Atorvastatin's Therapeutic Potential in Atherosclerosis: Inhibiting TGF-β-Induced Proteoglycan Glycosaminoglycan Chain Elongation through ROS-ERK1/2-Smad2L Signaling Pathway Modulation in Vascular Smooth Muscle Cells

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Abstract

Objective: According to the response-to-retention hypothesis, the inception of atherosclerosis is attributed to the deposition and retention of lipoprotein in the arterial intima, facilitated by altered proteoglycans with hyperelongated glycosaminoglycan (GAG) chains. Recent *studies* have elucidated a signaling pathway whereby transforming growth factor-β (TGF-β) promotes the expression of genes linked to proteoglycan GAG chain elongation (*CHSY1* and *CHST11*) via reactive oxygen species (ROS) and the downstream phosphorylation of ERK1/2 and Smad2L. Atorvastatin is known to exhibit pleiotropic effects, including antioxidant and anti-inflammatory. The purpose of the present research was to ascertain the influence of atorvastatin on TGF-β-stimulated expression of *CHST11* and *CHSY1* and associated signaling pathways using an *in vitro* model.

Materials and Methods: In this experimental study, vascular smooth muscle cells (VSMCs) were pre-incubated with atorvastatin (0.1-10 μM) prior to being stimulated with TGF-β (2 ng/ml). The experiment aimed to evaluate the phosphorylation levels of Smad2C, Smad2L, ERK1/2, the NOX p47phox subunit, ROS production, and the mRNA expression of *CHST11* and *CHSY1*.

Results: Our research results indicated that atorvastatin inhibited TGF-β-stimulated *CHSY1* and *CHST11* mRNA expression. Further experiments showed that atorvastatin diminished TGF-β-stimulated ROS production and weakened TGF-β-stimulated phosphorylation of p47phox, ERK1/2, and Smad2L; however, we observed no effect on the TGF-β-Smad2C pathway.

Conclusion: These data suggest that atorvastatin demonstrates anti-atherogenic properties through the modulation of the ROS-ERK1/2-Smad2L signaling pathway. This provides valuable insight into the potential mechanisms by which atorvastatin exerts its pleiotropic effects against atherosclerosis.

Keywords: Atorvastatin, Glycosaminoglycans, Smad Proteins, Transforming Growth Factor

Introduction

Atherosclerosis is a lipid-driven inflammatory disease that affects the blood vessel walls and is characterized by the formation of atherosclerotic plaques within the arterial walls (1). The response to retention hypothesis posits that the onset of atherosclerosis is initiated by the retention and deposition of lipoproteins in the arterial wall, facilitated by changed proteoglycans (2). Particularly, structural alterations in proteoglycans, such as hyperelongation of the glycosaminoglycan (GAG) chain, result in increased binding to lipoproteins, thereby promoting atherogenic lipoprotein trapping within the vessel wall (3-5). Transforming growth factor-β (TGF-β), a multifunctional cytokine implicated in vascular remodeling and atherogenesis, has been substantiated to stimulate the hyperelongation of GAG chains by inducing the expression of key enzymes involved in GAG chain elongation, such as chondroitin synthase 1 (CHSY1) and chondroitin 4 sulfotransferase-1 (CHST11/C4ST1) (6-8). Inhibiting GAG chain hyperelongation of proteoglycans has been proposed and demonstrated as a possible therapeutic approach for atherosclerosis (9, 10). TGF-β regulates numerous cellular events, such as biglycan synthesis and GAG chain elongation in vascular smooth muscle cells

(VSMCs), through both canonical and non-canonical signaling pathways (7, 11). In the canonical pathway, TGF-β attaches to the type II receptor (TβRII), subsequently activating the type I receptor (TβRI). Activated TGFβR1 induces the phosphorylation of Smad2 or Smad3 at the carboxy terminus, allowing them to form a heterocomplex with the Smad4 protein. This heterocomplex then translocates to the nucleus to activate the expression of target genes involved in GAG chain synthesis and structure. In contrast, the non-canonical signaling cascade involves mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-regulated kinase 1/2 (ERK1/2) (7, 12). TGF-β stimulates ERK1/2 to phosphorylate the Smad2 linker region (Smad2L), ultimately resulting in elevated expression of the *CHSY1* and *CHST11* genes (7, 13). In animal models of atherosclerosis, increased *CHST11* mRNA expression has been associated with lipoprotein retention and atherosclerosis acceleration (14). Reactive oxygen species (ROS), which function as secondary messengers in signal transduction, have been associated with increased *CHSY1* and *CHST11* expression. Mohamed et al. (15) demonstrated that in VSMCs, ROS derived from NADPH oxidase (NOX) foster *CHSY1* and *CHST11* expression by augmenting ERK1/2 activation and its downstream Smad2L phosphorylation. NOX, a prominent ROS source in vascular cells, exhibits up-regulation in various cardiovascular diseases, such as atherosclerosis (16). The NOX complex consists of two transmembrane subunits, namely p22phox and gp91phox, along with multiple cytosolic subunits, including p67phox, p47phox, p40phox, and Rac. When cells are activated, the cytosolic components undergo phosphorylation and translocation to the membrane, where they gather together with the component that is bound to the membrane (17). P47phox phosphorylation is necessary for NOX activation and is linked to ROS generation (18). NOX-derived ROS can activate MAPK (19), which in turn stimulates Smad2L phosphorylation to increase *CHSY1* and *CHST11* expression (15). Consequently, pharmacological interventions targeting these signaling cascades may be a promising potential therapeutic strategy against atherosclerosis.

Statins, such as atorvastatin, are a group of medications used to treat patients with hypercholesterolemia. Besides lowering cholesterol levels, statins are known to have various pleiotropic (cholesterol-independent) effects, including improved endothelial function, reduced vascular inflammation, decreased oxidative stress, and enhanced stability of atherosclerotic plaques (20, 21). In a related study, it was observed that statin exerts vascular protective effects by obstructing the ROS-p38/ERK1/2 in VSMCs (22). It has been demonstrated that statin exerts protective effects against VSMC migration and proliferation in vascular remodeling (23). Moreover, research has shown that atorvastatin has potential anti-atherogenic properties (24) and attenuates atherosclerosis progression (25).

The anti-atherogenic effects of statins have been linked to their pleiotropic effects, specifically their antioxidant, anti-thrombotic, and anti-inflammatory actions (26). Considering the anti-atherogenic effects of atorvastatin and its modulating effect on the TGF-β/Smad, in this research, we aimed to investigate whether atorvastatin can influence the process of GAG chain elongation in proteoglycans. For this purpose, we utilized TGF-β to stimulate human VSMCs and evaluated the phosphorylation levels of ERK1/2, Smad2L, Smad2C, the NOX p47phox subunit, ROS production, and the mRNA expression of *CHST11* and *CHSY1*, which are all involved in the GAG chain elongation.

Materials and Methods

The present research was approved by the Ahvaz Jundishapur University of Medical Sciences Ethics Committee (IR.AJUMS.REC.1399.459).

Reagents and antibodies

In this experimental study, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), antibiotics (streptomycin and penicillin), fetal bovine serum (FBS), and trypsin-EDTA used were all purchased from Bioidea (Tehran, Iran). In addition, we ordered from Sigma Aldrich (USA) TGF-β receptor 1 inhibitor SB431542, dichlorodihydrofluorescein diacetate (DCFH-DA), protease inhibitor cocktail, Tween 20, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and atorvastatin. We acquired from Pars Tous (Iran) the bicinchoninic acid (BCA) protein assay kit and enhanced chemiluminescence (ECL) detection kit. Acrylamide, TEMED, glycine, and tris were from Merck (Germany). Cell Signaling Technology (USA) supplied the human recombinant TGF-β, anti-rabbit immunoglobulin-G (IgG)-horseradish peroxidase (HRP) antibody, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, phospho-Smad2C (Ser465/467) antibody, the phospho-Smad2L (Ser245/250/255) antibody, and the phospho-ERK1/2 (Thr202/Tyr204) antibody. And last, we acquired our CHSY1, CHST11, and GAPDH primers from Avinstemgene (Ahvaz, Iran).

Cell culture and treatment

Human VSMCs cell line (CRL-1999) was sourced from the Pasteur Institute (Tehran, Iran) and grown in DMEM-F12 at 37°C with 5% CO_2, 10% FBS, and 1% antibiotics. The VSMCs were cultivated in 60-mm dishes until the cells had reached confluence. To initiate treatment, cells were serum-starved by incubating in DMEM/F-12 with 0.1% FBS for 16 to 24 hours. In our experimental setup, VSMCs were pre-treated with atorvastatin for 60 minutes before exposure to TGF-β. The concentration of atorvastatin (dissolved in DMSO) used in this research (0.1, 1, and 10 μM) was based on previous studies (27, 28). SB431542 (10 μM) (29),

a well-characterized TGF-β receptor 1 inhibitor, is dissolved in DMSO. In the untreated control group, an equivalent quantity of DMSO (0.01%) was added. In this experimental study, VSMCs were divided into the control group (VSMCs without any treatment), TGF-β group (VSMCs were incubated in 2 ng/ml TGF-β) (10), atorvastatin groups (VSMCs were pre-treated with varying concentrations of atorvastatin (0.1, 1, and 10 μM), and then stimulated with TGF-β), and TGF-β +SB431542 group (VSMCs were pre-treated with SB431542 for 30 minutes, and then stimulated with TGF-β). All the experiments were replicated three times.

Western blot analysis

Protein extraction was performed using radioimmuno-precipitation assay (RIPA) lysis buffer, and the protein concentration was ascertained using a BCA kit. Equal amounts of proteins (60 μg) from each lysate were analyzed through 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. Primary antibodies against phospho-NCF1/p47-phox (Ser359), phospho-ERK1/2 (Thr202/Tyr204), phospho-Smad2L (Ser245/250/255), and phospho-Smad2C (Ser465/467) were incubated with the membranes overnight after being blocked with 5% (w/v) BSA in Tris-buffered saline-Tween (TBST). An HRP-conjugated secondary antibody was then used to incubate the membranes for 1 hour at room temperature. After removing the primary and secondary antibodies, the blot was reprobed with GAPDH antibodies and incubated with an HRP-labeled secondary antibody overnight at 4°C. The Chemidoc imaging system (BioRad) was utilized with ECL for identifying the proteins of interest. Densitometry analysis was conducted using the ImageJ software program.

Quantification of intracellular reactive oxygen species

The level of ROS in cells was assessed using DCFH-DA. DCFH, on oxidation with ROS, converts into a highly fluorescent substance, 2',7'-dichlorofluorescein (DCF). To briefly elaborate, VSMCs were initially pre-treated with varying concentrations of atorvastatin (0.1, 1, and 10 μM) or SB431542 (10 μM), followed by stimulation with TGF-β at a concentration of 2 ng/ml for 30 minutes. Afterward, the VSMCs were washed in phosphate-buffered saline (PBS) and subjected to the incubation process with 10 μM DCFH-DA in a serum-free medium at a temperature of 37°C for a span of 30 minutes. DCF fluorescence was then measured employing a 488 nm excitation wavelength along with a 520 nm emission wavelength (30).

Quantitative real-time polymerase chain reaction

The levels of *CHST11* and *CHSY1* mRNA expression were calculated using quantitative real-time polymerase chain reaction (qRT-PCR). RNA was extracted from cultured VSMCs using the RNA FastPure™ kit.

The concentration and purity of RNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), which takes absorbance measurements at 260 and 280 nm wavelengths. The Quanti Tect reverse transcription kit (Qiagen, Germany) was applied to synthesize first-strand cDNA. QuantiFast™ SYBR® Green PCR kit (Qiage, Germany) together with specific primers were used to conduct RT-PCR according to the manufacturer's instructions. The expression value was normalized to the GAPDH housekeeping gene. The delta-delta cycle-threshold (ΔΔCt) method was applied for the quantification of the level of mRNA expression. The following sequences of primers were utilized in this investigation:

GAPDH-

F: GTCTCCTCTGACTTCAACAGCG
R: ACCACCCTGTTGCTGTAGCCAA

CHSY1-

F: CGACAGGAACTTTCTCTTCGTGG
R: GGTACAGATGTGTCAGAACCCTC

CHST11-

F: CCTTTGGTGTGGACATCTGCTG
R: CTGTTGGCTCGGCACGTGTCT

Statistical analysis

The analysis of data was carried out employing GraphPad Prism software, version 8.0.2 (GraphPad. Software Inc., La Jolla, CA, USA). Results were depicted as mean ± standard error of the mean (SEM). Statistical evaluation was conducted using a one-way analysis of variance (ANOVA) followed by an LSD post hoc test. Any P<0.050 was deemed statistically significant.

Results

Atorvastatin diminishes TGF-β-induced reactive oxygen species formation and phosphorylation of p47phox

Our research aimed to determine the relevance of atorvastatin to ROS formation and the phosphorylation of p47phox, a crucial step in NOX activation. Upon stimulation of VSMCs with TGF-β, intracellular ROS formation and p47phox phosphorylation increased compared to the non-treated control. Pretreatment of VSMCs with atorvastatin dose-dependently reduced TGF-β-mediated ROS formation and the maximal inhibitory effect was observed at 10 μM (n=3, P<0.050 vs. TGF-β alone) (Fig.1A). Moreover, atorvastatin pretreatment significantly attenuated p47phox phosphorylation (n=3, P<0.050 vs. TGF-β alone) (Fig.1B), which was consistent with the observed reduction of ROS production. The specific TGF-β receptor 1 inhibitor (SB431542) attenuated TGF-β-stimulated ROS formation and p47phox phosphorylation (P<0.050), as predicted.

A

Figure 2 demonstrates that after stimulating VSMCs with TGF-β, ERK1/2 phosphorylation was elevated (n=3, P<0.050 vs. non-treated control), while atorvastatin inhibited TGF-β-stimulated ERK1/2 phosphorylation (n=3, P<0.050 vs. TGF-β alone). SB431542 attenuated TGF-β-induced ERK1/2 phosphorylation (P<0.050), as predicted.
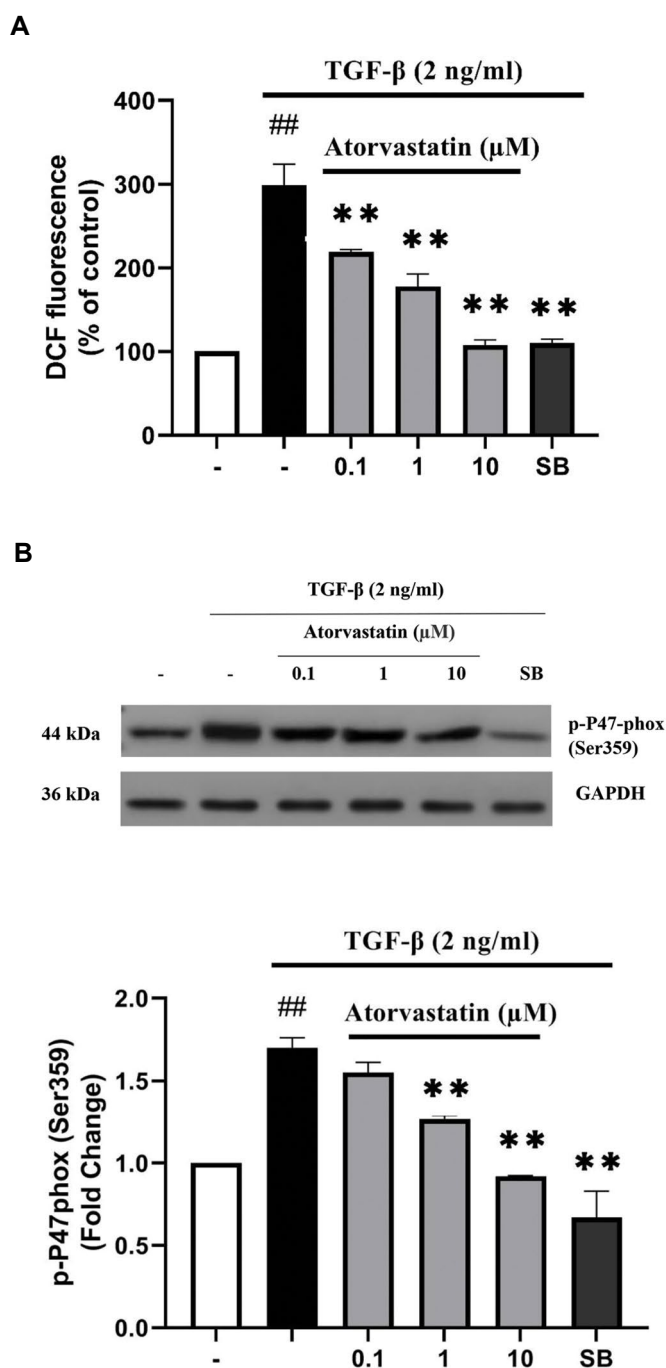
B

Fig.1: Impact of atorvastatin on TGF-β-stimulated ROS formation and p47phox phosphorylation. Cells were exposed with or without TGF-β (2 ng/ml) for a period of 15 minutes, in the presence or absence of atorvastatin (at concentrations of 0.1, 1, and 10 µM) or 10 µM of TGF-β receptor 1 inhibitor SB431542 (SB). **A.** Subsequently, the intracellular ROS levels and **B.** The phosphorylation state of p47phox were determined using a ROS assay kit and western blot analysis, respectively. The data expressed as mean ± SEM, were derived from three independent experiments. Statistical significance was defined as ##; P<0.010 against the non-treated control group and **; P<0.010 against the TGF-β group. TGF-β; Transforming growth factor-β, ROS; Reactive oxygen species, and DCF; 2',7'-dichlorofluorescein.
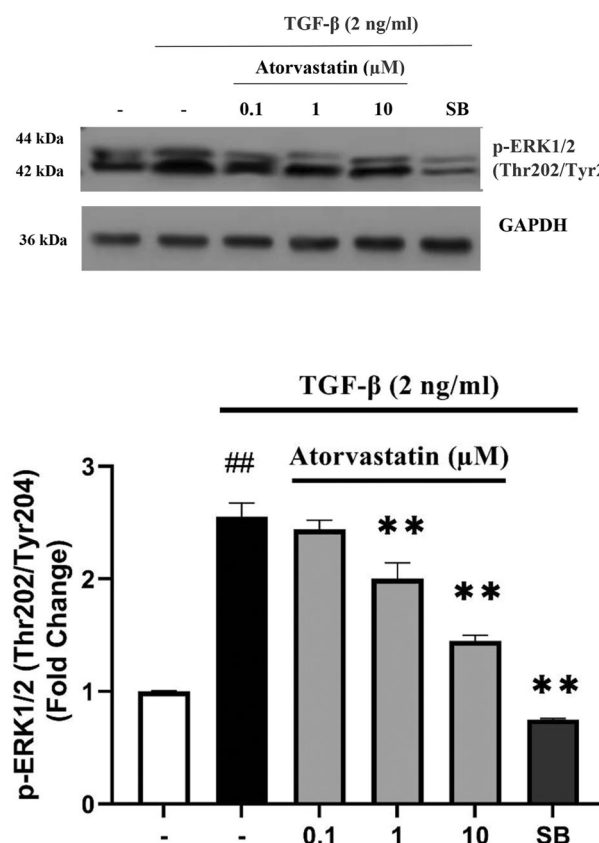
Fig.2: Influence of atorvastatin on TGF-β-stimulated ERK1/2 phosphorylation. Cells were pre-incubated with atorvastatin at concentrations of 0.1, 1, and 10 µM, or with 10 µM of TGF-β receptor 1 inhibitor SB431542 (SB) prior to stimulation with 2 ng/ml of TGF-β for 15 minutes. The degree of ERK1/2 phosphorylation was evaluated using western blot analysis. Data from three experiments, presented as mean ± SEM, indicated statistical significance as ##; P<0.010 against the non-treated control group and **; P<0.010 against the TGF-β group. TGF-β; Transforming growth factor-β.

Atorvastatin suppresses TGF-β-stimulated ERK1/2 phosphorylation

We examined whether atorvastatin pretreatment reduces TGF-β-mediated ERK1/2 phosphorylation in VSMCs.

Atorvastatin blocks TGF-β-induced Smad2L phosphorylation

The effects of atorvastatin on TGF-β-mediated phosphorylation of Smad2L were explored by western blot analysis. In VSMCs stimulated with TGF-β, Smad2L phosphorylation was elevated by 2.27-fold (n=3, P<0.050 vs. non-treated control). Preincubation of VSMCs with atorvastatin or SB431542 significantly attenuated Smad2L phosphorylation (n=3, P<0.050 vs. TGF-β alone) (Fig.3A).

Atorvastatin did not affect on theTGF-β-induced Smad2C phosphorylation

Western blot analysis was used to investigate the impact of atorvastatin on TGF-β-mediated Smad2C phosphorylation. As shown in Figure 3B, TGF-β treatment of VSMCs led to an increase in Smad2C phosphorylation (n=3, P<0.050 vs. non-treated control), while atorvastatin did not affect TGF-β-induced phosphorylation of Smad2C.

Atorvastatin mitigates TGF-β-stimulated *CHSY1* and *CHST11* mRNA expression

GAG chain hyperelongation has been linked to the pathogenesis of atherosclerosis, with CHSY1 and CHST11 serving as rate-limiting enzyme genes that mediate this process. TGF-β-stimulated phosphorylation of ERK1/2 and Smad2L has been linked to the mRNA expression of *CHSY1* and *CHST11*. As mentioned earlier, atorvastatin significantly attenuated TGF-β-induced phosphorylation of ERK1/2 and Smad2L, so we assessed its effect on the mRNA expression of *CHSY1* and *CHST11*. Figure 4A demonstrates that after stimulating VSMCs with 2 ng/ml TGF-β, mRNA expression of *CHSY1* was 4.3 fold elevated (n=3, P<0.050 vs. non-treated control). However, pretreatment with atorvastatin significantly decreased TGF-β-mediated *CHSY1* mRNA expression (n=3, P<0.050 vs. TGF-β alone). Similarly, TGF-β incubation caused a 3.5-fold increase in *CHST11* mRNA expression in VSMCs (n=3, P<0.050 vs. non-treated control), which was reduced by atorvastatin (n=3, P<0.050 vs. TGF-β alone) (Fig.4B). The effect of atorvastatin on TGF-β-stimulated mRNA expression of *CHST11* was dose-dependent, while the effect on mRNA expression of *CHSY1* was dose-independent. These findings reveal that atorvastatin can inhibit TGF-β-induced GAG chain hyperelongation.
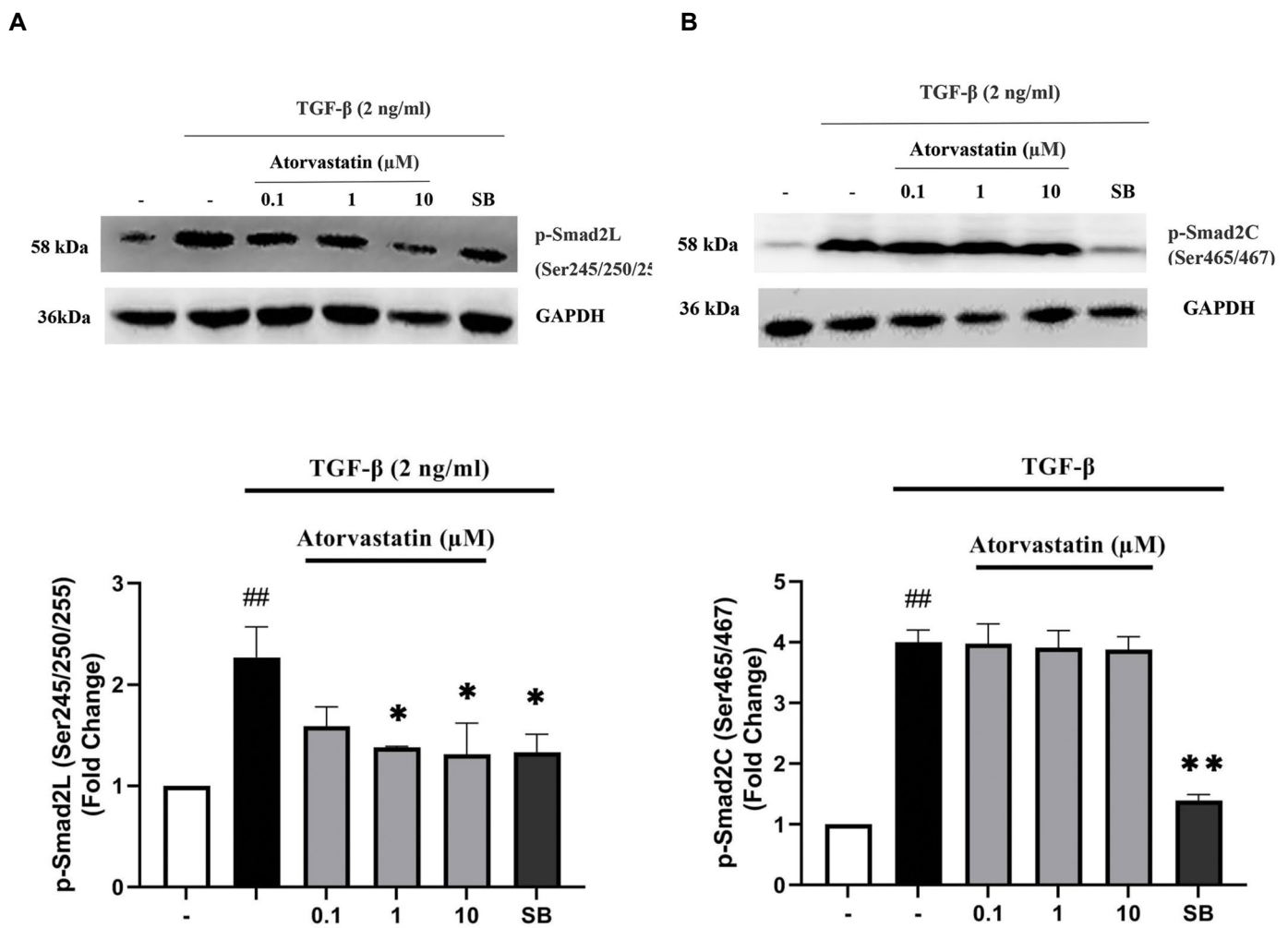

Fig.3: Role of atorvastatin in TGF-β-induced phosphorylation of Smad2L and Smad2C. VSMCs were pre-treated with atorvastatin (at concentrations of 0.1, 1, and 10 μM) or 10 μM of TGF-β receptor 1 inhibitor SB431542 (SB), followed by stimulation with TGF-β at a concentration of 2 ng/ml for 30 minutes. Expression levels of **A.** p-Smad2L and **B.** p-Smad2C were determined by western blot analysis. Data from three experiments, presented as mean ± SEM, denoted statistical significance as ##; P<0.010 against the non-treated control group, *; P<0.050, and **; P<0.010 against the TGF-β group. TGF-β; Transforming growth factor-β.
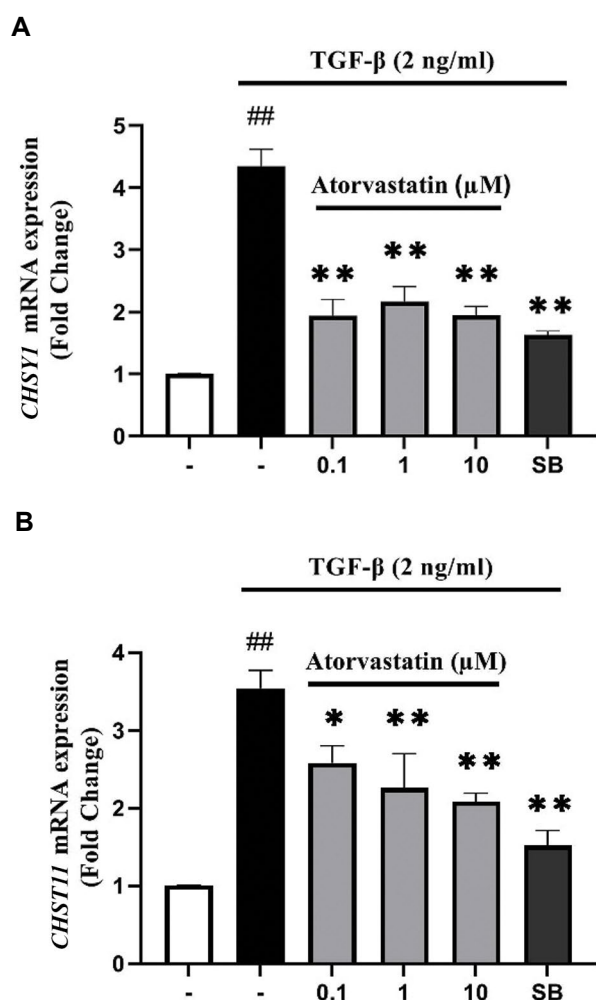
A

TGF-β (2 ng/ml)

CHSY1 mRNA expression (Fold Change)

Atorvastatin (μM)

B

TGF-β (2 ng/ml)

CHST11 mRNA expression (Fold Change)

Atorvastatin (μM)

Fig.4: Impact of atorvastatin on TGF-β-induced *CHSY1* and *CHST11* mRNA expression. cells were pre-incubated with varying concentrations of atorvastatin (0.1, 1, and 10 μM) or TGF-β receptor 1 inhibitor SB431542 (SB; 10 μM), followed by a 24-hour treatment with TGF-β (2 ng/ml). The mRNA expression levels of **A.** *CHSY1* and **B.** *CHST11* in VSMCs were evaluated by real-time polymerase chain reaction (PCR). Data from three experiments are presented as the mean ± SEM. ##; $P<0.010$ against the non-treated control group, *; $P<0.050$, and **; $P<0.01$ against the TGF-β group. TGF-β; Transforming growth factor-β.

Discussion

In this study, we focused on the effect of atorvastatin on GAG chain elongation of proteoglycans and associated signaling pathways using an *in vitro* model. We applied TGF-β to stimulate VSMCs and discovered that atorvastatin could inhibit the expression of *CHSY1* and *CHST11* in VSMCs. Additionally, we observed that atorvastatin reduced TGF-β-induced ROS formation and attenuated the phosphorylation of p47phox, ERK1/2, and Smad2L induced by TGF-β. We further demonstrated that atorvastatin had no effect on Smad2C phosphorylation.

The initial step in the progression of atherosclerosis is characterized by the accumulation and retention of lipoproteins within the arterial wall due to ionic interactions with the GAG chains of proteoglycans

(2). CHSY1 and CHST11 are rate-limiting enzymes that mediate GAG chain elongation of proteoglycans (31). Our findings show that TGF-β enhances the expression of *CHST11* and *CHSY1* in VSMCs, which corroborates with previous research (10). TGF-β activates the ERK1/2 signaling pathway, which stimulates the phosphorylation of Smad2L, subsequently driving *CHSY1* and *CHST11* expression in VSMCs (7, 13). Mohamed et al. (15) have established that ROS mediates *CHSY1* and *CHST11* expression via increasing ERK1/2 activation and Smad2L phosphorylation. This data substantiates that the ROS-ERK1/2-Smad2L signaling pathway is a significant driver of the expression of *CHSY1* and *CHST11*. The identification of potential inhibitors capable of impeding these signaling pathways and GAG chain elongation suggest a possible therapeutic strategy for atherosclerosis.

Atorvastatin is recognized to have various pleiotropic effects, including antioxidant, anti-inflammatory, and vascular endothelial function improvements (20, 21). This drug exerts its pharmacological actions by inhibiting a wide array of signaling pathways, including ERK1/2, P38, and Smad3 (27, 32). Our research revealed that atorvastatin inhibited the TGF-β-stimulated *CHSY1* and *CHST11* expression through the inhibition of ERK1/2 and Smad2L phosphorylation. This result is in line with prior research reporting the anti-atherogenic effects of atorvastatin (28, 33). For example, Peng et al. (33) examined the impact of atorvastatin on a mouse model of vulnerable atherosclerotic plaques and found that the drug reduced inflammation, decreased lipid deposition, and stabilized vulnerable atherosclerotic plaques. All these data suggest that atorvastatin can be considered as a potential therapeutic strategy to treat atherosclerosis.

Excessive ROS production has been linked to the pathogenesis of atherosclerosis (34). Previous studies have demonstrated atorvastatin's potent antioxidant capabilities. For instance, atorvastatin was found to exhibit antioxidant effects through the suppression of NOX subunit expression in VSMCs and in the vasculature of spontaneously hypertensive rats by Wassmann et al. (35). Another study has shown that atorvastatin can reduce aldosterone-induced ROS generation in VSMCs (36). Mohamed et al. (15) have revealed that the TGF-β-stimulated expression of genes connected with GAG chain elongation is mediated through NOX-dependent mechanisms, with ROS playing an important component in the TGF-β-induced expression of these genes. In our investigation, we assessed the impact of atorvastatin on this molecular pathway and discovered that it could diminish the elevated levels of ROS induced by

TGF-β in VSMCs, which is consistent with Bruder-Nascimento's study (36). Additionally, atorvastatin lessened the phosphorylation of the NOX p47phox subunit, a critical event in NOX activation. This result is in agreement with the previous report, which showed that another statin, rosuvastatin, attenuated angiotensin II-induced phosphorylation of p47phox (37). Furthermore, it has been shown that atorvastatin exhibits antioxidant effects via reduced expression of essential NOX subunits and increased expression of catalase (35). Since ROS are known to mediate TGF-β-stimulated *CHSY1* and *CHST11* expression in VSMCs (15), atorvastatin may attenuate TGF-β-stimulated *CHSY1* and *CHST11* expression, potentially through the reduction of ROS production in VSMCs.

ROS has the ability to induce the activation of different kinases, such as ERK1/2, by modifying the signaling pathway proteins through oxidative processes (38). Statins have been identified to manifest anti-atherosclerotic as well as anti-inflammatory effects through the inhibition of the ROS-p38/ERK1/2 signal pathway (22). Specifically, ERK1/2 is implicated in the phosphorylation of Smad2L in VSMCs (7). To further explore the underlying mechanism of atorvastatin suppression on TGF-β-stimulated GAG elongation, ERK1/2 and Smad2L phosphorylation (non-canonical TGF-β) were evaluated. Our findings show that TGF-β treatment caused a rise in the p-ERK1/2 level, which subsequently caused an increase in p-Smad2L levels. This is in accordance with the findings of Burch et al. (7). However, in the presence of atorvastatin, TGF-β-mediated phosphorylation of ERK1/2 and the subsequent phosphorylation of Smad2L were attenuated. A similar mechanism of statins inhibiting proliferation through a decrease in ERK1/2 phosphorylation was also demonstrated in VSMCs (32). TGF-β is known to stimulate ROS production via NOX activation, and ROS can modulate phosphorylation of ERK1/2 and Smad2L, leading to *CHSY1* and *CHST11* expression in VSMCs (17). All of those can be inhibited by atorvastatin.

It has been demonstrated that the phosphorylation of Smad2 at the carboxy terminus (canonical TGF-β signaling) is associated with proteoglycan GAG elongation in VSMCs (11). To further understand whether atorvastatin's suppression of *CHSY1* and *CHST11* expression happens through inhibiting the canonical TGF-β signaling, we evaluated Smad2C phosphorylation. Interestingly, our study demonstrated that atorvastatin doesn't affect TGF-β-mediated Smad2C phosphorylation. This finding suggests that atorvastatin might operate through a non-canonical pathway. The effect of statins on Smad proteins varies depending on the cell type and pathological conditions. For instance, in human tenon fibroblasts, lovastatin hinders TGF-β-mediated myofibroblast transdifferentiation by inhibiting MAPK activation without affecting Smad2/3 phosphorylation (39), Conversely, in alveolar epithelial cells, simvastatin reduces TGF-β1-stimulated epithelial-mesenchymal transition through inhibiting Smad2 and Smad3 phosphorylation (40).

Our study uncovers a novel mechanism by which atorvastatin exerts its therapeutic effects on atherosclerosis. We demonstrate that atorvastatin inhibits the TGF-β-induced proteoglycan GAG chain elongation through ROS-ERK1/2-Smad2L signaling pathway modulation. This finding provides a new understanding of the molecular basis of atorvastatin's anti-atherosclerotic activity and also highlights the potential for statins to be developed as more effective and versatile therapeutic agents. However, various limitations should be noted. We only carried out *in vitro* experiments; thus, more *in vivo* studies are needed to confirm the in vitro results. We have evaluated the effects of atorvastatin on the phosphorylation of ERK1/2 and its downstream Smad2L. However, the impact of atorvastatin on other kinases associated with Smad2L phosphorylation (i.e., P38) needs to be further investigated. Furthermore, the effect of atorvastatin on other sources of ROS production, such as nitric oxide synthase, lipoxygenase, and xanthine oxidase, must be further elucidated.

Conclusion

This study demonstrates that TGF-β stimulates the expression of genes linked to proteoglycan GAG chain elongation (*CHSY1* and *CHST11*) in VSMCs. However, atorvastatin attenuates TGF-β-induced *CHSY1* and *CHST11* expression via interfering with ROS production and subsequent suppression of ERK1/2 and Smad2L phosphorylation. According to these findings, the anti-atherogenic properties of atorvastatin could potentially be attributed to the modulation of the ROS-ERK1/2-Smad2L signaling pathway, providing a potential therapeutic strategy for reducing lipoprotein retention within the arterial wall and combating atherosclerosis progression.

Acknowledgements

Authors' Contributions

H.G.-Z.; Conception, Design of the manuscript, and Experimental work. G.M., M.R., M.A.; Data acquisition or Data analysis and Interpretation.

H.G.-Z., H.B.-R.; Experimental work and Drafting of the manuscript. H.B.R.; Final approval of the manuscript and repetation of experimental work. All authors read and approved the final manuscript.

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