to increase these genes occurs via the ET receptor. The increased expression of these genes is correlated with the sulfation and GAG chain hyperelongation in vivo. Recently, Kamato et al. demonstrated that thrombin stimulation of human VSMCs leads to the transactivation of the TβR1 to induce regulation of ChSy-1 and C4ST-1 mRNA expression involving Smad2 linker region phosphorylation. We wished to determine the possibility that the action of ET-1 on C4ST-1 and ChSy-1 gene expression may also be occurred by this mechanism. Here, to address the role of ET receptor-mediated transactivation of TβR1 on the mRNA expression of GAG synthesising enzymes, we assessed the phosphorylation of Smad2 in the linker region. Following ET-1 stimulation of human VSMCs, there was a rise in Smad2 linker region phosphorylation occurring as early as 30 min which was blocked by the TβR1 antagonist SB431542 with maximum inhibition. This data indicates that ET-1-mediated Smad2 linker region phosphorylation is mediated by transactivation of the TGF-β receptor. The expression of C4ST-1 and ChSy-1 stimulated by ET-1 were blocked by SB431542, strongly confirming that ET-1-mediated GAG synthesising enzymes mRNA expression can occur through the TGF-β receptor transactivation. These results suggest that ET-1 acting through its receptor leads to the transactivation of TGF-β receptor to induce regulation of ChSy-1 and C4ST-1 mRNA expression involving Smad linker region phosphorylation. Smad2 linker region contributes to GAG synthesising enzyme ChSy-1 and C4ST-1 mRNA expression but the upstream mediators differ. The linker region of Smad phosphorylated via the multiple alternate pathways, including MAP kinases that can be activated by TGF-β and many other growth factors. Several studies have demonstrated that activation of GPCRs via ET-1 could activate MAP kinases cascades to regulate various cellular responses including growth, proliferation, and cellular hypertrophy in several cell types. In human VSMCs, ERK and p38 are regulators of the mRNA expression of the
Our findings indicate that the activation of p38 MAP kinase by ET-1 leads to the phosphorylation of Smad2 linker region in human VSMCs. A number of studies reported that the activation of p38 MAP kinase cascade can occur via NOX/ROS in intracellular signal transduction pathways. Furthermore, one previous study has demonstrated an increase in phosphorylation of p38 MAP kinase in NOX4-overexpressing human endothelial cells. Our experiments support that NOX is involved in ET-1-induced p38 MAP kinase phosphorylation in human VSMCs. NOX is upstream of p38 MAP kinase because NOX inhibitors were able to block p38 MAP kinase phosphorylation. Among NOX isoforms (NOX1-5, Duox1/2), NOX1 and NOX4 isoforms are highly expressed in the VSMCs which are participated in the oxidation processes in the vascular wall. To address the role of NOX in ET-1 mediated expression of C4ST-1 and ChSy-1, we used NOX inhibitors in this response. The ET-1-stimulated expression of C4ST-1 and ChSy-1 was blocked in presence of NOX inhibitors in the present study. Since NAC scavenges ROS, the present findings also suggest that GAG synthesising enzymes mRNA expression is sensitive to ROS. These clearly demonstrate that NOX is involved in the expression of genes associated with GAG hyperelongation via the activation of p38 MAP kinase and Smad2 linker region. Other signalling pathways may also be involved in this process and further studies are necessary to explore these possibilities.

We have identified a highly specific signalling pathway from ET-1 to Smad2 linker region phosphorylation via transactivation of the TjIR1 which results to ChSy-1 and C4ST-1 mRNA regulation in human VSMCs. Our study shows a novel pathway of redox-sensitive signalling via NOX and p38 MAP kinase in this pathway. Smad linker region phosphorylation is a major contributor to ET-1 signalling to induce mRNA expression of GAG synthesising enzymes ChSy-1 and C4ST-1 that are closely associated with GAG chain elongation. These findings provide a better understanding of the signalling pathways controlling GAGs hyperelongation on proteoglycans and controlling these changes may be a therapeutic target for the prevention of atherosclerosis.

4 | MATERIALS AND METHODS

4.1 | Materials

Dulbecco's Modified Eagle Medium nutrient mixture-F12 (DMEM/F12), antibiotics (penicillin, streptomycin) and trypsin-EDTA were obtained from Bioidea (Tehran, Iran). Fetal bovine serum (FBS) was purchased from Gibco (Invtrogen, Carlsbad, CA, USA). The following chemicals were obtained from Sigma Aldrich (St Louis, MO, USA): SB239063, SB431542, apocynin, diphenyleleniodinium chloride (DPI), N-acetyl-L-cysteine (NAC), bosentan, endothelin-1. Anti-rabbit immunoglobulin-G (IgG)–horseradish peroxidase (HRP) antibody was obtained from Abcam (Cambridge, MA, USA). Primers (forward and reverse) for C4ST-1, ChSy-1 and GAPDH were purchased from Takapouzist (Tehran, Iran).

4.2 | Culture of human aortic smooth muscle cells

Human VSMCs were obtained from the Pasteur Institute (Tehran, Iran). These cells were maintained in DMEM/F12 (1:1) culture medium with 10% (v/v) FBS and 1% penicillin–streptomycin at 37 °C under 5% CO₂. For experiments, VSMCs were seeded into 35 mm dishes (qRT-PCR and Western blot experiments) at a density of 4 × 10⁵ well and grown to confluency and then confluent cultures were serum starved for 24 h by incubation in DMEM/F12 medium containing 0.1% (v/v) FBS and 1% penicillin–streptomycin before experimentation. VSMCs were pre-incubated with inhibitors (bosentan (20 μM), SB431542 (10 μM) and SB239063 (20 μM) for 30 min, NAC (10 mM) for 1 h, DPI (20 μM) and apocynin (20 μM) for 2 h and then exposed to ET-1 (100 nM) or TGF-β (2 ng/ml). VSMCs used in experiments were between passages 10–20. Treatment conditions are described in detail in the figure legends.

4.3 | Quantitative RT-PCR

The mRNA levels of GAG synthesising enzymes C4ST-1 and ChSy-1 were determined by quantitative real-time polymerase chain reaction.
levels. or using one-way ANOVA followed by post hoc Tukey’s test. error of the mean (SEM) and statistical significance was analysed All results were normalised and reported as the mean ± standard (q-RT-PCR). To measure gene expression, total RNA from cultured human VSMCs was extracted using RNeasy Mini kit (Yektatajhiz, Iran) according to the manufacturer’s protocol, and RNA concentration and purity was checked using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The first strand of CDNA was synthesised from 500 ng RNA using the cDNA synthesis kit (TAKARA, Shiga, Japan) according to the manufacturer’s protocol. Q-RT-PCR to determine the levels of human ChSy-1, C4ST-1 and housekeeping GAPDH mRNAs was performed using the SYBR Green qPCR Master Mix (Yektatajhiz, Iran) together with specific primers of the following sequences.

C4ST-1 Forward: 5′-GGGCCCTGCGCAAAG-3′, Reverse: 5′-GGGTTGTTGGTGATGAG-3′,
ChSy-1 Forward: 5′-CCCCGGCCAGAAGAGTC-3′, Reverse: 5′-TCTCAACCCATTCATACTGCAA-3′,
GAPDH Forward: 5′-CAGTTCAACGGCACAGTCAAG-3′,
Reverse: 5′-CATACTACGACCGACGACACC-3′. Data was normalised to the GAPDH housekeeping gene. All experiments were performed at least three times and analysis performed in duplicate for each experiment.

4.4 Western blotting

VSMCs were pre-treated with inhibitors followed by stimulation by Et-1 and TGF-β or left untreated as control. Cells were lased in RIPA buffer containing protease inhibitor cocktail on ice and protein extracted at 12,000 x g for 15 min at 4°C, protein concentrations measured with the Bradford assay. Total cell lysates with concentration of 50 μg of proteins were resolved on 10% polyacrylamide gels SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 3% skim milk at room temperature for 1 h and then incubated at 4°C overnight with primary anti-phospho-Smad2 (Ser245/250/255) rabbit polyclonal antibody (1:1000 dilution) followed by HRP-conjugated secondary antibody (1:1000 dilution) and then ECL detection. The membranes were reprobed with anti-GAPDH polyclonal antibody (1:10,000 dilution) and then ECL detection kit. GAPDH was used as loading control.

4.5 Statistical analysis

All data are averaged from three or more independent experiments. All results were normalised and reported as the mean ± standard error of the mean (SEM) and statistical significance was analysed using one-way ANOVA followed by post hoc Tukey’s test. p < 0.05 or p < 0.01 were accepted as statistically significant difference of levels.

ACKNOWLEDGMENTS

This work was supported by the Ahvaz Jundishapur University of Medical Sciences (Grant no. HLRC-9412). Open access publishing facilitated by The University of Queensland, as part of the Wiley - The University of Queensland agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST

The authors declare the absence of any conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the authors.

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How to cite this article: Babaahmadi-Rezaei H, Mohamed R, Dayati P, et al. Endothelin-1 dependent expression of GAG genes involves NOX and p38 mediated Smad linker region phosphorylation. Clin Exp Pharmacol Physiol. 2022;49(7):710-718. doi: 10.1111/1440-1681.13650