Irbesartan attenuates TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression through suppression of NF-κB pathway in HUVECs

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Abstract. – **OBJECTIVE:** It is widely recognized that atherosclerosis is a chronic inflammatory disease. Intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), and E-selectin play vital roles in inflammatory processes. ICAM-1, VCAM-1, and E-selectin expression is regulated by nuclear factor (NF)-κB signaling. It has been reported that irbesartan can decrease expression of atrial fibrillation-Induced atrial adhesion molecule and reduce secretion of inflammation associated cytokines from cultured human carotid atheroma. In this study, we examined whether irbesartan prevents TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS: HUVECs were cultured. The expression of ICAM-1, VCAM-1 and MCP-1 was measured by real-time quantitative PCR and ELISA. The expression of NF-κB and p-IκB-α was measured by Western blot.

RESULTS: It indicated that in HUVECs irbesartan inhibited expression and secretion of TNFα-induced ICAM-1, VCAM-1, and E-selectin. Furthermore, irbesartan inhibited TNF-α-induced IκB-α phosphorylation and NF-κB P65 nuclear translocation substantially. In conclusion, irbesartan attenuates TNFα-induced ICAM-1, VCAM-1, and E-selectin expression by way of suppressing the NF-κB pathways in HUVECs. Irbesartan might postpone the progression of inflammatory diseases, including atherosclerosis.

CONCLUSIONS: Irbesartan attenuates TNFα-induced ICAM-1, VCAM-1 and MCP-1 expression through the suppression of NF-κB pathways. These results suggest irbesartan would be of great benefit to delaying the progression of inflammatory diseases, including atherosclerosis.

Key Words:
  Atherosclerosis, Irbesartan, ICAM-1, VCAM-1, E-selectin.

Introduction

Atherosclerosis is the common cause of heart attacks, peripheral artery disease and strokes. More and more scholars obviously agree that atherosclerosis is a chronic inflammatory disease[1]. It has been broadly known that monocytes adhering to vascular endothelial cells and transforming into macrophage is a critical point at the atherosclerosis prophase[2]. Intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), and endothelial cell selectin (E-selectin) play vital roles in the process[3]. Presently, ICAM-1, VCAM-1, and E-selectin have been considered as biomarkers for the detection of endothelial dysfunction in patients with coronary heart disease (CHD)[4]. Thus, it may be a good way to inhibit the development of atherosclerosis by cutting down the expression of ICAM-1, VCAM-1 and E-selectin.

Nuclear factor kappa B (NF-κB) as a transcription factor is involved in many pathophysiological processes, including inflammatory diseases like atherosclerosis[5]. NF-κB increases the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin[6,7].

The renin-angiotensin-systerm plays a critical role in the regulation of the cardiovascular system. It is popularly accepted that angiotensin II (Ang II) induces cardiovascular events through activing angiotensin receptor (ATR)1. ATR1 blocking drugs (ARBs) can reduce the cardiovascular events, such as CHD. It has been accounted that treatment with an Ang II type 1 receptor antagonist (candesartan) decreased plasma levels of the immune markers such as TNF-alpha, IL-6, sICAM-1 and sVCAM-1 in patients with chronic heart failure[8]. Furthermore, irbe-

sartan can decrease expression of atrial fibrillation-induced atrial adhesion molecule and reduce secretion of inflammation associated cytokines from cultured human carotid atheroma[9]. Nonetheless, it is still in doubt whether irbesartan is against the TNF-α induced expression of ICAM-1, VCAM-1, and E-selectin through NF-κB pathways in HUVECs.

This study investigated the influences of irbesartan on TNFα-induced ICAM-1, VCAM-1 and E-selectin expression in HUVECs and its potential molecular connection with the NF-κB pathways. Our results stated clearly that irbesartan attenuates TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression via suppressing NF-κB pathways in HUVECs. These results might render valuable foundations for the treatment of clinical cardiovascular diseases.

Materials and Methods

Materials

Antibodies against NF-κB p65, IκB-α, proliferating cell nuclear antigen (PCNA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Abcam (Cambridge, UK). 1640 medium, fetal bovine serum (FBS) and 0.25% trypsin-EDTA were from Gibco (New York, NY, USA). ICAM-1 and VCAM-1 ELISA kits were from the Ha Ling company (Shang Hai, China). Irbesartan, TNF-α and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma Aldrich (St. Louis, MO, USA) and were all of analytical grade.

Cells Culture and Identification

HUVECs were isolated from a fresh umbilical cord, which was obtained from a healthy neonate under aseptic conditions in the Department of Gynaecology and Obstetrics of The Xinjiang Medical University. HUVECs were incubated in Roswell Park Memorial Institute (RPMI)-1640 medium and identified by morphology.

RNA Isolation and Real-time Polymerase Chain Reaction (PCR)

Total cellular RNA was isolated using the TRIzol® reagent according to the manufacturer's protocol. RNA (1.0 mg) was reverse-transcribed into complementary DNA (cDNA) using a PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa, Otsu, Shiga, Japan). The PCR primers used are listed in Table I and all primer sequences were determined through established GenBank sequences. Real-time PCR, using SYBR® Green detection chemistry, was performed on an ABI QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems; Carlsbad, CA, USA) under the following conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 30 s and 60°C for 30 s. The cycle threshold values (Ct values) were used to calculate the fold differences using the $^{\Delta\Delta}$CT method, and β-actin expression was used as the internal control.

ELISA

Intracellular ICAM-1, VCAM-1, and E-selectin levels were determined with an enzyme immunoassay kit according to the manufacture's instructions. The optical density of each well was determined using a microplate reader at 450 nm within 30 min.

Western Blot Analyses

Briefly, after treatment, 1×10^6 HUVECs were harvested, and protein extracts were prepared in lysis buffer containing 1 mM PMSF (Sigma, S. Louis, MO, USA). The protein concentration was calculated using a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). For Western blot analyses, 50 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore Inc., Billerica, MA, USA) and probed with anti-mouse monoclonal PCNA, GAPDH (1:1,000 dilution; Santa Cruz, Santa Cruz, CA, USA), NF-κB p65, phosphor-IκB-α antibodies (1:1,000 dilution; Cell Signaling, Danvers, MA, USA) followed by incubation with the HRP-conjugated secondary antibody. ECL plus system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used for detection of the protein signal. The expression level was determined by measurement of the corresponding band intensities by using a Gel Imaging System (Bio-Rad, Hercules, CA, USA), and the relative values were expressed relative to GAPDH signal.

Statistical Analysis

All data analyses were performed using the SPSS v 16.0 software (SPSS Inc., Chicago, IL, USA). Data were presented as means ± SE. Group differences were analyzed by Student's unpaired t-test or one-way analysis of variance. Statistical significance was assumed when the p-values were less than 0.05.

Table I. Primer sequences for gene expression analyses.

Gene	Primer sequences
VCAM-1	Forward： 5′-GATACAACCGTCTTGGTCAGCCC-3′ Reverse： 5′-CGCATCCTTCA ACTGGGCCTT-3′
ICAM-1	Forward： 5′-CAGTGA CCATCTACA GCTTTCCGG-3′ Reverse： 5′-GCTGCTACCACAGTGATGACAA-3′
E-selectin	Forward： 5′-GTCTAGCGCCTGGATGAAAG-3′ Reverse： 5′-ATYGCCACCAGATGTGTGTA-3′
β-actin	Forward： 5′-CCTAGAAGCATTTGCGGTGG-3′ Reverse: 5′-GAGCTACGAGCTGCCTGACG-3′

Results

Isolation and Identification of HUVECs

Von Willebrand factor (vWF) is specifically expressed in endothelial cells, immunofluorescence staining of vWF was conducted to identify HUVECs. The results indicated that approximately 100% of the isolated cells were vWF-positive (Figure. 1).

Irbesartan Attenuates TNF-α-induced Expression of ICAM-1, VCAM-1, and E-selectin in HUVECs

In order to estimate influences of irbesartan on TNFα-induced ICAM-1, VCAM-1, and E-selectin expressing in HUVECs, the cells were pre-treated with irbesartan for 30 min and then stimulated with 100 nM TNF-α for 6 h. Culture supernatants were gathered, and the levels of ICAM-1, VCAM-1, and E-selectin were detected by ELISA. In the meanwhile, the HUVECs were harvested, and the mRNA levels of ICAM-1, VCAM-1, and E-selectin were measured by real-time PCR. In comparison with the control, marked increase in ICAM-1, VCAM-1, and E-selectin mRNA expression and secretion resulted from TNFα treatment. However, in comparison with TNFα treatment alone, pretreatment with irbesartan inhibited expression and secretion of ICAM-1, VCAM-1, and E-selectin mRNA (Figure 2).

Irbesartan Inhibits TNF-α-induced IκB-α Phosphorylation and NF-κB Translocation in HUVECs

As the NF-κB pathway is a crucial mediator of inflammation, we evaluated the influence of irbesartan on the TNFα-induced NF-κB pathway. As Figure 3 obviously demonstrates that TNFα drastically increased the translocation of NF-κB p65 from the cytosol to the nucleus, and irbesartan inhibited this increase. The phosphorylation and degradation of IκB-α are important in translocation of NF-κB to the nucleus. Accordingly, we also investigated the phosphorylated IκB-α expression by western blot. As shown in Figure 3, irbesartan prevented the TNF-α-induced phosphorylation of IκB-α. These results revealed that irbesartan inhibited TNF-α-induced inflammatory responses by inhibiting TNFα-induced NF-κB translocation and IκB-α phosphorylation in HUVECs.

Effects of PDTC on TNFα-induced Expression of ICAM-1, VCAM-1, and E-selectin in HUVECs

To better examine whether the TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression is induced by the NF-κB pathways in HUVECs, we pretreated the cells with PDTC (an inhibitor of NF-κB). As exhibited in Figure 4, compared with the control, TNF-α leaded to a marked increase of ICAM-1, VCAM-1, and E-selectin expression.

Figure 1. Immunofluorescence staining of Von Willebrand factor (vWF). The figure *A,* is normal cultured HUVECs and *B,* is immunostaining of vWF. Approximately 100% of the isolated cells were vWF-positive.
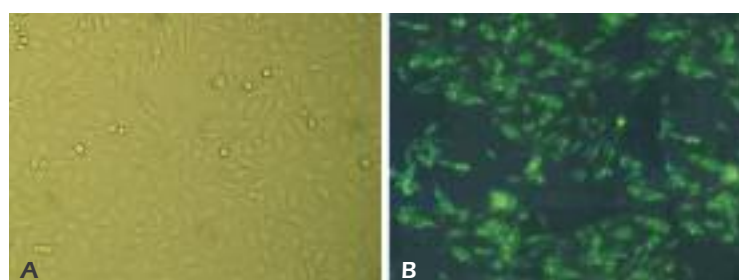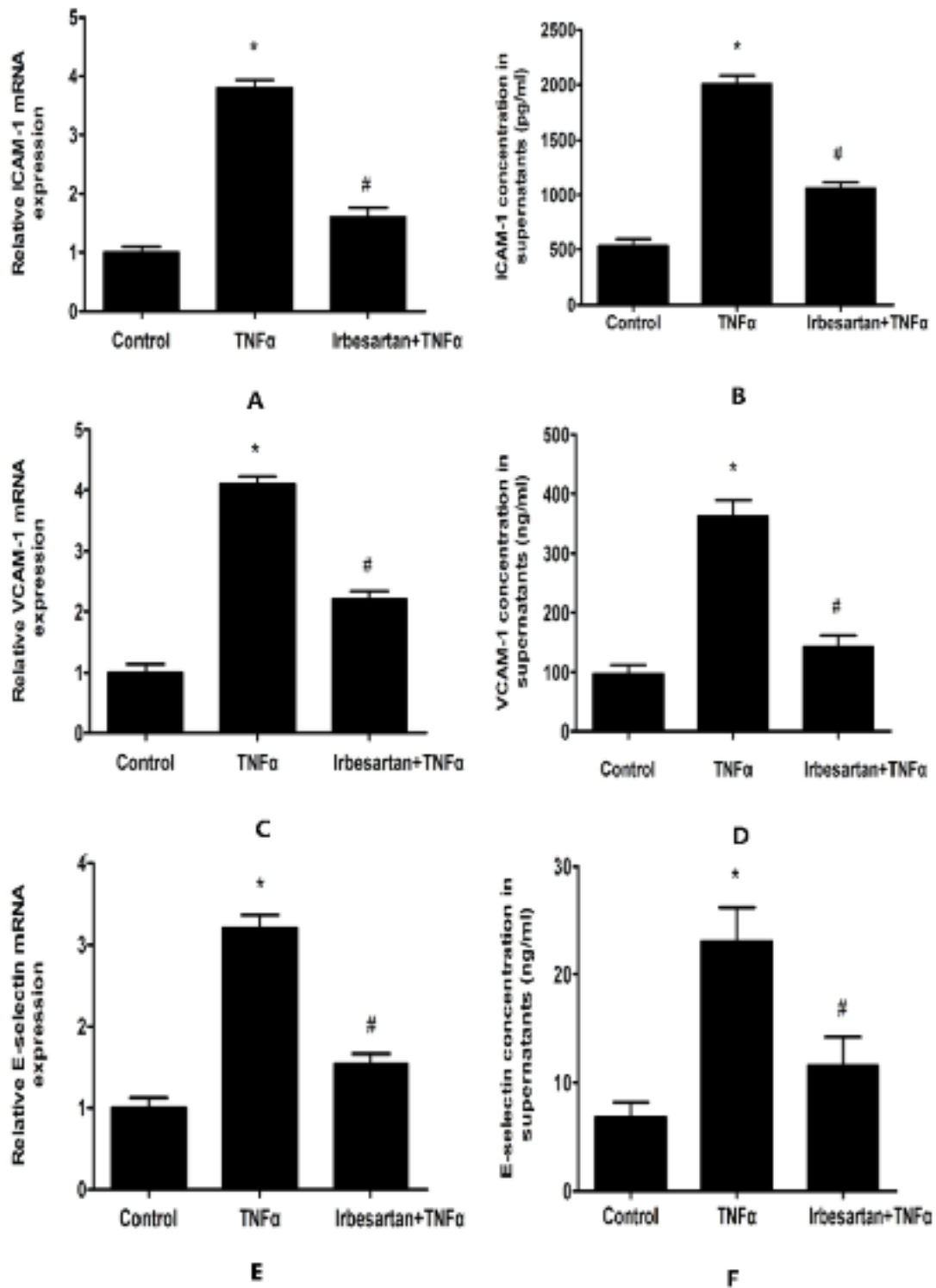
Figure 2. Irbesartan attenuates tumor necrosis factor-α (TNF-α)-induced intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), and E-selectin expression in HUVECs. HUVECs were incubated with 1 ng/mL TNFα alone for 6 h. Furthermore, HUVECs were pretreated with 2 μM irbesartan for 30 min before being incubated with 1 ng/mL TNF-α for another 6 h. **A, C,** and **E,** mRNA expression of ICAM-1, VCAM-1, and E-selectin was measured by real-time polymerase chain reaction (PCR). **B, D,** and **F,** Culture supernatants were collected, and the secretion levels of ICAM-1, VCAM-1, and E-selectin were determined by enzyme-linked immunosorbent assay (ELISA). Data are presented as the means ± standard deviation (SD) of three independent experiments. *$p<0.05$ compared with the control group; #$p < 0.05$ compared with the TNF-α group.
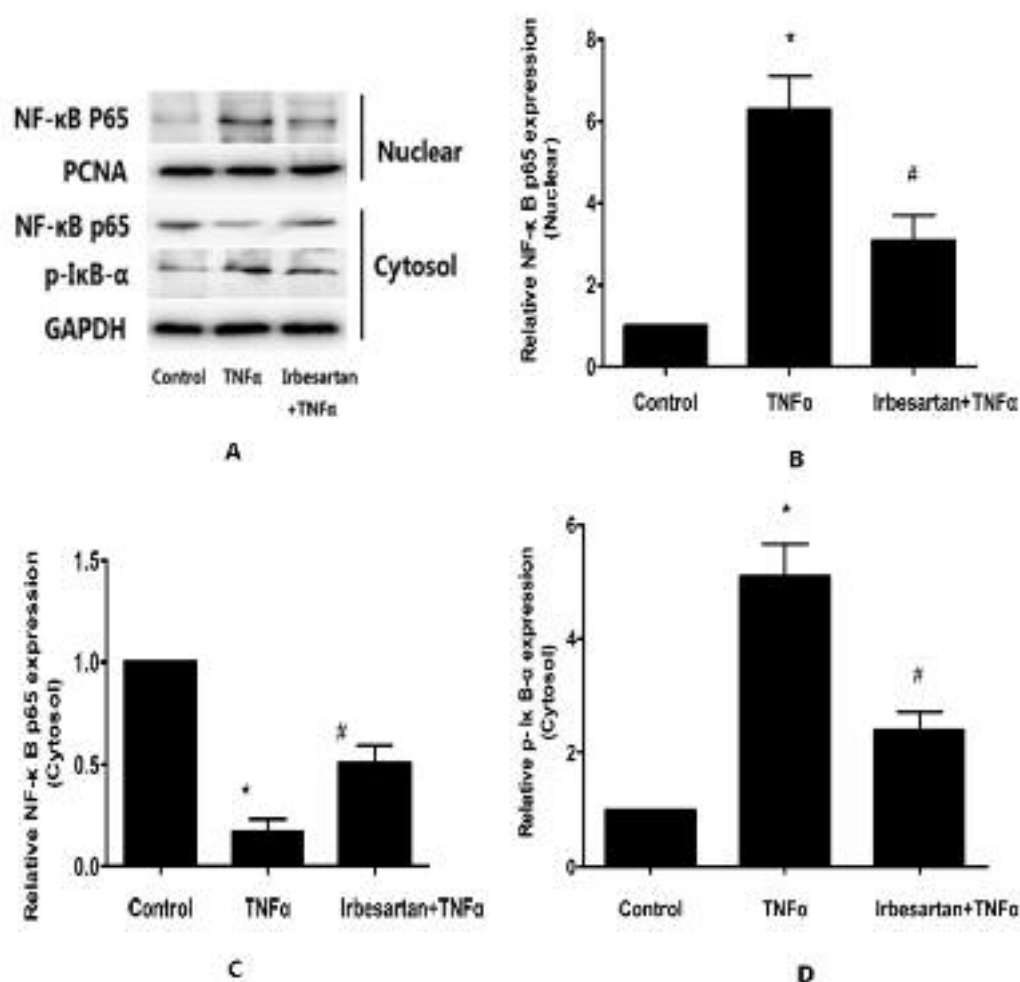
Figure 3. Irbesartan inhibits TNFα-induced IκB-α phosphorylation and nuclear factor (NF)-κB translocation in HUVECs. HUVECs were incubated with 1ng/mL TNFα alone for 30 min. Furthermore, HUVECs were pretreated with 2 μM irbesartan for 30 min before being incubated with 1ng/mL TNFα for another 30 min. The expression of nuclear factor (NF)-κB and p-IκB-α was measured by western blotting. Data are presented as the means ± SD of three independent experiments. *$p < 0.05$ compared with the control group; #$p < 0.05$ compared with the TNF-α group.

PDTC inhibited the influences of TNFα on ICAM-1, VCAM-1, and E-selectin expression (Figure 4). These results show that NF-κB may be indispensable to TNF-α-induced the expression of ICAM-1, VCAM-1, and E-selectin in HUVECs.

Discussion

Recently, it has been generally recognized that atherosclerosis is a chronic inflammatory process and several studies have already suggested that the expression of ICAM-1, VCAM-1, and E-selectin has closely associated with the development of atherosclerosis. Additionally, NF-κB signaling has been manifested to be deeply involved in inflammatory process, particularly in regulating the expression of ICAM-1, VCAM-1, and E-selectin.

Ang II plays an important role in the cardiovascular diseases such as myocardial infarction and atherosclerosis. It causes the proatherosclerotic effect that Ang II binds to AT1 receptor[10]. It has been proved that Ang II activates transcription factors NF-κB which induces the expression of the TNF gene in atherosclerotic lesions[11]. Reviewing recent studies, they have suggested that Ang II promotes ICAM-1, VCAM-1, and E-selectin expression in HUVECs via NF-κB pathways[12-14].
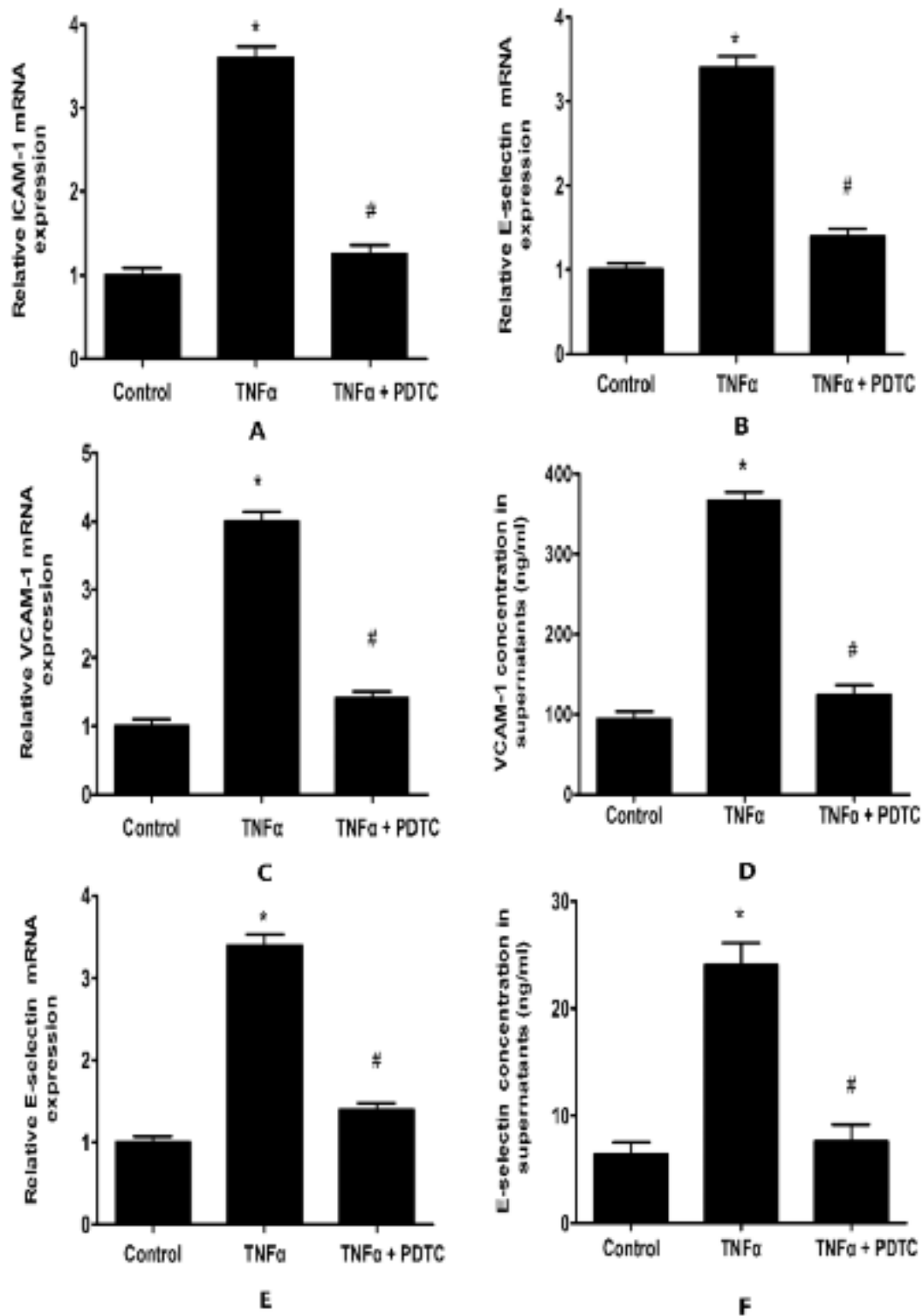
Figure 4. Effects of pyrrolidine dithiocarbamate (PDTC) on Ang II-induced expression of ICAM-1, VCAM-1, and E-selectin in HUVECs. HUVECs were incubated with 1 ng/mL TNF-α alone for 6 h. Furthermore, HUVECs were pretreated with 10 mM PDTC for 30 min before being incubated with 1 ng/mL TNFα for another 6 h. **A, C,** and **E,** mRNA levels of ICAM-1, VCAM-1, and E-selectin was estimated by real-time PCR. **B, D,** and **F,** Culture supernatants were collected and the secretion levels of ICAM-1, VCAM-1, and E-selectin were estimated by ELISA. Data are presented as the means ± SD of three independent experiments. *$p < 0.05$ compared with the control group; #$p < 0.05$ compared with the TNF-α group.

Irbesartan can block the effect of Ang II, which cause majority of the well-known physiological processes, via specific, selective non-competitive antagonism of AT1 receptors[15]. There is no doubt that irbesartan can provide good 24-h blood-pressure control and improve left atrium volume in patients with mild to moderate hypertension[16,17]. It has been known irbesartan brought positive results to patients with heart failure[18]. Several studies have also confirmed that irbesartan could suppress the inflammatory components and even platelets aggregation, which attenuate the atherosclerotic processes[19,20]. On the other hand, it remains unclear whether irbesartan attenuates TNF-α-induced the expression of ICAM-1, VCAM-1, and E-selectin via NF-κB pathway in HUVECs. So in this study, we first investigated the influence of irbesartan on the expression of ICAM-1, VCAM-1, and E-selectin. We noticed that irbesartan inhibited TNFα-induced mRNA and secretion of ICAM-1, VCAM-1, and E-selectin in HUVECs, which shows a possible advantageous effect on irbesartan through the attenuation of HUVECs activation and inflammation. As a result, irbesartan might retard the progression of inflammatory diseases.

NF-κB is definitely one of the most important regulators of inflammation. ICAM-1, VCAM-1, and E-selectin expressing is mediated by NF-κB. In unstimulated cells, NF-κB dimers are offered inactive by inhibitory proteins of the IκB family in the cytosol. IκB phosphorylation is the decisive step in NF-κB activation, which occurs owing to activation of the IκB kinase complex. The phosphorylation of inhibitory IκB proteins causes their ubiquitination and subsequent proteasomal degradation, followed by the release and nuclear translocation of active NF-κB[21]. To further illuminate the mechanisms that underlie the inhibitory impact of irbesartan on the expression of ICAM-1, VCAM-1, and E-selectin in HUVECs, we also examined the impact of irbesartan on the IκB-α and NF- κB p65. Although incubation of HUVECs with TNF-α caused significant cytosolic phosphorylation of IκB-α and NF-κB p65 translocation into the nucleus, pretreatment with irbesartan markedly inhibited IκB-α phosphorylation and NF-κB p65 nuclear translocation. These results stated plainly that irbesartan suppressed TNF-α-induced inflammatory responses through inhibiting TNFα-induced IκB-α phosphorylation and NF-κB p65 nuclear translocation in HUVECs.

Conclusions

Our data show that irbesartan inhibits TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression by NF-κB-dependent signaling in HUVECs. Therefore, irbesartan is a vital mechanism which reverses the inflammatory cascades that involved in atherosclerosis. Moreover, activation of this pathway may offer a novel therapeutic method for the treatment of atherosclerosis.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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