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In vitro Effects of Curcumin on Transforming Growth Factor-β-mediated Non-Smad Signaling Pathway, Oxidative Stress, and Pro-inflammatory Cytokines Production with Human Vascular Smooth Muscle Cells

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ABSTRACT

Transforming growth factor-β (TGF-β) induces pro-inflammatory cytokines expression including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and these cytokines are associated with the development of atherosclerosis. Curcumin has anti-atherogenic effects and anti-inflammatory properties in the vascular wall, but the relative mechanisms are almost unknown. In the present study, we investigate the effect of curcumin on modulating the pro-inflammatory action of TGF-β in human vascular smooth muscle cells (VSMCs) and its molecular mechanisms.

Cultured VSMCs were seeded into several groups: a control group (untreated group), a group treated with TGF-β, and several groups treated with TGF-β plus inhibitors. The cells were pre-treated with diphenyleneiodonium chloride, DPI, (20 μM), curcumin (5, 10 and 20 μM) and N-Acetyl-L-Cysteine, NAC, (10 mM) and then TGF-β (5 ng/mL) was added to the culture medium. The mRNA levels of IL-6 and TNF-α were detected by quantitative Real-Time Polymerase Chain Reaction. For monitoring the Smad2 linker region phosphorylation (pSmad2L), the western-blotting technique was applied and reactive oxygen species (ROS) generation was measured by utilizing 2',7'-dichlorofluorescein diacetate-based assay.

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TGF-β increased the mRNA expression of IL-6 (p=0.02 and p=0.001) and TNF-α (p =0.014 and p=0.001) in a time-dependent manner, ROS production (p=0.03) and Smad2L phosphorylation (p=0.015). Pre-treatment with curcumin, DPI and NAC inhibited TGF-β–induced IL-6 (p=0.04) and TNF-α (p=0.001) mRNA expression, Smad2L phosphorylation (p=0.02) and ROS production (0.03).

Pharmacological inhibition by Curcumin blocks TGF-β–induced ROS production, Smad2L phosphorylation, and IL-6 and TNF-α mRNA expression in human VSMCs.

Keywords: Curcumin; Interleukin-6; Smad2 protein; Transforming growth factor beta; Tumor necrosis factor-alpha

INTRODUCTION

Atherosclerosis, the major underlying cause of the cardiovascular disease (CVD), is currently responsible for about 31% of global deaths.[1] Recent studies demonstrated considerable evidence about the role of chronic inflammation as a major player in the pathogenesis of atherosclerosis.[2] Inflammation via the action of pro-inflammatory cytokines in the arterial wall ultimately leads to the development of atherosclerotic plaque.[3] Cytokines are small secreted proteins that are important in cellular signaling pathways to regulate adhesion molecules and chemokines, growth, proliferation, and migration of vascular smooth muscle cells (VSMCs) which promote to the progression of atherosclerotic plaques.[2,3] There is abundant evidence that pro-inflammatory cytokines, namely Interleukin-6 (IL-6) and Tumour Necrosis Factor-α (TNF-α) are produced by macrophages, endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) during atherogenesis and act (often synergistically) on these cell types in the atherosclerotic lesions.[4,5] In a mouse model of atherosclerosis, which has been studied in the Wallenberg Laboratory in Sweden, an increase in the expression of IL-6 and TNF-α correlated with an increase in plaque formation.[6,7] Induced pro-inflammatory cytokines expression by multiple signaling pathways increase the local inflammatory response in the arterial wall. Inhibition of these pathways can provide an accessible target for the prevention of atherosclerosis.[3,8]

Transforming growth factor-β (TGF-β) is a potent growth factor produced by many cell types including macrophages and regulates a range of cellular functions. In the vascular wall, TGF-β induces the expression of pro-inflammatory cytokines and contributes to the development of atherosclerosis.[9] The active TGF-β transmits its signals by binding to its specific receptors at the cell surface comprised of two types I and two types II transmembrane serine/threonine kinase receptors.[10] In the canonical pathway, the activated type I receptors directly phosphorylate R-Smads (receptor-activated Smads), Smad2 or Smad3, at relevant C-terminal regions. Phosphorylated Smad2 and Smad3 then form a heteromeric complex with Smad4 (Co-Smad) and the activated Smad-complex translocate into the nucleus, where they, in a cooperative manner with other nuclear cofactors, regulate transcription of many of target genes.[11] In addition to Smad signaling, TGF-β can also use several other intracellular signaling pathways (known as non-canonical or non-Smad signaling) such as the mitogen-activated protein kinase (MAPK) pathways to modulate a wide array of downstream cellular responses.[12,13] TGF-β induces many of its effects on different vascular wall cells by the involvement of redox-mediated signaling pathways.[14] Increased levels of reactive oxygen species (ROS) correlate with the vascular stress responses and pathophysiology of human atherosclerosis.[15] However, ROS has an important role as modulators of signaling molecules and transcription factors.[16] Accumulative evidence suggests that nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are major enzymes responsible for the production of ROS in the cardiovascular system and increased NOX expression and activity results in the generation of oxidative stress, which can amplify vascular inflammatory responses.[17]

The pharmacological inhibition of NOX-dependent signaling may prevent the advancement of atherosclerosis.[18] Curcumin, a safe natural polyphenolic section extracted from the Curcuma longa plant rhizomes (commonly known as turmeric), has been widely used as food additives but has also been used

for medicinal purposes as an anti-inflammatory and pain-relieving agent in Iran, India and some South East Asia countries.[19,20] Curcumin has a wide range of potential biological and pharmacological effects, including antioxidant effects, anti-cancer, anti-proliferative and anti-inflammatory activities.[19,21] Numerous *in vitro* and *in vivo* studies, especially clinical trials, have revealed that curcumin might be a possible remedial factor for preventing and cure of atherosclerosis.[22-24] Curcumin modulates signaling pathways and regulates the expression of numerous molecular targets, including inflammatory cytokines.[24] However, the principals of the molecular mechanisms of the anti-oxidant and anti-inflammatory effects of curcumin in human VSMC are not manifested. In this study, we determined the regulation of IL-6 and TNF-α expression by curcumin in TGF-β stimulated VSMCs and identified its molecular mechanisms.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen, Carlsbad, CA, USA). Both antibiotics (penicillin, streptomycin) and Trypsin-EDTA (Ethylenediaminetetraacetic acid) were bought from Bioidea (Tehran, Iran). Curcumin (purity over 98%), diphenyleneiodonium chloride (DPI), SB431542, N-Acetyl-L-Cysteine (NAC), 2′,7′- dichlorofluorescein diacetate (H2DCF- DA), sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME), anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and dimethyl sulfoxide (DMSO) were procured from Sigma Aldrich (St. Louis, MO, USA). Human recombinant TGF-β, anti-phospho-Smad2L (Ser245/250/255) and anti-rabbit Immunoglobulin-G (IgG)-horseradish peroxidase (HRP) antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Primers (forward and reverse) for IL-6, TNF-α, and GAPDH were procured from Takapouzist (Tehran, Iran).

Ethical Considerations

The Study Was approved and conducted in accordance with the Ethical Committee of Animal Breeding and Research of Jundishapur University of Medical Sciences (Ethical Code: IR.AJUMS.REC.1395.25). Ethical issues have been observed by the authors.

Preparation of CurcuminStock Solution

Curcumin stock solution was prepared in DMSO to obtain a concentration of 5 mM, and then was stored at −20°C, in the dark. For cell treatments, a stock solution of curcumin was diluted in culture medium to obtain the final working concentrations (5, 10 and 20 μM).[25]For cell treatment, VSMCs were divided into several groups: a control group (untreated group), a group treated with TGF-β, and several groups treated with TGF-β plus curcumin. The cells were pre-incubated with curcumin (5, 10 and 20 μM) for 1 h prior to the addition of TGF-β (5 ng/mL) to the culture medium.

Human VSMC Culture and Treatment

Human VSMCs were purchased from the Pasteur Institute (Tehran, Iran). The cells were cultured in DMEM containing 10% heat-inactivated FBS and 1% antibiotic (penicillin-streptomycin) and were grown in a humidified atmosphere of 5% CO2 at 37˚C. VSMCs that were between passage 3 to 10 were used for all experiments. VSMCs were seeded into 6-well dishes at a density of 5×10^5/well or onto 96 well plates (10,000 cells/well) and incubated until confluency at 37°C. Before the experimentation, confluent cultures were starved in DMEM containing only 0.1% FBS and 1% penicillin-streptomycin and were incubated for 20-24 h before treatment.

For experimentation, VSMCs were seeded into several groups: (1) a control group (untreated group), (2) a group treated with TGF-β, and (3) several groups treated with TGF-β plus inhibitors. The cells (the exception of the control group) were pre-treated with inhibitors for certain time periods and then TGF-β was added to the culture medium. The cells were pre-treated with the TGFβR inhibitor, SB431542, (20 μM, 30 min), the NOX inhibitor DPI (20 μM, 2 hours), antioxidant NAC (10 mM,2 hours) and curcumin (5, 10 and 20 Mm, 1 hour) and then TGF-β (5 ng/mL) was added to the culture medium. For the purposes of a time-course study, the human VSMCs were treated with TGF-β (5 ng/mL) and then harvested at the time points of 2, 4, 8 and 24 hours. Next, The effect of inhibitors and curcumin on TGF-β-mediated ROS production, pSmad2L and IL-6, and TNF-α expression was investigated.

Quantitative Real-time Polymerase Chain Response (q-RT-PCR)

To measure transcript levels of TNF-α and IL-6, total RNA from cultured VSMCs was isolated using RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's protocol, and RNA concentration and purity determined by measuring the absorbance A260 nm/A280 nm by utilizing Nanodrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from 1 µg of total RNA by reverse transcriptase according to the instructions of a commercial Takara RT kit (Takara, Japan). The primer pairs to detect human TNF-α, IL-6 and GAPDH were as follows: IL-6: forward, 5'-AGTCCTGATCCAGTTCCTGC-3; reverse, 5'-AAGCTGCGCAGAATGAGATG-3', TNF-α: forward, 5'-AGGACCAGCTAAGAGGGAGA-3; reverse, 5'-CCCGGATCATGCTTTCAGTG-3',[26] GAPDH: forward, 5′-ACCCAGAAGACTGTGGATGG-3′; reverse, 5′-AGTAGAGGCAGGGATGATGTT-3′.[27] QRT-PCR was performedviaQuantiFast™ SYBR Green PCR kit (Qiagen, Germany) together with specific primers. The mRNA levels were measured through real-time PCR using an ABI instrument (Applied Biosystem 7500 Fast Real-Time PCR System, USA). GAPDH primers were utilized as an internal reference gene (housekeeping gene). All experiments were performed at least three times and analyses performed in duplicate for each experiment. The comparative delta-delta cycle-threshold (ΔΔCt) approach is utilized to estimate the fold variation difference in the expression of mRNA from qRT-PCR tests.

Western Blot Analysis

Cells were collected and the total proteins were extracted from samples. The protein concentration of each sample was determined with the Bradford assay. Extracted proteins (50 µg) were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto a polyvinylidene difluoride membrane. Next, non-specific binding sites were blocked with 5% non-fat milk for 1 h at room temperature, and then independently incubated with primary antibodies against phospho-Smad2 (1:1000), and GAPDH (1: 5,000) overnight at 4°C. Blot was washed and then followed by an anti-rabbit secondary antibody (1: 10,000) conjugated with horseradish peroxidase at room temperature for 1 h and enhanced chemiluminescence (ECL) detection system. The membrane was stripped and incubated with a monoclonal antibody against GAPDH, which was used as an internal control to normalize protein expression levels.

Intracellular ROS Assay

The level of intracellular ROS was measured via 2',7'–dichlorofluorescein diacetate (DCFDA, also known as H2DCFDA) assay protocol, a fluorogenic dye, based on the ROS-dependent oxidation of H2DCFDA to the fluorescent DCF. In brief, human VSMCs at various time points after stimulation with TGF-β (5 ng/mL) were exposed with serum-free DMEM containing 10 µM H2DCFDA for 1 h at 37°C. At the end of incubation time, VSMCs were washed twice with PBS, detached and resuspended in 1 ml of PBS. The fluorescence intensity was immediately measured by spectrofluorometer (Cary, Australia) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Statistical Assessment

All results were from three independent experiments and shown as the mean ± standard error of the mean (SEM). Data management and analysis were performed by IBM SPSS Statistics, version 21 (IBM Corp., Armonk, N.Y., USA). Data were analyzed using One-Way Analysis of Variance (ANOVA) followed by least significant difference (LSD) *post-hoc* analysis and$p< 0.05$ and $p< 0.01$ were considered as statistically significant.

RESULTS

TGF-β Induces ROS Production in Human VSMCs

To investigate the effects of the TGF-β on ROS levels in human VSMCs, the cells were pre-incubated with SB431542 (20 µM), DPI (20 µM), NAC (10 mM) and then TGF-β (5 ng/mL) was added to the culture medium (Fig. 1A). Intracellular ROS production was analyzed by monitoring the DCF fluorescence intensity of each well-using spectrofluorometer. TGF-β treatment increased the ROS levels by 1.6-fold (p=0.037) at 24 hours compared with the control group and this increase was completely blocked in the presence of SB431542. DPI, a selective inhibitor of NOX, and NAC totally prevented the ROS levels caused by TGF-β (p=0.034) (Figure 1A). This result clearly indicates that TGF-β treatment of human

VSMCs results in intracellular ROS production and this effect is mediated through its receptor and probably activation of NOX enzyme.

TGF-β Mediated Smad2L Phosphorylation is ROS-dependent in Human VSMCs

Activation of TGF-β signaling was occurred by the phosphorylation of Smad2 and Smad3.[11] For clarifying the role of ROS in the phosphorylation of Smad2L, NAC (10 mM) was used. VSMCs were treated with TGF-β (5 ng/mL) for 1 hour and the phosphorylation of Smad2L measured by western blotting (Fig.1B). TGF-β treatment of cells caused an increase in the phosphorylation of Smad2L to 2.6-fold (p=0.015) at 1 hour compared to the non-treated group. In the presence of NAC (10 mM), the TGF-β mediated Smad2L phosphorylation was inhibited (p=0.027) (Figure1B). The TGF-β receptor inhibitor, SB431542 (20 μM), absolutely blocked the response to TGF-β (p=0.02) (Figure1B). These results declared that TGF-β mediated-Smad2L phosphorylation involves ROS.

TGF-β Treatment Stimulates the mRNA Expression of Pro-inflammatory Cytokines within Human VSMCs

We assessed the effect of TGF-β treatment on TNF-α and IL-6 expression in human VSMCs. To determine whether or not IL-6 and TNF-α mRNA expression is mediated by TGF-β signaling within human VSMCs, cells were exposed to TGF-β (5 ng/mL) and the IL-6 and TNF-α expression were measured by q-RT-PCR at 2, 4 and 8 and 24 h. Exposure of human VSMCs to TGF-β (5 ng/mL) enhanced IL-6 and TNF-α mRNA expression in a time-dependent manner. Following TGF-β treatment of cultured VSMCs, IL-6 mRNA expression increased 2.6 fold (p=0.02) after 8 hours in comparison to non-treated cells. This up-regulation was maintained until at least 24 hours (p=0.001) (Figure1C). Similarly, following the TGF-β treatment of cultured VSMCs, TNF-α mRNA expression increased 2.25fold after 4 hours compared with non-treated cells. This up-regulation was maintained until at least 24 hours (p=0.001) (Figure1D).Stimulation with TGF-β at 24 hours used in the further experiment.

TGF-β-stimulated pro-inflammatory cytokines mRNA expression is ROS dependent

The mechanisms regulating IL-6 and TNF-α mRNA levels in human VSMCs are not well understood. Here, we examined the contribution of NOX/ROS in the TGF-β mediated IL-6 and TNF-α expression. TGF-β treatment of cells caused an increase in IL-6 mRNA expression (p=0.034) at 24 hours compared to the non-treated group. The TGF-β induced IL-6 mRNA expression was totally blocked by DPI (20 μM) (p=0.037) and NAC (10 mM) (p=0.032). Treatment with SB431542 (20 μM) prevented the response to TGF-β (p= 0.026) (Figure 1E).

Similarly, TGF-β treatment of cells caused an increase in TNF-α mRNA expression to 3.5-fold (p=0.001) at 24 hours compared to untreated cells. Pre-incubation with DPI (20 μM), NAC (10 mM) and SB431542 (20 μM) totally prevented the TGF-β induced TNF-α mRNA expression (p=0.001) (Figure 1F). The results show that ROS may be involved in TGF-β stimulated the pro-inflammatory cytokines mRNA expression.

Curcumin Reduces TGF-β-induced ROS Production in Human VSMCs

The antioxidant activity of curcumin in TGF-β-stimulated VSMCs was measured using the H2DCFDA-based assay. According to our data, cell incubation with TGF-β (5 ng/mL) for 24 hours caused a significant increase in ROS production (p=0.024). As shown in Fig. 2A, pre-incubation with curcumin (5, 10 and 20 μM) dose-dependently attenuated the ROS level in the TGF-β-stimulated VSMCs (Figure 2A).

Curcumin Inhibits TGF-β-stimulated Smad2L Phosphorylation in Human VSMCs

Our results showed that TGF-β enhances Smad2L phosphorylation in human VSMCs. However, it is largely unknown if curcumin modulates TGF-β mediated Smad2L phosphorylation in human VSMCs. The effect of curcumin (10 and 20 uM) on the phosphorylation of Smad2L mediated by TGF- β was examined by western blotting. Fig. 2B shows that pre-treatment of curcumin (10 and 20 uM) significantly inhibited phosphorylation of Smad2L induced by TGF-β in human VSMCs (p=0.007).

Curcumin inhibits TGF-β-stimulated IL-6 and TNF-α Expression within Human VSMCs.

To investigate if curcumin inhibits TGF-β-induced IL-6 and TNF-α expression, human VSMCs were treated with TGF-β (5 ng/mL) in the presence and absence of curcumin and the mRNA IL-6 and TNF-α

expression examined via qRT-PCR.

As displayed in Fig. 2C, stimulation of VSMCs with TGF-β (5 ng/mL) for 24 hours caused a significant increase in the IL-6 mRNA expression. However, pretreatment with curcumin (10 and 20 μM) attenuated TGF-β-induced IL-6 mRNA expression in a concentration-dependent manner (Figure 2C). Similarly, as shown in Fig. 2D, stimulation of VSMCs with TGF-β (5 ng/mL) for 24 hours caused a significant increase in the TNF-α expression. However, pretreatment with curcumin (10 and 20 μM) attenuated TGF-β-induced the TNF-α mRNA expression in a concentration-dependent manner (Figure 2D). These results indicate that curcumin down-regulated the pro-inflammatory cytokines IL-6 and TNF-α expression stimulated by TGF-β in human VSMCs.

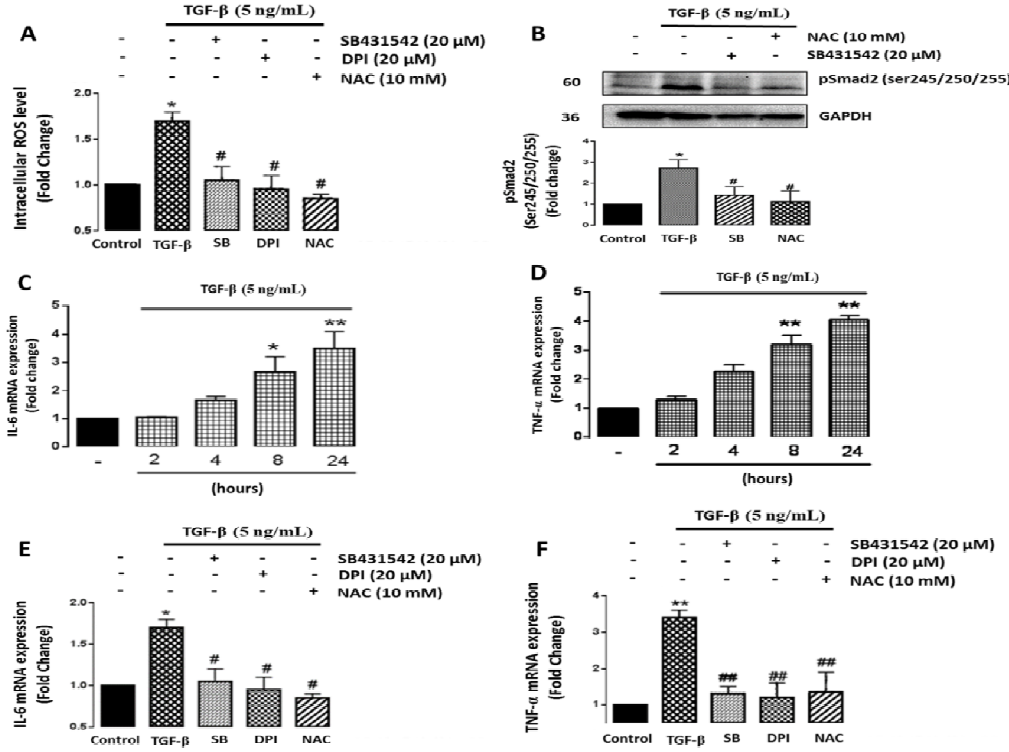

Figure1. Transforming growth factor-β (TGF-β) acting through its receptor leads to the reactive oxygen species (ROS) production, Smad2L phosphorylation, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) mRNA expression in human vascular smooth muscle cells (VSMCs). (A) Cells were incubated; using TGF-β (5 ng/mL) for 24hours in the presence and absence of TGF-β receptor antagonist (SB431542, 20 μM), diphenyleneiodonium chloride, DPI, (20 μM) and N-Acetyl-L-Cysteine, NAC, (10 mM) and then intracellular ROS generation was analyzed by monitoring 2′,7′-dichlorofluorescein intensity using fluorimeter. (B) Cells were treated with TGF-β (5 ng/ml) for 1 hour in the presence and absence of the TGF-β receptor antagonist (SB431542, 20 μM) and NAC (10 mM). Blots were exposed with anti-phospho-Smad2L (1:1000) antibody followed by anti-rabbit IgG labeled with peroxidase (1:10000) and enhanced chemiluminescence detection. Anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. VSMCs were exposed for 2, 4, 8 and 24 hours with 5 ng/mL of TGF-β. Total RNA was isolated and the mRNA of C) IL-6 and D) TNF-α was measured by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). VSMCs were pre-incubated DPI (20 μM), NAC (10 mM) for 2 hours and SB431542 (20 μM) for 30 minutes before exposed with TGF-β (5 ng/mL) for 24 hours. Total RNA was isolated and the mRNA of E) IL-6 and F) TNF-α was measured by qRT-PCR. GAPDH was used as a housekeeping gene. Results are presented as mean ± standard error of the mean(SEM) from three independent tests. Data were analyzed using One-Way Analysis of Variance followed by the least significant difference *post-hoc* analysis. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with untreated control, $^{\#}p<0.05$ and $^{\#\#}p < 0.01$ compared with TGF-β.

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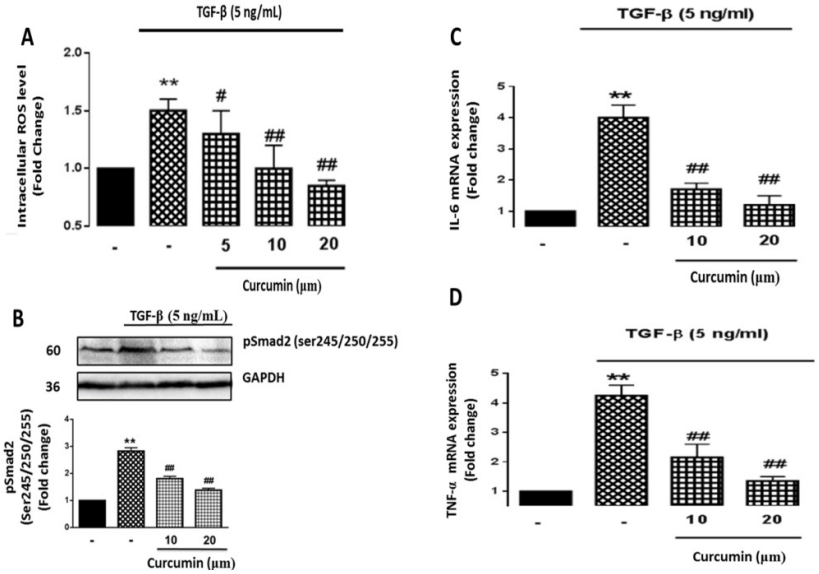

Figure2. Curcumin reduces Transforming growth factor-β (TGF-β)-stimulated reactive oxygen species (ROS) generation, Smad2L phosphorylation, interleukin-6 (IL-6) and tumor necrosis factor-alpha(TNF-α) mRNA expression within human vascular smooth muscle cells (VSMCs). (A) VSMCs were treated with TGF-β (5 ng/ml) for 24 hours in the presence and absence of the curcumin (5-20 µM) and then intracellular ROS generation was analyzed by monitoring 2′,7′-dichlorofluorescein intensity; using fluorimeter. (B) VSMCs were treated with TGF-β (5 ng/mL) for 1 hour in the presence and absence of the curcumin (10 and 20 Mm). Blots were incubated with anti-phospho-Smad2L (1:1000) accompanied by anti-rabbit IgG labeled with peroxidase (1:10000) and Enhanced chemiluminescence detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal control. Normalized data for every test is presented as mean ± SEM from three independent tests. VSMCs were incubated with curcumin (10 and 20 µM) for 1 hour before exposed with TGF-β (5 ng/ml) for 24 h. Total RNA was isolated and the mRNA of (C) IL-6 and (D) TNF-α was measured by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). GAPDH was used as a housekeeping gene. Results are presented as mean ± standard error of the mean (SEM)from three independent tests. Data were analyzed using One-Way Analysis of Variance followed by least significant difference *post-hoc* analysis. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the untreated control, $^{\#}p<0.05$ and $^{\#\#}p < 0.01$ compared with TGF-β.

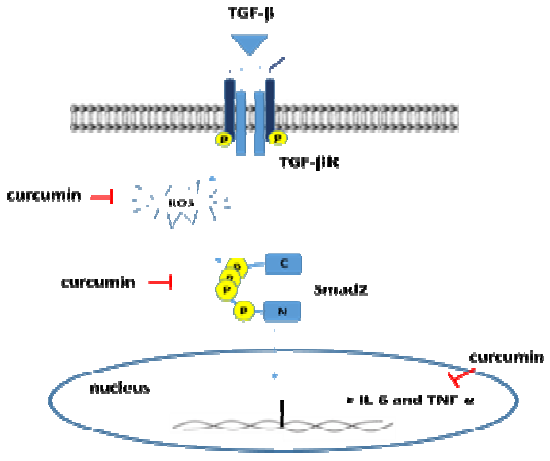

Figure3. Schematic showing of the inhibitory effect of curcumin on pro-inflammatory cytokines production by Transforming growth factor-β(TGF-β)-mediated non-Smad signaling pathway within human vascular smooth muscle cells (VSMCs).

DISCUSSION

This study provides an insight into a specific signaling pathway from TGF-β/TGFBR to NOX/ROS and then Smad2L phosphorylation leading to the mRNA expression associated with pro-inflammatory cytokines, IL-6 and TNF-α, in human VSMCs. In this research, we also evaluated the anti-inflammatory properties of curcumin within human VSMCs. Curcumin blocks TGF-β–induced IL-6 and TNF-α expression. Furthermore, this anti-inflammatory effect of curcumin is mediated via inhibition of ROS production and through inhibition of Smad2L phosphorylation.

The binding of TGF-β to TβR I and TβR II is the first step of TGF-β signaling, which then phosphorylates Smad2 and Smad3 transcription factors. Smad2 and Smad3 are key components of the TGF-β signaling cascade. In addition to TGF-β/Smads pathway, TGF-β receptors initiate various non-Smad signaling pathways.[10,28] Studies have shown that oxidative stress, characterized by overexpression of intercellular ROS, plays a vital role in the progression of atherosclerosis and the inhibition of ROS production may be a potential target for suppressing atherosclerosis.[15]Several reports suggest that ROS is an essential mediator of Smad2 transcription factor activation.[29,30] In the present work, we showed that TGF-β-stimulated phosphorylation of Smad2L involves ROS. We observed that blocking NOX/ROS with inhibitors, DPI and NAC, decrease the levels of Smad2L phosphorylation in human VSMCs.ROS are potential secondary messengers in cell signaling pathways, especially in the inflammatory responses. Therefore, there is a close relationship between inflammation and ROS production.[17] Several reports have indicated that ROS are involved in the expression of inflammatory cytokines. Recent studies have shown that NOX/ROS are involved in the regulation of IL-6 gene expression in NRK-52E cells and fibroblasts.[31,32]In this research, we found that TGF-β significantly up-regulated the inflammatory cytokines IL-6 and TNF-α in human VSMCs. DPI, an inhibitor of NOX, was able to block TGF-β-induced TNF-α and IL-6 expression. However, inhibition of TGF-β-induced TNF-α and IL-6 expression may be an efficient strategy to suppress the development of atherosclerosis.

Curcumin is a potent anti-inflammatory and anti-oxidant agent derived from Curcuma longa roots, and it is utilized as a therapeutic agent in patients with various chronic diseases including atherosclerosis.[22] Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in VSMC, it is possible that the anti-atherogenic effects of curcumin are attributable to its antioxidant and anti-inflammatory properties and its ability to inhibit proliferative and migratory signaling pathway. In vitro experiments have indicated that curcumin suppressed atherosclerosis development by inhibiting inflammatory markers expression.[22,23]

Curcumin mediates its effects against inflammation through interaction with numerous transcription factors, cytokines and redox status that can differentially modulate multiple cell signaling pathways involved in inflammation. Some recent studies show that curcumin is a potent inhibitor of transcription factors such as nuclear factor-κB (NF-κB), which plays an important role in cellular inflammation. Curcumin exerts anti-inflammatory effects by inhibiting the expression of IL-6, TNF-α, and IL-1β in the cells.[33,34] Atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular wall. In the cardiovascular system, curcumin could prevent the increase in TNF-α and the oxidative effects and prevent the progression of the atherosclerotic lesion.[35]

In this study, we show that curcumin inhibits the inflammatory activity of TGF-β within human VSMCs. Our data showed that pretreatment with curcumin significantly decreased TGF-β-induced TNF-α and IL-6 expression, two major inflammation mediators that are vital in the inflammatory responses within atherosclerotic plaques. The underlying mechanism of the anti-inflammatory curcumin effects on TGF-β-induced VSMCs was further investigated. Our data suggest that in VSMCs treated with TGF-β, the IL-6 and TNF-α levels were increased via a pathway dependent on ROS production and Smad2L phosphorylation. Growing evidence indicates that curcumin inhibits ROS production induced by various agonists including LPS and TNF-α.[9,33,36,37]

In this study, treatment with curcumin effectively inhibited the TGF-β-stimulated increase in intracellular ROS, which acts as an upstream regulator of the Smad2L pathway. In HK-2 Cells, curcumin suppressed TGF-β-induced Smad2 phosphorylation and Smad2 and Smad3 nuclear accumulation.[25] Our findings

demonstrated that the levels of Smad2L phosphorylation were significantly increased at 1 hour after treating with TGF-β in human VSMCs, and curcumin attenuated the TGF-β-induced increase of Smad2L phosphorylation. These findings highlight that the anti-inflammatory effects of curcumin occur through inhibition of the TGF-β/Smad2 and NOX/ROS signaling pathway.

This manuscript provides a better understanding of the signaling pathways controlling pro-inflammatory cytokines production within human VSMCs. TGF-β acting through its receptor leads to the ROS production and phosphorylation of the transcription agent Smad2L, a response associated with TGF-β-mediated mRNA expression of IL-6 and TNF-α that are closely associated with the development of atherosclerosis. Curcumin blocks TGF-β–induced expression of IL-6 and TNF-α via inhibition of ROS production and through inhibition of Smad2L phosphorylation.

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